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The goal of the project was to evaluate the role of the proteasome in prostate cancer biology and response to heat and radiation therapy. We have shown considerable variation between prostate cancers in their functional proteasome activity. In all cases heat and radiation inhibit activity, and this is accompanied by changes in signal transduction pathways that are regulated through the proteasome. Most notably, NF-κB is suppressed by heat and by low radiation doses due to stabilization of IκBα. In contrast, at high radiation doses NF-κB is activated, even in the presence of IκBα stabilization, suggesting an alternative pathway of activation. The response of the proteasome to these stressors is very rapid, suggesting the proteasome regulates the initial cellular response to challenge. It is redox sensitive and radiation can directly affect proteasome activity, suggesting that it is a direct target for radiation. Interestingly, the "immunoproteasome" containing interferon-inducible subunits appears to be most sensitive. We have shown that proteasome inhibitors cause apoptosis of prostate cancer cells and also sensitize them to the effects of radiation in vitro and in vivo. We have also identified the proteasome as a target for other drugs, such as anthracyclins and HIV protease inhibitors, suggesting that they might be used clinically in combination with radiation therapy to elicit improved therapeutic benefit.

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Final Report

Introduction:

The goal of the proposal was to investigate the novel hypothesis that the proteasome functions as a sensor for hyperthermia and/or irradiation and its response is important in determining the outcome of prostate cancer treatment. This topic has grown considerably in importance in the last few years as seen by the fact that we have been invited to publish several relevant reviews [1-3]. The proteasome has been found to have critical roles in selectively regulating many cell cycle progression, transcription, DNA repair, and cell death. We, and others, have shown that it is frequently dysregulated in cancer. We have also shown this in prostate cancer, leading to many interesting questions relating to tumor prognosis and response to therapy.

The major contribution resulting from this grant is the finding that hyperthermia and/or radiation decrease proteasome activity, and this influences the expression of cell death/survival molecules following treatment in a manner that might determines therapeutic outcome. Significantly, we have shown for the first time that the 26s proteasome appears to be a direct target for radiation [4, 5] and that its activity is regulated by changes in redox potential [6]. We have some remarkable preliminary evidence that different proteasomes respond differently to irradiation, suggesting that the nature of the proteasomes a cancer cell contains might determine therapeutic outcome (to be published).

We have also shown that proteasome-specific drugs and radiosensitize for prostate cancer in vitro and in vivo. One such drug PS-341 is in clinical trials in solid tumors and leukemias, and plans are now in place to use it as a radiosensitizer, most likely including in prostate cancer, as a result of our preliminary data [7-10]. Significantly, we have also shown that certain chemotherapeutic drugs [11], including HIV protease inhibitors [12, 13] modulate proteasome activity and this may be part of their therapeutic action, and that such drugs can act as radiosensitizers. These studies expand the scope of the proposal, but are of considerable therapeutic importance.

Progress will be considered under the headings of the original stated tasks. The narrative contains sections from last year's report with added sections dealing with further progress.

Body of the Report:

Task 1. To quantify proteasome activity in human prostate cancer cells and its modification by hyperthermia and/or radiotherapy.

We have examined the proteasome-associated chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activity in human PC3, LnCaP, DU-145, and a newly isolated human prostate line LAPC4, as well as the murine prostate cancer model TRAMP cell lines C1 and C2. We have compared this with activity in over 12 other types of human tumors. The clear picture that has emerged is one of considerable variation in constitutive steady state proteasome activity between cancer cell lines, which is what we predicted. This activity generally exceeds that in normal cells, indicating that cancer cells have dysregulated proteasome activity.

The variation between cancer cells is a reflection of the genotype of the cell and the tumor microenvironment. We think that this will impact tumor behavior and response to therapy. An example of how proteasome activity is altered is that hypoxia/reperfusion, levels of free radical scavenegers, and cytokine levels all affect proteasome function. As far as cytokines are concerned, we have shown that interleukin (IL)-6 and IL-3 [14], both of which can be constitutively produced by prostate cancer cells, can enhance proteasome activity. Serum growth factors and addition of interferon-γ can do the same. Importantly, these affect not only proteasome function, but also the composition of the proteasome subunits. Interferon, and other cytokines, cause changes in the basic enzymatic core subunits and in regulatory subunits, resulting in formation of what is often called an "immunoproteasome." Recently, we have shown that this influences their response to radiation. Immunoproteasomes are more sensitive to radiation-induced inhibition than constitutive proteasomes (to be published). There is an interesting corollary to this finding. It is that the immunoproteasome determines the nature of the peptide antigen that is presented to the immune system. Therefore the immunoproteasome content of a prostate tumor could determine whether or not it is recognized by the immune system. In addition, inhibition of the activity of this subset of

immunoproteasomes by radiation could be a novel mechanism of radiation-induced immune suppression. This is the topic of a new grant that we have just submitted to the D.O.D.

The response of PC-3, LnCaP, and DU-145 to heat has been examined. Hyperthermia at 42 or 44 degrees for 30 minutes decreased 26s proteasome activity to about 40% of untreated control cells, as assessed by proteolysis of specific fluorogenic substrates (chymotrypsin-like activity was decreased to 36.2 ± 3% (PC-3), 33.4 ± 8.4% (DU-145) and 45 ± 3.4% (LnCaP)). The response was very rapid, if not immediate and is clearly an important part of the stress response of cells to hyperthermia. We have shown that this response leads to stabilization of IκBα, which is known to be degraded through the proteasome, with a resultant decrease in constitutive and heat-induced NF-κB. Since NF-κB is a survival factor for many cell types, the consequence was apoptosis and radiosensitization of the cell lines. In fact, we believe that this provides and explanation for the long-observed phenomenon of heat-induced radiosensitization.

The effect of irradiation on proteasome activity has been assessed using the same prostate cancer cell lines. These studies have now been published [4, 5]. We have confirmed that the proteasome is inordinately sensitive to very low doses of irradiation and that inhibition is almost immediate. We have identified the proteasome as a novel target for the direct effects of radiation and, using free radical scavengers, we have shown that the effects are most likely due to changes in redox [10]. The consequences of these findings for radiation-induced gene expression are profound. P53, mdm2, p21, IκBα, and numerous other immediate radiation-induced responses may be mediated, or at the very least influenced by, proteasome inhibition. This indeed may be the prime mechanism by which radiation and heat rapidly up-regulate protein expression in cells, leading to an adaptive response with transcriptional activation of new genetic programs.

In the published papers, we address an apparent paradox, which is that several groups of investigators, including ourselves, have shown that radiation induces NF-κB expression. Our hypothesis would anticipate that radiation-induced inhibition of proteasome activity would decrease expression. We have shown that at low doses inhibition is paramount and that only at high doses is inhibition overriden and NF-κB is induced, in part by pathways that are alternate pathways that

involve tyrosine phosphorylation of IκBα. These finding provide a mechanism for the anti-inflammatory effects of low dose radiation that have been reported on numerous occasions and explain why radiation has long been used, especially in Europe, to treat inflammatory diseases.

We have now extended the studies on the effects of ultra-low doses of radiation on proteasome function to include responses to low doses subsequently treated with high doses of radiation 24 hours later. We have shown that ultra-low doses prevent responses to higher radiation doses. In recent years, several groups have shown adaptive responses to low dose radiation that result in increased cell survival or decreased radiation-induced carcinogenesis. Our observations provide a possible mechanism for these adative responses. We have applied for a D.O.E. grant to follow up on these observations.

Task 2. To identify pathways by which proteasome activity is altered in prostate cancer cells after hyperthermia and/or radiotherapy.

We have examined the response of 20s and 26s proteasomes to heat. To do this we have developed a gradient ultracentifugation system that isolates these with reasonable degrees of purity. Heat-induced inhibition of proteasome activity was largely associated with 26s fraction, did not requires transcriptiona, as shown using cyclohexamide. This differential response of the different major proteasome structures mimiced the response to hydrogen peroxide. The implication of these findings is that the cell makes a molecular "stress"response by inhibiting proteolysis of regulatory molecules through the 26s proteasome, while continuing to allow removal and degradation of heat-damaged or oxidised proteins that would otherwise be toxic and lethal. It also implies that 19s regulatory subunits that feed proteins into the catalytic core are the target.

One of our hypotheses is that heat caused inhibition of proteasome activity through activation of HSP90. We thought this because we have shown that the response to heat correlated with HSP90 induction, as assessed by PCR, in a temporal and temperature-related manner, but not with HSP70 or HSP27 expression. We showed that recombinant hsp90 does indeed inhibit proteasome activity, however geldanamycin, a hsp90 analog has the opposite effect and does not prevent heat-induced inhibition, suggesting that the mechanism of proteasome inhibition is not through this pathway.

We have shown that hsp90 is also not responsible for radiation-induced proteasoem inhibition. This is in keeping with our finding that irradiation of purified isolated proteasomes has the same level of effect on their function as does irradiation of cells followed by proteasome isolation. This suggests that the effects of irradiation are direct and not mediated by some soluble factor in the cell.

However, the fact that hsp90 inhibits proteasome activity led us to suggest that this might be a physiological mechanism that controls proteasome activity through other pathways. One of the pathways that we investigated was that mediated by the androgen receptor (AR). This exists in a multimolecular complex along with hsp90. We suggested that adrogen causes dissolution of this complex liberating hsp90 and this inhibits proteasome activity and initiates non-transcriptional pathways mediated by AR-androgen interactions. This hypothesis is currently being tested using hsp90 and anti-sense hsp90 overexpressing cells. However, as part of the studies, we have shown that heat downregulates expression of the androgen receptor (AR) on LNCaP cells and this appears to lead to a loss of androgen independent growth. This could have enormous importance as a mechanism for developing androgen independence in prostate cancer, although further studies are needed to confirm its importance.

Because both radiation and heat alter the redox status of cells, we have investigated whether or not the proteasome is redox-sensitive. In fact, the 26s proteasome appears to be very sensitive to N-acetyl cytseine, tempol, and glutathione treatment. Furthermore, at least the latter two free radical scavengers counteract radiation-induced proteasome inhibition. These studies been confirmed and published [6, 10], at least in part, and clearly indicate that the proteasome is a very sensitive sensor.

Furthermore we have shown that cytosolic and non-cytosolic (nuclear) extracts are both affected in a cell. This is important because the spatial location of the proteasomes is being increasingly shown to dictate their substrates and functions.

As discussed above, we have identified NF-κB as a pathway that is clearly affected by heat- or radiation-induced proteasome inhibition. Other targets are however likely and we have explored some of these. One possibility is that caspases are activated through inhibition of IAP degradation. Since the DNA repair enzyme DNA-PK is a substrate for caspase 3 we have investigated this as a

possible pathway for heat-induced radiosensitization. Our data indicate that caspase 3 activation is not required for proteasome-dependent apoptosis. There is still considerable debate as to the mechanism by which proteasome inhibitors, such as PS-341 and MG-132, cause apoptosis of cancer cells. We have data to suggest that apoptosis inhibitory factor (AIF) might be involved, but this is still preliminary and needs to be confirmed.

We have also developed an in vivo assay with PC3 cells transfected to express Ub-GFP. Using this assay, we have shown that heat causes accumulation of GFP in individual cells, in keeping with its effects on proteasome function. Surprisingly, radiation did not have the same effect. We believe that the assay is too insensitive to detect the radiation effects and have temporarily abandoned the use of this approach for the radiation studies.

In the last year we have focused on the cytokine-inducible proteasome subunits lmp7, lmp2, and Mec1. We have used human cell lines deficient in these subunits as well as cells from knock-out mice to show that the "immunoproteasome" that contains these subunits are particularly sensitive to radiation-induced inhibition. This explains why inhibition is incomplete following radiation exposure. It never reaches 100%. This observation is particularly important for the effects of radiation on the development of anti-tumor immunity. Radiation is an excellent cytotoxic agent, but the cell killing is never translated into tumor immunity. This is surprising since radiation also promotes the milieu that contains "danger" signals that are thought to be needed for immunity. Our observations provides an explanation why this does not occur. The proteasome is essential for antigen processing and presentation to cytotoxic T cells. We have shown that radiation inhibits this process in dendritic cells, which asre professional antigen presenting cells. In contrast, radiation enhances presentation of peptides added exogenously to dendritic cells. Radiation therefore alters the nature of any immunity that doeas develop and moves it away from the form needed for tumor rejection. We submitted an RO-1 grant on this topic and have been told it received a very high and fundable score.

Task 3. To determine the extent to which 26s proteasome activity determines the effectiveness of prostate cancer treatment by hyperthermia and/or radiotherapy.

We have now shown that hyperthermia inhibits proteasome degradation by prostate cancer cells and prevents radiation-induced NF-κB activation resulting in cell death by apoptosis. This mechanism could explain the radiosensitizing effect of heat given shortly prior to radiation treatment. We have shown that PS-341, a proteasome inhibitor, can radiosensitize prostate tumors in vitro and in vivo [9, 10]. This agent is in clinical trials alone in multiple myeloma and, in large part because of our findings, is being explored in combination with radiotherapy for treatment of prostate cancer. We have started studies to examine this in combination therapy with radiolabelled antibody and intend to initiate a clinical trial in refractory ALL using this approach.

We have shown that sequinavir, which is used clinically in HIV treatment is a direct proteasome inhibitor that can cause radiosensitization and may be a useful agent for the treatment of cancer in combination therapy. The reason is that the HIV protease has evolved a similarity with the mammalian proteasome, which is presumably one of its mechanisms of pathogenicity. This work has now been published In experiments designed to test this concept, we prepared the vectors encoding anti-sense HSP90, sense HSP90, and HSP70. [13]. Clinical trials are planned in Europe using this approach. We have pointed out that this may be one of the reasons why patients receiving this drug do so well follwoing radiation therapy for primary brain lymphoma [12].

We have also shown that there is similarity between multiple drug resistence gene product mdr1 has cross specificity with the 26s proteasome and that proteasome inhibtors may help in overcoming mdr resistance to therapy [15]. This indicates the utility of using proteasome inhibitors in combination with chemotherapy. We have also shown that several anthracyclins inhibit proteasome function and this may accentuate their anti-cancer action. This work has been submitted for publication.

Key Research Accomplishments:

- Determined proteasome function in prostate, and other, cancer cell lines.
- Demonstrated inhibition of proteasome function in prostate cancer cells in response to hyperthermia.
- Shown dependency of heat-induced proteasome inhibition on the 26S, but not 20S, proteasome.

- Shown that heat-induced proteasome inhibition is very rapid and does not require protein synthesis.
- Shown temporal and temperature-dependent relationship between heating and HSP90 expression.
- Shown that HSP90 directly inhibits proteasome function.
- Shown that HSP90 is not the mechanism of heat-induced proteasome inhibition, or at least not the only mechanism.
- Shown that heat leads to loss of androgen receptor and may result in androgen independent growth of prostate cancer.
- Shown heat stabilizes IκBα expression and decreases NF-κB expression.
- Shown that heat induces apoptosis and radiosensitization of prostate cancer cells and that this correlates with proteasome inhibition and loss of ability of radiation to activate NF-κB.
- Shown that radiation result in a very rapid loss of proteasome activty down to 50% of control values and that the proteasome is extremely sensitive to even very low doses of irradiation.
- Shwon that the proteasome responds to low doses of radiation by making an adaptive response that renders it refractory to further radiation exposure.
- Shown that radiation-induced proteasome inhibition results in increased IκBα expression and decreased NF-κB expression at low doses, but that this is circumvented at high doses, providing an explanation for the dual anti- and pro-inflammatory responses seen after irradiation.
- Shown that irradiation of cells and purified proteasomes result in the same level of proteasome inhibition, suggesting that the effects of irradiation are direct.
- Shown that radiation-induced inhibition is not dependent on hsp90.
- Shown that nuclear and cytoplasmic proteasomes are both inhibited by radiation.
- Developed methodology for independently measuring 20S and 26S functional proteasome activity in gels.
- Developed methods for further purifying proteasomes on glycerol density gradients.
- Shown that the 26S proteasome is more sensitive to radiation than the 20S.

- Shown that the "immunoproteasome" that contains lmp2, lmp7, and mecl1 is more sensitive to radiation than the constitutive proteasome in human and mice.
- Shown that radiation-induced proteasome inhibition may be one mechanism of radiation-induced immune suppression and that this alters the nature of the immune response that is generated.
- Developed Ub-GFP stable transfectants that respond to proteasome inhibition by heat and other proteasome inhibitors, but not radiation.
- Demonstrated that the proteasome is redox sensitive and that free radical scavengers can prevent radiation-induced proteasome inhibition.
- Ruled out our hypothesis that heat-induced inhibition of proteasome activity leads to caspase 3 activation, degradation of DNA-PKcs, and decreased DNA repair in response to ionizing radiation.
- Shown that caspase independent mechanisms, probably involving apoptosis inhibitory factor, are involved in apoptosis following proteasome inhibition.
- Shown that the proteasome inhibitor PS341 causes radiosensitization of prostate cancer cells in vitro and in vivo.
- Shown that HIV protease inhibitors cause proteasome inhibition and may be of value as radiosensitizers for prostate cancer.
- Shown that the proteasome and mdr1 have a degree of cross specificity and that proteasome inhibitors may be useful in eliminating multiple drug resistance and that anthracyclins may act in part by causing proteasome inhibition.

Reportable Outcomes:

Attached are 15 manuscripts that have been published, are in press, or submitted that deal with the topic of the grant. We have submitted 3 further grants stemming from these studies. One RO-1 has received a high fundable score, and we are waiting to hear about the others. We anticipate several clinical trials may come indirectly from observations made during this funding period. The salient findings of the study are summarized above.

Conclusions:

The proteasome has been identified as a direct target for radiation and heat. It responds rapidly to these insults by modulating important signal transduction pathways. Such responses may modulate some of the responses prostate cancer cells make to therapeutic treatments. Through this study, we have identified the proteasome as an unexpected target for drugs, such as anthracyclins and HIV protease inhibitors, that were not thought to act primarily through this mechanism. Since proteasome inhibition on its own can cause apoptosis and cell cycle arrest and these responses are augmented by radiation, the proteasome serves as a novel target for therapies that might improve the response of prostate cancer to radiation treatment.

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List of Personnel Who Received Pay From This Research Effort:

William H. McBride	Principal Investigator
Keisuke S. Iwamoto	Co-Investigator
Milena Pervan	Graduate Student Researcher
George Venegas, Jr. (until 8/31/02)	Lab Helper
Vaughan Greer (from 8/29/02)	Lab Helper

APPENDIX MATERIAL

For DAMD17-00-1-0076

William H. McBride, P.I.

In: Proteasome Inhibitors in Cancer Therapy,
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The Proteasome in Cancer Biology and Therapy

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1. Introduction

A perfect cancer treatment in a world of perfectly treatable cancers would target only the unique features of malignant cells and leave normal cells untouched. In the real world of real cancers, cancer specific alterations of common pathways have been identified that can offer opportunities for the development of cancer specific drugs. One of the most recent successful examples is STI 571, which has been used to target the deregulated tyrosine-kinase bcr-abl in chronic myelogenous leukemia (CML)[1]. This is recognized as a key translocation in this disease, but unfortunately most cancers do not have one distinct but many mutations, leading to a complex deregulated malignant phenotype. One of the best-studied examples is the adenoma-carcinoma sequence of colorectal cancers, in which a series of mutations drives normal epithelium to benign adenoma and finally to invasive cancer, which is generally accompanied by cellular de-differentiation[2]. Furthermore, recent data provide strong evidence that this process is not exclusively determined by mutations of the genome but is also driven by the tumor microenvironment and that, in principle, the process is reversible[3]. Understanding how tumors interact with their environment could be a new avenue to the discovery of powerful tools for cancer therapy.

A key regulator of many molecular pathways in eukaryotic cells is the ubiquitin/26s proteasome system[4]. Its function is frequently deregulated in cancer[5] suggesting that the proteasome is also a promising target for cancer therapy[6]. In this chapter we emphasize the contribution of this central protease to the malignant phenotype and how modulation of its function might help cure cancer.

2 The Proteasome and the Malignant Phenotype

In an oversimplified model of cancer one can break down the malignant phenotype to three different properties: decreased control over proliferation, invasive growth, and the ability to metastasize. The proteasome is often involved, even though these properties may originate from alterations in entirely different pathways.

2.1 Proliferation

In order to proliferate, cells have to leave the quiescent G_0/G_1-phase of the cell cycle and enter S, G2, and M-phases. Well-organized production and removal of positive and negative regulatory molecules, produced in response to external growth factors and internal programs, drive progression through the cell cycle. These guarantee that cellular homeostasis in non-malignant tissue proceeds in a timely fashion from G_1- to S-phase, exactly one round of DNA-replication per cell cycle is completed before entry into G2 and M-phase, and that the number of cell divisions within a specific time period is controlled. In cancer, this process is often deregulated. The pro-inflammatory tumor environment behaves like a wound with production of high levels of growth factors that might act directly on tumor cells through paracrine action to stimulate proliferation or indirectly by enhancing angiogenesis. Alternatively, tumors can develop autocrine loops involving growth factors and their receptors, which are frequently overexpressed in tumors, with a similar result. Also, genetic alterations of genes involved in cell cycle control have been described.

Removal of many cell cycle regulators is performed by ubiquitin-dependent proteolysis by the proteasome. The protein to be removed is usually first tagged by phosphorylation. Ubiquitin is activated by an E1-ubiquitin-activating enzyme and then transferred by an E2 enzyme to an E3 ubiquitin-ligase that specifically recognizes the targeted protein and covalently binds ubiquitin to its lysine residues. The mono-

by the 19s regulatory subunits of the proteasome. Inhibition of proteasome function using specific inhibitors like lactacystein, MG-132 or PS-341 prevents removal of cell-cycle regulators and thus blocks movement of cells through cell cycle transition checkpoints. Proteasome inhibitors can therefore be thought of, in one sense, as anti-proliferative drugs. However, long-time inhibition of proteasome function in cancer patients would also affect proliferation of normal tissues and would not be well tolerated.

2.2 Invasive growth and metastasis

The invasiveness of tumor cells depends on their ability to open junctions between normal cells and to spread into the resulting spaces. This ability is thought to be, at least in part, dependent on the expression of matrix-metallo-proteases (MMP's), which digest extracellular matrix proteins. One of the key regulators of expression of certain MMP genes, is the transcription factor NF-κB, which in activated by pro-inflammatory signals and is often highly expressed in cancers. NF-κB expression is under tight control of the proteasome. Preformed NF-κB is sequestered in the cytoplasm by inhibitor molecules of the IκB family. Stress or pro-inflammatory signals cause IκB to become phosphorylated at specific serine sites. This leads to polyubiquitination and subsequent degradation by the proteasome. This process frees NF-κB, allowing its translocation to the nucleus and initiation of transcription of NF-κB-responsive genes[7]. Blocking the NF-κB pathway using proteasome inhibitors prevents TNF-dependent MMP-1, MMP-3, ICAM-1, and COX-2 expression (Sakai, T, 2001) and is a possible mechanism for their anti-metastatic effects (Ikebe, T, 1998). In addition to proteasome inhibitors inhibiting MMP production, there is a report that metallo-protease inhibitors inhibit the proteasome[8].

growth and angiogenesis *in-vivo*[9] and stabilize tight-junctions, preventing cell-cell dissociation[10]. In contrast MMP-2 expression, which is controlled through the Akt pathway, was increased by proteasome inhibition leading to increased cellular invasiveness Park, B-K 2001, showing the multiple levels of control that are being exerted on pathways by this structure and the need to take into consideration the cellular context in which they are used.

3 Proteasome inhibitors – a new class of chemotherapeutic drugs

3.1 Apoptosis and Radiosensitization *in-vitro*

Given the multiple functions of the proteasome, which generally support the malignant phenotype of cancer cells, it is not surprising that proteasome inhibitors have anti-tumor effects including induction of apoptosis. Efficacy was demonstrated against tumor cells from many different tumor entities including Hodgkin's[12] and non-Hodgkin's lymphoma[13], multiple myeloma[14, 15], leukemia[16, 17], prostate cancer[11, 17], glioblastoma[17, 18], pancreatic cancer[19, 20], gastric cancer[21], cervical cancer (Pajonk et al., unpublished results), colorectal cancer[22, 23], ovarian cancer[24] and lung cancer[25]. Numerous *in-vitro* and *in-vivo* studies have shown that such effects did not necessarily depend on classical molecular pathways like those dictated by p53 and bcl-2[11] but the exact mechanism is still not fully understood.

In-vitro, proteasome inhibitors like MG-132, lactacystein, NLVS and PS-341 induce apoptosis by a mechanism that involves activation of initiator caspases[26]. However, depending on the specificity of the inhibitors used, activation of effector caspases by proteasome inhibitors[27] can not always be assumed. For example, drugs like MG-132 also inhibit calpains that are necessary for activation of caspase-

proteasome inhibitors often reassembles a picture of caspase-independent apoptosis previously described for other stimuli,[29] while cells of hematopoetic origin show full caspase-dependent apoptosis[26].

Proteasome-inhibitor-induced apoptosis might involve apoptosis inducing factor (AIF),[30] which seems to be stabilized by proteasome inhibition and co-immunoprecipitates with ubiquitinated proteins (Pajonk et al., unpublished results). AIF is normally located in the inner membrane space of the mitochondria and released into the cytoplasm upon mitochondrial damage. By a not fully understood mechanism, AIF activates a caspase-independent form of apoptosis which results in large scale fragmented DNA (50 kBp). One could propose a mechanism in which there is a permanent slow leakage of AIF into the cytoplasm where it is rapidly degraded by the proteasome. In the presence of proteasome inhibitors it would accumulate and initiate caspase-independent apoptosis. Another possible contributory mechanism that is less specific is based on the fact that many oncogenes and tumor repressor genes are regulated by the proteasome[5]. Proteasome inhibition might partly reconstitute a normal oncogene or tumor suppressor gene expression profile and thus reassemble a benign phenotype with an increased apoptotic potential. Escape from proteasome-inhibition-induced apoptosis also seems possible. At least in EL-4 murine thymoma cells, long term treatment with proteasome inhibitors induced resistance based on expression of tripeptidyl-peptidase II (TPPII) which could compensate for loss of proteasome function[31].

While the permanent presence of nanomolar concentrations of proteasome inhibitors efficiently prevents tumor cell growth,[12] short term treatment of cancer cells with these compounds sensitize tumor cells to ionizing radiation[12], suggesting the use of proteasome inhibitors in radiation therapy. The underlying mechanism is,

depend on inhibition of constitutive active NF-κB[32]. There is now strong evidence that the proteasome is involved in nucleotide excision repair[33]. Nucleotide excision repair is not considered to have a role in repair of radiation-induced DNA double-strand breaks, but involvement of the proteasome in other more relevant repair processes, such as non-homologous end joining (NHEJ) seems possible

3.2 Apoptosis and Radiosensitization in-vivo

At first sight, inhibition of proteasome function seems to be a new therapeutic approach in cancer therapy. A closer look at established tumor therapies reveals that many cancer therapeutic modalities already interfere with proteasome function: for example many chemotherapeutic agents, such as alkaloids[34] and anthracyclins (Pajonk et al., submitted) and hyperthermia treatment impair proteasome function (Pajonk et al., submitted). Even ionizing radiation partially inhibits proteasome cleavage activities directly[35]. Additionally, we (Pajonk et al., submitted) and others[36] could recently show inhibitory effects of proteasome inhibitors on the maturation and the pumping function of the multi-drug-resistance-1 gene product P-glycoprotein, which is responsible for the removal of many chemically unrelated anti-cancer agents from the cytoplasm and thus failure of chemotherapy.

Remarably, drugs like cyclosporine A[37], N-acetyl-L-cysteine[38] and HIV-I protease inhibitors[17, 39] are potent inhibitors of the proteasome. At least for the latter, it is known that AIDS-related Kaposi-sarcomas can regress when patients are treated with HAART (highly active anti-retroviral therapy) regimens[40]. In an elegant experimental setting, Sgadari et al. were able to demonstrate that this appeared due to inhibition of angiogenesis and not to regained immunological competence of the host[41]. This explanation is somewhat counterintuitive since inhibition of proteasome

enhances VEGF transcription. However, angiogenesis is complex and does not depend only on VEGF but also on endothelial cell proliferation and invasive growth. Oikawa et al. described complete inhibition of angiogenesis by lactacystein using a chorioallantoic membrane model. On the other hand, Pati and coworkers demonstrated a direct pro-apoptotic effect of HIV-I protease inhibitors on Kaposi sarcoma cells[42] and we have recently been able to show that HIV-I protease inhibitors are also potent inducer of apoptosis in leukemia, prostate cancer and glioma cells and unrelated to HIV. Additionally, we could show that HIV-I protease inhibitors sensitized surviving cells to ionizing radiation[17]. For AIDS-related primary central nervous system lymphoma, the combination of HAART with cranial irradiation increased survival of patients 30 fold when compared to cranial irradiation alone [43] suggesting that HIV-I protease inhibitors might be useful as sensitizers in radiation therapy of non-AIDS related cancers.

PS-341 is the first specific proteasome inhibitor to enter clinical trials for multiple myeloma[44]. PS-341 has direct anti-tumor,[6] as well as radiosensitizing effects[45, 46] on cancer cells *in-vitro* and *in-vivo*. It seems that other existing drugs, like the HIV-I protease inhibitors, or new proteasome inhibitors modeled on PS-341 will become valuable adjuncts to existing cancer therapies, especially in combination with conventional chemotherapeutic drugs or radiation therapy. At the same time investigations into the role of the proteasome in cancer is likely to suggest new targets within the ubiquitin/proteasome system, such as regulatory components of the proteasome, the E3 ligases, or the ubiquitination system itself that may be beneficially manipulated.

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The Role of the Ubiquitin/Proteasome System in Cellular Responses to Radiation

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Abstract

In the last few years the ubiquitin/proteasome system has become increasingly recognized as a controller of numerous physiological processes, including signal transduction, DNA repair, chromosome maintenance, transcriptional activation, cell cycle progression, cell survival, and certain immune cell functions. This is in addition to its more established roles in removal of misfolded, damaged, and effete proteins. This review examines the role of the ubiquitin/proteasome system in processes underlying the classical effects of irradiation on cells, such as radiation-induced gene expression, DNA repair and chromosome instability, oxidative damage, cell cycle arrest, and cell death.

Furhermore, recent evidence suggests that the proteasome is a redox-sensitive target for ionizing radiation, and other oxidative stress signals. In other words, the ubiquitin/proteasome system may not simply be a passive player in radiation-induced responses, but may modulate them. The extent of the modulation will be influenced by the functional and structural diversity that is expressed by the system. Cell types vary in the ubiquitin/proteasome structures they possess and the level at which they function, and this changes as they go from the normal to the cancerous condition. Cancer-related functional changes within the ubiquitin/proteasome system may therefore present unique targets for cancer therapy, especially when targeting agents are used in combination with radio- or chemotherapy. The peptide boronic acid compound PS-341, that was designed to inhibit proteasome chymotryptic activity, is in clinical trials for treatment of solid and hematogenous tumors. It has shown some efficacy on its own and in combination with chemotherapy. Preclinical studies have shown that PS-341 will also potentiate the cytotoxic effects of radiation therapy. In addition, other drugs in common clinical use have been shown to

affect proteasome function, and their activities may be valuably reconsidered from this perspective.

Introduction

The ubiquitin/proteasome system is at the heart of cellular proteolysis, and it uses its degradative capacity to spatially and temporally control and integrate numerous physiological processes in the cell. Unlike the endosomal-lysosomal pathway, which operates in an acidic membrane-bound compartment, the proteasome is found in the cytosol, endoplasmic reticulum, and nucleus and is responsible for degrading the majority of endogenous cellular proteins (Ferrell et al., 2000; Pajonk & McBride, 2001c). It integrates its functions with other cellular processes, including the action other proteases. Some of its functions can be assumed by the large oligopeptidase, tripeptidylpeptidase II (Wang et al., 2000) but targeted disruption of proteasome subunits has shown that, at least in yeast, it remains indispensable for cell viability (Fujiwara et al., 1990; Heinemeyer et al., 1991). The system may also play a role in extracellular protein degradation, at least in some cases (Sawada et al., 2002). The proteasome is intimately linked to a flexible, well developed, and highly efficient ubiquitinylation system that marks native proteins for ATPase-dependent destruction. Additionally, it removes misfolded and damaged proteins and closely cooperates with chaperonin systems, as well as itself performing chaperonin-like protein unfolding and folding activities.

In recent years, the importance of protein degradation for normal cellular physiology has become increasingly recognized. The rate at which the ubiquitin /proteasome system removes specific proteins will have a marked effect on cell behavior. The absolute nature of proteolysis will confer a directional quality to pathways. At the same time, the system has been recognized to display considerable plasticity that allows it to rapidly and dynamically adapt to multiple cellular challenges, including those posed by irradiation.

Because the activities of the proteasome are vital for life, the molecules responsible for enzymatic activity are well conserved. However, replacement variants have co-evolved with the development of specific immune systems. These features, plus rapid changes in the number, type and location of proteasome subunits lend adaptability to cellular responsiveness (Huang et al., 2002; Rock et al., 2002). Further, up-stream regulatory components that bind to the basic proteasome structure have evolved that modulate activity. Certain of these regulatory components, once considered integral proteasomal structures, have recently been suggested to have additional independent functions (Gillette et al., 2001). Modulation of proteasome activity has been shown to affect cellular processes as diverse as transcriptional activation (Ejkova & Tansey, 2002; Kang et al., 2002; Ottosen et al., 2002), cell cycle progression (Yamaguchi & Dutta, 2000), cell survival (Delic et al., 1998; Pajonk et al., 2000), DNA repair and chromosome stability (Arnold & Grune, 2002), receptor-mediated responses to external ligands (Strous & van Kerkhof, 2002), and antigen presentation through the MHC class I mediated pathway (Rock et al., 2002). The diversity and plasticity expressed by the proteasome allows the cell flexibility in making these responses.

There is increasing recognition that diversity within the proteasome-associated ubiquitin (Ub) system also contributes greatly to the nature of the proteolytic and other functions that are displayed. Further, novel functions of the Ub system are being discovered at a rapid rate, including in endocytosis (Carthew & Xu, 2000), protein trafficking, transcriptional control, chromosome unwinding, gene silencing, and DNA repair (Pickart, 2001). Often monoubiquitinylation rather than polyubiquitinylation of substrate is involved, the latter being largely, but perhaps not solely, reserved for targeted proteolysis. Families of Ub-like molecules (Ubl) are being discovered that act antagonistically to, or independently of, Ub to control the fate

and function of proteins (Yeh et al., 2000). A plethora of deubiquitinylating enzymes (Fontana et al., 1982) (Fontana et al., 1982) have been identified that must play key roles in moderating and reversing ubiquitinylation, although their physiological functions are still largely unknown (Chung & Baek, 1999; Wilkinson, 2000).

Perhaps the most obvious, but least understood, display of plasticity and diversity in the Ub/proteasome system is the number of times components show up as features in gene microarray data (Hu et al., 2002a). Clearly, the Ub/proteasome system has the potential to take a unique position as a master controller able to simultaneously integrate multiple physiological signals within a cell. It is not surprising that ubiquitinylation /deubiquitinylation and proteolysis are now seen to rival phosphorylation /dephosphorylation in importance in cellular physiology, and these processes interface and interact to ensure proper cell function.

This review will focus on how the Ub/proteasome system might regulate cellular responses to irradiation and other oxidative stresses. A characteristic of these responses is that the initial reaction is very rapid and involves post-translational mechanisms. Recent evidence (Pajonk & McBride, 2001c) suggests that the Ub/proteasome system may play a critical role in these, and later, responses to radiation. Discussion of the functional response of the Ub/proteasome system to radiation exposure, however, first requires a brief consideration of diversity within the structural elements of the system.

Structural Aspects of the Ub/Proteasome System

Proteasomes:

At the heart of the proteasome is a 20S barrel-shaped core structure comprising of α and β rings with α1-7, β1-7, β1-7, α1-7 symmetry (Fig. 1). The β1, β5, and β2 subunits catalyze threonine-dependent chymotrypsin-, capase-, and trypsin-like activities, respectively. The

enzymatically active sites are sequestered within the central core of the barrel (Coux, 2002) and are therefore relatively inaccessible. The core 20S structure is inactive in vivo and the transformation to a functionally active proteasome requires a pore in the α ring to be opened. One way this is achieved is by binding of 19S regulatory complexes to the ends of the 20S core to form a 26S proteasome that is 2 MDa in size. The 19S particle has 17 subunits that can be further resolved into a base that has 6 ATPase and several other units and a "lid" that is homologous to the cop-signalosome complex. With very few exceptions proteins are marked for destruction through the 26S complex by ubiquitinylation.

An alternative to the 19S particle as a partner for the 20S proteasome core unit is an 11S heteroheptamer activator complex (PA28 α/β) (Harris et al., 2001; Li & Rechsteiner, 2001). Binding of this also opens the pore in the alpha ring to allow substrate access, but this structure has a predilection for partially degraded proteins and peptides rather than intact polyubiquitinylated molecules. Degradation is not ATP dependent. Subcellular location of these structures may vary and critically determine function (Brooks et al., 2000). Hybrid proteasomes that contain one 19S and one 11S structure attached to the 20S core may play special roles (Tanahashi et al., 2000).

One specialized function of the proteasome is processing of endogenous molecules for presentation to cytolytic T cells (CTL) as MHC class I-bound antigens (Rock et al., 2002). Since CTL play a critical role in immune recognition of viruses and tumors, this is an important function. Proteasomal degradation can generate peptides that, after trimming by aminopeptidases, are of around 9 amino acids in size and able to bind in the cleft between the 2 alpha helices of the MHC class I molecule. So important is this process that alternative 20S core enzymes have co-evolved linked to activation of the immune system. Treatment of cells with

IFN-γ, TNF-α, and other pro-inflammatory "danger" signals, causes co-ordinated replacement of the 3 enzymatic subunits in the core (LMP2 for β1, MECL-1 for β2, LMP7 for β5) (Gaczynska et al., 1994) (Fig. 1). Cleavage favors production of sites that anchor peptides to MHC class I molecules and alters the nature of the epitopes that are presented to CTL (Rock et al., 2002). This has led to these structures being called "immunoproteasomes." PA28α and β components are also IFN-γ inducible and also, perhaps remarkably, play a major role in determining the nature of the epitope that is presented by the immunoproteasome (Sijts et al., 2002a; Sijts et al., 2000). A link with the chaperonin system is seen in that hsp90 can substitute in part for lack of PA28 and forms a PA28-independent pathway for MHC class I antigen presentation (Sijts et al., 2002b).

Ubiquitin:

Ubiquitinylation involves a hierarchical system requiring, initially, activation of the small 76 amino acid peptide Ub by an Ub-activating enzyme (E1) in an ATP-dependent reaction (Fig. 2). Ub is covalently bound through a high-energy thiolester bond to E1 and then passed to an Ub-conjugating enzyme (Ubc)(E2), where it forms a similar thiolester linkage. The final component of the system is an Ub–protein ligase (E3) that confers most of the specificity on the cascade and mediates transfer of Ub moieties to lysine residues in the target protein. E3 enzymes recognize cognate substrates through often ill-defined sequence elements referred to as "degrons." The thiolester components of this cascade make it susceptible to modification by redox active agents (Obin et al., 1998).

There are many groups of E3 ligases. E3α and E3β target basic and bulky hydrophobic N-terminal residues and uncharged N-terminal residues, respectively (N-terminal end rule substrates). The HECT class (homology to the E6AP carboxyl terminus) directly transfers Ub to

their substrates and their conserved carboxyl terminal domain identifies members. The anaphase promoting complex (APC), or cyclosome, group targets mitotic substrates that contain 'destruction box' sequences. The class formed by SCF (Skp1, Cullin, F-box) complexes has a hexameric structure linked to a variable F box protein that mediates substrate recognition and targets substrates are diverse as the NF-κB inhibitor, IκBα, the CDK inhibitor, $p27^{Kip1}$, cyclin E, and the transcription factor, β-catenin. The class of Ring finger E3 ligases includes c-Cbl that targets CSF-1R, EGFR, and PDGFR, and mdm2 that targets p53, as well as the DNA repair protein BRCA1, and members of the inhibitors of apoptosis (IAP) family. Then there is an ECS (ELONGIN-C-CULLIN-SOCS) group, typified by the von Hippel Lindau protein, pVHL, that also includes the suppressors of cytokine signaling (SOCS), which regulate cytokine receptor kinase signaling pathways and are characterized by a C-terminal SOCS box motif (Kile et al., 2002).

Efficient protein degradation through the 26S proteasome requires that protein target molecules are polyubiquitinylated. On the other hand, monoubiquitinylation has recently been shown to redirect proteins into specific pathways, such as receptor internalization and endosomal sorting (Carthew & Xu, 2000). Monoubiquitinylation has also been implicated in chromatin and gene regulation and DNA repair (Haglund et al., 2002; Pickart, 2001; Salghetti et al., 2001).

Deubiquitinylation is perhaps as important a regulatory process as ubiquitinylation (reviewed in (Chung & Baek, 1999; Wilkinson, 2000)). Genomic sequencing has recognized more than 90 deubiquitinylating cysteine protease enzymes (Fontana et al., 1982). DUBs play roles in Ub recycling, can reverse ubiquitinylation, and edit inappropriately ubiquitinylated proteins. Other roles are still largely speculative, but DUB1 and DUB2 are induced by cytokines such as IL-3 and IL-5, and play roles in the cell cycle (Jaster et al., 1999). Recently a

proteasome subunit has been found that deubiquitinates substrates immediately prior to their degradation (Borodovsky et al., 2002; Yao & Cohen, 2002).

Another regulatory mechanism is attachment of Ub-like molecules (Ubl) such as SUMO ("small Ub-related modifier"), NEDD8, and Apg12, in place of Ub. Different conjugating enzymes are involved and the fate of the protein is affected (Yeh et al., 2000). For example, while activation of NF-κB is generally associated with ubiquitinylation and destruction of its natural inhibitor IκBα, SUMOylation of IκBα stabilizes the protein and prevents NF-κB activation (Desterro et al., 1998). Similarly, the rapid turnover of p53 protein following genotoxic stress (Maki et al., 1996) is mediated largely by the E3 ligase mdm2 (Bottger et al., 1997). Degradation is inhibited by phosphorylation of p53 or the interaction of mdm2 with $p19^{ARF}$ (Honda & Yasuda, 1999). In contrast, SUMOylation of p53 appears to be an alternative route to its activation that may function by relocating the protein to transcriptionally active subnuclear sites (Rodriguez et al., 1999). It is possible that the mutual antagonism between SUMO-1 and Ub could also result in a molecule existing in stable and unstable pools within different cellular compartments.

The main, but probably not the only, proteasomal subunit to recognize polyubiquitinylated proteins is Rpn10. Following binding, the 19S base unit performs the critical step of ATP-dependent unfolding the substrate and "feeding" it into the degradation chamber (Ferrell et al., 2000). The role of chaperonins, such as heat shock proteins, in the recognition and unfolding process has yet to be fully evaluated, but the relationship is close and has probably co-evolved. What is clear is that many proteins have developed an affinity for the proteasome and bind to various subunits, most often within the 19S regulatory structure, to interfere with the degradative process. Since yeast two-hybrid screening are often used to detect

such potential interactions, some may be artefactual, but many are real and have considerable potential for modulating proteasome function (Ferrell et al., 2000).

This brief overview serves to indicate that there is considerable structural diversity within the Ub/proteasome system. Major forces have obviously been at work to conserve enzyme function, while co-evolution has occurred with the immune system, the chaperonin system, and the E2/E3-based regulatory systems that has added diversity and flexibility in making responses to external challenge. The evolutionary battle that has been waged can be evidenced by the many strategies that viruses and cancers have developed to avoid immune surveillance or to subvert cellular metabolism that specifically target the Ub/proteasome system. Discussion of these interactions is not within the province of this review but they are dealt with elsewhere (Pajonk & McBride, 2001c). They are worth noting because they illuminate the complexity of the ongoing regulatory interactions.

Another complexity when studying the Ub/proteasome system is the marked functional variation between cell types. For example, cancers and rapidly growing embryonic cells generally have higher levels of proteasome activity than their normal well differentiated counterparts (Ichihara et al., 1993; Kanayama et al., 1991; Kumatori et al., 1990; Shimbara et al., 1992). Proteasome function decreases with age (Carrard et al., 2002; Ponnappan et al., 1999) and is severely affected by microenvironmental factors and disease processes. The close relationship between the cytokines IFN-γ (Rock et al., 2002) and TNF-α (Pallares-Trujillo et al., 2000) and proteasome structure and function that has already been mentioned extends to other cytokines and growth factors and their receptors. IL-3 increases proteasome activity (McBride et al., in press). Proteasome inhibition increases production of IL-6 (Pritts et al., 2002) and IL-8 (Hipp et al., 2002; Wu et al., 2002b) through NF-κB independent pathways (Wu et al., 2002b).

franconia's anemiaTGF-β signaling pathways are closely regulated through the Ub/proteasome (Zhang et al., 2002). Muscle wasting in response to burn injury, cachexia, or sepsis with associated myofibril proteolysis has been associated with increased proteasome activity, an effect that is mediated at least in part by TNF-α, IL-6, and possibly other cytokines (Costelli et al., 2002).

This plasticity and diversity has to be taken into account when considering how the system behaves when encountering challenges such as those provided by irradiation and oxidative stress.

The Ub/Proteasome System and Radiation Responses

The classical effects of irradiation involve activation of DNA repair, cell cycle arrest, and cell death pathways. Perhaps not surprisingly, there is increasing recognition of interconnections between molecular pathways leading to these apparently distinct functional endpoints and evidence that they are co-ordinately regulated in order that cells can make the response most appropriate to the circumstances (Fig. 3). Since the Ub/proteasome system is intimately involved in signal transduction, DNA repair, cell cycle, and cell death, and since many of the effects of irradiation involve changes in protein stability, it seems reasonable to ask what roles this system plays in modulating and co-ordinating cellular responses to radiation damage (Pajonk & McBride, 2001c; Rolfe et al., 1997). This question is made all the more pertinent by the discovery that the proteasome may be a direct, redox-sensitive target for ionizing radiation and other oxidative stresses resulting usually in a slowed rate of proteolysis (Pajonk & McBride, 2001a).

DNA repair:

The Yeast Genome Deletion Project recently examined the global relationship between the Ub/proteasome system and repair of damage caused by ionizing radiation (Bennett et al., 2001). Of the 130 plus genes (out of 3,670) that were identified as influencing radiation responses, around a quarter were involved in protein transcription, trafficking, translation, or degradation. The first report of an intimate relationship between DNA repair and ubiquitinylation was made in 1987. The protein encoded by the RAD6 gene from S. cervisiae, which is required for post-replicative DNA repair, amongst other functions, was shown to have Ubc (E2) activity (Jentsch et al., 1987). Since then, ubiquitinylation has often been implicated in DNA repair through mechanisms that, interestingly, often seem unrelated to protein degradation. For example, RAD6 is required for the E3 ligases RAD18 and RAD5 to ubiquitinate PCNA (Hoege et al., 2002). Neither monoubiquitinylation or polyubiquitinylation of PCNA appears to result in its degradation. In contrast, SUMOylation by Ubc9 of the same K164 PCNA residue that is normally ubiquitinylated, inhibits repair (Hoege et al., 2002), indicating that DNA repair may be regulated by the balance between SUMOylation and ubiquitinylation.

A negative regulatory influence of SUMOylation on DNA repair has also been suggested in human cells. Yeast two hybrid analyses showed that the human homolog of Ubc9 binds SUMO-1 and the DNA double strand break repair proteins RAD51 and RAD52 (Shen et al., 1996) to form nuclear foci which are a hallmark of DNA damage. Overexpression of SUMO-1 down-regulates DNA double-strand break-induced homologous recombination and lowers cellular resistance to ionizing radiation (Li et al., 2000). Ubc9 is present predominantly in the nucleus and at the nuclear pore complex and its relocation to the cytoplasm prevented the

formation of RAD51 foci, suggesting that SUMOylation may play a role in RAD51 trafficking (Saitoh et al., 2002).

BRCA1, a tumor specific suppressor gene that is mutated in familial forms of breast and ovarian cancer, also tangibly links Ub/proteasome system to DNA repair by homologous recombination. It forms a heteromeric complex with BARD1. Both molecules have Ring-finger domains and express E3 ligase activity. Loss of BRCA1 is associated with sensitivity to ionizing radiation and DNA cross-linking agents, as well as with spontaneous chromosome breakage (Deng & Scott, 2000). Cancer-predisposing mutations within the BRCA1 RING domain abolish its E3 ligase activity. Unlike wild type genes, such mutants are unable to reverse radiation hypersensitivity of BRCA1-null human breast cancer cells or to restore radiation-induced G2/M arrest (Ruffner et al., 2001). The radioprotective effects of BRCA1 are therefore mediated by its E3 ligase activity. The targets for BRCA1 are not fully known, but it interacts with RAD51 and has a role in homologous recombination (Scully, 2001). It also co-localizes with the Fanconi Anemia gene product FANCD2 at sites of DNA damage and monoubiquitinates it (Taniguchi et al., 2002). The suggestion is that FANCD2/ BRCA1/ RAD51 complexes participate in S-phase-specific cellular processes, including DNA repair by homologous recombination.

A role for histone ubiquitinylation in DNA repair has been suggested in numerous reports. For example, BRCA1 co-localizes in nuclear damage foci with the histone variant H2AX, which can serve as its ubiquitinylation substrate, at least in vitro (Mallery et al., 2002). Since H2AX mice are sensitive to DNA damage and express chromosome instability (Celeste et al., 2002), the suggestion is that histone ubiquitinylation by BRCA1 has a role in chromatin unwinding perhaps by increasing access of repair enzymes. Disruption of histone ubiquitinylation is known to lead to defects in DNA repair (Robzyk et al., 2000). The roles that

have been proposed for histone ubiquitinylation are however multiple and complex, extending from chromatin uncoiling and gene transcription to gene silencing (Sun & Allis, 2002), and remain controversial (Jason et al., 2002).

DNA base excision repair in response to DNA damage also comes under close scrutiny from the Ub/proteasome system. The proteasome-associated rpn4 that controls expression of genes encoding many proteasome components also modulates expression of a group of DNA base excision repair genes, hinting at a degree of co-ordinate regulation (Jelinsky et al., 2000). In addition, poly-(ADP-ribose) polymerase-1 (PARP-1) has been implicated in base excision repair. Its activation is an early event following cell irradiation and automodified PARP-1 has been found to enhance the ability of proteasomes to remove and degrade oxidatively damaged histones (Arnold & Grune, 2002). This suggests a link between nucleoprotein turnover and DNA damage and repair that could be important for restoring chromatin structure damaged by irradiation. Physical association of proteasomes with sites of DNA damage has been reported, as has a novel 200kDa nuclear protein that activates 20S proteasome degradative activity and is expressed in a radiation-induced punctate fashion that is similar to the distribution of DNA repair proteins (Ustrell et al., 2002).

The nucleotide excision repair (NER) pathway is less involved in repair of damage caused by ionizing radiation, but is important for removal of various forms of bulky base damage from DNA, such as occurs following UV exposure. It contains several proteins that interact with the 26S proteasome, including the Rad4/Rad23 DNA binding complex. The amino-terminal region of RAD23 protein contains a Ub-like domain (Ubl) that is required for physical interaction between RAD23 protein and the 26S proteasome in yeast (Schauber et al., 1998). The Ubl site is required for optimal levels of NER in vivo and links NER to the Ub/proteasome

system. RAD4 is ubiquitinylated by RAD23 and its degradation negatively regulates repair (Lommel et al., 2002). The 19S regulatory complex of the proteasome has also been reported to affect NER, independent of RAD23, of proteolysis, and of binding to the 20S component (Gillette et al., 2001), indicating that this complex may have unexpected roles in addition to regulating proteolysis.

Cell Cycle:

Cells normally progress in a timely fashion through the G1 cell cycle phase, DNA replication, sister chromatid separation, and exit from mitosis. Transitions through these phases are controlled by checkpoints that monitor intracellular preparedness for each move. Irradiation can cause cell cycle checkpoint arrest in G1/S, S, and G2/M phases. Cells in G2/M phase are particularly vulnerable to radiation exposure, while cells in S phase are markedly more resistant. Cells arrested at the G1/S checkpoint display greater sensitivity than in the rest of G1 (Dewey et al., 1972; Dewey et al., 1971). These differences in radiosensitivity are large. It requires perhaps 3 times the dose to achieve the same level of cell kill in the most sensitive compared with the most resistant cell cycle phase. Cell cycle synchronization of tumor cells could therefore significantly increase the clinical efficacy of radiotherapy. Drugs that have been used to achieve this in preclinical animal models, such as hydroxyurea (Gillette et al., 1970), have unfortunate adverse toxicity when used clinically, but synchronization may be better achieved by targeting specific elements within the Ub/proteasome system.

Proteolysis is essential for cell cycle progression and is thought to impose order on the complex series of events that are involved. Proteasome inhibitors therefore have similar effects to irradiation in causing cell cycle checkpoint arrest in G1/S, S, and G2/M phases (Hashemolhosseini et al., 1998; Kumeda et al., 1999; Machiels et al., 1997; Wojcik et al., 1996).

Interestingly, cell cycle arrest in response to TGF-β has been reported to require proteasome function and inhibition promotes G1 to S transition under such conditions (Zhang et al., 2002).

Cell cycle progression is regulated by periodic activation of a family of protein kinases known as cyclin-dependent kinases (CDKs). CDK activity is regulated positively through the interaction of CDKs with their cyclin counterparts resulting in phjosphorylation of target proteins, and negatively through binding of CDK inhibitors (CKI) of the Cip/Kip and INK4 families (Yew, 2001) and by inhibitory phosphorylations such as on Thr14 and Tyr 15 of Cdk1. Dephosphorylation of CDKs by phosphatases of the Cdc25 family, of which there are 3 homologs A, B, and C (Nilsson & Hoffmann, 2000) results in their activation. Cdc25B and C are regarded as mitotic regulators.

The most obvious manifestation of involvement of the Ub/proteasome pathway in the cell cycle is the periodic oscillation in expression levels of cyclins. The levels of cyclins that are expressed in a cell are rate limiting and their removal by the Ub/proteasome system upon completion of their mission is critical for cell cycle progression. For example, phosphorylation of cyclin E allows its recognition by hCdc4, a member of the F box family of proteins (Spruck & Strohmaier, 2002; Strohmaier et al., 2001). This bridges to an SCF E3 ligase that mediates its degradation. If the level of cyclin E is insufficient, cell cycle arrest at G1/S occurs, while too much cyclin E results in premature entry into S phase, mutations, and genomic instability (Spruck et al., 1999). Irradiation of hematopoietic cancer cells was found to increase cyclin E levels in a time and dose dependent manner (Mazumder et al., 2000; Mazumder et al., 2002) and this could influence cell cycle arrest. Importantly, mutations in the F box protein hCdc4 have been implicated in the pathogenesis of various forms of cancer, and may be responsible for some cases of elevated cyclin E levels that are frequently associated with human cancers (Koepp et al.,

2001; Spruck et al., 2002). Similarly, degradation of cyclin B in anaphase is needed to allow cells to exit from mitosis and genetically engineered stable cell cycle proteins will block this progression (Pines & Rieder, 2001; Wheatley et al., 1997). Cyclin A is degraded before cyclin B in prometaphase and exceessively high levels of cyclin A can delay mitotic alignment and anaphase (den Elzen & Pines, 2001).

A major feature involved in degradation of mitotic cyclins is a large multiprotein E3 ligase complex known as the anaphase-promoting complex/cyclosome (APC) (Sudakin et al., 2001), which is active during M and G1 phases. APC has numerous targets including cyclin B, cyclin A, mitotic kinases, inhibitors of anaphase, spindle-associated proteins, and inhibitors of DNA replication (Cohen-Fix & Koshland, 1997). Their destruction is normally a prerequisite for cell cycle progression to proceed. The mechanism whereby this class of E3 ligases recognize substrates is not clear, but recently proteins containing WD40 repeats of the Cdc20 family and Cdh1 that were found to associate with APC at the metaphase to anaphase transition and in G1 respectively (Visintin et al., 1997) have gained attention for their ability to recruit substrates for destruction (Peters, 2002; Vodermaier, 2001). The Cdh1–APC complex is inactivated from S phase until the mid-mitotic phase by phosphorylation by cyclin A–cdk2 allowing timely accumulation of other APC targets before mitosis (Lukas et al., 1999).

Levels of cyclins are frequently dysregulated in cancer and defective proteasomal degradation has been proposed as one mechanism for cyclin D1 upregulation in breast cancer (Naujokat & Hoffmann, 2002). Overexpression of cyclin D1 has been reported to radiosensitize cancer cells, a finding that was ascribed to effects on the G2-M transition (Coco Martin et al., 1999). Caspase cleavage of both cyclin D1-Cdk4 and cyclin A-Cdk2 have been suggested to promote apoptosis in embryos following ionizing radiation exposure (Finkielstein et al., 2002),

indicating that cell cycle arrest may be linked to apoptosis. Links between cyclin D and transcription have also been suggested (Coqueret, 2002).

In addition to cyclins, many other positive regulators of cell cycle, such as cdc25A, cdc6, and E2F1 (Bastians et al., 1999; Diehl et al., 1997; Koepp et al., 2001; Naujokat & Hoffmann, 2002; Yew, 2001) are degraded by the Ub/proteasome system. The same is true for many CDKI negative regulators, such as $p21^{Cip1/WAF1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Blagosklonny et al., 1996; Sheaff et al., 2000) and $p19^{INK4d}$ (Thullberg et al., 2000). Clearly, radiation-induced alterations in protein stability will alter the rate of degradation of critical substrates for cell cycle progression - as might cancer-related alterations in cell cycle-related proteins.

The first indication of how cell cycle arrest is achieved after ionizing radiation was reported almost a decade ago (el-Deiry et al., 1993) with the discovery that irradiation stabilized p53 expression allowing it to transcriptionally activate CDKI $p21^{Cip1/WAF1}$ which interferes with cyclinE-cdk2 mediated events required for the S phase transition (reviewed in (Iliakis, 1997; Shackelford et al., 1999). ATM (mutated in ataxia telengiectasia) and ATR (AT mutated and Rad3 related), are essential transducers of the radiation-induced p53-mediated response; and of most DNA damage checkpoint responses. Their relative contribution varies with the nature of the DNA lesion (Gatei et al., 2001). ATM is involved more in responses to ionizing radiation. ATR seems more focused on responses to replication blocks and UV damage (Cliby et al., 2002; Cliby et al., 1998). AT cells display cell cycle checkpoint defects as well as hypersensitivity to ionizing radiation. Under normal circumstances, p53 turns over rapidly in a cell with a half life of about 30 minutes as a result of ubiquitinylation mediated by Ubc5 and the E3 ligase mdm2 (Bottger et al., 1997; Maki et al., 1996). Following DNA damage, degradation of p53 is inhibited by several proposed mechanisms including phosphorylation by ATM (Shieh et al.,

1997), or acetylation (Ito et al., 2002), both of which inhibit the ability of mdm2 to negatively regulate expression, or by increased the interaction of hmdm2 with $p14^{ARF}$ (Bothner et al., 2001). The half-life of p53 increases to around 3.5 hours and ubiquitinylated forms increase (Maki & Howley, 1997). The CDKIs $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ are also subject to rapid proteasomal degradation following phosphorylation. This enhances activation of cdk2 and promotes cell cycle progression. An interesting inhibitory effect of NSAIDs on cell cycle progression was recently shown to be caused by inhibition of proteasome subunit production and function resulting in up-regulation of $p27^{KIP}$ (Huang et al., 2002).

Proteasome inhibitors, not surprisingly, stabilize p53 and p21 expression (Hideshima & Anderson, 2002) resulting in G1/S arrest (Machiels et al., 1997; Yew, 2001). Radiation-induced impairment of proteasome function may therefore be involved in the rapid radiation-induced increase in p21 levels that precedes maximum p53 expression (Daino et al., 2002) and as well as p53 protein stabilization (Maki & Howley, 1997). A rapid p53-independent pathway to G1 arrest in response to DNA damage was recently suggested to result from cyclin D1 proteolysis (Agami & Bernards, 2000). Tumor necrosis factor alpha (TNF-α) may cause G1 arrest through a similar mechanism (Hu et al., 2002b). These rapid checkpoint arrests are tightly linked to proteolysis and may allow the cell time to mount transcriptional p53-mediated responses.

Recognized targets of ATM activated by ionizing radiation, other than p53, include Nbs1, which is involved in DNA repair, and Chk2 (checkpoint kinase 2). An important target for Chk 2 is the Cdc25 phosphatase. After ionizing radiation exposure, ATM-dependent, Chk2-mediated phosphorylation of Cdc25A on Ser123 (Falck et al., 2002), results in its rapid removal by the Ub/proteasome system (Mailand et al., 2000). Since dephosphorylation of Cdk2 is required for its activity, removal of Cdc25A phosphatase can block G1 and intra-S phase progression.

Defective function of any member of the Chk2-Cdc25A-Cdk2 cascade results in radioresistant DNA synthesis (RDS), which has long been considered a hallmark of AT cells. However, cells from mice lacking Chk2 did not display the RDS phenotype and it therefore was suggested that other factors like Chk1 (checkpoint kinase 1) may compensate for lack of Chk2 (Takai et al., 2002). Recently, it was found that Chk1 also regulates both normal S phase progression and the intra-S phase checkpoint in response to ionizing radiation via its phosphorylation of Cdc25A (Zhao et al., 2002).

RDS can be caused also by defects in Nbs1 or the Nbs-associated repair protein MRE11, which is another target for ATM (Falck et al., 2002). The pathway that is involved in cell cycle arrest may be determined in part by the nature of the DNA damage and may evolve with time after initiation of damage. Following UV radiation damage, a G1 arrest pathway that is downstream of ATR and independent of p53-p21 has been described whose salient feature is proteasome-dependent removal of the Cdc25A phosphatase that is activated by Chk1 (Mailand et al., 2000), although ATR may also be activated slowly after ionizing radiation (Zhou et al., 2002). Again, because the Chk pathways are post-translational, they would be expected to be rapid.

The master controller for the G2 to M transition is cyclinB-Cdk1 (mitosis promoting factor). Potentially active cyclin B-Cdk1 accumulates during S and G2 phases. It is phosphorylated at Thr161, but is maintained inactive by phosphorylation at Thr14 and Tyr15 by wee1 and Myt1 until the end of G2 (Norbury et al., 1991; Russell & Nurse, 1987). Dephosphorylation by Cdc25C activates Cdk1 (Blasina et al., 1997). Ionizing radiation phosphorylates Cdc25C through Chk1 and Chk2, which causes it to bind 14-3-3sigma and to be sequestered in the cytoplasm, which prevents it from performing its function and results in G2

arrest (Chaturvedi et al., 1999; Poon et al., 1997; Russell & Nurse, 1986). Cdc25A or Cdc25B may be able to compensate for Cdc25C since the checkpoint appears normal in cells from mice lacking Cdc25C (Chen et al., 2001). Chk1 phosphorylation of Cdc25A is also important for the radiation-induced G2 checkpoint (Zhao et al., 2002) and Chk1 and Chk2 appear to play complementary roles in the G2 checkpoint. Chk1 is required for the initiation of G2 arrest following DNA damage (Liu et al., 2000), while Chk2 is required for its maintenance (Hirao et al., 2000). The G2 checkpoint is also dependent on the E3 ligase BRCA1 (Xu et al., 2001), which has been linked to activation of Chk1 (Yarden et al., 2002).

Cell Death:

Cells lethally injured by radiation typically execute one or more divisions before undergoing 'mitotic death.' The number depends upon the size of the radiation dose, but after a clinically relevant dose of 2 Gy, 2 to 3 attempts may be made. In contrast to 'mitotic death', certain cells in certain locations, including some lymphocytes, spermatogonia, oligodendrocytes, and cells in the salivary gland, thyroid, intestinal crypt, and hair follicles, undergo rapid 'interphase death' within hours of irradiation. Interphase death is now acknowledged to represent rapid apoptosis. Multiple pathways can trigger different forms of cell death and proteolysis plays a major role in all death pathways. This review will be limited to discussing the role of the Ub/proteasome system in apoptosis induced by irradiation or similar stresses. An excellent more general review of the role of the Ub/proteasome system in apoptosis is available elsewhere (Jesenberger & Jentsch, 2002).

In certain normal cell types, the proteasome plays a pro-apoptotic role early in the endogenous pathway to apoptosis induced by irradiation. Primary mouse thymocytes are partially rescued from apoptosis when treated with proteasome inhibitors 1 hour, but not 3 hours,

after γ-radiation (Grimm et al., 1996). Involvement is upstream of central apoptotic events, such as disruption of mitochondrial transmembrane potential, release of cytochrome *c*, and activation of caspases (Dallaporta et al., 2000). In contrast, proteasome inhibition had no inhibitory effect on apoptosis induced via the CD95 pathway (Dallaporta et al., 2000). Similar protective effects of proteasome inhibitors were found in differentiated neuronal cells (Sadoul et al., 1996) and rat cerebellar neurons (Bobba et al., 2002; Canu et al., 2000) undergoing apoptosis in response to deprivation of nerve growth factor and potassium, respectively, but not in cells undergoing necrosis (Bobba et al., 2002).

One possible explanation for why proteasome inhibition delays radiation-induced apoptosis in thymocytes is that XIAP and c-IAP1, which are E3 ligase members of a family of inhibitors of apoptosis (IAP) autoubiquitinylate and are degraded following irradiation (Yang et al., 2000). IAPs have multiple roles in apoptosis (Jesenberger & Jentsch, 2002). For example, XIAP blocks activation of caspase 3, 7, and 9 and ubiquitinylates caspase 3 targeting it for proteasomal degradation (Suzuki et al., 2001a; Suzuki et al., 2001b). By preventing removal of IAPs, proteasome inhibitors could slow the apoptotic process. Proteasome inhibition might also block caspase cleavage of the deubiquitinylating enzyme HAUSP, which has been shown to be involved in thymocytes apoptosis in response to various signals (Vugmeyster et al., 2002).

In contrast to the pro-apoptotic role proteasomes play in these normal tissue systems, in tumors and transformed cells proteasomes function to prevent apoptosis. Therefore, treatment of tumor cells with proteasome inhibitors almost invariably activates rapid apoptosis within hours. This process is often accentuated by irradiation, and proteasome inhibitors such as MG132 and PS-341 act as radiosensitizers in vitro and in vivo (Pajonk et al., 2000; Pervan et al., 2001a; Pervan et al., 2001b; Russo et al., 2001; Teicher et al., 1999). The difference between normal

and cancer cells in response to proteasome inhibitors suggests that there may be a therapeutic differential to be derived from their use that can be exploited to clinical advantage in cancer treatment.

One theory as to why this apparent difference exists between normal and cancerous cells in their response to proteasome inhibitors is that apoptosis is the natural default pathway for rapidly cycling cells that are unable to remove used components of the cell cycling apparatus. Levels and cleavage products of various cyclins have been implicated in apoptosis induction (Finkielstein et al., 2002; Mazumder et al., 2002). Also, accumulation of CDKI $p27^{KIP1}$ and cyclin E in mice deficient in its E3 ligase Skp2 results in increased spontaneous apoptosis (Nakayama et al., 2000) and the level of Skp2 in gastric carcinomas modulates the phenotype of the cancer, presumably by affecting p27 expression (Masuda et al., 2002).

On the other hand, it is tempting to link the difference in response of normal and cancerous cells to proteasome inhibition to the fact that cancers and rapidly growing embryonic cells generally have higher levels of proteasome components and activity than their normal counterparts (Ichihara et al., 1993; Kanayama et al., 1991; Kumatori et al., 1990; Pajonk et al., 2000; Shimbara et al., 1992). Interestingly, when human myelogenous leukemic cells are induced to terminally differentiate, proteasome inhibitors no longer induced apoptosis (Drexler, 1997). The reason for the enhanced proteasome activity in cancer cells is unknown, but it could be due to increased levels of cytokines and growth factors, reactive oxygen species, or heat shock factors, and consequent increased proliferation rate, metabolic stress, and dependency on cell survival pathways that are associated with the cancer state. Cells might increase their degradative abilities to cope with crises caused by mutational events and chromosomal instability. Proteasome inhibition would therefore be more likely to precipitate cell death. This

theory suggests involvement of a critical survival pathway for each cancer, but this will vary from cancer to cancer. Levels of expression of many important regulators of apoptosis have been shown to be tightly linked to the function of the Ub/proteasome system. Proteasome inhibition will affect the degradation rate of these critical molecules that may have opposing effects or be differentially expressed by different cell types. It is therefore not surprising that the pathway that appears most involved in apoptosis following proteasome inhibition will vary with the cell type, and other influences.

One apoptotic pathway that might be affected by proteasome inhibition is that involving p53. As has already been mentioned, expression of p53 is regulated in large part by the E3 ligase mdm2 that modulates its degradation rate and its nuclear location (Haupt et al., 1997a; Kubbutat et al., 1997). Binding of mdmx, $p19^{ARF}$, or other inhibitors to mdm2 further moderates degradation (Fuchs et al., 1998). Following stress-induced activation, p53 down-regulates various anti-apoptotic proteins, such as Bcl-2 (Deveraux et al., 2001; Miyashita et al., 1994) and induces expression of various pro-apoptotic proteins, including Bax, Apaf-1, Fas, etc., whose degree of involvement in the apoptotic process appears to vary with the tissue type (Deveraux et al., 2001). Non-transcriptional mechanisms may also mediate p53-induced apoptosis (Haupt et al., 1997b). Cell death following proteasome inhibition has however been demonstrated in some circumstances that is independent of p53 (Herrmann et al., 1998; Pajonk et al., 2000).

The relative amounts or equilibrium between members of the Bcl-2 family can either promote cell survival (Bcl-2, Bcl-XL, A1, Mcl-1, and Bcl-W) or cell death (Bax, Bak, Bcl-XS, and Bok) and this is another possible mechanism for regulating proteasome-mediated apoptosis. Bcl-2 is specifically degraded by the 26S proteasome and although there are ambivalent reports about the role of Bcl-2 phosphorylation in apoptosis, there is a clear link with proteasome

degradation (Breitschopf et al., 2000; Dimmeler et al., 1999). The pro-apoptotic factor Bax has also been shown to be under proteasome control (Li & Dou, 2000). There are reports both for (Grimm et al., 1996; Soldatenkov & Dritschilo, 1997) and against (Herrmann et al., 1998) involvement of a Bcl-2 pathway in apoptosis induced by proteasome inhibitors. In lymphoma cells, proteasome inhibition by lactacystin differentially elevated a pro-apoptotic member of this family, Bik, which accumulated in a ubiquitinylated form in the mitochondria (Marshansky et al., 2001). Involvement of the Bcl-2 family in radiation-induced apoptosis of Ewing's sarcoma has also been suggested (Soldatenkov & Dritschilo, 1997).

Another survival pathway that is frequently up-regulated in cancer is directed by NFκ-B, a transcription factor that is sequestered in the cytoplasm by IκB inhibitors until activated to translocate into the nucleus (Bussell, 2001; Pahl, 1999; Wang et al., 1997). Activation of NFκ-B most often involves phosphorylation, ubiquitinylation, and subsequent proteasomal degradation of IκB (reviewed in (Karin et al., 2002; Karin & Lin, 2002)) although alternative pathways exist (Imbert et al., 1996; Raju et al., 1998). Since NF-κB is induced by ionizing radiation, it may activate a survival pathway that offers a potential target for tumor radiosensitization (Jung & Dritschilo, 2001), although this appears not always the case (Pajonk et al., 1999). NFκ-B may provide survival signals in cells by transactivating various anti-apoptotic genes, most notably IAPs (Lee & Collins, 2001; Ueda et al., 2001). NFκ-B complexes have recently also been linked to downregulation of the c-Jun amino-terminal kinase (JNK) cascade in mouse embryo fibroblasts responding to TNF-α, which involves transcriptional up-regulation of the growth arrest gene *GADD45* (De Smaele et al., 2001). Additionally, radiation-induced NF-κB expression up-regulates Bcl-2 in PC3 cells and this could be down-modulated by a pro-apoptotic protein PAR-4, which was found to confer radiosensitivity (Chendil et al., 2002).

Microarray analysis and mechanistic studies on multiple myeloma cells treated with the proteasome inhibitor PS-341 showed decreased levels of several anti-apoptotic proteins and activation of a dual apoptotic pathway of mitochondrial cytochrome c release and caspase 9 activation, as well as a Jun kinase (JNK) and a Fas/caspase 8 dependent pathway (Mitsiades et al., 2002a). Heat shock proteins, p53, and mdm2 levels increase and caspase 3 and 8 may be activated (Hideshima & Anderson, 2002). Caspase inhibitors were able to prevent DNA fragmentation but not apoptosis caused by lactacystin inhibition of proteasome function in MO7e human myeloid progenitor cells (Wu et al., 1999), suggesting that caspase activation was a secondary effect of apoptosis rather than being directly involved. MG-132, which inhibits calpain as well as proteasome activity, did not cause caspase 3 activation in PC-3 prostate cancer cells, but the cells still died by apoptosis (Pajonk and McBride, submitted for publication). Since MG132 treatment radiosensitized cells, we examined DNA-PKcs levels and DNA-PK activity following MG-132 treatment and irradiation of PC3 cells, but were unable to ascribe radiosensitization to alterations in this DNA repair pathway (Pajonk and McBride, unpublished).

Overall, it seems likely that there is no single survival pathway that is targeted by proteasome inhibitors that results in apoptosis and is responsible for radiosensitization of cancer cells. Rather there are likely to be a number. This broad specificity of killing of cancer cells could confer advantages to the clinical use of proteasome inhibitors, especially in combination with cytotoxic agents such as radiation.

Radiation-Induced Modification of Proteasome Activity

Recently, Pajonk and McBride showed that ionizing radiation had a rapid inhibitory effect on proteasome function in a variety of cell types, as assessed by degradation of specific fluorogenic substrates (McBride et al., 2002; Pajonk & McBride, 2001b). The inhibitory effect

on proteasome activity that is achieved with exposure to ionizing radiation is not as complete as it is with drugs that target proteasomal enzymatic activity, but in many cell lines a 40 - 50% impairment in chymotrypsin-like activity was found within 15 minutes of exposure to doses as low as 5cGy, and over a wide dose range up to 20Gy. Because of evidence discussed earlier on the role of the proteasome in DNA repair, cell cycle arrest, and cell death, these findings have obvious potential implications with respect to radiation-induced cellular responses.

Most of the radiation-induced impairment of proteasome function was associated with 26S activity, with minor effects on the 20S core, suggesting that the 19S regulatory subunit was the main target for radiation. Bulteau has shown similar rapid impairment of 26S proteasome function after exposure of human keratinocytes to UV-A and UV-B radiation (Bulteau et al., 2002). The inhibition following UV radiation became progressively greater with time, unlike that following ionizing radiation, which recovered to a large extent over a 24-hour period of culture (Pajonk, unpublished).

One possible explanation for radiation-induced impairment of proteasome function is an increase in expression levels of endogenous inhibitors of proteasome activity. Hsp90 (Conconi & Friguet, 1997)) and PI31 (Zaiss et al., 2002) have been shown to inhibit proteasome function, as have other undefined factors found in low molecular weight cytosolic extracts (Pajonk, unpublished). We have failed to detect any change in the level of endogenous inhibitors following irradiation, as measured in proteasome function assays with fluorogenic substrates. Also, while treatment of cells with geldanamycin, the hsp90 antagonist, increased proteasome function, the inhibitory effects of ionizing irradiation were still observed in the presence of the drug (Pajonk & McBride, 2001b), suggesting that hsp-90 was not responsible. On the other hand, Bulteau provided evidence that extracts of UV irradiated keratinocytes, including 4-

hydroxy-2-nonenal modified proteins, inhibited degradation by the proteasome (Bulteau et al., 2002), suggesting that the mechanism may vary with the type of radiation, and perhaps also the dose and therefore the extent of protein damage.

An alternative hypothesis for radiation-induced proteasome impairment is that free radical damage to proteasome-associated molecules blocks substrate processing. Evidence for a direct effect was obtained almost immediately after irradiating isolated purified proteasomes. This impaired their functional activity to an extent similar to that achieved by irradiation of whole cells (Pajonk & McBride, 2001b). The 26S proteasome, or molecules tightly associated with the 26S proteasome, therefore appear to serve as direct targets for ionizing radiation.

The extent to which irradiation impairs proteasomal proteolysis may depend upon the composition of the proteasomes. We have recently found that proteasomes from T2 cells, which lack Lmp2 and Lmp7 (and TAP1 and TAP 2), appear to be more resistant to the inhibitory effects of irradiation than T1 parental cells (Pervan, unpublished), suggesting that immunoproteasome structures may be more sensitive, although this conclusion requires to be confirmed in a more direct manner. If proteasome composition is important in radiation-induced impairment of proteasome function, this could help explain differentials between different cell types with respect to radiation-induced gene expression patterns, cell cycle arrest, and apoptosis. It might also have implications for radiation-induced immune suppression, since immune cells, which are involved in MHC class I-mediated antigen processing and presentation, will be affected more than non-immune cells, which would contain less immunoproteasomes. The concept that molecular substitutions in proteasome structure could redirect and fine-tune cellular responses is indirectly supported by the finding that hsp90 affects constitutive and not

immunoproteasomes (Lu et al., 2001). Mechanisms that protect specific proteasome structures against oxidative damage may exist.

The impairment of proteasome function following exposure to ionizing radiation appears to involve free radical generation. In fact, the proteasome may be a prime sensor of redox changes in the cell. We have recently shown that N-acetyl-L-cysteine (Pajonk et al., 2002b), tempol, and glutathione (Pervan, unpublished) treatment inhibit proteasome function. Furthermore, concentrations of tempol that were minimally inhibitory could prevent radiation-induced inhibition (Pervan, unpublished), indicating that the effect of ionizing radiation on 26S proteasome function is mediated by free radicals. The Ub system may also sense redox changes, since intracellular reduced glutathione is required for E1 and E2 enzymes to form the Ub thiol-esters required for ubiquitinylation (Jahngen-Hodge et al., 1997; Obin et al., 1998).

Response of the Proteasome to Other Oxidative Stresses

Since ionizing radiation appears to affect proteasome function through the generation of free radicals, it is worth briefly examining how the Ub/proteasome system responds to oxidative stress. Cells use reactive oxygen and nitroxide species for multiple important physiological processes, but this has required evolution of means to moderate their toxicity. Antioxidant defense mechanisms include production of enzymes that neutralize free radicals and specific pathways to rapidly remove damaged molecules. The latter involves proteolysis, mainly through non-ATP dependent proteasomes. Recognition of damaged proteins by proteasomes may be through exposed hydrophobic moieties. Oxidatively modified proteins that are toxic to cells increase with age and in certain pathologic conditions and this been ascribed to decreased proteasome function (Carrard et al., 2002; Grune, 2000).

An expected effect of 26S proteasome inhibition is an increase in the level of polyubiquitinylated molecules, whereas inhibition of non-ATPase dependent proteasomes might lead to accumulation of damaged proteins. One manifestation of protein accumulation is formation of aggresomes, the main components of which are misfolded proteins, Ub, proteasomes, and heat shock proteins (especially hsp70, hsp90). Cells appear to attempt to protect themselves from toxic intracellular protein overload by activating stress kinases (Marcu et al., 2002; Meriin et al., 1998) and increasing expression of cytosolic heat shock proteins, which is associated with acquisition of thermal tolerance (Bush et al., 1997). A number of disease states have as their hallmark accumulation of ubiquitinylated proteins, especially neurodegenerative diseases, such as Parkinson's (Ii et al., 1997), Alzheimer's (Keller et al., 2000), Huntington's (Peters et al., 2002) and Angelman's syndrome (Ii et al., 1997; Ishii et al., 1997).

The response of the Ub/proteasome system following exposure of cells to various oxidative stressors, including hydrogen peroxide, has been examined in some detail (Grune, 2000; Grune et al., 1995). ATP-dependent degradation of fluorogenic substrates through the 26S proteasome is much more sensitive to hydrogen peroxide treatment than the ATP- and Ub-independent 20S degradation pathway (Reinheckel et al., 1998; Reinheckel et al., 2000; Shringarpure & Davies, 2002; Shringarpure et al., 2002). Indeed, modest levels of oxidative stress increase the degradation rate of modified proteins (Grune et al., 1995), as oxidized proteins are preferentially removed by the more resistant 20S proteasome pathway (Shringarpure et al., 2002). Current evidence therefore suggests a division of labor between the 20S and 26S proteasome in response to oxidative stress that allows the 26S proteasome to slow down degradation of ubiquitinylated proteins and activate pathways leading to appropriate cellular

responses without compromising the need to remove potentially cytotoxic non-functional proteins, which is performed independently.

In addition to the proteasome minimizing oxidative damage through proteolysis, proteasome inhibition is associated with an increase in reactive oxygen and nitrogen species that may mediate subsequent biological effects (Lee et al., 2001; Wu et al., 2002a). Both proteasome inhibition and oxidative stress induce production of heat shock proteins (Ding & Keller, 2001). Hyperthermia treatment of cultured myotubes increases degradation of short and long-lived proteins through the proteasome, with a maximal effect at 41°C (Luo et al., 2000). On the other hand, in other studies heat shock impaired proteasome activity (Bush et al., 1997; Mathew et al., 1998). Kuckelkorn and colleagues have shown that a one hour exposure of cells to 44° C "locks" 20S proteasomes in their inactive state and does not allow *de novo* proteasome maturation or further activation of the 26S proteasome by ATP (Kuckelkorn et al., 2000). They also showed rapid intracellular redistribution of proteasomes after heat shock. Our data show that heat exposure preferentially inhibits 26S proteasome function in prostate cancer lines and that heat-induced impairment of proteasome function could be prevented by induction of immunoproteasomes using interferon-γ (Pajonk et al, in press). Variation in the responses of different cells to heat shock are well known and some of this variation may be due to varying levels of different types of heat shock proteins and proteasome structures. The inhibition of proteasome function experienced by cells following heat exposure could also be responsible for the ability of hyperthermia to inhibit DNA repair processes when administered shortly before ionizing radiation (Locke et al., 2002).

Cellular Consequences of Modulation of Proteasome Activity by Radiation

The evidence discussed earlier that ionizing radiation affects proteasome function has obvious potential implications with respect to radiation-induced DNA repair, cell cycle arrest, and cell death, although currently these are somewhat speculative. Decreased rates of proteasome degradation were observed less than 15 minutes after exposure to radiation in a number of different cancer cell lines (Pajonk & McBride, 2001b). This may be the first adaptive cellular response to damage and it may result in radiation-induced expression of immediate early genes, such as jun, fos, and TNF-α, which occurs within minutes of exposure (Hong et al., 1996; Hong et al., 1997). It should be noted that proteasome inhibition could result in increased expression of these genes at the mRNA, as well as at the protein, level. The reason is that many cytokine, growth factor, and proto-oncogene mRNAs have AU rich elements (ARE) in their 3' noncoding region. Association of this region with factors such as tristetraprolin or AUF1 promotes their rapid degradation through Ub/proteasome pathways (Laroia et al., 2002). Radiation-induced impairment of proteasome activity could also be involved in hypersensitivity, adaptive responses, and bystander effects that have been observed following low dose irradiation, and about which there is currently little mechanistic information (Joiner et al., 1999; Joiner et al., 2001; Mothersill & Seymour, 2001).

If proteasome degradation pathways are affected by irradiation, one might expect levels of ubiquitinylated proteins to be altered. Indeed, ubiquitinylated cellular p53 levels increase following irradiation (Maki & Howley, 1997). Expression levels of the cyclin kinase inhibitor p21, which is known to be degraded through the Ub/proteasome system, are also elevated after irradiation, but it is not in a ubiquitinylated form (Maki & Howley, 1997) unlike the case following treatment with proteasome inhibitors. The difference may be that p21 can be unphosphorylated or dephosphorylated as a result of pathways activated by DNA damage, and

this would inhibit ubiquitinylation (Fukuchi et al., 2002). Irradiation increased Ub mRNA expression and ubiquitinylated nuclear proteins in human lymphocytes (Delic et al., 1993) and in Ewing's sarcoma cells (Soldatenkov & Dritschilo, 1997), leading to the suggestion that functional changes in the Ub-proteasome system were involved in the radiation-induced apoptosis. Proteins targeted by the N-end rule pathway appeared to be particularly important. On the other hand, deubiquitinylation of nucleosomal histones has been reported following treatment of cancer cells with proteasome inhibitors, and this was ascribed to depletion of unconjugated Ub (Mimnaugh et al., 2000). Given the complexity of the interactions between proteins linked to the Ub system, the relationship of ubiquitinylated proteins to proteasome function will also be complex. A general increase in expression of all ubiquitinylated proteins is therefore not an expected consequence of radiation-induced proteasome inhibition, but alterations in specific molecular pools would be expected and occurs.

One of the most obvious effects of proteasome inhibition on a molecular pathway is inhibition of NF-κB activation, which is a major mediator of gene transcription for oxidative stress, pro-inflammatory cytokines, immune, and cell survival responses (reviewed in (Karin et al., 2002; Karin & Lin, 2002)). This is a complex pathway under multiple levels of regulatory control (Ladner et al., 2002). The Ub/proteasome system is involved in three ways. First, NF-κB1 (p50) and NF-κB2 (p52) have to be processed from p105 and p100 precursor proteins, respectively, and this is achieved by partial degradation through the Ub/proteasome. The complete mechanism still has to be elucidated, but a glycine-rich stop region has been reported to interfere with degradation of the amino-terminal region of the p105 molecule allowing the carboxy-terminal to be cleaved. In addition, p105 is targeted for degradation by two unique Ub system recognition motifs, one of which is probably an E3 recognition site. These seem to

function under different conditions to provide cells with a low or high amount of p50 (Ciechanover et al., 2001; Cohen et al., 2001). Second, ubiquitinylation is required for activation of IκB kinase that phosphorylates IκB. Third, the E3 ligase β-TrCP specifically ubiquitinylates phosphorylated IκBα, targeting it for degradation. Inhibition of proteasome function prevents the generation of new NF-κB molecules and stabilizes IκBα expression, preventing NF-κB nuclear localization (Pajonk & McBride, 2001b). Since NF-κB is involved in both inflammatory responses and as a survival factor for cancer cells, proteasome inhibitors are anti-inflammatory agents with potential anti-tumor activity, in particular for tumors that are addicted to the NF-κB pathway for survival.

Radiation-induced impairment of proteasome function therefore presents s a paradox with respect to NF-κB activation. Proteasome inhibition would be expected to prevent NF-κB activation, but numerous studies have shown irradiation to activate it (Li & Karin, 1998; Raju et al., 1998). Activation of NF-κB is considered to mediate radiation-induced pro-inflammatory responses and irradiation of cells and tissues increases expression of pro-inflammatory chemokines (Johnston et al., 2002) and cytokines such as TNF-α (Chiang et al., 1993; Hallahan et al., 1989), IL-1α and β (Hong et al., 1994; Hosoi et al., 2001), IL-5 (Lu-Hesselmann et al., 1997), IL-6 (Abeyama et al., 1995; Beetz et al., 1997), GM-CSF (Zhang et al., 1994), IFN-α (Woloschak et al., 1990), bFGF (Haimovitz-Friedman, 1991) and VEGF (Gorski et al., 1999; Park et al., 2001), as well as pro-inflammatory cell adhesion molecules (ICAM-1 (Behrends et al., 1994; Gaugler et al., 1997; Hong et al., 1994), E-selectin (Hallahan et al., 1995), and VCAM-1 (Heckmann et al., 1998)), prostaglandins and leukotrienes (Eisen et al., 1977; Iwamoto & McBride, 1992), proteases (Fittkau et al., 2001; Hong et al., 1994; Patel et al., 1998), and pro-oxidant species. If the damage is not too severe, this is normally counterbalanced in time by

production of anti-inflammatory cytokines, anti-proteases, anti-oxidants, and heat shock proteins leading to resolution of the inflammation (Barcellos-Hoff, 1993; Broski & Halloran, 1994; Roedel et al., 2002; Sadekova et al., 1997; Sierra-Rivera et al., 1993).

The apparent paradox extends to the clinic. Although ionizing radiation has recognized pro-inflammatory effects, ithas been used, especially for the first half of the last century, in the treatment of many benign inflammatory as well as hyperproliferative diseases and in many European countries it is still a popular treatment modality for such conditions (Trott & Kamprad, 1999). Such treatments, however, generally use considerably lower doses of radiation than are used in cancer therapy. These considerations prompted investigation into the radiation dose response relationship for NF-κB activation and expression of its inhibitor IκBα (Pajonk & McBride, 2001b).

In ECV304 cells, NF-κB was activated only in response to ionizing radiation exposure in the high dose range (>=8 Gy)(Pajonk & McBride, 2001b). The same was generally true for radiation-induced ICAM-1 expression, which is considered a downstream readout of NF-κB activity (>=4 Gy; Pajonk, unpublished). IκBα expression did not decrease at any dose, and in fact after 25 cGy IκBα expression was increased, in keeping with what would be expected if irradiation induced proteasome inhibition. ICAM-1 expression was decreased after doses in the range 25 to 150 cGy.

Currently the exact target of ionizing radiation that leads to NF-κB activation is unknown and the mechanism is discussed controversially (Li & Karin, 1998; Raju et al., 1998). In the system described above, an IκB super-repressor gene that contains serine-to-alanine mutations at position 32 and 36 prevented the NF-κB response, indicating that the classical pathway was

involved, even though the level of IκBα expression was not decreased after any radiation dose, as it is following, for example, TNF-α treatment.

The interpretation of the dose-response data is that irradiation induced proteasome inhibition over a wide dose range and that this stabilizes expression of IκBα, and has an anti-inflammatory effect, but that at high doses a pathway is activated that can overcome this inhibition. Failure to demonstrate a decrease in IκBα when NF-κB is clearly active may be due to slower turnover following irradiation. A recent report showed that inducible nitric oxide synthase (iNOS), which is a key mediator of inflammation downstream of NF-κB, is inhibited by low dose ionizing radiation and superinduced by high doses, and is in keeping with the above concept (Hildebrandt et al., 1998).

Radiation-Induced Immunomodulation

The effects of radiation on the immune system have been extensively investigated. Generalized immunosuppressive effects, even after local radiation therapy, are well known, although some studies have shown that radiation, especially at low doses, can be immunostimulatory (Cao et al., 2002; North, 1984). Immune suppression is most often ascribed to lymphocytes being prone to radiation-induced apoptosis. Immunostimulation at low doses may be due to the relatively high radiosensitivity of suppressor T cell subsets compared with other lymphocytes (North, 1984).

If radiation impairs proteasome function, this could affect immune function by pathways other than apoptosis. Maturation of dendritic cells (DC), the most powerful antigen presenting cell known, is dependent on proteasome function (Macagno et al., 2001). Maturation of DCs is an NF-κB-dependent process that is associated with a switch to immunoproteasome expression and is likely to be affected by radiation or oxidative stress (Lutz & Schuler, 2002; Macagno et

al., 2001; Morelli et al., 2000). This could be important because mature DC may present a different spectrum of epitopes than immature DC (Morel et al., 2000; Schultz et al., 2002; Sun et al., 2002). Irradiation may therefore differentially affect mature and immature DC and constitutive and immunoproteasomes, which could result in a switch in the nature of the epitopes being presented to the immune system.

Also, proteasomal processing is required for the production of peptides presented by MHC class I molecules to generate CTL. Recently, evidence has been presented for a surprising increase in the ability of irradiated DC pulsed in vitro with MART-1 immunodominant peptides to activate tumor-specific T cells (Liao et al., 2002). This was ascribed to a radiation-induced loss of endogenous processed self-antigen and vacation of MHC class I molecules on the DC for more efficient exogenous loading. The extent of MHC occupancy by peptides is thought important for T cell stimulation, in particular poor binders.

One would expect irradiation to block the endogenous pathway leading to processing and presentation of endogenous antigen by DC, as is seen using proteasome inhibitors (Rock et al., 2002). Radiation would also be expected to also affect expression of the target antigens on tumor cells that are recognized by the immune system, and specifically by CTL, although this concept is still speculative.

Clinical Exploitation of Proteasome Inhibition in Radiation Therapy

The Ub system, specifically E2 Ubc and E3 ligases represent a range of specific molecular targets for intervention that are too extensive to consider here (Fang et al., 2003; Garber, 2002; Shah et al., 2002). However, proteasome inhibitors affect multiple targets and represent a broader based approach to cancer therapy. As discussed earlier, they seem to

precipitate apoptosis in tumor cells more readily than in normal cells, and therefore have potential to result in a therapeutic benefit.

The peptide boronic acid compound PS-341 (pyrazlcarbonyl-Phe-Leu-boronate) recently entered clinical trials (Adams, 2002). Anti-tumor activity has been demonstrated in murine models of cancer (Cheson, 2002) and in human prostate cancer and multiple myeloma (L'Allemain, 2002; Mitsiades et al., 2002b). In the latter disease, patients with relapsed refractory disease had a high objective response rate with acceptable toxicity. A Phase III trial comparing PS-341 with dexamethasone in patients with relapsed disease is underway, as are several Phase II investigations (Adams, 2002). The focus is on hematological malignancies, which is in keeping with high constitutive levels of NF-κB that appear to serve as a survival pathway in these cells and as a target for PS-341. It is unlikely, however, that this is the only target, given the considerations expressed earlier. The side effects of PS-341 are related to dose and timing. In rodents and primates, anorexia, vomiting, and diarrhea (Adams et al., 1999) that has been observed are presumed due to effects on rapidly proliferating cells in the gastro-intestinal tract. However, 80% inhibition of in vivo 20S proteasome activity can be achieved using PS-341 before serious complications arise (Adams et al., 1999). Avoiding daily delivery can minimize side effects. Optimal scheduling has yet to be firmly established.

Targeting the proteasome is a novel strategy though it is likely to be limited in effectiveness without addition of a cytotoxic agent. Because proteasome inhibition targets tumor cell survival and DNA repair pathways, proteasome inhibitors generally sensitize to the effects of radiation (Pajonk et al., 2000; Pervan et al., 2001a; Pervan et al., 2001b; Russo et al., 2001; Teicher et al., 1999) and chemotherapy (Mitsiades et al., 2002b). PS-341 is being combined with gemcitabine or irinotecan in Phase I trials in advanced solid tumors (Adams, 2002).

Although PS-341 is the only specifically designed proteasome inhibitor to reach clinical trials, there are other drugs that directly affect the Ub/proteasome system that have been used clinically for other purposes. Recently, the HIV protease was shown to share cleavage specificities with the 20S core proteasome (Flexner, 1998) and the HIV protease inhibitors ritonavir (Lebbe et al., 1998) and saquinavir (Pajonk et al., 2002a) inhibit proteasome function and have anti-tumor effects. At least the latter radiosensitizes cancer cells and may have clinical utility in this setting (Pajonk & McBride, 2002). Interestingly, dramatically improved survival rates for AIDS patients suffering from primary central nervous system lymphoma (PCNSL) were found using cranial irradiation along with highly active antiretroviral therapy (HAART), which involves HIV protease inhibitors (Hoffmann et al., 2001). It is tempting to think that this was achieved by proteasome inhibition resulting in radiosensitization.

Inhibitors of multiple drug resistance gene product 1 (mdr1), like cyclosporine A (Meyer et al., 1997), rapamycin (Pajonk & McBride, 2000), vinblastine (Piccinini et al., 2001), verapamil, and other anthracyclin inhibitors have also been found to inhibit 26S and 20S proteasome function, suggestion an overlap between mdr1 and proteasome specificities that might be usefully exploited (Pajonk et al., in press). It is tempting to think that some of the immunosuppressive effects of cyclosporine A are achieved through its inhibitory effects on proteasome activity. Interestingly, NSAIDs have also been shown to inhibit proteasome function (Huang et al., 2002). Although this aspect of their action has yet to be investigated in detail, decreases were found in the immunoproteasome subunits within 24 hours of treatment. The immunoproteasome has been linked by others to NF-κB activation (Hayashi et al., 1990; Hayashi & Faustman, 2000) and the relationship between NF-kB and the proteasome activity may in the future prove to be more intimate and reciprocal than is currently evident.

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FIGURE LEGENDS

Figure 1: Proteasome structures and alternative IFN-γ inducible forms.

Figure 2: The ubiquitin system. Ubiquitin is a 76-residue protein that is attached through a C-terminal glycine to an ε-amino group of lysine on the substrate following a series of reactions involving activating (E1), conjugating (E2), and ligating (E3) enzymes. The polyubiquitinylated product is then recognized by the 26S proteasome. The Ub is recycled by isopeptidases.

Figure 3: Some of the molecules involved in radiation-induced DNA repair, cell cycle arrest, and apoptosis and their relationship to the Ub/proteasome system. ✶ = E3 ligase activity ■ = ubiquitinylated

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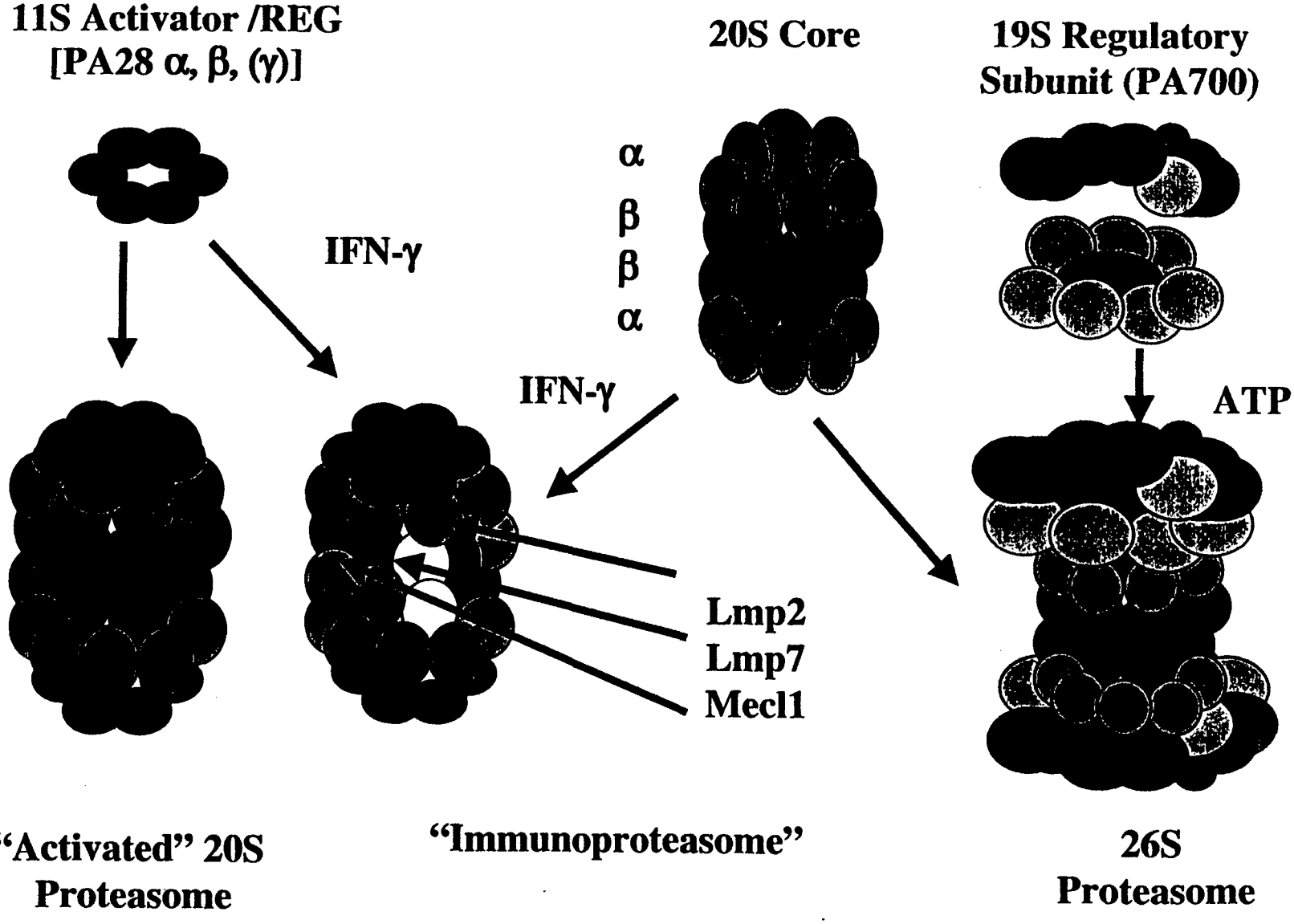
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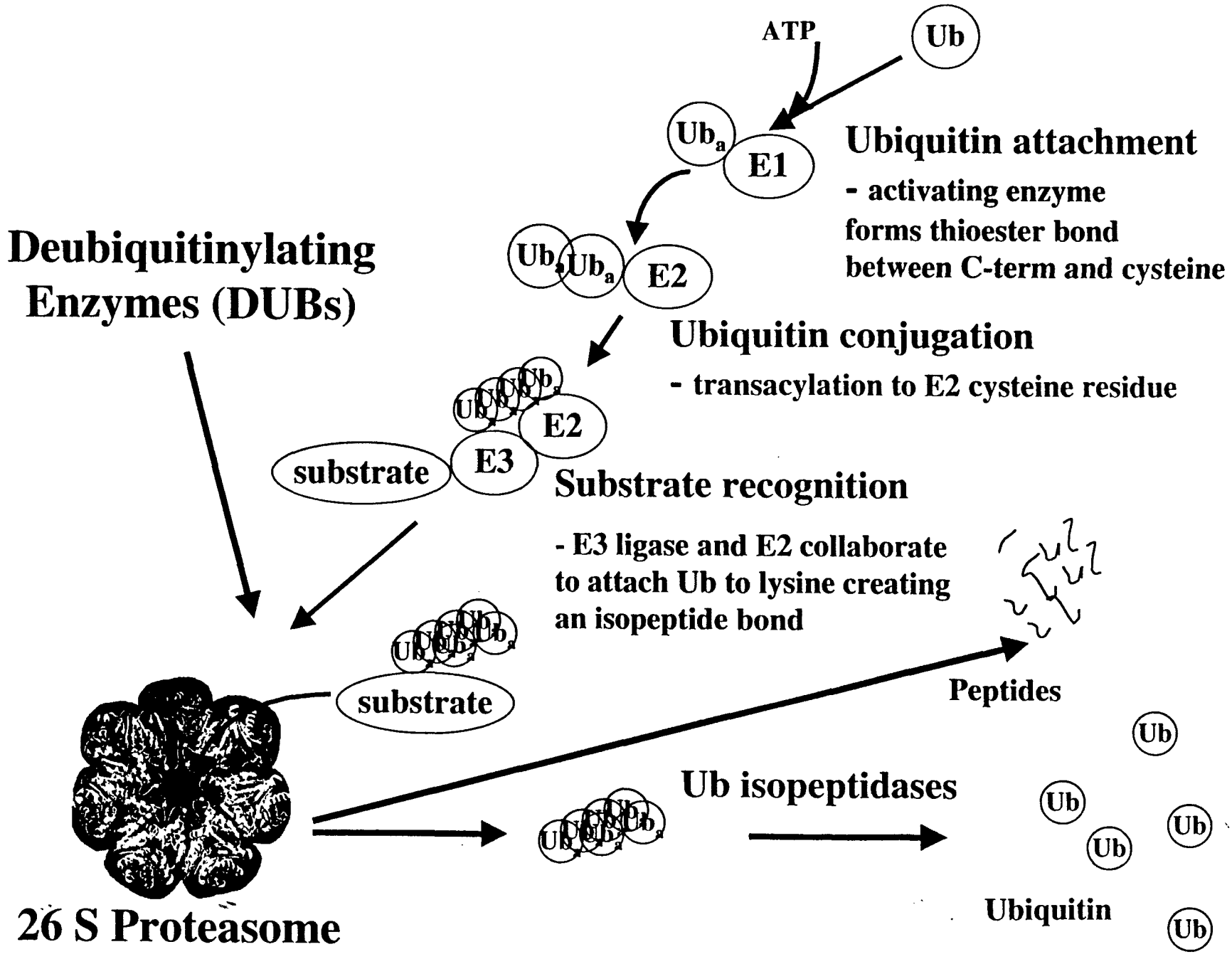
[PA28 α, β, (γ)]
20S Core
19S Regulatory
Subunit (PA700)
α
β
β
α
IFN-γ
IFN-γ
ATP
Lmp2
Lmp7
Mecl1
"Activated" 20S
Proteasome
"Immunoproteasome"
26S
Proteasome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Ub
Ub$_a$
E1
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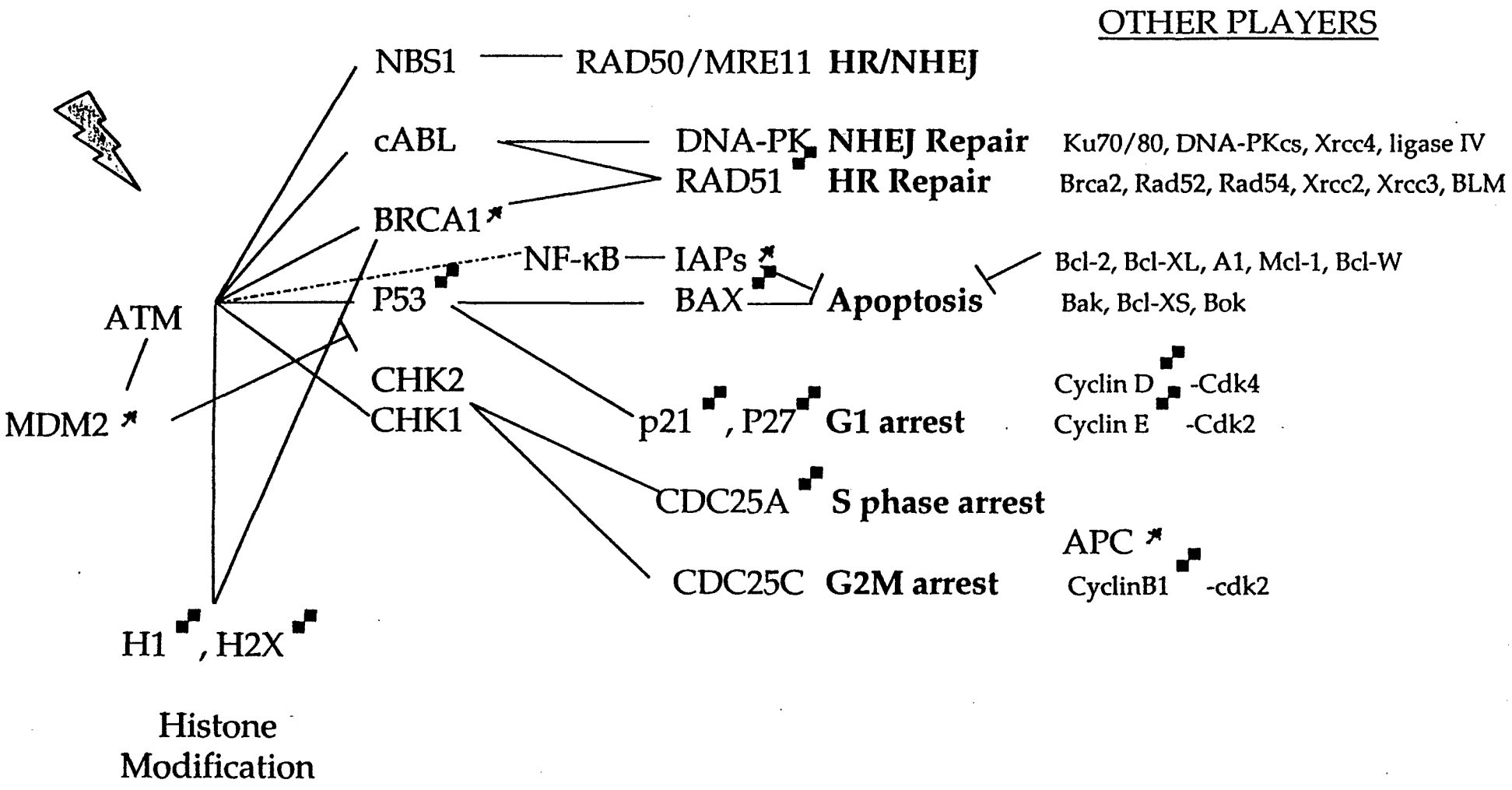
- activating enzyme
forms thioester bond
between C-term and cysteine
Ub$_a$Ub$_a$
E2
Deubiquitinylating
Enzymes (DUBs)
Ubiquitin conjugation
- transacylation to E2 cysteine residue
E2
E3
substrate
Substrate recognition
- E3 ligase and E2 collaborate
to attach Ub to lysine creating
an isopeptide bond
substrate
Peptides
Ub isopeptidases
Ub
Ubiquitin
26 S Proteasome


OTHER PLAYERS
NBS1
RAD50/MRE11 HR/NHEJ
cABL
DNA-PK NHEJ Repair
Ku70/80, DNA-PKcs, Xrcc4, ligase IV
RAD51 HR Repair
Brca2, Rad52, Rad54, Xrcc2, Xrcc3, BLM
BRCA1
NF-κB
IAPs
Bcl-2, Bcl-XL, A1, Mcl-1, Bcl-W
P53
BAX
Apoptosis
Bak, Bcl-XS, Bok
ATM
CHK2
Cyclin D -Cdk4
MDM2
CHK1
p21, P27 G1 arrest
Cyclin E -Cdk2
CDC25A S phase arrest
APC
CDC25C G2M arrest
CyclinB1 -cdk2
H1, H2X
Histone
Modification

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REVIEW

The Proteasome in Cancer Biology and Treatment

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Pajonk, F. and McBride, W. H. The Proteasome in Cancer Biology and Treatment. *Radiat. Res.* 156, 447–459 (2001).

During the last 30 years, investigation of the transcriptional and translational mechanisms of gene regulation has been a major focus of molecular cancer biology. More recently, it has become evident that cancer-related mutations and cancer-related therapies also can affect post-translational processing of cellular proteins and that control exerted at this level can be critical in defining both the cancer phenotype and the response to therapeutic intervention. One post-translational mechanism that is receiving considerable attention is degradation of intracellular proteins through the multicatalytic 26S proteasome. This follows growing recognition of the fact that protein degradation is a well-regulated and selective process that can differentially control intracellular protein expression levels. The proteasome is responsible for the degradation of all short-lived proteins and 70–90% of all long-lived proteins, thereby regulating signal transduction through pathways involving factors such as AP1 and NFKB, and processes such as cell cycle progression and arrest, DNA transcription, DNA repair/misrepair, angiogenesis, apoptosis/survival, growth and development, and inflammation and immunity, as well as muscle wasting (e.g. in cachexia and sepsis). In this review, we discuss the potential involvement of the proteasome in both cancer biology and cancer treatment. © 2001 by Radiation Research Society

INTRODUCTION

Most potentially malignant tumor cells, like their normal counterparts, will die as they move outside the environment that nurtures them. Only a small number of cells in a small proportion of individuals will develop mutations that allow them to overcome these limitations and evolve into a malignant metastatic phenotype. This transition requires multiple mutations in multiple genes. It is logical to assume that targeting therapies to individual cancer-related genes or pathways will leave other pathways intact and allow at least a proportion of the tumor cells to survive. A superior tactic for cancer therapy would be to target molecules or complexes of molecules that control nodes where multiple pathways converge rather than molecules that function primarily in one pathway. One promising nodal regulator of cellular function is the proteasome.

For cells to perform their functions appropriately, they must control the rates of both synthesis and destruction of their proteins. During the last two decades, our understanding of the pathways involved in protein synthesis has grown enormously. Only recently has the importance of controlled proteolysis in dictating the level of protein expression, in functionally activating precursor proteins to their mature form, and in antigen processing been recognized. Importantly, proteolysis has also been shown to play a major role in the cellular response to stimulation. Here it has a major advantage over protein synthesis in the speed at which responses can be activated. Targeting pre-existing proteins for rapid cleavage is a common mechanism by which inhibitory proteins can be destroyed and signal transduction pathways can be activated. The 26S proteasome is the multimolecular structure most responsible for the controlled degradation of short- and long-lived proteins in eukaryote cells. This review emphasizes how cancer mutations affect molecular processing through this multicatalytic proteasome complex, the role of the functional alterations in the activity of the complex in cancer biology, and its potential as a target for therapeutic intervention in cancer treatment.

Proteolytic Systems

Proteolytic systems are highly conserved in eukaryotic cells (*1–6*). The major division is into lysosomal and nonlysosomal systems. While the former is dependent on cathepsin B, D, H and L, the latter can be subdivided into energy-independent and energy-dependent mechanisms. The major mediators of energy-independent proteolysis are calpain I and II. Most intracellular molecules, however, are degraded through the proteasome. For many years, proteasomes were considered as being involved only in normal housekeeping events that required protein turnover. More

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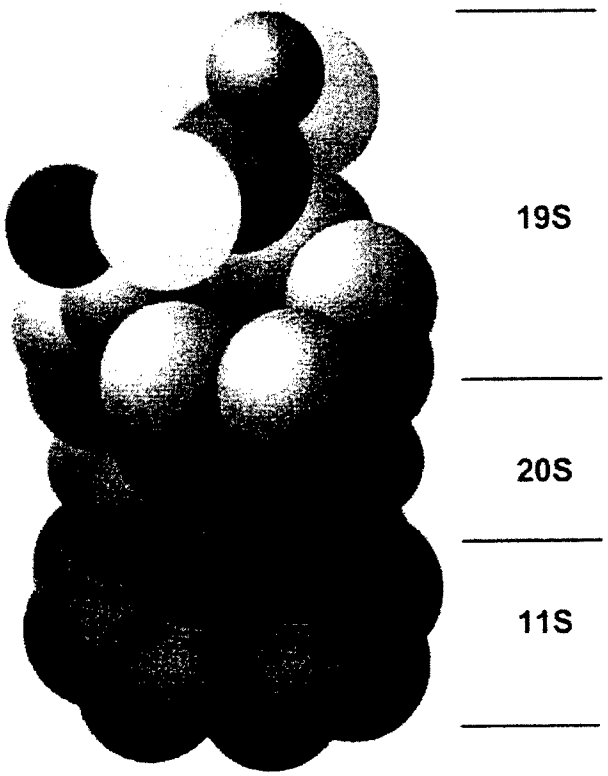

FIG. 1. Structure of a 19S–20S–11S hybrid proteasome as described in ref. (*23*). The 20S core unit consists of four rings of seven α and β units, respectively. The inner two rings of beta subunits form the catalytic center of the protease. Attached to both ends of the 20S core unit are 11S and 19S regulatory units. See the text for details.

recently, their roles in cell cycle progression, transcription and DNA repair, as well as in cellular responses to stress, have come to the fore. Several structural forms of proteasomes have evolved that have the same basic central core structure and that specialize in specific functions. The ATP- and ubiquitin-dependent 26S proteasome is responsible for controlled degradation of all short-lived proteins (*7*) and 70–90% of all long-lived proteins (*7, 8*). Non-ATP and non-ubiquitin proteasomal pathways are also present that tend to specialize in the removal of degraded proteins.

Structure and Function of Proteasomes

The proteasome consists of a large barrel-shaped 20S core unit of about 700 kDa that may have two 19S regulatory units (also known as PA700), or two 11S activator units (also known as PA28 or REG), or one 19S and one 11S unit (hybrid) attached to both ends (Fig. 1). The 19S regulator unit is formed from at least 18 different subunits. Some (Rpt1–6; human genes *PSMC1–6*) have ATPase activity and form a "base" to the 19S structure; others (Rpn1–12; human genes *PSMD1–12*) do not, and form the "lid." The 11S activator unit is formed from two subunits, α and β (PSME1 and PSME2, also known as PA28 α and β, or REG α and β). The 11S activator subunits share approximately 50% homology to a nuclear protein of unknown function, the Ki auto-antigen [recently identified as PSME3, also known as PA28γ (*9*)]. The 20S core is inactive unless it is activated by the 19S or 11S caps. Experimentally, it can be activated by SDS treatment.

The 20S core is a barrel-shaped structure of four protein complexes. The two outer rings have seven α subunits. The two inner two rings are built from seven β subunits, which form the catalytic sites. Proteins for degradation have to be unfolded and passed into the central core of the proteasome. Constitutive β subunits (β1, β2 and β5; human genes *PSMB6, PSMB7* and *PSMB5*) in the inner rings can be replaced by interferon γ-inducible subunits β1i, β2i, and β5i [LMP-2 (gene name *PSMB9*), MECL-1 (*PSMB10*), and LMP-7 (*PSMB8*), respectively] (*10–14*). The inducible subunits are coded in the class II region of the MHC locus. The 11S activator (PA28) subunits are also inducible by interferon γ. Structures containing inducible subunits are often called "immunosomes" because of their potency in processing antigen into peptides for immune presentation.

The 26S proteasome, which contains 19S regulatory subunits, is specialized in the recognition and destruction of proteins specifically targeted to it by the process of ubiquitinylation. A concerted cascade of three enzymes is generally involved in this process. First, ubiquitin, a 76-residue polypeptide found in all eukaryotic cells, is activated when its carboxy-terminal glycine is transformed into a high-energy thiolester intermediate by the ubiquitin-activating enzyme E1. A family of ubiquitin-conjugating (E2) enzymes conjugates ubiquitin to a diverse set of substrate recognition (E3) factors. These E3 ubiquitin ligases catalyze the last step, which is ligation of activated carboxy-terminal ubiquitin to amino groups of lysine residues in the targeted protein to form a polyubiquitin chain that is recognized by PSMD10 in the 19S regulatory unit. The E3 ligases are largely responsible for conferring specificity at the level of substrate recognition. Ligases belong to one of two major families, the HECT domain ligases (homologous to the E6 accessory protein carboxy terminus) and the RING finger ligases. E2 may also transfer activated ubiquitin directly to the substrate, and this may occur on the proteasome. Recognition of oxidized, damaged or excess proteins, at least in most cases, does not appear to require ubiquitinylation or proteasomes that contain the 19S regulator.

Proteasomal Function and the Immune System

Recognition of endogenously processed antigens by cytotoxic T lymphocytes requires epitopes to be presented in the context of major histocompatibility complex class I (MHC I) molecules. The α1 and α2 domains of MHC I molecules form a groove that holds peptides of 7–13 amino acids. While alternate nonproteasomal pathways exist for processing some antigens, most endogenously produced an-

tigens that generate cytotoxic lymphocytes are processed through the proteasome. The peptides are produced by the proteasome and delivered to nascent MHC I molecules in the lumen of the endoplasmic reticulum (ER) by specialized transporters that are associated with antigen processing (TAP) proteins. The affinity of binding of the peptides is determined by the nature of the interacting residues; binding requires the presence of one or more proline-rich anchor residues. Binding stabilizes the MHC I complex in association with the β2-microglobulin in a trimeric complex that moves to the cell surface so that the antigenic epitope can be presented to the cognate T cell [reviewed in refs. (*15–17*)]. The mechanism by which the proteasome cleaves proteins to generate products whose modal size, after N-terminus trimming, is a nonapeptide is not known. Immunoproteasomes formed under the influence of cytokines, in particular interferon γ, which is produced during activation of T cells specialized in cellular immunity, are particularly efficient at this cleavage process (*18–22*). The relationship between the 26S proteasome and the immunoproteasome in the processing of ubiquitinylated antigens is still uncertain, and hybrid proteasomes containing 19S and 11S subunits may be particularly effective in this situation.

Molecular Interactions with the Proteasome System and Evasion

In addition to indirect targeting of proteins for destruction through ubiquitinylation, two hybrid screens and other techniques have identified a number of proteins that interact directly with proteasomal subunits, in particular with those that form the 19S regulatory cap and that have ATPase activity (*23*). A number viral proteins such as Ad E1A, SV-40T, E7 and HVI-1 tat can interact in this manner, suggesting that viruses, including herpes simplex virus, cytomegalovirus, human papilloma virus, and human immunodeficiency virus type 1, have evolved strategies of replication and immune evasion that target the proteasome (*24–29*). Other interacting molecules are certain cell surface receptors, such as TNFRSF1A (TNFR1), TR, EGFR, ERα, RARA, and RXR. This may provide a very rapid mechanism by which cells can control responses to external stimuli. Demonstration of direct interactions with proteins involved in DNA repair (RAD23) and the cell cycle [CDC28, PSMD9 (p27)] indicate the importance of the proteasome in these processes. Finally, the proteasome is intimately linked with chaperones, such as HSP90, which play a role in unfolding proteins for insertion into the internal catalytic chamber and may also regulate proteasomal activity (*30*).

It is not surprising that tumors, as well as viruses, have evolved mechanisms to interfere with proteasome function. The extent of this interference is uncertain, but interference with molecules that are required for efficient antigen processing and presentation has been reported. Many tumors have down-regulated TAP1, TAP2, PSMB9 (LMP2) or PSMB8 (LMP7) expression (*31, 32*); re-establishing these pathways by gene transfer often leads to immune recognition of these tumors. One mechanism by which tumors may escape immune recognition is the release of high levels of the immunosuppressive cytokine interleukin 10, which inactivates TAP and proteasomal function, leading to decreased presentation of peptide-epitopes on MHC I molecules on the cell surface (*33*). Modulation of proteasome activity by cytokines may also enhance tumor antigen presentation. Studies in our laboratories showed that *IL3* gene transfection of fibrosarcoma cells enhanced proteasomal activity and the immunogenicity of immunogenic fibrosarcoma cells when compared to nonimmunogenic control cells expressing a control vector (Pajonk and McBride, unpublished data).

In addition to cancer and viral infections, there is growing evidence that the proteasome/TAP system plays a crucial immunomodulatory role in benign inflammatory diseases. Recent studies have linked polymorphism of 26S proteasome subunits and TAP to Sjoergren's syndrome, streptococcal-related polyarthritis, rheumatoid arthritis, myositis, systemic lupus erythematosus, HLA-B27-associated juvenile rheumatoid arthritis, and juvenile ankylosing spondylitis (*34–40*). The critical role of the proteasome in the generation of immunity is supported by recent studies identifying the frequently used immunosuppressive drugs cyclosporin A and rapamycin as potent and direct inhibitors of proteasome activity (*41, 42*).

PROTEASOMES AND CANCER

Tumor Suppressor and Oncogenes

It is widely accepted that most, if not all, forms of malignancy are caused by genetic mutations in oncogenes and/or tumor suppressor genes. It is of interest to note that proteasomal degradation regulates the level of expression of the products of many of these genes (Tables 1 and 2) (*43–45*), and any cancer-related modifications in proteasome function may affect the degradation pathways. Furthermore, some gene products interact directly, or indirectly through the ubiquitin pathway, with proteasomal subunits. Mutations in ubiquitin or proteasome binding sites could alter protein stability and contribute to carcinogenesis. In addition, some tumor suppressor gene or oncogene products actually participate in substrate ubiquitinylation as E3 ligases. The potential implications of these interactions can be seen by examination of a number of examples.

Mutations in the tumor suppressor gene *APC* have been associated with the development of familial polyposis coli and spontaneous colon carcinomas. One function of the APC gene product is the control of β-catenin levels. Mutation of the *APC* gene leads to accumulation of β-catenin in the cytosol, and increased β-catenin levels have been linked to human cancer (reviewed in ref. *46*). The mechanism by which APC controls β-catenin levels is not fully understood, but APC is involved in its ubiquitination (*47*),

TABLE 1
Human Tumor Suppressor Genes Interacting with the Ubiquitin-26S Proteasome Pathway

Gene	Tumor	Type of interaction	Reference(s)
APC	Familial adenomatosis polyposis	Blocks β-catenin degradation by the 26S proteasome	(*47*)
DCC	Colon carcinoma	Degraded by the 26S proteasome	(*52, 175*)
TP53	Breast, colon, lung carcinomas, osteosarcoma, astrocytoma, etc.	Degraded by the 26S proteasome	(*176–178*)
RB1	Retinoblastoma, osteosarcoma, breast, bladder, lung carcinomas	Degraded by the 26S proteasome	(*29*)
VHL	von Hippel-Lindau syndrome (renal carcinoma, pheochromocytoma, hemangioblastoma)	Targets HIF1A for 26S proteasome-dependent degradation under well-oxygenated conditions	(*66*)

and *APC* mutation may interfere with its phosphorylation (*48*).

The tumor suppressor gene *DCC* (deleted in colon cancer) spans a 1.4-Mbp region of the human genome (*49*), and deletions in this region are observed in about 70% of colorectal cancers (*50*). The functions of its gene products are not clear, but the main product is a large transmembrane protein of four immunoglobulin-like and six fibronectin type III-like extracellular domains with a 325-amino-acid cytoplasmic domain (*51*). The cytoplasmic domain has recently been shown by yeast two-hybrid screening to bind SIAH proteins, the human homologues of the *Drosophila* seven in absentia (sina) gene. SIAH regulates the stability of DCC by interacting with ubiquitinating enzymes that target DCC for degradation (*52*).

The *TP53* tumor suppressor gene is mutated in about half the cases of human cancer. It is expressed at low levels in normal cells, but levels are elevated after mutation or in response to DNA damage, such as that caused by ionizing radiation. The level of TP53 is determined by nontranscriptional mechanisms. TP53 is targeted for destruction by the E3 ubiquitin ligase MDM2, and disruption of this autoregulatory loop has profound effects on cell survival and tumorigenesis. Conjugation of UBL1 (also known as SUMO-1) with MDM2 prevents self-ubiquitination and degradation of MDM2, increasing the rate of destruction of TP53 (*53*). Stabilization of TP53 in response to radiation is associated with inhibition of MDM2-mediated degradation, reduction in MDM2 sumoylation, and phosphorylation of TP53 (*53*). Expression of TP53-induced CDKN1A (also known as $p21^{WAF1/Cip}$), which is responsible in large part for radiation-induced G_1/S-phase arrest through CDK inhibition, and GADD45, which is involved in G_2/M-phase arrest, is controlled directly and indirectly by proteasomal degradation. Stabilization of mutated TP53 seems to be the result of two independent phenomena, both of which cause its impaired ubiquitination: The loss of wild-type TP53 function acts to stabilize mutated TP53 by affecting MDM2-mediated ubiquitination. In addition, mutated TP53 can be stabilized by binding of HSP90, which could be overcome by treatment with the HSP90 inhibitor geldanamycin (*54*).

The protein product of the gene mutated in retinoblastoma (*RB1*, also known as RB, pRB or p105), is a negative regulator of the G_1- to S-phase transition. The hypophos-

TABLE 2
Human Proto-Oncogenes Interacting with the Ubiquitin-26S Proteasome Pathway

Gene/ gene family	Tumors with abnormal expression	Type of interaction	Reference(s)
ABL	Chronic myelocytic leukemia, acute lymphatic leukemia, chronic neutrophilic leukemia	BCR-ABL targets ABI for proteasomal degradation, BCR-ABL expression depends on 26S proteasome function	(*77, 78*)
FOS	Breast, ovarian, prostate, cervical cancer, skin	Degraded by the 26S proteasome	(*93, 94, 96*)
MOS	Plasmocytoma	Degraded by the 26S proteasome	(*179*)
MYB	Myeloid and lymphoid leukemia	Degraded by the 26S proteasome	(*99*)
MYC	B-cell lymphomas, promyelocytic leukemia	Degraded by the 26S proteasome	(*106*)
RAF		Raf-B binds to PA28α activates the 26S proteasome; RAF1 is degraded by the 26S proteasome	(*110, 180*)
RAS	Lung, colon, bladder, breast and teratocarcinoma, neuroblastoma, leukemia, fibrosarcoma, melanoma, rhabdomyosarcoma	Inhibits the proteasome-dependent degradation of *MYC*	(*44*)
REL	B-cell lymphomas, multiple myeloma, Hodgkin's disease, non-small cell lung carcinoma, squamous head and neck carcinoma, breast cancer, colon cancer, stomach cancer, thyroid carcinoma	Subunit of the transcription factor NFKB, activated by 26S proteasome-dependent degradation of NFKBI	(*114*)
SRC	Brain tumors	Targets ABI for proteasomal degradation	(*77*)

phorylated form of RB1 binds to the E2F family of transcription factors that controls expression of essential cell cycle proteins like DNA polymerase α. Upon phosphorylation by cyclin-dependent kinases (CDKs), RB1 releases E2F, allowing transcriptional activation of E2F-dependent genes and cell cycle progression (*55*). RB1 is ubiquitinated and degraded (*29, 56–59*). Additionally, free E2F itself is degraded in a ubiquitin-dependent manner by 26S proteasome [for a review of E2F regulation, see (*60*)]. Mutated RB1 that has been deleted in at the carboxy-terminal end (position 1–792) lacks E2F stabilizing function and is unable to protect E2F from ubiquitination and subsequent degradation by the 26S proteasome (*56*).

In response to low oxygen tension, cells activate the transcription factor HIF1, which binds to specific DNA sequences in the promoter regions of genes like erythropoietin and vascular endothelial growth factor (*VEGF*). HIF1 is a heterodimer of HIF1A and HIF-1β (now known as aryl hydrocarbon receptor nuclear translocator, ARNT). In addition to binding to HIF1A, ARNT is able to bind to the aryl hydrocarbon receptor (AHR) and subsequently activates genes of the xenobiotic detoxification system (*61*). Under well-oxygenated conditions, HIF1A is rapidly inactivated by 26S proteasome-dependent degradation (*62*). Under hypoxic conditions, HIF1A is stabilized and active (*63*). Hypoxia inhibits proteasome function (*64*), indicating one of the mechanisms by which this stabilization might occur. The von Hippel-Lindau tumor suppressor gene product (VHL), which is thought to be regulated by a ferro-protein oxygen sensor (*65*), is a component of the E3 ligase complex that leads to degradation of HIF1A (*66*). Two recent reports indicate that HIF1A is hydroxylated at a proline residue under well-oxygenated conditions and that this post-translational modification targets HIF1A for ubiquitination by VHL (*67–69*). Mutations in *VHL* are found in patients with dominant inherited VHL syndrome; this is the most common genetic defect associated with kidney cancer in humans. Such defects cause accumulation of HIF1A followed by uncontrolled angiogenesis as a result of HIF1 transcriptional activity. The data suggest a direct link between the VHL tumor suppressor gene product and the process of ubiquitination and its dysregulation in kidney cancer.

ABL is the cellular homologue of the transforming gene of Abelson murine leukemia virus. Activation of the oncogenic potential of *ABL* occurs as a consequence of a translocation event that results in the expression of chimeric fusion proteins like BCR-ABL and ETV6 (also known as TEL)-ABL (*70*) in chronic myelogenous leukemia (*70*), acute lymphocytic leukemia (*71*), and chronic neutrophilic leukemia (*72, 73*). ABL codes for a tyrosine kinase that carries 3 DNA-binding domains and is a negative regulator of cell growth (*74*). Intranuclear proteins such as RB1 negatively regulate ABL kinase activity, and proteasomal degradation of RB1 releases ABL to phosphorylate RNA polymerase II. After irradiation, ABL is phosphorylated by ATM (reviewed in ref. *75*). In cells carrying the BCR-ABL translocation, the fusion protein relocates from the nucleus to the cytoplasm and shows greatly enhanced tyrosine kinase activity, resulting in positive regulation of cell growth (*76*). Dai and coworkers recently reported that BCR-ABL targets ABL-interactor proteins (ABI1 and ABI2), which are inhibitors of the tyrosine kinase activity of ABL, for ubiquitin-dependent proteolysis by the 26S proteasome (*77*). The dependence of BCR-ABL on the 26S proteasome pathway for its growth-promoting function is further supported by the observation that proteasome inhibition leads to inactivation of BCR-ABL function, reduced BCR-ABL expression, and apoptosis in K562 cells (*78*). Mild hyperthermia, which also inhibits proteasome function, has a similar effect on BCR-ABL expression (*79*).

Abnormal expression levels of FOS have been found in breast cancer (*80*), ovarian cancer (*81*), prostate cancer (*82*), cervical cancer (*83*), and skin carcinomas (*84*). FOS, the gene product of the *FOS* proto-oncogene, is a major subunit of the activator protein 1 (AP1) transcription factor complex, a pathway involved in cell growth (*85*), differentiation (*86*) and transformation (*87*). AP1 activation results from signaling through MAP kinase pathways. Activation of the MAPK8 (also known as JNK/SAPK) pathway leads to up-regulated transcription of FOS and phosphorylation of JUN at serine 63 and 73 (*88*). This leads to formation and enhanced transcriptional potential of the AP1 transcription factor complex (*89–92*). These pathways are counterbalanced by ubiquitin-dependent proteolysis of FOS (*93–96*) and JUN (*94, 95, 97*) by the 26S proteasome. Although the conjugating enzymes responsible for ubiquitination of FOS have been identified (*96*), the pathways and signals that target FOS and JUN for ubiquitination are not fully understood, but deletion of the C-terminal PEST sequence, which is altered in the proto-oncogene *FOS*, greatly enhances its stability (*48*).

MYB is the human homologue of the avian gene that was first described in avian myeloid leukemia viruses, and it is associated with development of myeloid leukemia in humans. It is up-regulated during the G_1 phase of the cell cycle in hematopoietic cells. In these cells, MYB functions as a transcription factor involved in proliferation and differentiation (reviewed in ref. *98*). MYB proteins have a half-life of less than 1 h (*99, 100*). This is achieved by ubiquitin-dependent degradation by the 26S proteasome. Myeloid leukemia-specific forms of MYB that are truncated at the COOH-terminal exhibited increased stability (*99*).

The *MYC* gene family consists of cellular *MYC, MYCN,* which is associated with neuroblastomas (*101*) and retinoblastomas (*102*), and *MYCL,* which is detected in small cell lung cancers (*103, 104*). In the presence of RAS, MYC has transforming activity, and dysregulated levels of MYC have been described for many malignancies. MYC has a very short half-life of 30 min (*105*), and MYC and MYCN are both degraded by the 26S proteasome in a ubiquitin-dependent manner (*106*). This process is promoted by the human

papilloma virus protein E6, which is expressed by the highly oncogenic strain HPV16 (*106*).

Many extracellular survival signals converge in activation of the RAS/RAF1/MAP kinase pathway (reviewed in ref. *107*), leading to cell differentiation (*108*) or proliferation (*109*). RAF1 is normally complexed in a cell with CDC37 (p50), which recruits HSP90 to stabilize RAF1. RAF1 is rapidly degraded by the 26S proteasome in its absence (*110, 111*). Disruption of RAF1-HSP90 complex formation using the HSP90 inhibitor geldanamycin, a benzoquinone, or ansamycin or by overexpression of a dominant negative CDC37 (p50) that is unable to recruit HSP90 causes failure of signal transduction (*112*). RAF1 mutations have been described in human lymphomas and leukemias (*113*), but it is not known whether these mutations lead to the stabilization of RAF1 by the blocking of proteasome-dependent degradation.

The members of the REL/NFKB family of transcription factors share a highly conserved DNA-binding domain called the REL homology domain. Homo- or heterodimers of this family bind to 10-bp DNA sites (κ-sites). Mammals have five different NFKB subunits: p50 (TNFRSF5)/p105 (NFKB1), p52/p100 (NFKB2), REL, p65/RELA and RELB. The p65/p50 heterodimer is most important for most responses. It is sequestered in the cytoplasm bound to its inhibitor molecule NFKB1A. The classical pathway of NFKB activation involves phosphorylation of NFKB1A by IKK kinases, causing its ubiquitination and subsequent degradation by the 26S proteasome. This frees NFKB for translocation into the nucleus and allows transcriptional activation of NFKB-dependent genes (reviewed in ref. *114*).

Constitutive activation of the NFKB signal transduction pathway has been implicated as promoting cell survival in many malignancies, including non-Hodgkin's lymphoma, Hodgkin's disease (*115*), myeloma, breast cancer, prostate cancer (*116*), melanoma (*117*), and squamous cell carcinoma of the head and neck (*118*). Activation can be the result of gene amplification or rearrangements of the *REL* gene (lymphomas), *REL* overexpression (non-small cell lung carcinomas), *RELA* translocation, overexpression or amplification (lymphomas and leukemias, squamous cell carcinoma of the head and neck, adenocarcinoma of the breast and the stomach, and thyroid carcinomas), overexpression of TNFRF5 (non-small cell lung carcinomas), or mutations in NFKB1A (Hodgkin's disease) (reviewed in ref. *119*). One potential additional mechanism leading to constitutive NFKB activation is increased 26S proteasome activity, which could enhance the rate of degradation of NFKBI, or increase the production of TNFRSF5 from NFKB1. Proteasome activity and NFKB activity correlated well in a panel of human tumor cell lines (*120*).

The *SRC* oncogene encodes for the tyrosine kinase $pp60^{SRC}$, which was first described by Duesberg and Vogt (*121*). It is the prototype for a family of related kinases that are involved in many signal transduction pathways. Abnormal expression of this gene has been described in human brain tumors (*122*). The gene product $pp60^{SRC}$ interacts with ubiquitin/26S proteasome pathway in two ways: Oncogenic forms of $pp60^{SRC}$ phosphorylate ABI proteins and target them for ubiquitination and degradation (*77*). ABI proteins in turn antagonize the oncogenic potential of ABL, giving rise to a contribution of *SRC* to the progression of BCR-ABL-positive leukemias. Additionally, wild-type $pp60^{SRC}$ is itself degraded through the 26S proteasome (*123, 124*).

PROTEASOMES AND CELL FUNCTION

Cell Cycle Regulation

The eukaryotic cell cycle is coordinated by the interaction of families of cyclins with cyclin-dependent kinases (CDKs). Cyclin levels vary throughout the cell cycle, and their regulated degradation by the proteasome is essential for cell cycle progression. Degradation is facilitated by polyubiquitination by a family of E3 ubiquitin ligases termed the SKP1-CDC53-F-box protein (SCF) complex (*125*). Rapidly proliferating cells, whether they are progenitor cells or cancer cells, generally show increased levels of expression of proteasome subunits (*120, 126, 127*). Inhibition of proteasome function arrests cells in G_1 (*128–130*), late S (*131*), and G_2/M phase of the cell cycle (*132*).

The Role of the Proteasome in Catabolic States

Cancer is frequently associated with an increased rate of catabolism, as are several other pathological conditions, including chronic renal failure and sepsis. The increased catabolism is often linked to altered cytokine profiles, in particular increased levels of TNF and other proinflammatory cytokines, and/or to acidosis, and is characterized by progressive muscle protein loss (*133, 134*) and negative nitrogen balance (*135*). An inherent component of the imbalance appears to be increased proteasome activity. The cause of the increase is not known, but cytokines and acidosis may directly alter proteasome structure and function. This may explain why correction of low pH by bicarbonates corrects muscle protein loss in patients with chronic renal failure (*135, 136*).

The Proteasome in Angiogenesis and Erythropoiesis

Tumor growth requires the induction of new blood vessels to provide oxygen and nutrients. The process of angiogenesis depends on two critical steps: the sensing of low oxygen tensions and the subsequent transcriptional activation of pathways leading to production of growth factors that initiate angiogenesis and stimulate erythropoiesis. Both steps depend critically on proteasome function. As mentioned earlier, low oxygen tensions cause HIF1A to dimerize with ARNT. At present, it is not clear whether HIF1A must be activated in response to hypoxia or whether hypoxia-mediated inhibition of proteasome-dependent degradation is sufficient to regulate its activity, but there is strong evidence that the latter is important (*63*). Translocation of

HIF1A/ARNT to the nucleus causes transcription that includes the *VEGF* and erythropoietin (*EPO*) genes. The former acts on endothelial cells to initiate angiogenesis, while the latter promotes oxygen delivery. Signaling through the erythropoietin receptor (EPOR) and expression of the receptor itself are regulated by controlled proteolysis through the 26S proteasome (*137, 138*). Since angiogenesis within tumors through the HIF1A pathway is regulated by the ubiquitin/proteasome pathway, this pathway offers a promising target for future therapies directed at tumor-related anemia and tumor anti-angiogenesis.

The Proteasome and Apoptosis

One of the most striking observations in proteasome research is the fact that inhibitors of the proteasome induce apoptosis in almost every malignant cell line (*78, 120, 139–147*). Some normal cell types, such as thymocytes, are initially rescued by proteasome inhibitors from apoptosis induced by ionizing radiation, glucocorticoids or phorbol ester (*148*). Long-term inhibition of proteasomes will eventually result in cell death, but there appears to be a difference between normal and cancer cells that may be due to their proliferative status or to cancer cells relying more on stress pathways, both of which would be affected rapidly by proteasome inhibition.

Life without proteasome function is usually impossible, although compensatory mechanisms have been observed after extensive selection of EL-4 mouse lymphoma cells (*149*) and human PC-3 prostate carcinoma cells (Pajonk and McBride, unpublished results) that survived in the presence of a proteasome inhibitor. In EL-4 cells, TPPII, a giant protease, was shown to compensate for the loss of proteasome activity and to allow cells to escape cell death resulting from proteasome inhibition (*149, 150*).

The mechanism of apoptosis induced by proteasome inhibitors is incompletely understood and may vary with the cell line [for a recent review, see ref. (*151*)]. Drexler, as well as Soldatenkov and Dritschilo (*152*), suggested that activation of a BCL2-sensitive pathway is involved. In contrast, Hermann and coworkers excluded any involvement of BCL2 (*142*). We and others (*120, 142*) have demonstrated TP53-independent cell death using the MG-132 proteasome inhibitor. Caspase inhibitors were able to prevent DNA fragmentation but not apoptosis caused by lactacystin treatment of MO7e human myeloid progenitor cells, suggesting that caspase activation was a secondary effect rather than a direct effect (*141*). In our own studies, MG-132, which also inhibits calpain, did not cause caspase 3 activation in PC-3 prostate cancer cells (Pajonk and McBride, submitted for publication), which might be because calpain activity is necessary to cleave procaspase 3 to its active form (*153*). However, the cells still died by apoptosis.

Proteasome Function and Cancer Treatment

1. Proteasome function and radiation therapy

Studies in our laboratory recently demonstrated that proteasome inhibition radiosensitizes SiHa cervical cancer cells (Pajonk *et al.*, unpublished results), PC-3 prostate cancer cells (Pajonk and McBride, submitted for publication), and HD-MyZ Hodgkin's lymphoma cells (*120*). Comparable results have been reported for EMT-6 tumors (*154*). In our experiments, radiosensitization did not depend on the level of expression or activity of PRKDC (also known as DNA-PKcs) or on TP53 status. The mechanism of radiosensitization is unclear. One possibility is inhibition of the action of CDC25A, a phosphatase that is usually rapidly degraded by the 26S proteasome after exposure of cells to ionizing radiation and is required for the G_1- to S-phase transition of the cell cycle. Overexpression of CDC25A leads to enhanced DNA damage and decreased cell survival (*155*), but its role in radiosensitization induced by proteasome inhibitors has not been established. Recent observations also suggest that the proteasome is intimately involved in the control of DNA repair, although how this process is affected by proteasome inhibition is not known. In any event, these observations suggest that proteasome inhibitors might be an interesting new class of radiosensitizing drugs. The reversible proteasome inhibitor PS-341 has entered clinical trials as a single agent with some success, in particular in multiple myeloma and leukemia (J. Adams, personal communication). Toxicity does not seem to be a major problem, provided that the agent is given every four days to spare the gastrointestinal tract. The fact that a therapeutic differential can be obtained may be explained by proliferating or "stressed" cells being preferentially targeted.

Ionizing radiation itself can lead to rapid, dose-dependent inhibition of proteasome function to 60% of baseline levels (*156*). In ECV304 cells, this decrease occurred within the dose range of 0.2 to 2 Gy and was not increased further with higher doses up to 20 Gy. Inhibition could be achieved almost immediately after irradiation, and the effect lasted for up to 24 h. The implications of this finding are potentially important when considering radiation-induced protein expression. Expression of many proteins, such as TP53, JUN, FOS, TNF and NFKB, is rapidly up-regulated after irradiation by post-transcriptional mechanisms. Rapid inhibition of proteasome function provides a means by which this could be achieved.

The interplay between nontranscriptional and transcriptional control mechanisms in protein expression is of interest and could help provide an explanation for nonlinear dose–response curves. For example, the transcription factor NFKB, the major mediator of inflammatory responses, is activated by radiation. If proteasome function is inhibited by radiation, one would expect NFKB activation to be inhibited, not activated, because NFKBI degradation would be blocked. This apparent paradox can be resolved, because in many cells NFKB is activated only after high doses and in some situations may use a nonclassical pathway and does not involve a decrease in NFKBI expression (*157*). At lower doses, inhibition of NFKB activation has been detected, at least in one cell line (*156*). The universality of the effect has yet to be established, but if it is, it is possible that the clinically established daily fractions of 2 Gy were chosen in part because this was a dose that minimized inflammation, in addition to sparing late-responding normal tissues.

It is of interest that for treatment of benign inflammatory

and hyperproliferative diseases like insertion tendonitis (*158*) arthrosclerosis (*159*), vascular restenosis (*160*), arteriovenous malformations (*161*), endocrine ophthalmopathy (*162, 163*), pterygium (*162, 164*), induratio penis plastica (*165*), keloids and heterotopic ossifications (*161*), the doses of radiation that were used were often lower than those used for cancer treatment. The anti-inflammatory action of ionizing radiation has not been fully explained, but if the proteasome is inhibited at low doses, this could inhibit NFKB activation in this dose region. Also, given the observation that inhibition of proteasome function has a radiosensitizing effect on cancer cells, our findings could help to explain the hypersensitivity of mammalian cells to low-dose irradiation described by Joiner and coworkers (*166*) and others. Radiation-induced DNA damage might be amplified by the radiosensitizing effect of the proteasome inhibition caused by low-dose irradiation.

2. Proteasome function and chemotherapy

Proteasome inhibition is a promising way to induce cell death by apoptosis and to radiosensitize chemotherapy- and radiation-resistant cancers (*120, 143, 145–147*). Additionally, recent data indicate the possible direct involvement of the proteasome in the mechanism of action of chemotherapeutic agents and in resistance to chemotherapy: For example, the anthracycline antibiotic doxorubicin accumulates rapidly in the nucleus of malignant cells. Doxorubicin is known to bind to high-molecular-weight proteins that exhibit chymotrypsin-like proteolytic activity. It has been suggested that these are proteasome subunits and that, because many proteasome subunits contain nuclear translocation signals and proteasome subunit expression is increased in malignant cells, the proteasome acts as a carrier for nuclear uptake of doxorubicin (*167*).

Bleomycin hydrolase, which deamidates the anti-cancer drug bleomycin, is a neutral cysteine protease with structural similarity to the 20S proteasome. Yeast two-hybrid studies identified the human homologue of yeast ubiquitin-conjugating enzyme 9 (UBC9) as a binding partner for bleomycin hydrolase, linking the ubiquitin system to chemotherapy resistance (*168*). Also, preclinical studies have shown that the proteasome inhibitor PS-341 (*169*) has additive antitumor effects when combined with 5-fluorouracil, cisplatin, paclitaxel and Adriamycin (*154*). Furthermore, it is remarkable that resistance to chemotherapy based on expression of the P-glycoprotein (P-gp), coded by the multidrug resistance gene 1 (*ABCB1*, also known as *MDR1*), can be overcome by co-administration of substances like cyclosporin A (*170*), and HIV-1 protease inhibitors like ritonavir (*171*), MG-132 (*172*) or PS-341 (*173*), which share an inhibitory effect on proteasome function. In the presence of MG-132, ubiquitinated forms of P-glycoprotein accumulate, but since lactacystin failed to inhibit P-gp function, the exact functional interaction with the proteasome pathway is not clear (*172*). As with radiation therapy, the utility of proteasome inhibitors with chemotherapeutic agents will depend on the therapeutic benefit that can be obtained. The first clinical trials using the proteasome inhibitor PS-341 seem promising, because this drug is surprisingly well tolerated when applied systemically (J. Adams, personal communication), although the basis for any differential effect remains elusive, other than perhaps a tendency to target cycling cells (*174*).

CONCLUDING REMARKS

Our understanding of how protein expression is regulated within a cell has improved dramatically in recent years. While most attention has been focused on the pathways of gene transcription, it is now well accepted that post-transcriptional mechanisms are also important. The role of the proteasome pathway as a post-translational control mechanism has been extended so that it now appears to leave almost no area of biological research untouched. Once thought to contain little specificity, the proteolytic process mediated by the proteasome now is seen as an exceptionally well-regulated pathway with high specificity, most of which lies in the family of E3 ubiquitin ligases, many of which have yet to be identified. In the future, targeting these E3 enzymes using competitive inhibitors might be an efficient and highly specific pharmacological way to manipulate almost any pathway involved in the pathophysiology of cancer.

Other levels of control over proteolysis also operate, and it is clear that proteasome function can be modified by cytokines, ionizing radiation, heat, hypoxia/reperfusion, and other oxidative stresses. The proteasome itself is therefore a highly responsive system that functions in concert with phosphorylation and dephosphorylation to allow cells to make rapid and appropriate initial responses to a wide variety of insults. Its role in carcinogenesis and cancer treatment is only beginning to be understood, but it is going to be a prime topic for proteomic research in the immediate future.

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Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses

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Abstract

Background and purpose: Ionizing radiation is known to activate certain signal transduction pathways, the regulation of which could involve post-transcriptional as well as transcriptional mechanisms. One of the most important post-transcriptional pathways in eukaryotic cells is the ATP- and ubiquitin-dependent degradation of proteins by the 26s proteasome. This process controls initiation of many cellular stress responses, as well as inflammatory responses under control of the transcription factor NF-κB. The literature on the relationship between radiation and inflammation seems somewhat paradoxical. At high doses, radiation is generally pro-inflammatory. On the other hand, low dose radiation has a long history of use in the treatment of inflammatory disease. This suggests the involvement of multiple mechanisms that may operate differentially at different dose levels.

Materials and methods: In this paper, the ability of different doses of ionizing radiation to directly affect 26s proteasome activity was tested in ECV 304 cells. Proteasome activity, IκBα protein levels, and NF-κB activation were monitored.

Results: Inhibition of chymotrypsin-like 20s and 26s proteasome activity was observed immediately after low- and high-dose irradiation either of cells or purified proteasomes. The inhibitory effect was independent of the availability of the known endogenous proteasome inhibitor heat shock protein 90 (hsp90). Levels of IκBα, a physiological 26s proteasome substrate, were increased only at low doses (0.25 Gy) and unaltered at higher doses whereas only the highest doses (8 and 20 Gy) activated NF-κB.

Conclusions: We conclude that the proteasome is a direct target of ionizing radiation and suggest that inhibition of proteasome function provides a molecular framework within which low dose anti-inflammatory effects of radiation, and radiation-induced molecular responses in general, should be considered.

Keywords: Gene regulation; Transcription factors; Signal transduction; Acute phase reactants; Inflammatory mediators

1. Introduction

In recent years considerable interest has been shown in the mechanisms by which ionizing radiation induces early molecular responses and how these might influence subsequent radiation-related events [8,16]. Cellular gene expression can be re-orchestrated by radiation within minutes to hours. The most frequently reported immediate early responses include up-regulation of expression of the transcription factors JUN, FOS, and NF-κB, [2,4,14,15,20,28, 43,44,47,50] and of molecules implicated in recognition and repair of damaged DNA, in responses to oxidative stress, and in cell cycle arrest and death [16]. These immediate early responses co-ordinately link radiation damage to pathways that promote wound healing and tissue remodeling [8].

Gene expression can be modified by post-transcriptional as well as transcriptional mechanisms. The former are generally the more rapid. NF-κB, which is the prime player linking radiation and other signals to inflammatory responses, is a good example. NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105). Activation of this pathway is normally achieved by phosphorylation of one of the most important inhibitors, IκBα, at two serine-sites (Ser-32 and Ser-36) by IκB kinases. This marks IκBα for polyubiquitination and subsequent degradation by the 26S proteasome. Degradation of IκBα frees NF-κB for translocation to the nucleus and activation of its target genetic programs (reviewed in [3]).

NF-κB activation in response to pro-inflammatory agents such as TNFα is a redox-sensitive process [42]. It is therefore not surprising that ionizing radiation can activate NF-κB [31] through the classical ubiquitin/proteasome-depen-

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dent pathway [7,26], although in astrocytes and human brain tumor cells, phosphorylation of IκBα at tyrosine residues without its subsequent degeneration has been suggested as an alternate pathway [39]. In lymphocytes, NF-κB activation that is non-linear with dose has been reported [31,26], and this is the case for certain other radiation-induced molecular responses [43]. It seems likely that this non-linearity is the result of multiple mechanisms that operate differentially with dose.

Because ubiquitination and degradation of negative regulatory molecules through the proteasome is often a first critical step for activation of many molecular pathways, we have investigated the possibility the proteasome itself is a redox-sensitive target for irradiation. The 26s proteasome is responsible for the controlled ATP-and ubiquitin-dependent degradation of all short-lived [9] and 70–90% of all long-lived proteins [9,25], including key molecules in signal transduction, cell cycle control, and immune response [41]. Recently, it has become clear that this activity can be intrinsically regulated. Here, we provide evidence that the 26s proteasome complex is a direct target of ionizing radiation and that this mechanism operates functionally to inhibit activation of NF-κB at low radiation doses. It is of interest that although high dose radiation is generally pro-inflammatory [30], the history of radiation therapy for benign diseases in particular is replete with examples where ionizing radiation was used to terminate preexisting inflammatory conditions [45]. The molecular basis for this paradox is unexplained, although low radiation doses have been shown to inhibit nitric oxide production by macrophages, while high doses result in super-stimulation [18]. The relative contributions of post-transcriptional and transcriptional control mechanisms could help to explain some of the immediate early molecular effects of ionizing radiation at different dose levels.

2. Materials and Methods

2.1. Cell culture

Cultures of ECV 304 human bladder carcinoma cells (ATCC) and RAW 264.7 murine macrophages (a generous gift of Dr G. Hildebrandt, Department of Radiation Oncology, University Leipzig) were grown in 75 cm^2 flasks (Falcon) at 37°C in a humidified atmosphere at 5% CO_2. The medium used was DMEM medium (Gibco BRL) supplemented with 10 % FCS, 1 % penicillin/streptomycin (Gibco BRL), and 0.5 mg/ml fungizone (amphotericin B, Gibco BRL).

2.2. Irradiation

EVC 304 cells were trypsinized, counted and 1×10^5 cells were plated into culture dishes (Falcon, 5 cm). After 24 h plates were irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shepherd, Mark I) at a dose rate of 5.527 Gy/min. Corresponding controls were sham irradiated. Partially purified proteasome fractions were resuspended and immediately irradiated on ice. Control samples were sham irradiated.

2.3. Proteasome function assays

Proteasome function was measured as described previously [12] with some minor modifications. To obtain crude cellular extracts, cells were washed with PBS, then with buffer I (50 mM Tris (pH 7.4) 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation ($1000 \times g$, 5 min, 4°C). Glass beads and homogenization buffer (50 mM Tris (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at $1000 \times g$ for 5 min and $10\,000 \times g$ for 20 min at 4°C.

Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Partially purified proteasomes were prepared as described previously [22] with some minor modifications. Crude cellular extracts were subjected to ultra-centrifugation at $100\,000 \times g$ at 4°C for 3 h. The supernatant was transferred into fresh tubes and the resulting pellet was resuspended in homogenization buffer.

To measure 26s proteasome activity, 10 μg protein of crude cellular extracts or 1 μg protein of partially purified proteasomes of each sample was diluted with buffer I to a final volume of 200 μl. For assessment of 20s proteasome activity, 1 μg of protein was diluted to a final volume of 200 μl in a buffer consisting of 50 mM Tris–HC1 (pH7.9), 0.5 mM EDTA and 0.05% SDS. PS-34 1 [1] was kindly provided by Julian Adams, ProScript Inc., MA and was solubilized in acidified ethanol and stored in small aliquots at a concentration of 0.5 mg/20 μl at −80°C. PS-341 was added to the reaction buffers at a final concentration of 5 μM to prove the specificity of the cleavage reaction. The fluorogenic proteasome substrate SucLLVY-MCA (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 100 μM in 1% dimethyl sulfoxide (DMSO). Proteolytic activity was continuously monitored by measuring the release of the fluorescent group 7-amido-4-methylcoumarin (AMC) in a fluorescence plate reader (Spectrafluor, Tecan, 37°C) at 380/460 nm.

2.4. Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris–HC1 (pH 7.2), 150 mM NaC1, 1% Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodium vanadate). Protein concentrations were determined using the BCA protocol (Pierce) with BSA (Sigma) as standard. 50 μg of protein were electrophoresed in a SDS gel (0.1% SDS/12% polyacrylamide) and blotted to PVDF membranes at 4°C. Uniformity of loading was confirmed by Coomassie staining. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, 5%

skim milk) for 1 h at room temperature the membranes were incubated with a polyclonal antibody against human IκBα (0.5 μg/ml, Santa Cruz Biotechnologies) for 1 h at room temperature. A secondary HRP-conjugated antibody and the ECLplus system (Amersham) were used for visualization.

2.5. Cell extracts and electrophoretic mobility shift assays

For preparation of total cytosolic extracts, normal and treated cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX-buffer (20 mM HEPES (pH 7.9), 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, PMSF and aprotinin) for 30 min on ice. Lysate was centrifuged at 12 000 × *g* for 5 min at 4°C. Protein concentration was determined using the BCA protocol (Pierce) and bovine serum albumin (BSA, Sigma) as standard. 15 μg protein of the resulting supernate was incubated for 25 min at room temperature with 2 μl BSA (10 μg/μl), 2 μl dIdC (1 μg/μl), 4μl Ficoll-buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF), 2 μl buffer D + (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40, 2 mM DTT, 0.1 mM PMSF) and 1 μl of the [γ^{32}P]-ATP labeled oligonucleotide (Promega, NF-κB: AGT-TGA GGG GAC ITT CCC AGG). For a negative control, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5 % TBE gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphor imager (Storm 860, Molecular Dynamics).

2.6. Transduction experiments

The recombinant replication-deficient adenoviruses Ad5-IκB and Ad5-LacZ were generously provided by Dr. R. Batra (UCLA/VAGLAHCS, Los Angeles, CA). The vectors had been generated and quality tested at the Vector Core at the Gene Therapy Center of the University of North Carolina, School of Medicine and are described elsewhere [27]. Ad5-IκB contains a gene for a NF-κB super-repressor IκBα under control of a CMV-promoter/enhancer. The encoded protein contains serine-to-alanine mutations in residues 32 and 36, preventing its phosphorylation, ubiquitination, and subsequent degradation by the 26s proteasome. The Ad5-LacZ is a control virus that contains the gene for β-galactosidase instead of IκBα. Transduction was performed as described previously [37]. Briefly, cells were plated into culture dishes (10 cm, Falcon). After 24 h, the medium was changed and viral vectors containing the non-phosphorable IκBα or β-galactosidase gene was added at a multiplicity of infection (MOI) of 1000. After 2 h incubation, the virus-containing medium was replaced by fresh medium and cells were incubated for additional 48 h to allow gene expression.

2.7. Drug treatment

The 26s proteasome inhibitor MG-132 (Calbiochem) was dissolved in DMSO (10 mM) and small aliquots (30 μl) were stored at −20°C. Three hours before irradiation, growth medium was replaced by medium containing MG-132 (50μM, 0.5% DMSO). Control cells were subjected to DMSO treatment alone (final concentration 0.5%). In order to activate NF-κB RAW 264.7 cells were stimulated by addition of 0.1 μg/ml lipopolysaccharide (LPS from *Eschericia coli*; Serotype 0111:B4) and 100 U/ml murine recombinant interferon-gamma to the complete medium for 6 h before irradiation.

3. Results

3.1. Ionizing radiation inhibits 20s and 26s proteasome function

There are numerous reports on the use of the ECV 304 cell line as a model for human endothelium. Although this cell line has been recently identified to be a variant of the T24 bladder carcinoma cell line [11], it mimics an endothelial phenotype. In this study, it served as model for cells in an inflammatory environment by exhibiting constitutive and inducible NF-κB and ICAM-1 activity. RAW 264.7 murine macrophages are also a well accepted model for studying inflammatory responses [18].

In order to explore the 26s proteasome as a possible direct target of ionizing radiation, cells were irradiated with different doses and incubated for 30 min, 3 or 24 h. Chymotrypsin-like 26s proteasome function was assessed in crude extracts of ECV 304 cells by the rate of release of the fluorogenic compound 7-amido-4-methylcoumarin (AMC) from the proteasome substrate Suc-LLVY-AMC with continuous monitoring.

Doses from 0.17 to 2 Gy gave a rapid, dose-dependent decrease in chymotrypsin-like 26s proteasome activity from 73% to 53% of baseline levels at 3 h after irradiation. Higher radiation doses of 4, 8, or 20 Gy did not increase the extent of inhibition (Fig. 1A). Inhibition was observed as early as 30 min after irradiation and had not completely recovered by 24 h (data not shown).

The possible contribution of heat-shock protein 90 (hsp90) to the inhibitory effect, which has been described as a preformed endogenous inhibitor of proteasome function [46], was also investigated. Binding of hsp90 to any of its dimerization partners can be selectively disrupted by the benzoquinone ansamycin geldanamycin [24]. Preincubation of ECV 304 cells with geldanamycin increased proteasome activity, in keeping with it blocking hsp90 activity, but it failed to prevent radiation-induced proteasome inhibition when compared to non-irradiated geldanamycin-treated controls, excluding the possibility of radiation-

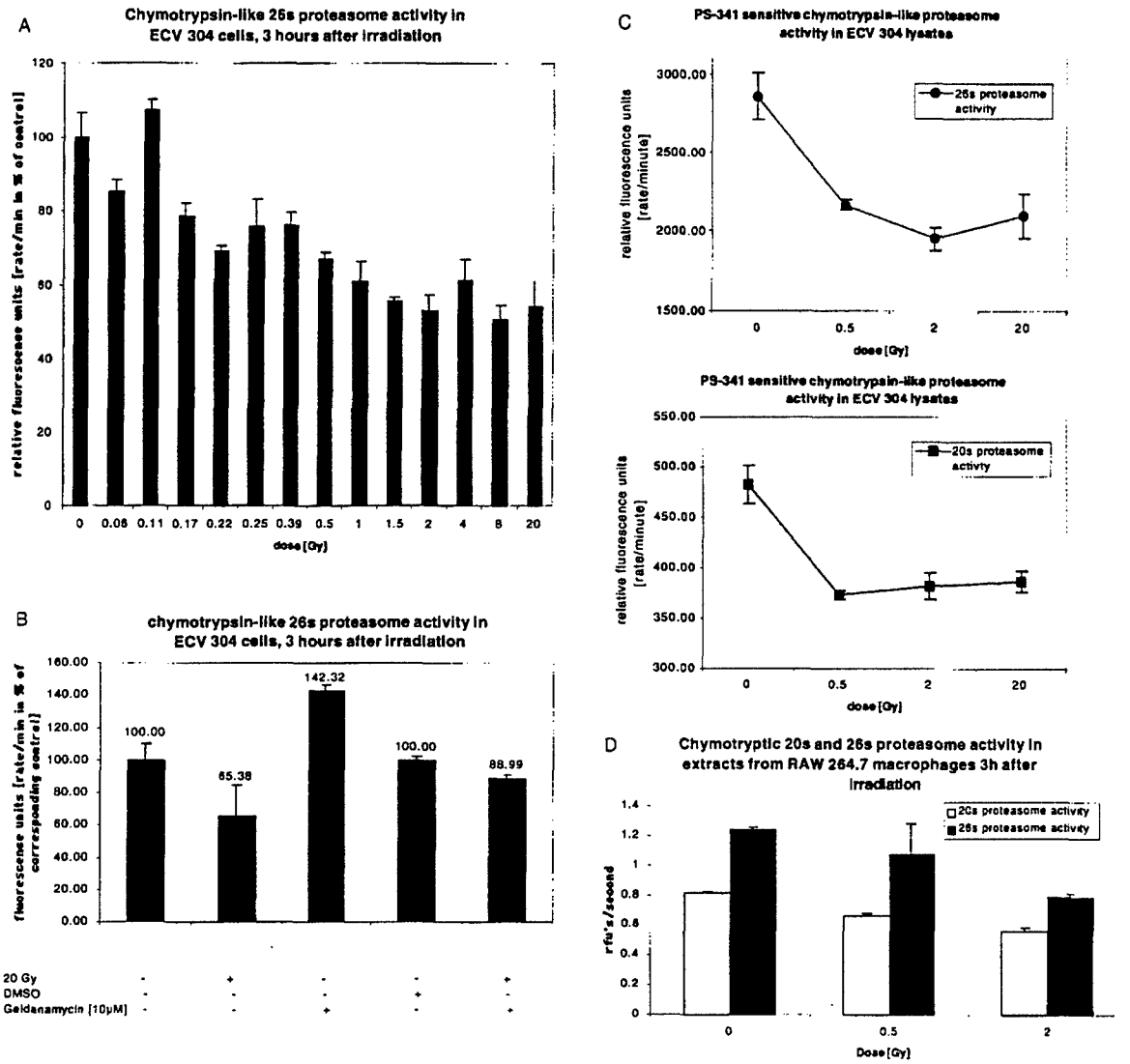

Fig. 1. (A) 26s proteasome function assay for chymotrypsin-like proteolytic activity in crude lysates of ECV 304 cells, 3 h after application of different dose of ionizing irradiation ($n = 4$, means ± standard deviation). Low dose ionizing radiation (0.17–2 Gy) caused a significant reduction of 26s proteasome cleavage activity (2 Gy: 52.97% ± 4.3 of untreated control cells, $P < 0.001$, Student's t-test). Application of higher doses (4–20 Gy) could not further enhance this effect. (B) Chymotrypsin-like 26s proteasome activity in crude lysates of ECV 304 cells, 3 h after irradiation ($n = 4$, means ± standard deviation). Application of 2Gy ionizing radiation significantly reduced the proteolytic activity of the complex (65.4% ± 6.7, $P < 0.001$, Student's t-test) when compared to sham irradiated controls. Inactivation of hsp90 by pretreatment of cells (30 min before irradiation) with geldanamycin (10 µM) increased the baseline cleavage rate 1.4 fold (142.3% ± 4.4, $P < 0.001$, Student's t-test, compared to DMSO treated controls), but failed to prevent radiation induced proteasome inhibition. (C) 26s and 20s proteasome activity in purified proteasome preparations from ECV 304 cells. The purified complex was irradiated on ice. The baseline activity of the 26s proteasome complex was about 6-fold higher than the activity of the 20s proteasome complex. Application of 0.5, 2 and 20 Gy immediately and significantly inhibited 26s and 20s proteasome activity when compared to sham irradiated control preparations (upper graph. 26s proteasome activity: 0.5 Gy: 75.8% ± 1.1, $P < 0.01$; 2 Gy: 68.3% ± 2.5, $P < 0.01$; 20 Gy: 73.4% ± 5.1, $P < 0.05$, Student's t-test. Lower graph. 20s proteasome activity: 0.5 Gy: 77.4% ± 0.9, $P < 0.01$; 2 Gy: 79.3% ± 2.7, $P < 0.01$; 20 Gy: 80.2% ±2.2, $P < 0.01$, Student's t-test ($n = 2$, means ± standard deviation).

induced liberation of this inhibitor from intracellular binding partners as a mechanism of inhibition (Fig. 1B).

The contribution of cytoplasmic components to the inhibitory effect was further excluded by showing that irradiation of purified 26s proteasome complexes from ECV 304 cells on ice directly inhibited their activity. Application of 0.5 to 20 Gy caused a highly significant reduction of both 26s and 20s chymotrypsin-like proteasome activity (Fig. 1C; 2 Gy, 26s: 68.3 ± 2.5%; 20s: 79.3 ± 2.7%). The pelleted complexes that were used in these experiments contained practically all of the cellular chymotrypsin-like cleavage activity, and activity could be inhibited by the

specific proteasome inhibitor PS-341 [1] at a concentration of 5 μM. It seems therefore that the effect of radiation on the proteasome is direct.

In order to show that this effect is not restricted to human ECV cells, we extended our experiments to murine RAW 264.7 macrophages. RAW 264.7 were irradiated with 0.5 and 2 Gy and incubated for 3 h at 37°C. Control cells were sham-irradiated. Chymotryptic 20s and 26s proteasome activity was monitored in subsequently prepared lysates at different substrate concentrations (25, 50, 100 and 200 μM SucLLVY-AMC). 20s as well as 26s proteasome activity was reduced in a dose-dependent manner (Fig 1D/F; 20s: 0.5 Gy 81.5 ± 1.7%, 2 Gy 69.1 ± 2.6%; 26s: 0.5 Gy 86.5 ± 17.3%, 2 Gy 63.5 ± 1.9%, 200 μM SucLLVY-MCA).

3.2. Ionizing radiation stabilizes target molecules of the 26s proteasome

Activation of NF-κB in response to a variety of stimuli is generally characterized by disappearance of IκB as a result of its phosphorylation and subsequent proteasome-mediated degradation. Proteasome inhibitors can attenuate NF-κB induction by blocking this degradative pathway [26]. The observed radiation-induced inhibition of proteasome function by radiation is at odds with radiation-induced NF-κB activation. We therefore considered the possibility that radiation has both inhibitory and enhancing effects on NF-κB activation, that multiple mechanisms operate, and that the outcome depends on radiation dose.

To investigate the relationship between radiation-induced inhibition of proteasome function and expression of IκBα, immunoblotting studies were performed using a polyclonal antibody against IκBα with total cellular extracts of ECV 304. IκBα expression was increased 30 min after irradiation with the lowest doses (0.25–0.5 Gy), in keeping with an effect of proteasome inhibition (Fig. 2), but was essentially not altered after higher doses.

3.3. Radiation-induced NF-κB activity

To examine NF-κB activity, a gel shift assay was used 3 h after various radiation doses. Analysis of eight independent gel-shift experiments showed no significant change in NF-κB DNA-binding activity after 0.25 Gy (1.02-fold ± 0.06) or 0.5 Gy (1.03-fold ± 0.03). Irradiation with 1, 1.5, 2 and 4 Gy caused a slight consistent, but not significant, elevation in NF-κB activity. Significant increases were achieved only after application of 8 Gy (1.24-fold ± 0.03, $P < 0.01$, Student's *t*-test) or 20 Gy (1.3-fold ± 0.05, $P < 0.001$, Student's *t*-test) (Fig. 3A,B).

These experiments with ECV 304 cells dealt with constitutive NF-κB DNA-binding activity, rather than with an induced inflammatory response. In order to investigate this aspect of the effect of ionizing radiation on inflammatory responses, we activated NF-κB in RAW 264.7 macrophages using LPS and IFN-α. Gel-shifts from total cellular lysates prepared 3 h after irradiation revealed a dose dependent decrease in NF-κB DNA-binding activity (Fig. 3C), showing that radiation could suppress, as well as activate, NF-κB activity.

The dependence of NF-κB activation by radiation on proteasome activity and IκB turnover has been questioned [39], largely because of observations that IκBα expression does not decrease in the same manner as it does in response to TNF stimulation [32]. At least in ECV 304, NF-κB activation still appears to depend on this pathway, although the kinetics of IκB turnover may be altered. Expression of the 'super repressor' IκBα mutant, which contains alanines at positions 32 and 36, prevented constitutive and radiation-induced activation of NF-κB (Fig. 4A), as did treatment with MG-132 (Fig. 4B) at doses that inhibited proteasome activity completely [36]. Transduction with the β-galactosidase gene did not decrease, and in fact increased, NF-κB activity, in keeping with the pro-inflammatory nature of the adenoviral vector, and NF-κB activity was still inducible by irradiation (Fig. 4A).

4. Discussion

Knowledge about molecular mechanisms activated after application of ionizing radiation is a key to understanding early and late side effects of radiation therapy. Also, identification of the sub-cellular targets of ionizing radiation might uncover new approaches to improve treatment outcome and to minimize toxicity.

Certain aspects of the molecular response to ionizing radiation can appear paradoxical. On one hand, signal transduction pathways leading to production of pro-inflammatory cytokines and cell adhesion molecules can be rapidly activated [19]. A major player in this response is the transcription factor NF-κB [26] (reviewed in [29]). One the other hand, ionizing radiation has anti-inflammatory and immunosuppressive effects in certain situations and the use of ionizing radiation in the treatment of benign inflammatory as well as benign hyperproliferative conditions is almost as old as the knowledge about radiation itself [48].

At present there are about 150 stimuli known to activate the NF-κB, including ionizing radiation, TNFα, and LPS, each causing transcriptional activation of about the same number of NF-κB dependent genes (reviewed in [34]). The exact mechanism of how radiation activates NF-κB is not known, but the initial steps are redox sensitive (reviewed in [42]). The classic mechanism of serine phosphorylation and degradation of IκB through the ubiquitin proteasome system [26] may be involved, although alternative pathways have been reported [39]. Some of the more paradoxical effects of radiation could be explained by multiple mechanisms operating at different dose levels to give what are essentially non-linear responses. Furthermore, many radiation-induced immediate early gene effects could be explained by alterations in proteasome processing.

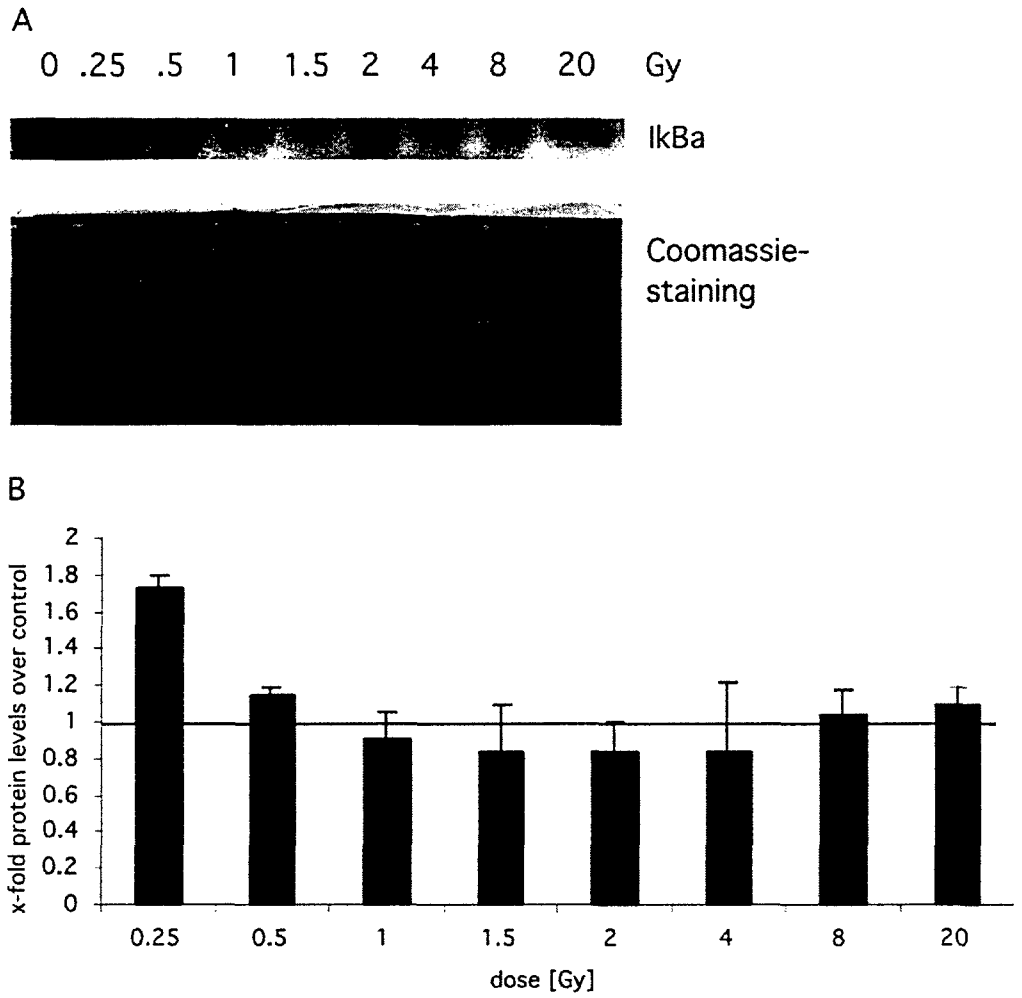

Fig. 2. (A,B) Western blot analysis of total cellular lysates from ECV 304 cells 30 min after irradiation using a polyclonal antibody against IκBα. Equity of loading was confirmed by coomassie staining. Application of 0.25 Gy significantly increased IκBα protein levels (173.2% ± 6.2, $P < 0.05$, Student's t-test) while higher doses did not cause significant changes ($n = 2$, means ± standard deviation).

We have demonstrated that the proteolytic activity of the 20s and 26s proteasome was directly compromised by ionizing radiation and that this inhibitory effect was not species- or cell-type-dependent. The observed effect was immediate and lasted for at least 24 h. After low dose (<2 Gy) irradiation of cells, 26s proteasome function declined to about 60% of baseline levels. Higher doses did not further decrease the activity. This inhibitory effect was also observed when purified proteasome preparations were irradiated on ice, indicating the involvement of a radiochemical rather than a biological mechanism. Reinheckel et al. [40] have reported that proteasome activity is sensitive to oxidative damage following direct addition of hydrogen peroxide. About 60% inhibition was achieved with concentrations of hydrogen peroxide up to 1 mM. Higher hydrogen peroxide concentrations did not lead to a further significant decrease of proteolytic activity. This suggests that the proteasome is sensitive to redox changes. Our finding that proteasome activity is inhibited by low concentrations of *N*-acetylcysteine (Pajonk, unpublished) is consistent with this view. The finding that radiation affected both 26s and 20s proteasome activity, suggests that ionizing radiation inhibits the catalytic sites in the 20s core complex, though effects on the regulatory cap molecules can not be excluded. It is also possible that within the cell additional mechanisms play a role, although our experiments tend to exclude participation of the known endogenous inhibitor hsp90.

In ECV 304 cells, expression of IκBα, the most important member of the IκB molecular family, was enhanced after application of low doses of radiation that blocked protea-

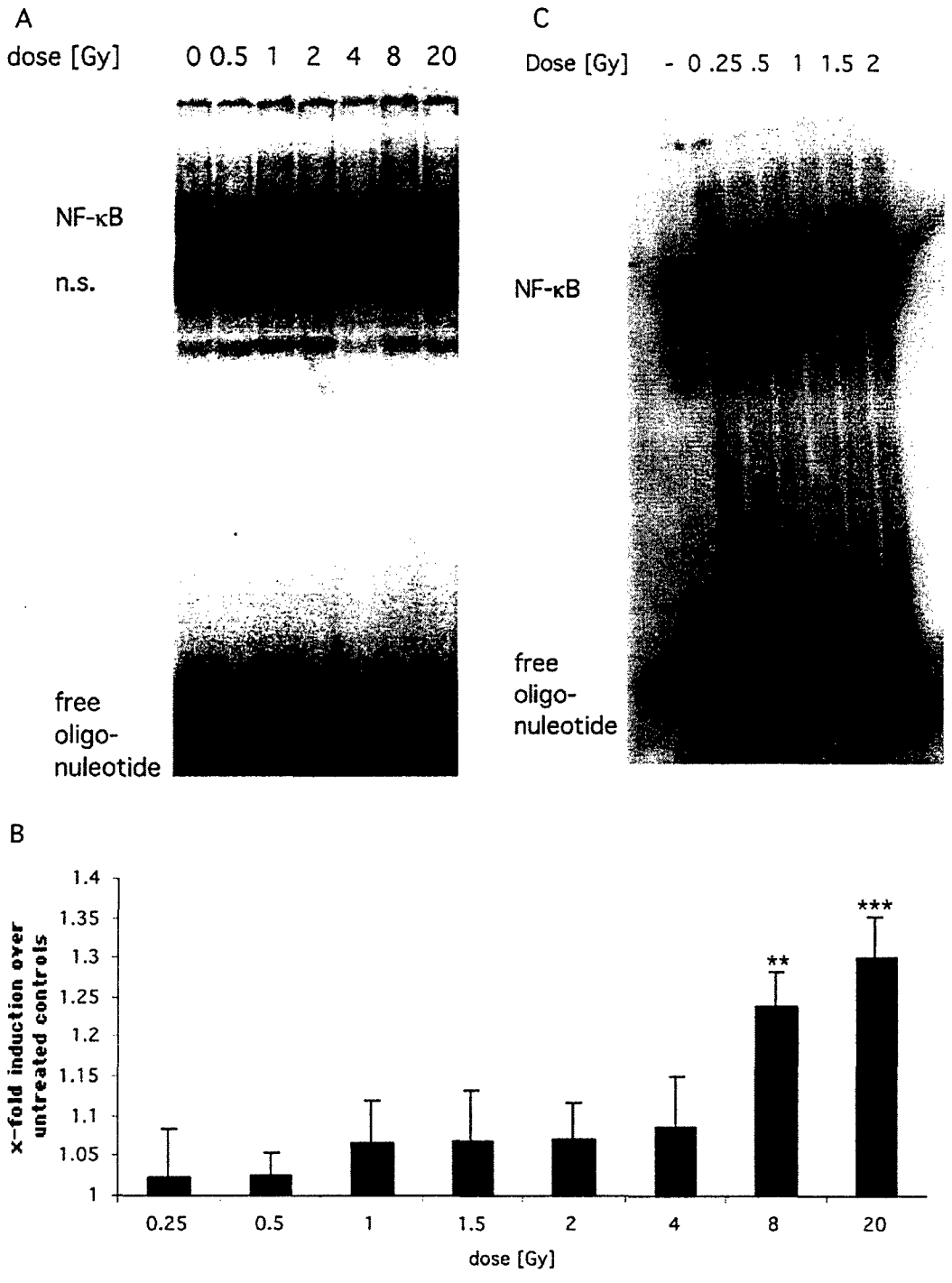

Fig. 3. (A) Representative gel-shift experiment and densometric analysis (B) of NF-κB DNA-binding activity in lysates from ECV 304 cells 3 h after irradiation (means ± standard deviation from eight independent experiments). Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2: untreated control cells. Lane 3 to 10: NF-κB activity after application of 0.25–20 Gy ionizing radiation. NF-κB DNA-binding activity in response to ionizing radiation is retarded and increases over baseline levels only after application of 8 and 20 Gy. (C) Representative gel-shift experiment of NF-κB DNA-binding activity in lysates from RAW 264.7 murine macrophages 3 h after irradiation. Cells have been pre-stimulated using LPS- and IFN-γ-supplemented media for 6 h. Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2–7: NF-κβ activity after application of 0 (lane 2), 0.25 (lane 3), 0.5 (lane 4), 1 (lane 5), 1.5 (lane 6) and 2 Gy (lane 7) of ionizing radiation.

some activity and was not decreased after higher doses, even though they caused NF-κB activation. Inhibition of proteasome activity may explain the failure of others to demonstrate decreases in IκBα levels after irradiation [39].

Radiation doses up to 4 Gy failed to significantly alter constitutive NF-κB DNA-binding activity in ECV cells and decreased activity in stimulated RAW macrophages, in keeping with a low dose inhibitory effect. A number of studies have shown that radiation activates NF-κB in a fashion that is non-linear with dose [17,26,31,38,39], as is the case in our study. Previous reports showed NF-κB activation to be maximal at 0.5 Gy in EBV-transformed 244B human lymphoblastoid cells [31,38], while primary human B cells show a strong activation of NF-κB at 15 Gy [49].

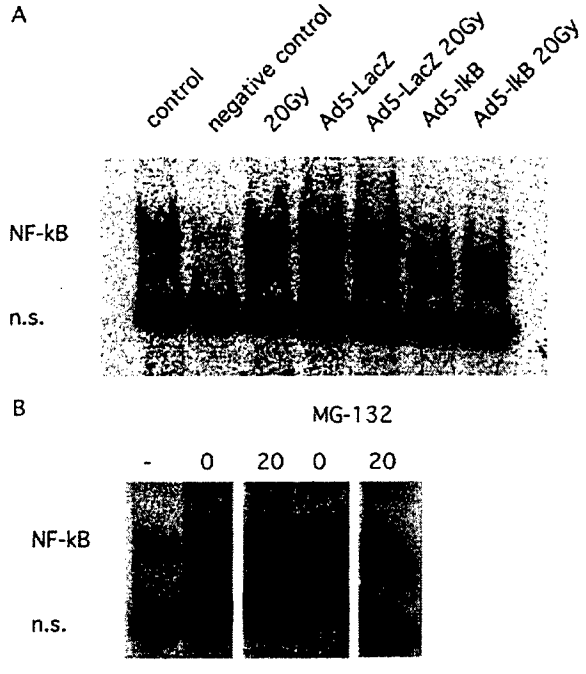

Fig. 4. (A) Representative gel-shift experiment with lysates of ECV 304 cells. Cells were transduced with an adeno-viral vector containing either a gene for B-galactosidase (Ad5-LacZ) or a gene for an IκB superrepressor (Ad5-IκB). The IκB superrepressor has serine-to-alanine mutations at position 32 and 36 and is thus unphosphorable by IκB kinases. 48 h after transduction the cell were treated with 20 Gy ionizing radiation and incubated for additional 3 h. Constitutive and radiation induced activation of NF-κB was blocked by the expression of the IκB superrepressor but not by the B-galactosidase gene. Additionally, transduction with the Ad5-LacZ caused an increase in constitutive NF-κB activity. Lane 1: untreated control cells. Lane 2: negative control; the unlabeled oligonucleotide was added in 50-fold excess to show specificity of binding. Lane 3: non-transduced cells after 20 Gy. Lane 4 and 5: cells transduced with Ad5-LacZ after 0 and 20 Gy. Lane 6 and 7: cells transduced with Ad5-IκB after 0 and 20 Gy. (B) Gel-shift experiment for NF-κB in ECV 304 cells 3 h after application of ionizing irradiation with and without preincubation with the proteasome inhibitor MG-132 (50 μM, 3 h). Proteasome inhibition prevents constitutive and radiation induced activation of NF-κB. Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2 and 3: NF-κB activity in ECV 304 cells 3 h after irradiation with 0 Gy (2) or 20 Gy (3). Lane 4 and 5: NF-κB DNA-binding activity in ECV 304 cell 3 h after irradiation with 0 Gy (4) or 20 Gy (5) and preincubation with the proteasome inhibitor MG-132.

NF-κB activation following ionizing radiation and other stimuli generally requires degradation of IκBα as an obligatory step [23,26]. NF-κB activation without degradation of IκBα has been reported for UV radiation [6,26] and anoxia [21], and in a recent study following exposure of brain cells to ionizing radiation [39]. The latter study suggested tyrosine-phosphorylation of IκB as a mechanism, although this was not demonstrated directly and the effects of erbstatin could have been on proteins upstream of IκB as has been described for NF-κB activation after TNFα treatment [32]. In our study, classic processing of serine phosphorylated IκB through the proteasome following irradiation seems to be responsible for NF-κB activation. NF-κB activation was blocked by addition of the proteasome inhibitor MG-132, as Hallahan et al. [17] also reported. In addition, the IκB super-repressor gene, which contains serine-to-alanine mutations at position 32 and 36, preventing its serine-phosphorylation by IκB-kinases and subsequent degradation by the 26s proteasome, blocked constitutive and radiation-induced NF-κB. Transduction with the vector carrying the gene for β-galactosidase actually activated NF-κB in ECV 304 cells. This activation could be the result of an ER-overload response [33,35] or a direct pro-inflammatory effect of the vector as described previously [10].

Although the classic pathway of NF-κB activation appeared to operate, like Raju et al. [39], we did not find a decrease in IκBα after ionizing radiation. A possible explanation is that the increased rate of degradation after phosphorylation was offset by inhibition of the rate of proteasome degradation, resulting in little change in expression. Thus, the dose dependency of the response is most likely determined at the level of phosphorylation and the outcome is impacted at all doses by inhibition of the rate of degradation and alteration in the kinetics of IκBα turnover. Further studies are needed to determine if this is the case.

Maximal proteasome inhibition by radiation in this study was achieved even with low doses such as have been used in the treatment of benign disorders. Furthermore, our findings might provide a theoretical basis for empirically established daily fractions of 1.8–2 Gy in the treatment of malignant disorders, resulting in maximal therapeutic benefit with minimized acute reactions. It is intriguing to note that, in addition to NF-κB, expression of p53, p21, p27, pRb, and several cyclins is regulated by proteasomal degradation [41]. The inhibitory effect of ionizing radiation on proteasome activity therefore should be taken into account when considering many of the radiation induced molecular changes, including those leading to cell cycle arrest. Also, since the proteasome is responsible for much of the processing of antigen for presentation by MIHC-I molecules (reviewed in [5,13]), part of the immunosuppressive effects of radiation could be mediated by through this pathway.

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NF-κB, Cytokines, Proteasomes, and Low-Dose Radiation Exposure

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Ionizing radiation shares with proinflammatory cytokines a pathway that involves reactive oxygen species and activation of the redox-sensitive nuclear transcription factor NF-κB, which leads to expression of inflammatory and cell survival programs. NF-κB activation normally requires phosphorylation of its inhibitor IκB and the inhibitor's subsequent degradation by the proteasome. Nonlinear dose-response curves have been reported for both radiation-induced cytokines and NF-κB and IκB expression with maximum exposures of less than 2 Gy and greater than 4 Gy, respectively. Radiation-inhibited proteasomes function over a wide dose range, suggesting that the proteasome is a redox-sensitive target for radiation that may function along with transcription to cause nonlinear dose-response relationships for early expression of many molecules, including NF-κB and cytokines. These pathways are relevant to low-dose radiation effects, adaptive responses, and carcinogenesis.

Introduction

Numerous reports indicate that radiation alters gene expression.[1] Few studies have examined responses systematically over a wide dose range, but there is evidence that certain responses can be elicited by doses of less than 1 Gy, whereas others require higher doses.[2,3] The spectrum of radiation-induced genes can be viewed within the functional context in which they might operate.[4] Thus, molecules that act as immediate early gene products or that signal cell cycle arrest or apoptosis are commonly induced. In addition, cytokines and cell adhesion molecules are expressed that serve to generate inflammation, tissue repair, and recovery—in other words, a wound-healing response. Associated functions of these molecules may include priming surrounding cells for adaptive survival responses, inducing genomic instability, and mediating radiation injury.

Ionizing radiation displays an interesting homology with certain cytokines and stress signals that has its roots in their common use of reactive oxygen species (ROS), which most likely accounts for the cross-talk between these signals. Two possible but diametrically opposed outcomes of ROS-mediated pathways are (1) induction of apoptosis and (2) development of resistance to further ROS effects. The outcome depends on the molecular wiring intrinsic to the cell, the magnitude of the signal, and other variables.

Integral to cellular ROS responses is NF-κB. NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered in the cytosol by inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105).[5] The classic pathway for activation of NF-κB is by phosphorylation of the inhibitor IκBα by IκB kinases, which marks IκBα for polyubiquitination and subsequent degradation by the 26S proteasome. The pathway has been reported to protect cells from apoptotic death,[6,7] but in some situations it is associated with apoptosis.[8] NF-κB activation has been reported after irradiation of human lymphoblastoid cells at doses as low as 0.25 to 2.0 Gy; the maximum is after 50 cGy[9] and ROS have been implicated.[10]

Further research is needed to determine what the target is for these low-dose radiation effects, how it differs from the target at higher doses, and how it relates to induction of pro-inflammatory cytokines and stress molecules. In this paper we propose the proteasome as a target for radiation that is particularly important in the low-dose range. The proteasome is responsible for degradation of short-lived regulatory molecules in the cell, including IκB, p21, AP-1, p53, and cyclins.[11] Many radiation-induced molecules are targeted for degradation through this mechanism, and inhibition of proteasome function could lead to their up-regulation. Working in concert with transcription, this mechanism could result in nonlinear dose-response curves for radiation-induced molecular expression.

Experimental Design and Results

In C3H/HeN mice, lung irradiation induced tumor necrosis factor-α, interleukin (IL) 1α, IL-1β, and IL-6 mRNA expression, as assessed by an RNase protection assay (Fig. 1). IL-2, IL-3, IL-4, IL-5, and interferon-γ levels were barely detectable. The responses were dose and time dependent. The dose response was nonlinear with both lower and higher doses being more effective than clinically relevant 2 Gy doses. Responses subsided within 24 hours.

Nonlinear dose- and time-dependent responses were also seen for NF-κB after irradiation of ECV304 cells (F. Pajonk and W.H. McBride, unpublished data). At 3 hours after irradiation, gel shift analyses showed activation of NF-κB after doses of 1 to 20 Gy with a sharp increase above 4 Gy. IκBα levels, measured by Western blot 30 minutes after irradiation, were elevated in the low-dose range of 25 to 50 cGy, around 2 Gy, and after high doses. Because early NF-κB activation does not require protein synthesis,[5] nontranscriptional mechanisms must be affected by irradiation. To test proteasome involvement, we measured activity in extracts of irradiated cells using a fluorogenic substrate specific for chymotrypsin-like activity.[12] The results showed that cellular irradiation decreased 26S proteasome activity even when the radiation doses were in the low range of 25 to 60 cGy (Fig. 2).

Discussion

Radiation therapy was once used more for the treatment of nonmalignant disease than for cancer treatment.[13] Relatively

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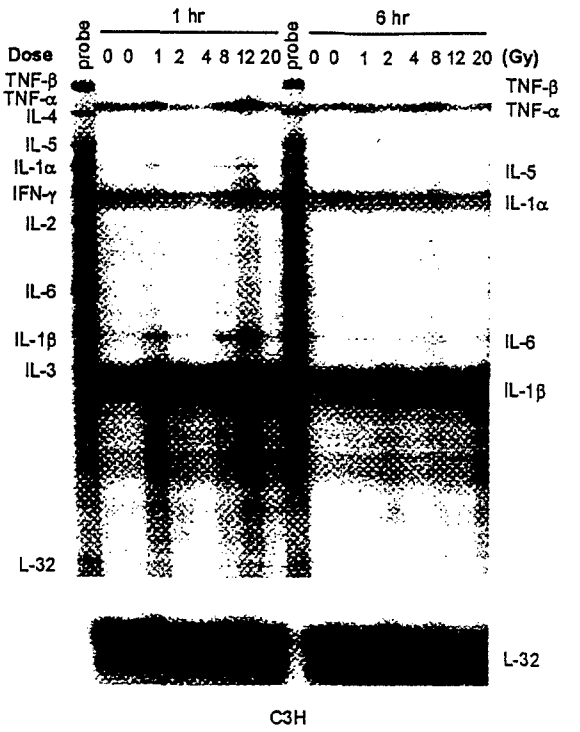

Fig. 1. Effect of lung irradiation on cytokine expression measured by RNase protection assay.

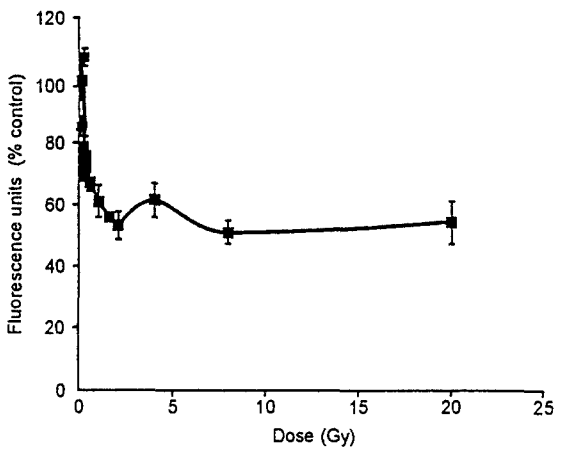

Fig. 2. Inhibition of proteasome activity 3 hours after irradiation as measured by cleavage of a fluorogenic substrate specific for chymotrypsin-like activity.

low radiation doses were frequently used to treat a variety of inflammatory and hyperproliferative conditions. This putative anti-inflammatory response contrasts with the proinflammatory response observed after higher radiation doses. The data presented here and by others[9,10] demonstrate a nonlinear dose-response curve for induction of NF-κB and proinflammatory cytokines, with a minimum of approximately 2 Gy. This dose may have been chosen for clinical use in part because of its relatively low proinflammatory potential. Both lower and higher doses were better able to stimulate these pathways.

The mechanisms underlying nonlinear radiation dose responses are complex. Gene expression is controlled at several different levels. In addition to transcriptional activation, mRNA and protein stability are under regulatory control. Many, if not all, of the short-lived regulatory proteins induced by radiation are degraded through the proteasome pathway.[11]

Conclusion

In this study, we report that radiation, even at doses as low as 50 cGy, slows the rate of degradation of fluorogenic peptides by proteasome-rich extracts. This suggests that the proteasome itself is a redox-sensitive target for radiation. The relative contributions of post-transcriptional and transcriptional control mechanisms may vary at different dose levels, which may explain the nonlinear dose- and time-response curve that is seen for some radiation-induced pathways. The findings also suggest that the proteasome might be a novel target for modification of radiation responses.

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Original Contribution

N-ACETYL-L-CYSTEINE INHIBITS 26S PROTEASOME FUNCTION: IMPLICATIONS FOR EFFECTS ON NF-κB ACTIVATION

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Abstract—Ionizing radiation shares with cytokines, such as TNF-α, an ability to generate free radicals in cells and activate downstream proinflammatory responses through NF-κB-dependent signal transduction pathways. Support for the role of free radicals in triggering such responses comes from the use of free radical scavengers like N-acetyl-L-cysteine (NAC). The nature of the link between free radical generation and NF-κB activation is, however, unclear. In this study, we explore the possibility that scavenging of free radicals by NAC might not be the mechanism by which it inhibits NF-κB activation, but rather that NAC acts through inhibition of proteasome function. The effect of NAC on the chymotryptic function of the 26s and 20s proteasome complex was measured in extracts from EVC 304 bladder carcinoma cells by assessing degradation of fluorogenic substrates. NAC inhibited 26s but not 20s proteasome activity, suggesting that it interferes with 19s regulatory subunit function. NAC blocked radiation-induced NF-κB activity in ECV 304 cells and RAW 264.7 macrophages, as measured by a gel shift assay, at doses that inhibited proteasome activity. This provides a possible mechanism whereby NAC could block NF-κB activation and affect the expression of other molecules that are dependent on the ubiquitin/proteasome system for their degradation, other than by scavenging free radicals. © 2002 Elsevier Science Inc.

Keywords—NF-κB, Radiation effects, N-acetyl-L-cysteine, Radical scavenger, Free radicals

INTRODUCTION

The free radical scavenger N-acetyl-L-cysteine (NAC) is used clinically for a broad spectrum of indications including mucolysis, detoxification after acetaminophen poisoning, adult respiratory distress syndrome (ARDS), hyperoxia-induced pulmonary damage, HIV infection, cancer, and heart disease [1–4]. Free radicals are critical in the determination of protein structure, regulation of enzyme activity, protein phosphorylation, and control of transcription factor activity and binding, and NAC is often used to explore their role in these effects. However, despite its frequent use and the enormous clinical knowledge about this drug, free radical scavenging is often assumed to be the mechanism by which it brings about its effects although its exact targets are unknown. For example, it is unclear how NAC acts to downregulate expression of the transcription factor NF-κB, which is a major mediator of inflammatory responses and controls expression of a large variety of genes encoding cytokines, growth factors, acute phase proteins, and cell adhesion and immunoregulatory molecules [5,6].

The findings that NAC prevents NF-κB activation in response to a variety of signals, including TNF-α and ionizing radiation [7,8], are generally taken as support for the involvement of free radicals in the process. TNF-α is thought to mediate both signal transduction and its cytotoxic effects through reactive oxygen intermediates (ROI). However, there is evidence that the lipoxygenase pathway may be more important than free radicals in mediating the latter [9]. Furthermore, some of the effects of NAC, like the G1-arrest described by Sekharam and colleagues [10], are not easily explained through a simple mechanism involving direct scavenging of free radicals.

NF-κB is a family of homo- or hetero-dimers of proteins of the RelA/NF-κB family. They pre-exist in the cytosol, bound to inhibitor molecules (IκB) that prevent

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nuclear translocation of the complex. Classically, upon appropriate signaling, IκB is phosphorylated at two serine sites (ser32, ser36) by specific kinases (IKK), poly-ubiquitinated and degraded by the 26s proteasome. This releases NF-κB and allows nuclear translocation followed by initiation of transcription of dependent genes (for a review see [11]). Degradation of IκB by the 26s proteasome is a mandatory step for NF-κB activation in response to most signals. The 26s proteasome is a large protease of 2MDa that consists of a cylindrical 20s core particle formed by four rings each with seven alpha and beta subunits. The inner two rings form the catalytic site of the complex. These exhibit five distinct cleavage activities [12]. Activity is regulated over a wide range by substitution of constitutive beta-subunits by interferon-inducible subunits LMP2, LMP7, and MECL-1 [13,14] and by 19s regulatory and 11s activator units that control substrate access, de-ubiquitination, and substrate linearization [15].

ATP- and ubiquitin-dependent protein degradation by the 26s proteasome is one of the most important degradation pathways of mammalian cells. The rate of degradation, as well as the rate of synthesis, regulates intracellular levels of proteins like p53, IκB, cJun, cFos, and cyclins A, B, and E, p21 and p27 [16,17]. It therefore controls cellular responses in many physiological and pathophysiological conditions [12]. It plays an additional important role in the immune system by determining the peptides that are expressed on the cell surface in association with MHC class I molecules [18–20]. Similarities between many of the effects attributed to NAC and those that follow inhibition of the ubiquitin-proteasome pathway led us to investigate a possible direct effect of NAC on 26s proteasome function using a well-established in vitro model for inflammatory responses. Our findings highlight an additional, and possibly major, pharmacological aspect of this frequently used drug.

MATERIALS AND METHODS

Cell culture

Cultures of ECV 304 human bladder carcinoma cells (DSMZ, Braunschweig, Germany), SiHa cervical carcinoma cells (ATCC, Manassas, VA, USA), and RAW 264.7 murine macrophages (a generous gift of Dr. Guido Hildebrandt, Department of Radiation Oncology, University Leipzig) were grown in 75 cm^2 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C in a humidified atmosphere at 5% CO_2. ECV 304 cells are a variant of the T-24 bladder carcinoma [21]. It exhibits many endothelial characteristics [22] and is often used as a model for inflammatory responses [23–25] because its response to inflammatory stimuli is reminiscent of that of endothelial cells. It shows constitutive activation of the NF-κB signal transduction pathway [26]. The media used was DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS, 1% penicillin/streptomycin (Gibco BRL).

Transfection

ECV 304 cells were maintained in DMEM (10% FCS, 1% penicillin/streptomycin). 12 h before transfection cells were trypsinized and plated at a density of 250,000 cells/well into six well plates. Cells were transfected with 5 μg of a plasmid (pEGFP-N1, Clontech, Palo Alto, CA, USA) coding for an ubiquitin-R-GFP fusion protein under control of a CMV promoter [27] (a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen, Santa Clarita, CA, USA) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10% FCS, 1% penicillin/streptomycin) supplemented with 500 μg/ml G418 (Sigma, St. Louis, MO, USA) and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-H, *FACSCalibur*, Becton Dickinson, Mountain View, CA, USA) using *CellQuest* Software before and after treatment with the proteasome inhibitor MG-132 (50 μM, Calbiochem, San Diego, CA, USA) for 10 h at 37°C. Clone #10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

Drug treatment

NAC (Sigma) was dissolved in phenol-red-free PBS. The pH of the NAC solution was adjusted to pH 7.5. In proteasome function assays, the drug was added to the reaction mixture immediately before measurements were started. In gel shift experiments, media was removed 60 min prior to irradiation and replaced by phenol-red-free PBS supplemented with the drug or carrier.

Proteasome function assays

Proteasome function was measured as described previously [28] with some minor modifications. Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1000 × *g* for 5 min and 10,000 × *g* for 20 min. Protein concentration was deter-

mined by the Micro BCA protocol (Pierce, Rockford, IL, USA) with BSA (Sigma) as standard. Twenty μg protein of each sample was diluted with buffer I to a final volume of 200 μl. The fluorogenic proteasome substrates Suc-LLVY-MCA (chymotrypsin-like; Sigma), Z-Leu-Leu-Leu-AMC (Calbiochem) and Boc-Val-Leu-Lys-AMC (trypsin-like; Sigma) were dissolved in DMSO and added in a final concentration of 80 μM (0.8% DMSO). Proteolytic activity was monitored continuously by the release of the fluorescent group 7-amido-4-methylcoumarin (AMC) measured in a fluorescence plate reader (Spectrafluor, Tecan, and Spectra Max Gemini XS, Molecular Devices, 37°C) at 380/460 nm. In control experiments using lactacystin (10 μM) and MG-132 the chymotryptic activity of the lysates was always inhibited by more than 90%, indicating that the observed cleavage activity was mainly based on proteasome function.

Irradiation

Cells were plated into petri dishes. After 24 h the cells were preincubated with NAC for 60 min. Cells were irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shephard, Mark I, dose rate of 5.80 Gy/min and IBL 637, CIS bio international, dose rate of 0.78 Gy/min). Corresponding controls were sham irradiated.

Electrophoretic mobility shift assays

Cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX-buffer [20 mM HEPES (pH 7.9), 0.35 mM NaCl, 20% glycerol, 1% NP-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, PMSF and aprotinin] for 30 min on ice. Lysates were centrifuged at 12,000 × *g* for 5 min. Protein concentration was determined using the BCA protocol (Pierce) with BSA (Sigma) as standard. Fifteen μg protein of the resulting supernatant was incubated for 25 min at room temperature with 2 μl BSA (10 μg/μl), 2 μl dIdC (1 μg/μl), 4 μl Ficoll-buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF), 2 μl buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1 mM PMSF), and 1 μl of the [γ^{32}P]-ATP labeled oligonucleotide (Promega, Madison, WI, USA, NF-κB: AGT TGA GGG GAC TTT CCC AGG). For negative controls, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5% TBE gels. Dried gels were placed on a phosphor-imaging screen, which was analyzed 24 h later (Storm 860, Molecular Dynamics and IPR 1500, Fuji, Takaoka, Japan).

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, SDS, 1% protease inhibitor cocktail (P8340, Sigma)]. Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin as standard. Twenty μg of protein was subjected to SDS gel electrophoresis (0.1% SDS/7.5% polyacrylamide) and blotted to PVDF membranes. After blocking with 5% skim milk in PBS, equity of loading was confirmed using a monoclonal antibody against α-tubulin (mouse anti-human, CP06, 1:20,000, Oncogene Science, Cambridge, MA, USA). Membranes were stripped and incubated with a monoclonal antibody against human p53 (mouse anti-human, OP43, 0.1 μg/ml, Oncogene), a polyclonal antibody against murine and human IκBα (rabbit anti-mouse, 554135, 1:20,000, BD) and a monoclonal murine antibody against poly-ubiquitinated proteins (1:5000, clone FK2, Affinity). Secondary HRP-conjugated antibodies [rabbit anti-mouse (Dianova) and goat anti-rabbit (DAKO), 1:20,000] and the ECL Plus System (Amersham, Arlington Heights, IL, USA) were used for visualization.

RESULTS

NAC is a free radical scavenger that has been shown to prevent NF-κB activation in response to a variety of stimuli [7,29–31]. We confirmed this in ECV 304 and RAW 264.7 cells, using a gel shift assay. As shown in Fig. 1, NF-κB was activated in ECV 304 (A) and RAW 264.7 (B) cells 1 h after irradiation with 30 Gy. Pretreatment of cells with NAC (0, 7.5, 15, and 30 mM) for 60 min inhibited radiation-induced NF-κB activity in ECV 304 and RAW 264.7 cells in a dose-dependent manner. The activation of NF-κB by ionizing radiation in RAW 264.7 macrophages was restricted to an increase of free p50/p65 heterodimers. Preincubation of RAW 264.7 cells with NAC prevented this induction but left constitutive p50/p50 homodimer activity unchanged. Similar inhibition could be achieved by using the proteasome inhibitor MG-132 (Fig. 1C).

To investigate a possible relationship between NAC and proteasome function, we examined its effects on the chymotryptic cleavage activity of the 20s and 26s proteasome in ECV 304 cells. An assay previously published by Glas and colleagues [28] was used to monitor proteasome activity. This assay utilizes fluorogenic peptides to measure the specific cleavage activities of the 26s proteasome. Addition of NAC at concentration of 0, 0.5, 1, 2, 4, 7.5, 15, and 30 mM to proteasome extracts from ECV 304 cells resulted in a dose-dependent inhibition ($r^2 = 0.85$) of chymotrypsin-like 26s proteasome

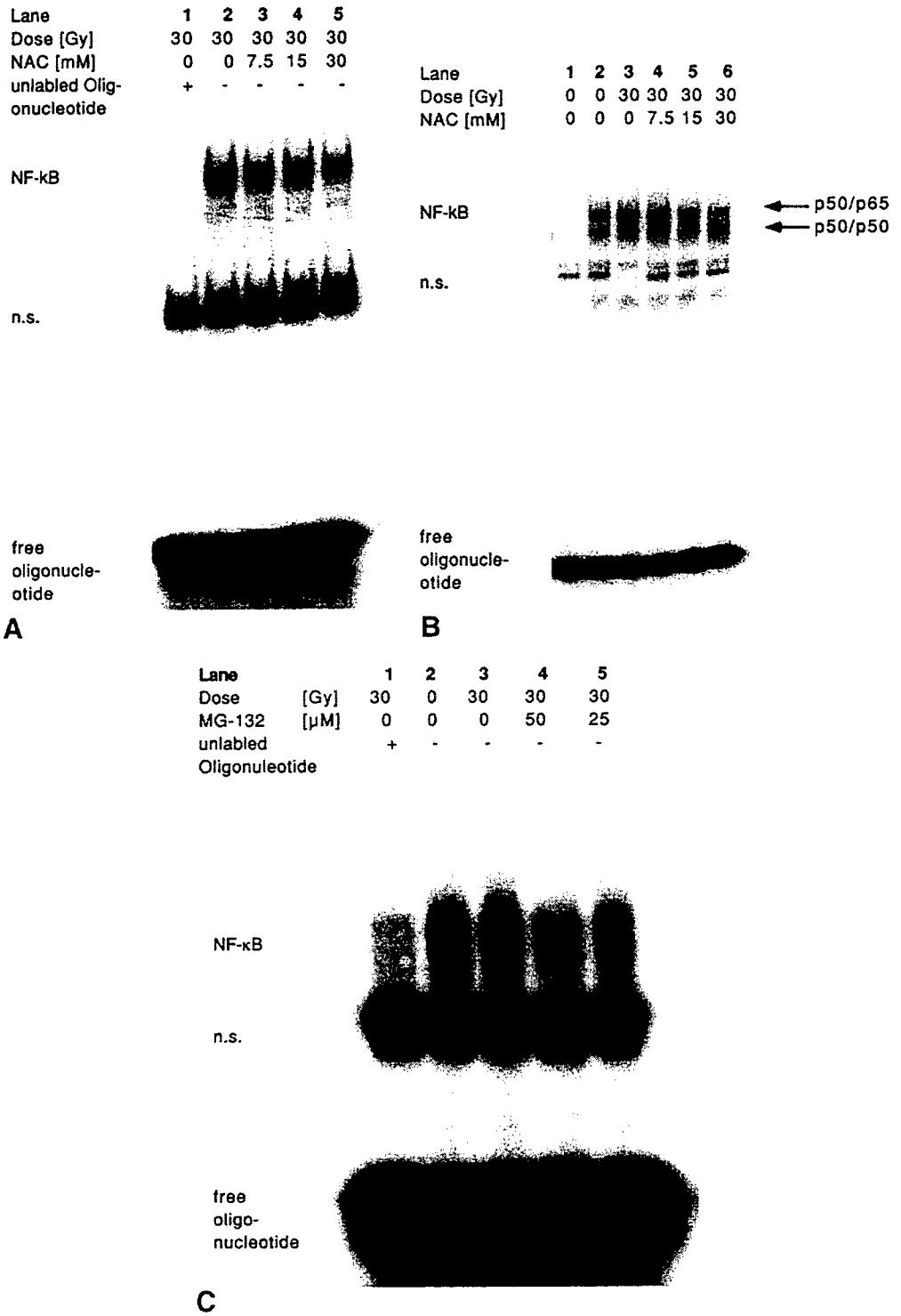

Fig. 1. Gel shift experiments using 15 μg of cytosolic extracts of ECV 304 (A) and RAW 264.7 (B) murine macrophages and a labeled double-stranded oligonucleotide containing a consensus binding motif for NF-κB. Unlabeled oligonucleotide in 50-fold molar excess was used as a negative control to demonstrate the specificity of the binding (lane 1, A, B, and C). (A) Pretreatment of ECV 304 cells with NAC for 1 h decreased radiation-induced (30 Gy) NF-κB activation 1 h after irradiation in a dose-dependent manner. Lane 1: negative control; lane 2: NAC 0 mM; lane 3: NAC 7.5 mM, lane 4: NAC 15 mM; lane 5: 30 mM. (B) Lane 1: negative control. Application of 30 Gy ionizing radiation caused an increase in p50/p65 heterodimer DNA-binding activity (lane 3) when compared to untreated controls (lane 2). This increase was prevented by preincubation with NAC in a dose-dependent manner (lane 4: 7.5 mM; lane 5: 15 mM; lane 6: 30 mM). In contrast DNA-binding activity of p50/p50 homodimers remained unchanged. (C) Pretreatment of ECV 304 cells with MG-132 for 3 h decreased radiation induced NF-κB activity. Lane 1: negative control; lane 2: control; lane 3: 30 Gy, lane 4: 30 Gy MG-132 50 μM; lane 5: 30 Gy MG-132 25 μM.

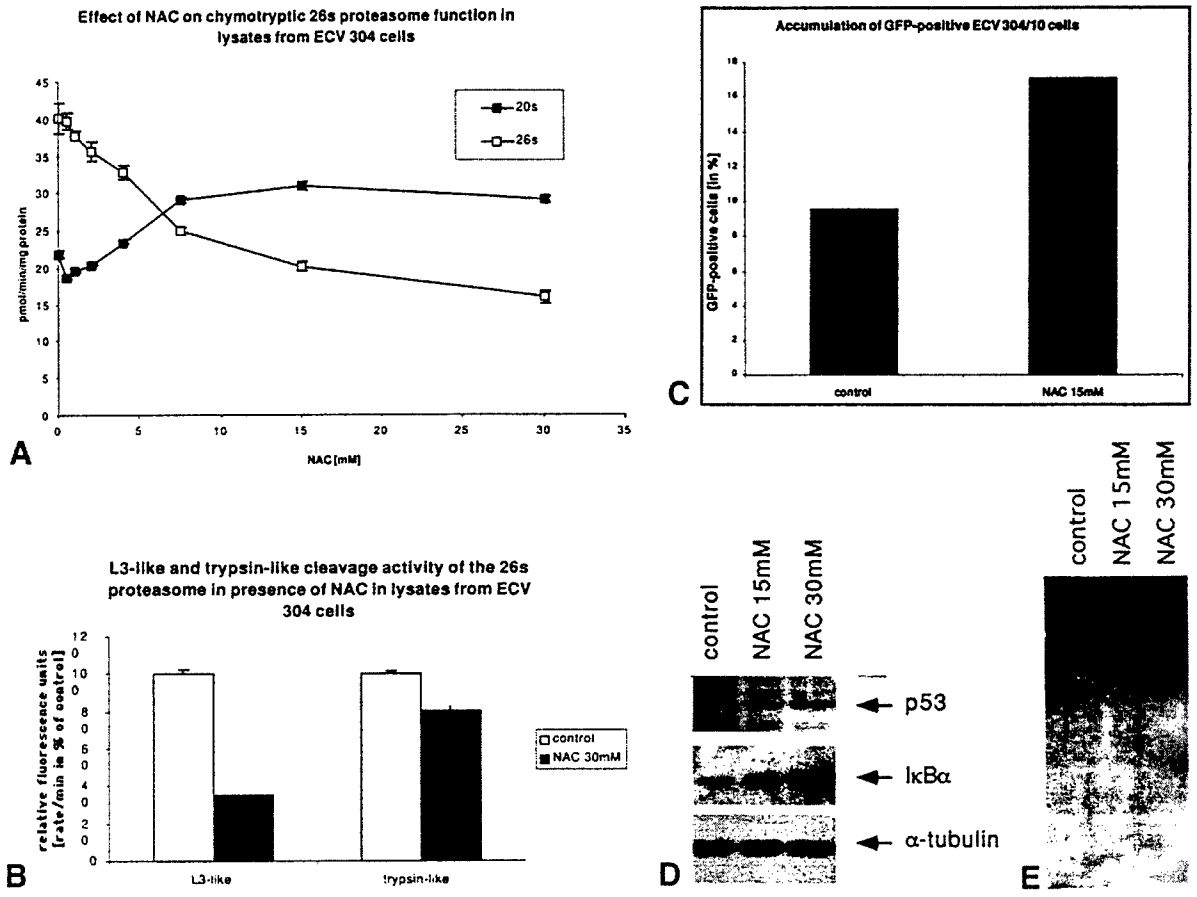

Fig. 2. (A) Effect of NAC on chymotryptic 20s (open symbols) and 26s (filled symbols) proteasome activity. Cleavage rate of SucLLVY-MCA (80 μM) was assessed with a fluorogenic peptide assay (excitation 380 nM, emission 460 nm). 26s proteasome activity in crude extracts of ECV 304 cells was inhibited by N-acetyl-L-cysteine in a dose-dependent manner (means from 4 measurements ± standard deviation [pmol/min/mg protein]) 0 mM NAC: 40.2 ± 2, 0.5 mM NAC: 39.8 ± 1.1, 1 mM NAC: 37.8 ± 0.6, 2 mM NAC: 35.6 ± 1.3, 4 mM NAC: 32.8 ± 0.9, 7.5 mM NAC: 25.0 ± 0.5, 15 mM NAC: 20.2 ± 0.6, 30 mM NAC: 16.0 ± 0.9, $p < .001$ t-test). In contrast, 20s was not only unaffected by NAC treatment but seemed to be slightly increased at NAC concentrations of 4 mM and higher. (B) Proteasome function assay using the fluorogenic proteasome substrates Z-Leu-Leu-Leu-AMC (L3-like) and Boc-Val-Leu-Lys-AMC (trypsin-like). L3- and tryptic-cleavage activity of the 26s proteasome were inhibited to 34.8 ± 0.5% and 80.2 ± 5.1% (mean ± standard deviation) of DMSO-treated controls, respectively, by 30 mM NAC. (C) Flowcytometric analysis of EVC 304 cells, stable transfected with an expression plasmid for a ubiquitin/GFP fusion protein. NAC treatment (15 mM) for 24 h increased the GFP-positive population of cells from initially 9.6 to 17.1%. (D) and (E) Western blot analysis of SiHa cervical carcinoma cells treated with N-acetyl-L-cysteine (0, 15, and 30 mM) for 20 h. A monoclonal antibody against α-tubulin was used to demonstrate equity of protein loading. Membrane was stripped and re-probed with antibodies against p53, IκBα, and poly-ubiquitinated proteins. NAC treatment caused accumulation of p53, IκBα, and poly-ubiquitinated proteins.

activity. At 30 mM NAC inhibited chymotryptic activity to 43.9 ± 1.2% (mean ± standard deviation, $p < .01$, two-sided t-test) of baseline cleavage rates (closed symbols, Fig. 2A) and tryptic- and L3-cleavage activity of the 26s proteasome to 80.2 ± 5.1% and 34.8 ± 0.5% (mean ± standard deviation) (Fig. 2B). 20s proteasome activity can be monitored by performing the assay in the presence of 0.03% SDS and in the absence of ATP [32]. NAC did not decrease cleavage activity under these conditions, but rather slightly increased it at higher concentrations (open symbols, Fig. 2A).

To confirm the effects of NAC on proteasome inhibition, ECV304/10 cells stably transfected with an expression plasmid for an Ub-R-GFP fusion protein were used. Under standard conditions the GFP protein is rapidly degraded by the 26s proteasome. After inhibition of proteasome function GFP accumulates in up to 20% of the cells [27]. Incubation with NAC (15 mM) for 24 h caused an increase of the GFP-positive fraction from initially 9.6 in untreated to 17.1% in NAC-treated cells (Fig. 2C) with a 1.3-fold overall increase of the mean fluorescence.

Experiments were performed to confirm that the effects of NAC could be extended beyond NF-κB activation to include p53. SiHa cells express the HPV E6 protein, which mediates degradation of p53 by the ubi-

quitin/26s proteasome pathway. Total cellular protein extracts from SiHa cervical carcinoma cells were incubated with NAC (0, 15, 30, and 60 mM) for 20 h and analyzed by Western blotting using an antibody against p53, IκBα and poly-ubiquitin. Incubation with NAC caused an increase of p53 and IκBα protein levels and accumulation of poly-ubiqitinated proteins consistent with inhibition of the ubiquitin/26s proteasome pathway (Fig. 2D and 2E).

DISCUSSION

Most previous studies on NAC have focused on its antioxidative effects. It was shown to prevent activation of NF-κB induced by TNF-α and ionizing radiation [8,30]. Both treatments are known to induce the formation of ROI and generation of these radicals could be prevented by NAC treatment. Together, these results have been taken to imply a role for ROI in NF-κB activation in response to TNF-α or radiation [33]. However, the exact mechanism of TNF-α-induced ROI formation is still not clear, nor is the link between ROI and NF-κB activation [34,35] or the cytotoxic action of TNF-α. Indeed, a direct role for ROI in mediating TNF-α-related cytotoxicity was recently seriously questioned [9].

The classical pathway for activation of NF-κB involves IκB-kinases that phosphorylate IκB at two serine sides, which leads to polyubiquitination and subsequent degradation of IκB by the 26s proteasome. It is this degradation that releases the NF-κB dimer from its inhibitor IκB and allows NF-κB translocation into the nucleus to initiate gene transcription [11]. NF-κB dependent genes are thought to be required for radiation-induced inflammatory responses that could play roles in both tumor control and normal tissue damage after radiotherapy [36].

Inhibition of 26s proteasome function has been shown to prevent TNF-α-induced activation of NF-κB [37]. In agreement with the findings of Hallahan and coworkers [8], we have recently shown that radiation-induced activation of NF-κB is dependent on 26s proteasome function since it was prevented by the specific proteasome inhibitor MG-132 [38], although an alternate pathway of radiation- and hypoxia-induced NF-κB activation has been described [39,40].

In this study radiation-induced NF-κB activation was clearly prevented by pretreatment of RAW 264.7 cells with NAC. Furthermore NAC had a direct effect on proteasome function at a concentration that was optimal for prevention of NF-κB activation in response to TNF-α treatment [7]. NAC acted as an inhibitor of 26s chymotryptic, as well as L3- and, to a lesser extent, trypsin-like, proteasome activity, but failed to decrease 20s chymotryptic-like activity. Attempts to generate Lineweaver-Burk diagrams using 10, 20, 40, and 80 μM concentrations of the fluorogenic substrate failed to identify a classical inhibition type. Together these observations give rise to the assumption that NAC targets one or more subunits of the 19s regulatory unit rather than interfering with the β-subunits of the 20s core unit, which are the catalytic sites of this protease complex. Our observation that NAC treatment causes accumulation of p53 in human cervical cancer cells confirmed an earlier report of an interference of NAC with this pathway [41]. Additionally, NAC treatment led to accumulation of IκBα, a ubiquitin/GFP fusion protein that is rapidly degraded by the 26s proteasome in untreated control cells and poly-ubiquitinated proteins in general. This supports the conclusion that NAC targets the proteasome directly. In addition to providing a possible mechanism for the inhibitory effects of NAC on NF-κB activation, inhibition of proteasome function easily explains the G1-arrest observed after NAC-treatment [10] as a functional proteasome is required for G1/S transition [42].

The data presented in this study suggest that cellular responses to NAC treatment result, at least in part, from 26s proteasome inhibition, although our experimental setting did not allow determination of the contribution of radical scavenging, as opposed to proteasome inhibition, to the total observed effect.

Some previous studies have questioned the role of ROI scavenging in mediating the effects of NAC. For example, it has been pointed out that H_2O_2 is not rapidly scavenged by NAC [43]. This study even reported that H_2O_2 could be generated as a result of the auto-oxidation process of NAC in the presence of O_2. Previous studies have shown that proteasome function can be inhibited by H_2O_2 [32,44] treatment and ionizing radiation [38]. However, the major site of these inhibitory effects appears to be the 20s core unit of the proteasome, rather than the 19s regulatory unit, which appears to be the site of action of NAC. This suggests that the proteasome is a highly redox-sensitive structure and that distinct structures may be the target for different chemical species.

Steps in this proteolytic degradation pathway, other than those mediated by the 19s regulatory subunit, might also be affected by in vivo treatment of cells with NAC, or other scavengers. For example, a necessary step for 26s proteasome-dependent proteolysis is the tagging of proteins with ubiquitin, a 76-residue polypeptide involving a three-enzyme cascade. Ubiquitin is activated when its carboxy-terminal glycine is transformed into a high-energy thiol-ester intermediate by the ubiquitin-activating enzyme E1. The ubiquitin-carrier protein E2 transfers ubiquitin to a ubiquitin-protein ligase, E3, that catalyzes the conjugation of the activated carboxy-terminal ubiquitin to ε-amino groups of lysine residues of the tar-

geted protein (reviewed in [16]). Intracellular reduced GSH is required for E1 and E2 enzymes to form ubiquitin thiol-esters [45] and is critical for the process of ubiquitination. NAC increases the level of GSH in cells [46] and this could affect the degradation process [47]. Therefore, both stimulatory and inhibitory effects of NAC on degradation rates would seem possible. Use of the Ub-GFP construct and the fluorogenic assay bypasses any stimulatory effects, but inhibition must predominate if the end result is NF-κB inhibition or p53 activation. This balance may not, however, be the same under all conditions.

Finally, this study raises a cautionary note regarding the use of free radical scavengers to implicate ROI in signaling events following exposure of cells to ionizing radiation and other signals. The proteasomal degradation pathway should be considered as an alternative to direct free radical scavenging as a target for the observed effects.

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BIOLOGY CONTRIBUTION

APOPTOSIS AND RADIOSENSITIZATION OF HODGKIN CELLS BY PROTEASOME INHIBITION

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Purpose: Malignant cells from Hodgkin's disease have been reported to be defective in regulation of NF-κB activity. Ionizing radiation is known to activate NF-κB, and it has been suggested that this pathway may protect cells from apoptosis following exposure to radiation and other therapeutic agents. Defective NF-κB regulation in Hodgkin cells could therefore dictate the response of this disease to therapy, as well as be responsible for maintaining the malignant phenotype. The purpose of this study was to explore whether NF-κB activity could be modulated in Hodgkin cells and whether it determines the response of these cells to treatment with ionizing radiation and/or dexamethasone.

Methods and Materials: Activation of NF-κB in cells is accomplished in large part by degradation of its inhibitor IκB through the 26s proteasome. HD-My-Z Hodgkin cells were treated with the proteasome inhibitor MG-132 or transduced with a dominant negative super-repressor IκBα. Clonogenic survival, apoptosis, proteasome activity, and NF-κB binding activity were monitored in response to ionizing radiation and/or dexamethasone treatment.

Results: HD-My-Z Hodgkin cells had modest NF-κB levels but, unlike other cell types, did not decrease their level of constitutively active NF-κB in response to proteasome inhibition with MG-132. In contrast, transduction with a non-phosphorable IκBα construct abolished expression. MG-132 did, however, induce apoptosis in HD-My-Z cells and sensitized them to ionizing radiation. Dexamethasone treatment had no effect on NF-κB activity or clonogenic survival of Hodgkin cells, but protected them from irradiation.

Conclusion: We conclude that inhibition of 26s proteasome activity can induce apoptosis in HD-My-Z Hodgkin cells and radiosensitize them, in spite of the fact that their constitutively active NF-κB levels are unaltered. The proteasome may be a promising new therapeutic target for intervention in this disease. In contrast, the use of glucocorticoids in conjunction with radiation treatment for this tumor may require re-evaluation. © 2000 Elsevier Science Inc.

Hodgkin's disease, 26s Proteasome, NF-κB, MG-132, Dexamethasone.

INTRODUCTION

The treatment of Hodgkin's disease has improved greatly over the last three decades. In the last 10 years, stage-adapted radio/chemotherapy protocols have been introduced that reduce overall treatment failure to less than 25 % (1–5). Even salvage therapies of relapsed Hodgkin's disease are highly effective, with long-term remission rates of more than 50% (6–8). In spite of this success, there are a number of patients with primary or relapsed Hodgkin's disease whose tumors are resistant to both radiotherapy and multi-agent chemotherapy. Others with relapsed disease cannot be treated because of hematological or local complications caused by prior treatment. New treatment modalities, preferably based on insights into the pathophysiology of Hodgkin's disease, are needed for these patients.

The malignant cells in Hodgkin's disease, the Hodgkin cell and the multinuclear Reed-Sternberg cell have recently been shown to belong to the B-lymphocyte lineage (9–11). However, in contrast to most B-lymphocytes, Hodgkin's lymphoma cells appear relatively resistant to apoptosis. Recently, Bargou and coworkers (12) observed a feature of Hodgkin cell lines that might explain this resistance. All Hodgkin cell lines tested showed constitutive activation of nuclear factor kappa B (NF-κB). Activation of NF-κB is known to protect many cell types from apoptotic death (13–15).

NF-κB is a hetero- or homodimer of proteins that recognize a specific DNA-binding motif in the promoter region of many genes, especially those involved in inflammatory responses. In its inactive form, the binding region (NLS) is

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covered by inhibitor proteins called IκB. NF-κB activation most often results from phosphorylation of IκB by IκB kinases (Iκ), its ubiquitination, and subsequent degradation by the multicatalytic 26s proteasome. This degradation enables nuclear translocation of NF-κB and initiation of gene expression (16, 17). While many events could cause constitutive activation of NF-κB, there are reports that Hodgkin cell lines may have mutated IκBα, resulting in a nonfunctional IκBα protein (18) or an almost complete lack of IκBα gene expression (19). Although only a few cell lines are available for study, it is possible that unregulated expression of NF-κB is responsible for the pathogenesis of this disease. In this study, we explored the status of NF-κB in the HD-My-Z Hodgkin cell line, the mechanism underlying its constitutive expression, and whether abnormalities in this pathway affect cell death following exposure to proteasome inhibitors and/or ionizing irradiation and steroids.

METHODS AND MATERIALS

Cell culture

The Hodgkin cell line HD-My-Z (DSMZ, Braunschweig) has been described in detail elsewhere (19). This, as well as PC3 and LnCaP human prostate carcinoma (ATCC), SW 1088 astrocytoma (ATCC), ECV 304 bladder carcinoma cells, and A549 non-small cell lung cancer (ATCC) cell line, were grown in 75-cm^2 flasks (Falcon) at 37°C in a humidified atmosphere at 5% CO_2. The medium was DMEM medium (Gibco/BRL) supplemented with 10% FCS (Sigma), 1% penicillin/streptomycin (Sigma), and 0.5 g/mL fungizone (amphotericin B, Gibco/BRL). For studies of proteasome and NF-κB functional activity, 10^6 cells were plated into 10-cm culture dishes 24 h before the start of the experiment.

Cell extracts and electrophoretic mobility shift assays

For preparation of total cellular extracts, cells were mechanically scraped from the plate, washed with ice-cold PBS, and lysed in TOTEX-buffer (20 mM HEPES [pH 7.9], 0.35 mM NaCl, 20% glycerol, 1% NP-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, PMSF, and aprotinin for 30 min on ice). Lysate was centrifuged at 12,000 *g* for 5 min. Protein concentration was determined using the BCA protocol (Pierce) with bovine albumin as standard. Fifteen micrograms of protein of the resulting supernate were incubated for 25 min at room temperature with 2 μl BSA (10 μg/μl), 2 μl dIdC (1 μg/μl), 4 μl Ficoll-buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF), 2 μl buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1 mM PMSF) and 1 μl of the [γ^{32}P] ATP-labeled oligonucleotide (Promega, NF-κB: AGT TGA GGG GAC TTT CCC AGG). For a negative control, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5% TBE gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphor imager (Storm 860, Molecular Dynamics).

Irradiation

HD-My-Z cells, which grow as a loosely adherent monolayer, were dislodged by shaking, counted, and diluted to a concentration of 10^6 cells/mL. The cell suspension was immediately irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shephard, Mark I) at a dose rate of 580 rad/min. Corresponding controls were sham irradiated.

Clonogenic survival

Colony-forming assays were performed immediately after irradiation by plating an appropriate number of cells (2×10^3–2×10^4) into Petri dishes, in triplicate. After 14 days' culture, cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Data shown resulted from a minimum of three independent experiments.

Proteasome function assays

Proteasome function was measured as described previously (20). Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl2, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl^2$, 2 mM ATP, 250 mM sucrose) were added and vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1,000 *g* for 5 min and 10,000 *g* for 20 min. Protein concentration was determined by the BCA protocol (Pierce). Ten micrograms of protein of each sample was diluted with buffer I to a final volume of 100 μl. The fluorogenic proteasome substrate SucLLVY-7-amido-4-methylcoumarin (chymotrypsin-like, Sigma) and the proteasome inhibitor MG-132 (Calbiochem) were dissolved in DMSO and added in a final concentration of 100 μM in 1% DMSO. Controls received diluent only. Samples were incubated for 45 min at 37°C. The reaction was stopped by the addition of 1 mL SDS (1%) and free 7-amido-4-methylcoumarin was determined using a fluorescence plate reader (fmax, Molecular Devices) at 380/460 nm.

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodiumvanadate). Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin as standard. One hundred micrograms of protein were subjected to SDS gel electrophoresis (0.1% SDS in Tris-HCl/10% polyacrylamide, pH 8.8 and a 4% polyacrylamide stacking gel, pH 6.8) and blotted overnight to PVDF membranes (4°C). After blocking with Blotto-buffer (PBS, 2% skim milk) membranes were incubated with a polyclonal antibody against human IκBα (Santa Cruz Biotechnologies). A secondary HRP-conjugated antibody and DAB (Sigma) were used for visualization.

Transduction experiments

The recombinant replication-deficient adenoviruses Ad5-IκB and Ad5-LacZ were kindly provided by Dr. R. Batra

(VA Hospital, Los Angeles, CA) and are described elsewhere (21). Ad5-IκB contains a gene for a NF-κB superrepressor IκBα under control of a CMV-promoter/enhancer. The encoded protein contains serine-to-alanine mutations in residues 32 and 36, preventing its phosphorylation, ubiquitination, and subsequent degradation by the proteasome. The Ad5-LacZ is a control virus that contains the gene for β-galactosidase in place of IκBα. Cells were plated into Petri dishes (10 cm, Falcon). After 24 h, the medium was changed and viral vectors containing non-phosphorable IκBα or β-galactosidase genes were added at different multiplicities of infection (MOIs). After a 2-h incubation, the virus-containing medium was replaced by fresh medium and cells were incubated for an additional 48 h to allow gene expression.

Determination of apoptosis

Apoptotic cells were detected using the *In Situ* Cell Death Kit (Boehringer Mannheim). The manufacturer's protocol was followed with some minor modifications. Briefly, attached and detached cells were collected, centrifuged, fixed with 100% ethanol, washed with PBS, and pelleted by centrifugation for 5 min at 2,000 *g*. Cells were permeabilized by resuspension in 0.1% Triton X-100 in 0.1% sodium citrate and incubation for 2 min on ice. Cells were washed twice in PBS, resuspended in TUNEL reaction mixture, and incubated for 60 min at 37°C. After three washes with PBS, fluorescence was measured at 518 nm using a flow cytometer (FACScan, Becton Dickinson) and analyzed using the CellQuest software (Becton Dickinson).

Cell cycle analysis

A sample consisting of 5×10^5 cells per sample were washed, fixed with 70% ethanol, and stained by propidium iodide (0.1 mg/mL), and 0.1% Nonidet NP-40. After treatment with RNAse (1 mg/mL), the cell cycle parameters were determined from the DNA content using a flow cytometer (FACScan).

Statistics

For statistical analysis of the survival plots, the data was fitted using a generalized linear model and the statistical software package JMP (version 3.2 for Macintosh Computers, SAS). Two curves were assumed to be significantly different from each other if F-distribution values showed significance at a *p*-value of 0.05.

RESULTS

NF-κB activity in HD-My-Z Hodgkin cells

It has been suggested that Hodgkin cells have high constitutive NF-κB levels (12, 22). To test this in HD-My-Z cells, gel shift assays for functionally active NF-κB were performed on cell extracts and the results compared with those for other cancer cell lines (A549, ECV 304, LnCaP, PC3, and SW1088). Surprisingly, constitutive NF-κB levels in HD-My-Z cells were lower than those in most of the other cell lines. For example, NF-κB DNA-binding activity in extracts from PC3 cells was almost three times higher than that in HD-My-Z cells (Fig.1: lanes 2, 4, 6, 8, 10, and 12).

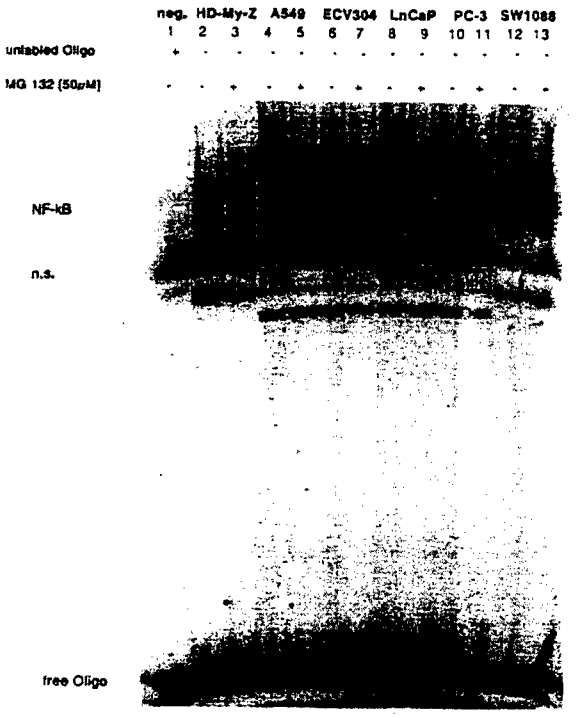

Fig. 1. The electrophoretic mobility shift assay shows constitutive NF-κB DNA-binding activity in cytosolic protein extracts (15 μg) from six different human tumor cell lines: HD-My-Z (lane 2), A549 (lane 4), ECV304 (lane 6), LnCaP (lane 8), PC3 (lane 10), and SW 1088 (lane 12) 24 h after plating. To show specificity of the binding reaction, a 50-fold molar excess of the unlabeled consensus oligonucleotide was added in lane 1. Preincubation of the cells for 3 h with the reversible proteasome inhibitor MG-132 (50 μM) decreased constitutive NF-κB binding activity in all cell lines except HD-My-Z (lanes 3, 5, 7, 9, 11, and 13).

Inhibition of the 26s proteasome does not decrease NF-κB activity in HD-My-Z cells

NF-κB activation is normally controlled by degradation of its inhibitor, IκB, through the proteasome. Blocking proteasome activity using the specific inhibitor MG-132 should therefore lead to a decrease in NF-κB activity. This was found to be the case for A549, ECV 304, LnCaP, PC3, and SW1088 cells treated for 3 h with 50 μM MG-132 (Fig. 1: lanes 5, 7, 9, 11, and 13). In contrast, the same treatment did not affect NF-κB activity in HD-My-Z cells (lane 3), indicating that constitutive NF-κB activity in this cell line is not regulated by this mechanism.

Clonogenicity of HD-My-Z cells is reduced by expression of a dominant negative IκBα

Because NF-κB activity was not decreased by MG-132 treatment of HD-My-Z cells, its ability to be modulated by

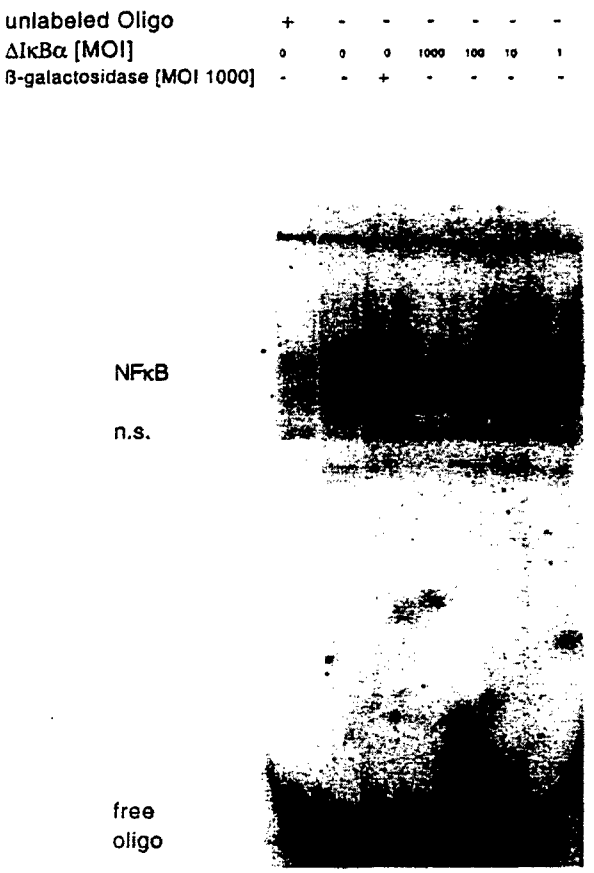

Fig. 2. Gel-shift experiments with extract of HD-My-Z Hodgkin cells after transduction with adenoviral vector carrying a truncated IκBα gene. An MOI of 1,000—equivalent to more than 99.9% cell transduction efficiency—resulted in a complete loss of NF-κB binding activity. Transduction with a β-galactosidase (MOI 1,000) gene did not change NF-κB activity.

IκB was tested by introducing a dominant negative IκBα, which does not undergo degradation through the proteasome. There was an MOI-dependent decrease in NF-κB activation 48 h after transduction (Fig. 2), consistent with regulation by the introduced IκBα. Clonogenicity of the HD-My-Z cells also was decreased by expression of dominant negative IκBα in a dose-dependent manner (Fig. 3). The control vector containing a β-galactosidase gene did not alter plating efficiency.

Proteasome activity is normal in HD-My-Z cells

The above experiments showed that HD-My-Z cells are unable to regulate NF-κB expression through the IκB degradation pathway. To exclude the proteasome as the defect, chymotrypsin-like cleavage activity was measured by fluorogenic assay in HD-My-Z cells and the result was compared with activity in other cancer cell lines. In general, proteasome activity correlated with NF-κB activity for the six cell lines tested (r = 0.863). PC3 cells had the highest activity; SW1088 cells had the lowest. The other cell lines, including HD-My-Z, were in-between these two extremes

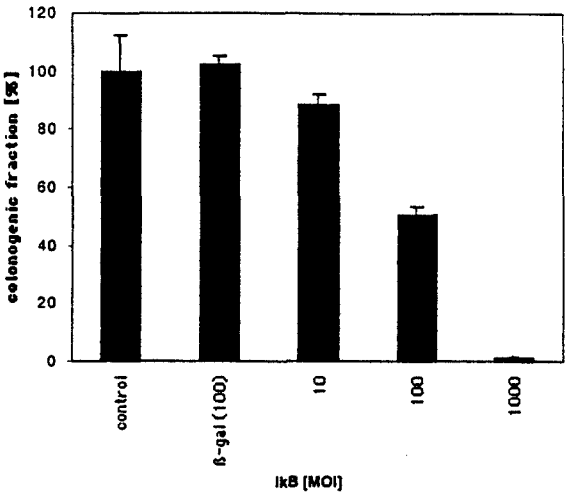

Fig. 3. Decrease in clonogenic fraction of HD-My-Z Hodgkin cells, assayed by measurements of plating efficiency, after expression of a dominant negative IκB. The decrease in clonogenicity in HD-My-Z cells was correlated to the number of viral particles used. The control vector, containing a β-galactosidase (MOI 100) gene, did not affect clonogenicity.

(Fig. 4). Extracts from HD-My-Z cells that were incubated with different concentrations of MG-132 showed dose-dependent inhibition of cleavage activity; 0.25 μM MG-132 inhibited to 50% after a 45-min incubation (Fig. 5). These experiments show that failure of MG-132 to affect NF-κB levels in HD-My-Z cells cannot be attributed to an inability of the drug to block proteasome activity, which appears normal in this cell line.

Further attempts were made to alter NF-κB activity in HD-My-Z cells using MG-132. Three hours' preincubation of cells with 3–50 μM MG-132 had no effect (Fig. 6a), nor did the continuous presence of 50 μM MG-132 over a period of 48 hours' cells (Fig. 6b). Twenty-four and 48 h

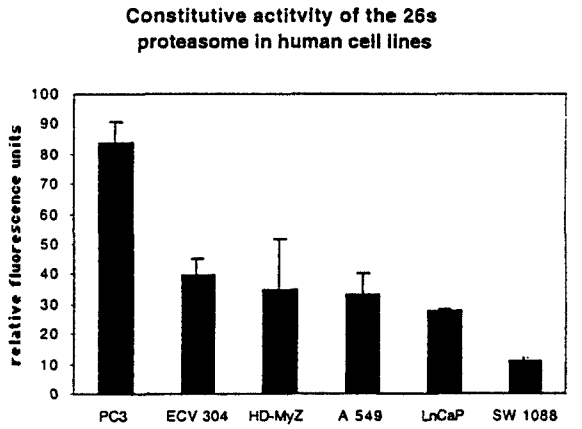

Fig. 4. Constitutive (chymotrypsin-like) proteolytic activity of the 26s proteasome in six different human cell lines. Measurements are expressed in relative fluorescence units and reflect the release of 7-amido-4-methylcoumarin (AMC) from the fluorogenic proteasomal substrate SucLLVY-AMC into the buffer (excitation 380 nm, emission 460 nm).

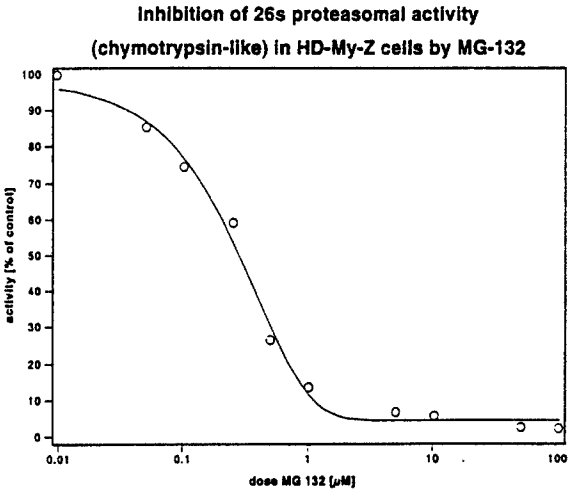

Fig. 5. Inhibitory effect of different concentrations (0, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 50, 100 μM) of the specific 26s proteasome inhibitor MG-132 on chymotrypsin-like cleavage activity in cell free extracts of HD-My-Z Hodgkin cells. Release of AMC from the fluorogenic peptide SucLLVY-AMC to the buffer was measured (excitation 380 nm, emission 460 nm) and expressed in percent of cleavage activity of extracts from untreated control cells.

after the start of drug treatment, there was a decrease in NF-κB, but this was associated with cell death. By 24 h, a pro-G_1 population (42%) was observed by flow cytometry that was almost absent (7%) in the control.

Inhibition of proteasome activity in HD-My-Z cells induces apoptosis

The ability of MG-132 treatment to cause death of HD-My-Z cells in the absence of any effect on NF-κB activity was explored further. Three hours' incubation in MG-132 (0–50 μM), followed by washing and incubation for an additional 24 h, caused dose-dependent apoptosis, as assessed by a TUNEL assay (Fig. 7a). The continuous presence of MG-132 in concentrations as low as 31.25 nM, which corresponds to a 10% inhibition of proteasome cleavage activity in cell-free protein extracts, reduced clonogenicity to 40% when compared to the untreated control (Fig 7b). The general correspondence between the dose of MG-132 required to cause cell death and the dose required for inhibition of 26s proteasome activity argue in favor of these processes being mechanistically linked.

Inhibition of proteasome activity in HD-My-Z cells sensitizes them to radiation

Because MG-132 induced apoptosis in HD-My-Z cells without affecting constitutive NF-κB activity, its effect on their response to radiation was examined. HD-My-Z cells were moderately radiation resistant, with a S.F. 2 Gy of 0.56 ± 0.032.(Fig. 8) They were 1.4 times more radioresistant than PC3 cells (S.F. 2 Gy = 0.39 ± 0.026, data not shown). Transient inhibition of proteasome function by 3 hours' preincubation of HD-My-Z cells in 50 μM MG-132 significantly sensitized them to ionizing irradiation, as measured by clonogenic survival (Fig. 8: S.F. 2 Gy = 0.42 ± 0.064, $p < 0.0001$, F-test). MTT assays performed 5 days after short-term incubation (3 h) of HD-My-Z cells confirmed the radiosensitizing effect of MG-132 with lower (1 and 10 μM) doses (data not shown).

In contrast to MG-132, exposure of HD-My-Z cells to dexamethasone (100 μM) for 3 h protected them from irradiation (Fig. 8: S.F. 2 Gy = 0.58 ± 0.042, $p < 0.0001$, F-test; see figure legend for radiobiological parameters). Glucocorticoids are a standard treatment for Hodgkin's disease. They also have been reported to decrease NF-κB in a number of cell lines by increasing IκB levels (23, 24). In this study, NF-κB levels did not decrease in HD-My-Z, PC3, or A459 cells under the conditions of treatment (data not shown). The plating efficiency in HD-My-Z cells was little changed, from 17.2% to 19.8%, by dexamethasone exposure, and there was no apoptosis (data not shown).

DISCUSSION

The malignant cell in Hodgkin's disease is most frequently reported to belong to the B-cell lineage (9–11). In general, B-cells are prone to undergo apoptosis. This is counteracted during immune activation by NF-κB-dependent pathways. It is therefore not surprising that Hodgkin and Reed-Sternberg cells generally show levels of NF-κB DNA-binding activity comparable to activated B-cells (22). In activated B-cells, NF-κB activation depends on CD40-ligand engagement (26). In Hodgkin cells, the factors that maintain NF-κB activity are less clear (18, 26–28).

Constitutive NF-κB activity is not a characteristic restricted to Hodgkin cells. In this study, extracts from other tumor cell lines had constitutive levels of active NF-κB, nor is the level of constitutive activity necessarily high in Hodgkin cells, as has been suggested (12, 22). The HD-My-Z Hodgkin cell line showed lower constitutive activity than most cell lines tested. What was unusual about the HD-My-Z Hodgkin cell line compared to the other cell lines was that the NF-κB activity was not down-regulated by treatment with MG-132, a reasonably specific proteasome inhibitor. It was, however, down-regulated by overexpression of a dominant negative IκB. Proteasome activity in HD-My-Z cells was inhibitable by MG-132 and was in the mid-range compared with the tumor cell lines that were tested. These findings indicate that HD-My-Z cells are most likely defective in IκB. Other Hodgkin cell lines have been reported to have low IκBα mRNA and protein levels (22) that appear to be non-functional (18). Although the HD-My-Z cell line has been reported to lack IκBα expression (19), Western blot analysis of extracts of the HD-My-Z cells detected a specific 38-kDa band for IκBα and degradation of IκBα was blocked by treatment with MG-132 (50 μM) for 3 h (data not shown). It remains to be investigated whether the IκBα is mutated, leading to a nonfunctional protein that is specifically unable to inhibit NF-κB.

Although treatment with MG-132 did not down-regulate NF-κB activity in HD-My-Z cells, it could induce apoptosis and decrease clonogenicity. The mechanism underlying

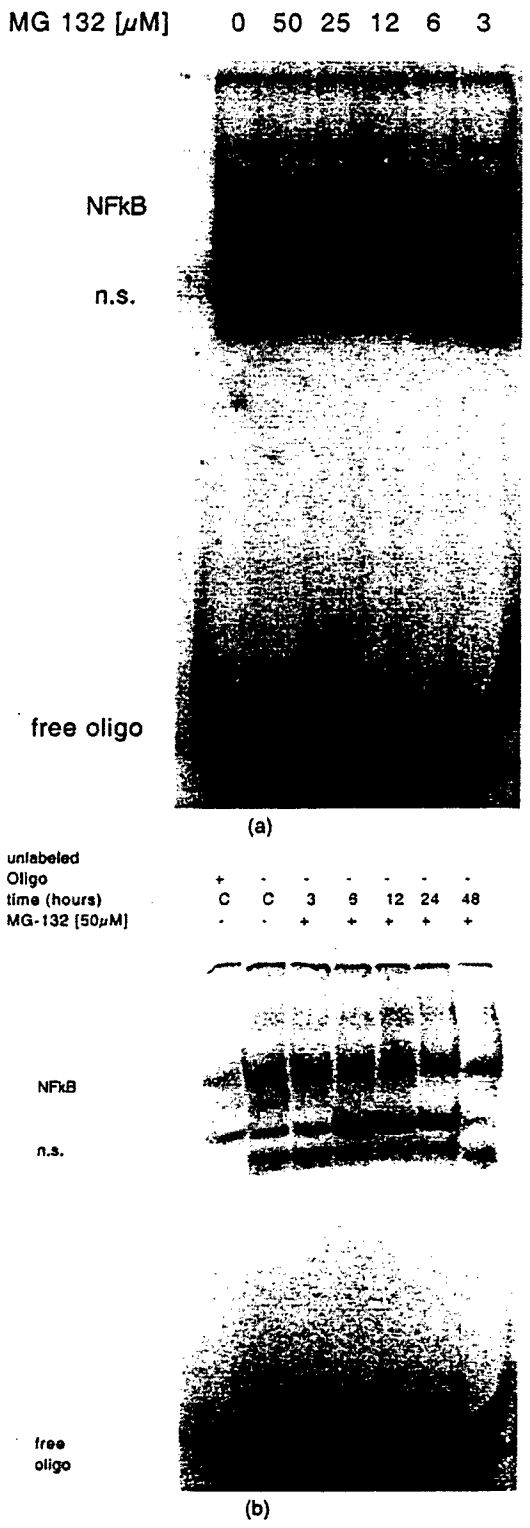

Fig. 6. (a) Gel-shift experiment with cytosolic protein extracts (15 μg) from HD-My-Z cells after 3-h preincubation with MG-132 (50, 25, 12.5, 6.25, and 3.125 μM). Constitutive high NF-κB activity was not affected by inhibition of proteasome function. (b) Incubation of HD-My-Z cells with MG-132 (50 μM) over a time period of 48 h did not change the NF-κB DNA-binding activity during the first 12 h. NF-κB activity decreased only when cells started to die, 24–48 h after start of incubation.

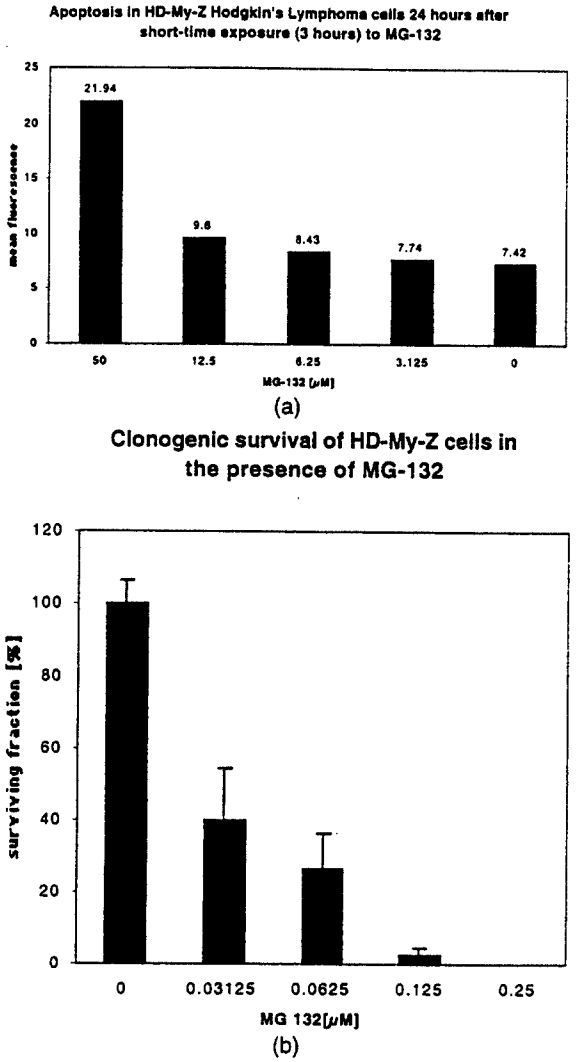

Fig. 7. (a) Apoptosis in HD-My-Z Hodgkin's lymphoma cells 24 h after transient inhibition (3 h) of proteasome function assessed by a TUNEL-assay. MG-132 increased the mean fluorescence signal in a dose-dependent manner. (b) Clonogenicity of HD-My-Z Hodgkin cells, assayed by measurements of the plating efficiency, was drastically decreased by the continuous presence of even low concentrations of MG-132.

these effects remains unclear. It is unlikely to be due to inhibition of proteasome degradation of p53, because HD-My-Z cells constitutively express p53 mRNA and are probably mutated in the p53 gene (19). Recent studies by Herrmann and coworkers excluded any involvement of p53, the JNK kinase pathway, or bcl-2 (29) in MG-132-induced apoptosis, although this in part contradicts an earlier report (30). Interestingly, clonogenic survival of HD-My-Z cells was completely abolished by maintaining them in the presence of concentrations of MG-132 as low as 0.5 μM. This suggests that the proteasome inhibition may be an effective new treatment modality for Hodgkin's disease, providing this level can be attained in humans without adverse toxicity.

There is surprisingly little knowledge on how Hodgkin cells respond to modalities that are classically used to treat

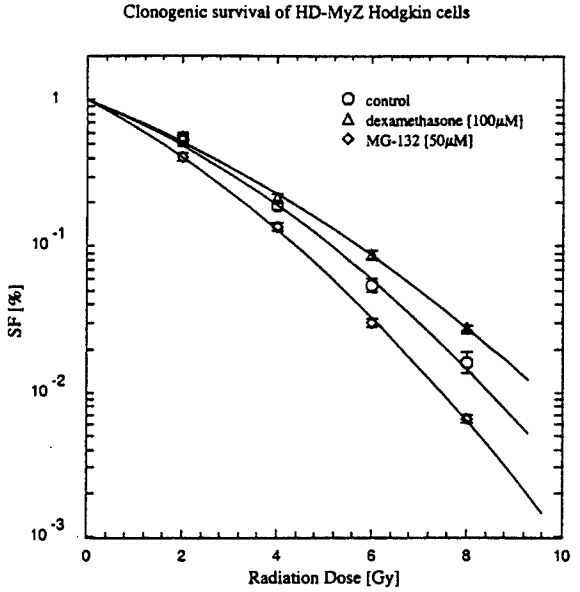

Fig. 8. Survival plots derived from colony forming assays with HD-My-Z cells after treatment with MG-132 or dexamethasone. Intrinsic radiosensitivity of HD-My-Z Hodgkin lymphoma cells was decreased ($p < 0.0001$, F-test) by inhibition of proteasome function by preincubation in MG-132 (50 μM) for 3 h before irradiation. Dexamethasone (100 μM) under the same conditions was a radioprotector ($p < 0.0001$, F-test) (control: $\alpha = 0.298 \pm 0.052$, β 7equal; 0.02893 ± 0.0072, $\alpha/\beta = 10.3$; MG-132: $\alpha = 0.3885 \pm 0.033$, $\beta = 0.03048 \pm 0.0041$, $\alpha/\beta = 12.7$; Dexamethasone: $\alpha = 0.2898 \pm 0.043$, $\beta = 0.01982 \pm 0.0048$, $\alpha/\beta = 14.6$).

this disease. Two such treatments are glucocorticoids and radiation. Glucocorticoids are a standard component of state-of-the-art chemotherapy protocols in Hodgkin's disease. Short-term treatment of HD-My-Z cells with glucocorticoids did not change their constitutive NF-κB activity. It also failed to induce apoptosis. If HD-My-Z cells are representative of the disease, these findings suggest that the tumor shrinkage seen after application of glucocorticoids in patients with Hodgkin's disease may be caused more by death of the inflammatory cells infiltrating the tumor than by death of tumor cells themselves. HD-My-Z was, however, isolated from the pleural effusion of a patient with relapsed and therapy-refractory Hodgkin's disease (19). Thus, this cell line might reassemble the phenotype of relapsing Hodgkin's disease rather than that of cases that are easily cured with standard therapy.

Radiation therapy often causes rapid shrinkage of bulky lymphomas after only a few treatment fractions. To our knowledge, there have been no reports on the radiosensitivity of Hodgkin cell lines. In this study, HD-My-Z cells were found to be moderately radiation resistant. In comparison, PC3 prostate cancer cells were 1.4 times more sensitive. Although it is difficult to extrapolate from these *in vitro* findings to the clinical situation, the results contrast with the doses of irradiation needed clinically to achieve tumor control in these two diseases. One possible explanation for the relative sensitivity of Hodgkin's lymphomas is that the number of malignant cells they contain is usually below 5%, and comparably lower doses would be required to achieve cure.

Interestingly, the proteasome inhibitor, MG-132, radiosensitized HD-My-Z cells. We had a similar result with SiHa cervical cancer cells (Pajonk *et al.*, unpublished data). Although MG-132 also inhibits calpain, there is strong evidence that the effects described in this study are the result of proteasome rather than calpain inhibition. First, MG-132 induces apoptosis in MOLT-4 and L5178Y cells, while Z-leu-leucinal, a specific calpain inhibitor, does not (31). Second, calpain is activated during apoptosis and calpain inhibition prevents apoptosis (32–34). Third, MG-132 is a relatively specific inhibitor for the proteasome (Ki for IκB 3 μM). Inhibition of calpain and cathepsin by peptide aldehydes occurs after doses much lower than those used in this study (5–12 nM) (35) and that are ineffective at inducing apoptosis. Finally, calpain is less important than the proteasome in eukaryotic proteolysis. Almost all short-lived (36) and 70–90% of all long-lived proteins (35, 37) are degraded by the 26s proteasome and its activity is up to 1,000-fold higher than the activity of calpain.

The mechanism for the radiosensitizing effect of proteasome inhibitors seen in this study also has yet to be elucidated. Proteasome inhibitors have been shown to activate caspase-3 indirectly (38). Caspase-3 targets DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase responsible for repair of DNA double strand breaks (39), which is one possible pathway for radiosensitization. In contrast to the effect of proteasome inhibition, treatment with dexamethasone clearly protected HD-My-Z cells from irradiation, as has been reported for other cell lines (40, 41). There is no doubt that steroids are useful in combination with other chemotherapeutics in Hodgkin's disease because shrinkage of bulky tumors will enhance the effect of cytotoxic drugs by reperfusion and/or reoxygenation of malignant cells. However, if the cell line HD-My-Z can be taken as *pars pro toto* for Hodgkin's disease, steroids in combination with radiation therapy should be used with caution. In addition, the use of proteasome inhibitors, perhaps in combination with radiation therapy, has potential as a novel strategy for cases of Hodgkin's disease that resist conventional therapies.

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NOTES

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Inhibition of NF-κB, Clonogenicity, and Radiosensitivity of Human Cancer Cells

Frank Pajonk, Katja Pajonk, William H. McBride

Background: **Activation of the transcription factor NF-κB is part of the immediate early response of tissues to ionizing irradiation. This pathway has been shown to protect cells from tumor necrosis factor-α, chemotherapy, and radiation therapy-induced apoptosis (programmed cell death). However, because the role of NF-κB as a modifier of the intrinsic radiosensitivity of cancer cells is less clear, we have studied the impact of NF-κB on the intrinsic radiosensitivity of human cancer cells.** ***Methods:*** **We used PC3 prostate cancer cells and HD-MyZ Hodgkin's lymphoma cells transduced with an adenovirus vector that contains a gene encoding a form of IκB (an inhibitor of NF-κB) that cannot be phosphorylated. This form of IκB will remain bound to NF-κB; thus, NF-κB cannot be activated. We monitored NF-κB activity with a gel-shift assay and used a colony-forming assay to assess clonogenicity and radiosensitivity.** ***Results:*** **Constitutive DNA-binding activity of NF-κB was dramatically decreased in PC3 cells transduced with the IκB super-repressor gene. The clonogenicity of transduced PC3 cells declined to 19.6% of that observed for untreated control cells, a finding similar to one we have previously demonstrated for IκB-transduced HD-MyZ cells. However, inhibition of NF-κB activity in the surviving PC3 and HD-MyZ cells failed to alter their intrinsic radiosensitivity.** ***Conclusions:*** **We conclude that activation of NF-κB does not determine the intrinsic radiosensitivity of cancer cells, at least for the cell lines tested in this study. [J Natl Cancer Inst 1999;91: 1956–60]**

The immediate early response of mammalian cells to ionizing irradiation includes activation of transcription factors, such as AP-1, p53 (also known as TP53), and NF-κB *(1,2)*. NF-κB activation is an obligatory step *(3)*, leading to expression of almost all genes involved in the inflammatory response generated by irradiation *(4)*, as for other proinflammatory signals.

Activation of NF-κB does not require protein synthesis. Homodimers and heterodimers of its subunits p50, p52, p65/RelA, c-Rel, and Rel-B are located in the cytosol preformed and bound to inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105). Activation of NF-κB requires that IκB is phosphorylated at two serine residues (Ser-32 and Ser-36) by IκB kinases, polyubiquitinated, and subsequently degraded by the 26S proteasome. This process frees NF-κB for translocation to the nucleus and activation of its target genetic programs [reviewed in *(5)*].

Although it is widely accepted that ionizing irradiation can cause a typical inflammatory response by activating NF-κB, the role of this transcription factor as a survival factor for cells after ionizing irradiation remains unclear. In general, activation of NF-κB has been reported to protect cells from apoptosis (programmed cell death) *(6–8)*. However, this is not always the case; cells from patients with ataxia telangiectasia are one exception *(9)*. Although radiation therapy-induced apoptosis has been reported to be associated with radiotherapeutic cure of murine tumors *(10,11)*, its contribution in radiation therapy remains controversial. In most cases, cells in a tumor survive initial damage caused by therapeutic doses of radiation therapy and traverse several cell cycles before finally dying or producing clonogenic survivors *(12)* that cause tumor recurrence. The success of cancer treatment depends mainly on eliminating these tumor stem cells, by whatever pathway.

Activation of NF-κB and consequent inhibition of apoptosis might be expected *a priori* to increase cell survival after irradiation, but the possible relationships of these events to the elimination of clono-

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See "Note" following "References."

genic stem cells after irradiation need further clarification. In this study, we investigated the role of NF-κB in modulating the intrinsic radiosensitivity of two human cancer cells lines, PC3 prostate cancer cells and HD-MyZ Hodgkin's lymphoma cells. These cell lines were chosen because, for different reasons, they have high constitutive levels of NF-κB that might confer relatively high resistance to radiation therapy.

Materials and Methods

Cell Culture

Cultures of PC3 human prostate carcinoma (American Type Culture Collection, Manassas, VA) and of the Hodgkin's cell line HD-MyZ (DSMZ, Braunschweig, Germany) were grown in 75-cm^2 flasks (Falcon Becton Dickinson and Co., Lincoln Park, NJ)) at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Dulbecco's modified Eagle medium (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) was used supplemented with 10% fetal calf serum and 1% penicillin–streptomycin (Life Technologies, Inc.).

Transduction Experiments

The recombinant replication-deficient adenoviruses Ad5-IκB and Ad5-LacZ were provided by R. Batra (University of California/West Los Angeles-Veterans Administration Medical Center). The vectors had been generated and quality tested at the Vector Core at the Gene Therapy Center of the University of North Carolina School of Medicine and are described elsewhere *(13)*. Ad5-IκB contains a gene for IκBα, an NF-κB superrepressor, under control of a cytomegalovirus promoter/enhancer. The encoded IκBα contains serine-to-alanine mutations at positions 32 and 36, preventing the phosphorylation, ubiquitination, and subsequent degradation by the proteasome. Ad5-LacZ is a control virus that contains the gene for β-galactosidase instead of IkBα. Cells were plated in 10-cm culture dishes (Falcon Becton Dickinson and Co.). After 24 hours, the medium was changed and viral vectors containing the nonphosphorable IκBα or β-galactosidase gene were added at a multiplicity of infection (MOI) of 1000. After 2 hours of incubation, the virus-containing medium was replaced by fresh medium, and cells were incubated for an additional 48 hours to allow gene expression. Successful transduction was confirmed by staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside.

Irradiation

PC3 cells were trypsinized, counted, and diluted to a final concentration of 10^6 cells/mL. HD-MyZ cells were dislodged mechanically, counted, and diluted to a final concentration of 10^6 cells/mL. The cell suspensions were immediately irradiated at room temperature with a ^{137}Cs laboratory irradiator (Mark I; J. L. Shepherd and Associates, San Fernando, CA) at a dose rate of 580 rads/minute. Corresponding control cells were sham irradiated.

Cell Extracts and Electrophoretic Mobility Shift Assays

For preparation of total cellular extracts, normal and treated cells were dislodged mechanically, washed with ice-cold phosphate-buffered saline (PBS), and lysed in TOTEX buffer (20 m*M* HEPES [pH 7.9], 0.35 m*M* NaCl, 20% glycerol, 1% Nonidet P-40 [NP40], 0.5 m*M* EDTA, 0.1 m*M* ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 0.5 m*M* dithiothreitol [DTT], 50 μ*M* phenylmethylsulfonyl fluoride [PMSF], and aprotinin [90 trypsin inhibitor U/mL]) for 30 minutes on ice. Lysate was centrifuged at 12 000*g* for 5 minutes at 4 °C. Protein concentration was determined with the BCA protocol (Pierce Chemical Co., Rockford, IL). Fifteen micrograms of protein from the resulting supernatant was incubated for 25 minutes at room temperature with 2 μL of bovine serum albumin (10 μg/μL), 2 μL of poly[d(I–C)] [poly-deoxyinosinic-deoxycytidylic acid] (1 μg/μL), 4 μL of Ficoll buffer (20% Ficoll 400, 100 m*M* HEPES, 300 m*M* KCl, 10 m*M* DTT, and 0.1 m*M* PMSF), 2 μL of buffer D+ (20 m*M* HEPES, 20% glycerol, 100 m*M* KCl, 0.5 m*M* EDTA, 0.25% NP40, 2 m*M* DTT, and 0.1 m*M* PMSF), and 1 μL of the [γ-^{32}P]adenosine triphosphate-labeled oligonucleotide (Promega Corp., Madison, WI; NF-κB: AGTTGAGGGGACTTTCCCAGG). For a negative control, unlabeled oligonucleotide was added to 50-fold excess. Gel analysis was carried out in native 4% polyacrylamide/0.5× TBE (Tris–boric acid–EDTA) gels. Dried gels were placed on a phosphor screen for 24 hours and analyzed on a phosphor imager (Storm 860; Molecular Dynamics, Sunnyvale, CA).

Clonogenic Survival

Colony-forming assays were performed immediately after irradiation by plating an appropriate number of cells into culture dishes in triplicate. After 14 days, cells were fixed and stained with crystal violet, and the number of colonies containing more than 50 cells were counted. The surviving fraction was normalized to the surviving fraction of the corresponding control, and survival curves were fitted by use of a linear-quadratic model.

Determination of Apoptosis

Apoptotic cells were detected with an *In Situ* Cell Death Kit (Boeringer Mannheim GmbH, Mannheim, Germany). The manufacturer's protocol was followed with some minor modifications. Briefly, attached and detached cells were collected, centrifuged at 500*g* for 5 minutes at 4 °C, fixed in ice-cold 75% ethanol, washed with PBS, and pelleted by centrifugation at 500*g* for 5 minutes at 4 °C. Cells were

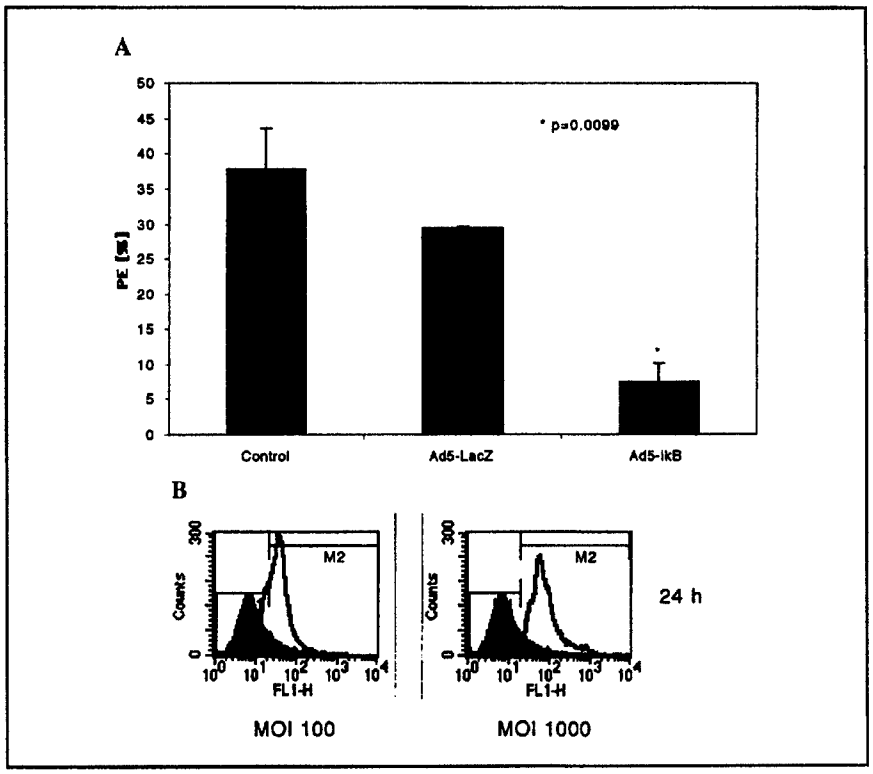

Fig. 1. A) Clonogenicity of PC3 prostate cancer cells after transduction with an IκB super-repressor gene (Ad5-IkB) or a gene for β-galactosidase (Ad5-LacZ) assessed by plating efficiency (PE) in a colony-forming assay. Error bars = 95% confidence interval. *Two-sided P = .010 versus control. B) Flow cytometric analysis of apoptosis in Ad5-IkB-transduced PC3 cells, replated 48 hours after transduction. Twenty-four hours after replating, PC3 cells were analyzed with a TUNEL (terminal deoxynucleotidyltransferase-mediated-uridine triphosphate nick-end labeling) assay. Transduction increased the apoptotic fraction from initially 15% (untreated control cells) to 73% (multiplicity of infection [MOI] = 100) and 90% (MOI = 1000) 24 hours after replating. **Solid areas** = control cells; **open areas** = transduced cells. FL1-H = intensity of the fluorescence signal (excitation = 488 nm/emission = 518 nm).

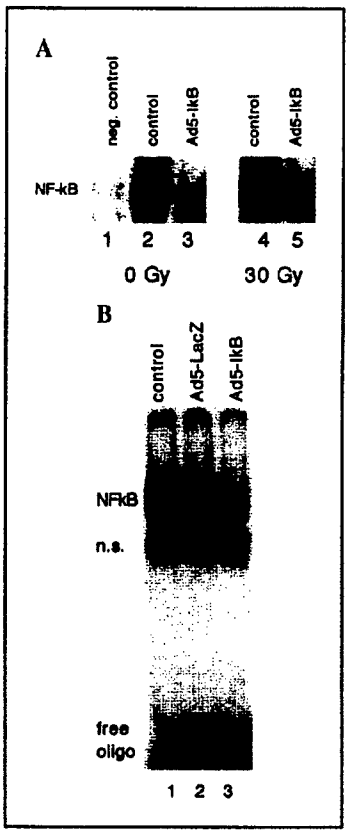

Fig. 2. A) Gel-shift experiment with cytosolic extracts of PC3 prostate cancer cells 48 hours after inhibition of NF-κB activity by transduction of an IκB superrepressor gene. Fifteen micrograms of protein was used for each lane. Negative (neg.) control cells with unlabeled oligonucleotide in a 50-fold excess (**lane 1**), control cells (**lane 2**), and cells transduced with Ad5-IkB vector (**lane 3**) are shown as well as control cells (**lane 4**) and Ad5-IkB-transduced cells (**lane 5**) 3 hours after a 30-Gy irradiation. **B**) The IκB super-repressor gene product inhibits radiation-induced NF-κB activation. Control cells (**lane 1**), cells transduced with control Ad5-LacZ vector (**lane 2**), and cells transduced with Ad5-IkB vector (**lane 3**) are shown. The mutated IκB gene product decreases constitutive NF-κB activity, whereas the control vector has no effect. n.s. = nonspecific; oligo = oligonucleotide.

permeabilized by resuspension in a solution of 0.1% Triton X-100 and 0.1% sodium citrate and incubated for 2 minutes on ice. Cells were washed twice in PBS, resuspended in TUNEL (terminal deoxynucleotidyltransferase-mediated-uridine triphosphate nick-end labeling) reaction mixture, and incubated for 60 minutes at 37 °C. After three washes with PBS, fluorescence was measured at 518 nm in a flow cytometer (FACScan System: Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed with the CellQuest software (Becton Dickinson Immunocytometry Systems).

Statistics

All data are means ± 95% confidence intervals. A P value of <.05 from Student's t test was considered to be statistically significant. All statistical analyses were carried out with the JMP (version 3.2) software package from SAS (SAS Institute, Inc., Cary, NC) for Macintosh. All P values are two-sided.

Results and Discussion

We have recently shown that PC3 prostate cancer cells contain high levels of constitutive NF-κB activity, almost five times higher than HD-MyZ Hodgkin's cells under comparable conditions. In PC3 cells, high constitutive NF-κB activity is, at least in part, due to a high level of 26S proteasome activity (Pajonk F, Pajonk K, McBride WH: unpublished results). In HD-MyZ cells, NF-κB is also constitutively active, although the reason remains unclear *(14)*.

As we have shown for HD-MyZ cells (Pajonk F, Pajonk K, McBride WH: unpublished results), adenoviral vectors were highly efficient at inserting genes into PC3 prostate cancer cells. Transduction rates in excess of 99%, confirmed by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside, were achieved at

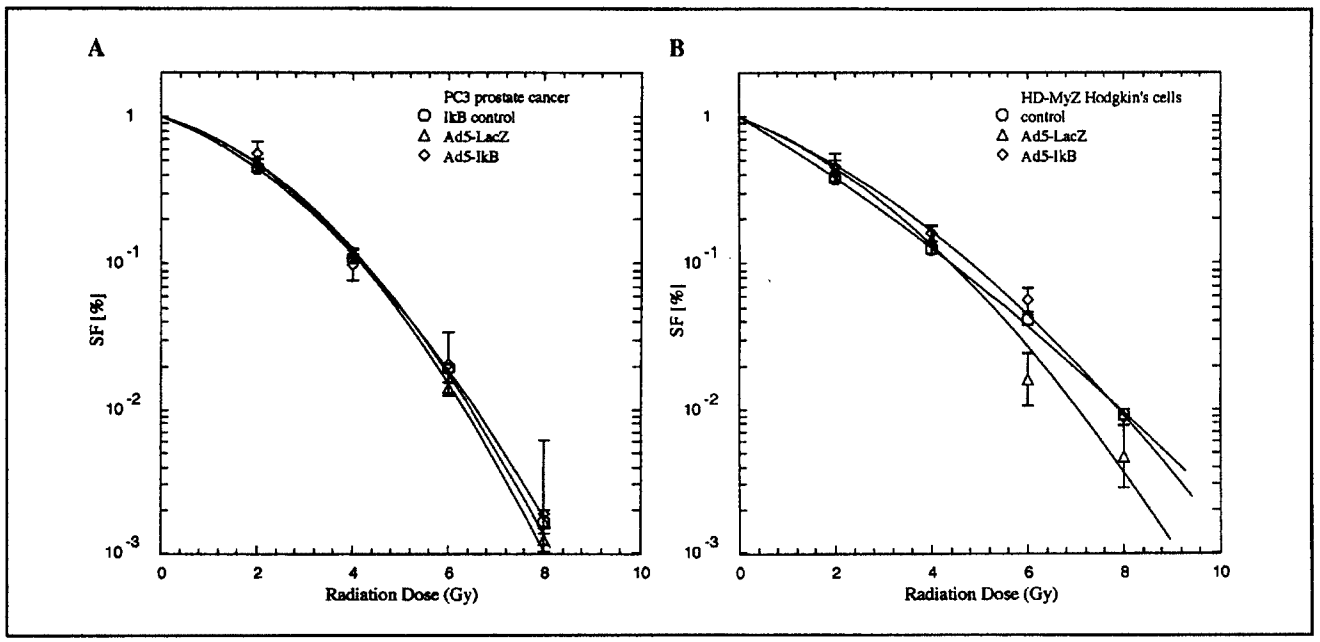

Fig. 3. Clonogenic survival of PC3 prostate cancer cells (**A**) (multiplicity of infection [MOI] = 1000) and HD-MyZ Hodgkin's lymphoma cells (**B**) (MOI = 100) 48 hours after transduction with a gene for the IκB super-repressor cells. Cells were irradiated, and 2000–20 000 cells were plated into culture dishes. Transduction with Ad5-LacZ and Ad5-IkB did not alter the sensitivity of PC3 cells and HD-MyZ cells to radiation therapy. SF = surviving fraction. Data are means ± 95% confidence intervals.

Table 1. Radiobiologic parameters*

	Control	Ad5–LacZ	Ad5–IkB
PC3 prostate cancer cells			
Alpha	0.279 ± 0.084	0.205 ± 0.065	0.208 ± 0.55
Beta	0.064 ± 0.012	0.082 ± 0.011	0.078 ± 0.078
Alpha/beta	4.4	2.5	2.7
HD-MyZ Hodgkin's lymphoma cells			
Alpha	0.449 ± 0.067	0.3 ± 0.27	0.31 ± 0.12
Beta	0.017 ± 0.009	0.05 ± 0.039	0.035 ± 0.015
Alpha/beta	27	6	8.9

*Data are means ± 95% confidence intervals. Alpha and beta coefficients are obtained from curve fitting the data points in Fig. 3 with a linear-quadratic model (LQ model).

a MOI of 1000 (data not shown). This MOI caused no visible transduction-related toxicity after 48 hours, but when PC3 cells transduced with the control vector were trypsinized and replated at that time, their plating efficiency was slightly reduced to 29.5% ± 0.24% (78% of the nontransduced control level [37.9% ± 5.63%]; $P = .11$; Student's t test). However, the clonogenicity of cells transduced with the adenoviral vector containing the IκB superrepressor gene was greatly reduced to 7.4% ± 2.67% (19.6% of the nontransduced control level; $P = .010$; Student's t test; Fig. 1, A). These findings are almost identical to our results for HD-MyZ cells (Pajonk F, Pajonk K, McBride WH: unpublished results).

After 24 hours, 16% of nontransduced PC3 cells had entered apoptosis. Cells transduced with the IκB superrepressor gene had an increased apoptotic index of 73% (MOI = 100) and 90% (MOI = 1000) (Fig 1, B).

DNA-binding activity of NF-κB in HD-MyZ cells has been reported to be high in comparison to other tumor cell lines *(14)* because of mutated IκB. We had previously shown that NF-κB levels in HD-MyZ cells decreased after transduction with the IκB super-repressor gene (Pajonk F, Pajonk K, McBride WH: unpublished results). We examined whether PC3 cells responded in the same way. The same vector containing the gene for β-galactosidase was used as the control for changes in NF-κB activity caused by the vector itself. DNA-binding activity of NF-κB was measured with a gel-shift assay. Radiation therapy-induced (30 Gy, 3 hours after exposure; Fig. 2, A) and constitutive NF-κB activity was dramatically decreased in PC3 cells transduced with the IκB super-repressor gene but not in cells transduced with the β-galactosidase gene 48 hours after transduction (Fig. 2, B).

To study whether activation of the NF-κB survival pathway alters the radiation response of cancer cells, we exposed PC3 and HD-MyZ cells to 0–8 Gy of ionizing irradiation and measured clonogenic survival in a colony-forming assay. Transduction of either cell line with Ad5-LacZ and Ad5-IkB did not change the sensitivity of these cells to radiation therapy. There was no statistically significant change in the alpha or beta parameters obtained from a linear-quadratic fit (Fig. 3; Table 1).

Even though ionizing radiation has been repeatedly reported to activate NF-κB *(15–18)*, the role of this pathway in preventing radiation-induced cell death is less clear. Jung et al. *(9)* reported that inhibition of NF-κB activation in cells derived from a patient with ataxia telangiectasia group D by transfection with an IκB that cannot be phosphorylated restored normal sensitivity to radiation therapy. In contrast, other groups have shown that activation of NF-κB protects against apoptosis induced by chemotherapy, tumor necrosis factor-α, or ionizing irradiation in several different cell types *(6–8)*. Nakshatri et al. *(19)* showed that loss of hormone dependency and progression to a more aggressive tumor phenotype coincides with constitutive activation of NF-κB in breast cancer. Another study *(20)* reported that activation of this pathway is related to chemotherapy resistance.

So far, there has been only one report describing a possible relationship between NF-κB activity and the intrinsic radiosensitivity of human cancer cells *(21)*. In that study, the authors selected cell clones from p53-negative glioma cell lines with high-level expression of wild-type IκBα messenger RNA after transfection with an expression plasmid for this gene. Inconsistently, only two of the clones that were selected had both high IκBα protein levels and increased sensitivity to radiation therapy. The possibility of selection of radiosensitive clones rather than IκB-related radiosensitization cannot be excluded as an explanation for these findings.

In this study, we used an adenoviral vector to insert a gene for the IκB superrepressor into PC3 prostate cancer cells and HD-MyZ Hodgkin's lymphoma cells; this IκB has been shown to efficiently inhibit constitutive, radiation therapy-induced, and tumor necrosis factor-α-induced activation of NF-κB (Pajonk F, Pajonk K, McBride WH: unpublished data). Both cell lines most likely carry a mutated p53. Transduction rates of more than 99% guaranteed inhibition of NF-κB in almost all cells. The data from this study show that radiosensitivity of two human cancer cell lines with high levels of constitutively activated NF-κB is not dependent on this pathway. Comparable results were recently reported for NF-κB and cytotoxic drugs *(22)*. However, inhibition of NF-κB binding to DNA drastically decreased the clonogenicity in both cell lines, emphasizing the importance of NF-κB activation for survival of these human cancer cells.

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NOTE

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Glutathione *S*-Transferase Mu and Theta Polymorphisms and Breast Cancer Susceptibility

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Background: **The enzymes encoded by the glutathione *S*-transferase mu 1 (GSTM1) and theta 1 (GSTT1) genes are involved in the metabolism (mainly inactivation, but activation is possible) of a wide range of carcinogens that are ubiquitous in the environment; the enzyme encoded by the GSTT1 gene may also be active in endogenous mutagenic processes. Homozygous deletions of the GSTM1 and GSTT1 genes are commonly found in the population and result in a lack of enzyme activity. This study was undertaken to evaluate the association between GSTM1 and GSTT1 gene polymorphisms and breast cancer risk.** ***Methods:*** **Our study included 466 women with incident cases of breast cancer occurring from May 1989 through May 1994 and 466 matched control subjects. These individuals were part of a prospective cohort of U.S. women (i.e., the Nurses' Health Study). Odds ratios (ORs) and 95% confidence intervals (CIs) from conditional logistic regression models were used to estimate the association between genetic polymorphisms and breast cancer risk.** ***Results:*** **The GSTM1 and GSTT1 null genotypes were not associated with an increased risk of breast cancer (OR = 1.05 [95% CI = 0.80–1.37] for GSTM1 null; OR = 0.86 [95% CI = 0.61–1.21] for GSTT1 null). On the contrary, a suggestion of a decreased risk of breast cancer associated with the GSTT1 null genotype was observed among premenopausal women. When considered together, no combination of the GSTM1 and GSTT1 genotypes was associated with an increased risk of breast cancer. The relationship between GSTM1 and GSTT1 gene deletions and breast cancer risk was not substantially modified by cigarette smoking.** ***Conclusions:*** **Our data provide evidence against a substantially increased risk of breast cancer associated with GSTM1 and/or GSTT1 homozygous gene deletions. [J Natl Cancer Inst 1999;91:1960–4]**

Recognized risk factors for breast cancer cannot fully explain the observed variation in breast cancer incidence over time and across geographic locations *(1,2)*. Environmental carcinogens, such as polycyclic aromatic hydrocarbons, could be responsible for some of the unexplained variation *(3,4)*. Many chemical carcinogens are activated or inactivated through metabolic reactions. Genetically determined differences in the activity of metabolizing enzymes involved in these reactions might contribute to host susceptibility to cancer; thus, taking these genetic factors into account may improve our ability to determine if environmental chemicals contribute to breast cancer *(5)*.

The glutathione *S*-transferase mu (GST-M1) and theta (GST-T1) are separate isoforms of glutathione transferase enzymes that participate in the metabolism of a wide range of chemicals, including possible carcinogens *(6)*. The known substrates for the GST-M1 enzyme in-

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Molecular Pathways That Modify Tumor Radiation Response

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Aberrant expression of signal transduction molecules in pathways controlling cell survival, proliferation, death, or differentiation are a common feature of all tumors. The identification of the molecules that are involved allows the development of novel tumor-specific strategies. Not surprisingly, targeting these pathways often also results in radiosensitization. The efficacy of such directed therapies may, however, be limited by the heterogeneity and the multiple mutations that are associated with the cancerous state. A more robust alternative may be to target global mechanisms of cellular control. The ubiquitin/proteasome degradation pathway is one candidate for such therapeutic intervention. This pathway is the main posttranscriptional mechanism that controls levels of many short-lived proteins involved in regulation of cell cycle progression, DNA transcription, DNA repair, and apoptosis. Many of these proteins are involved in various malignancies and/or radiation responses. In recent years, proteasome inhibitors have gained interest as a promising new group of antitumor drugs. PS-341, a reversible inhibitor of proteasome chymotryptic activity, is currently being tested in phase I clinical trials. In this study, we show that proteasome inhibition by PS-341 can alter cellular radiosensitivity in vitro and in vivo, in addition to having direct antitumor effects.

Key Words: Signal transduction pathways—Radiation response—Radiosensitization—Proteasome—PS-341.

In recent years, numerous studies have demonstrated that the cellular response to irradiation can be modified by intracellular or extracellular manipulation of signal transduction pathways.[1–6] Those studies dealing with the effects of cytokines/growth factors, cell–cell contact, and extracellular matrix interactions often reflect classic radiobiologic experiments demonstrating repair of potentially lethal damage. The primary role of these extracellular signals in organized tissues is to act in concert with the programmed expression of specific receptors to define a sense of position and to control cell proliferation and differentiation under physiologic and damage conditions. Mispositioning of a cell leads to homelessness (or "anoikesis") and a tendency toward death by "neglect" because of a lack of appropriate signals. It also tends to result in a state of relative radiosensitivity.[7,8] In vivo, this can be seen in a frequent coincidence of the spatial distribution of apoptotic cells after tissue irradiation with areas of active proliferation.[9] Indeed, radioresponsiveness in general is likely to be spatially dependent and governed by both the signal transduction pathways preexisting in a cell before irradiation, and those activated by irradiation.

The difference between tumors and normal tissues is that the former have mutated genes that affect the signaling pathways that control cell proliferation, differentiation, or death. These mutations allow them to circumvent positional control mechanisms and survive in a state of relative positional independence. Signal transduction pathways become reequilibrated, and the cells become addicted to specific pathways for survival. The therapeutic advantage is that tumor cells are very sensitive to blockade of these pathways and they may serve as an "Achilles heel" for that cancer. Identification and characterization of these pathways in individual cancers is therefore very important for selection of an appropriate specific therapy. New gene discovery techniques will be extremely useful in this regard. Already a number of molecular cell pathways that are mutated in cancer and that appear to determine survival or death after irradiation have been identified, and various agents, primarily designed as sole therapies, have been devised that also serve to radiosensitize.[10] Although optimism for pathway-specific therapies for cancer is high, it should be noted that: (1) overexpression of a known survival factor does not necessarily indicate its involvement in an addictive pathway or characterize a radioresistant tumor;[11] (2) the pathways are, to an extent, cell type-specific; and (3) there is much yet to be learned about the apparently intimate relationship between proliferative/growth arrest and survival/death pathways and radiosensitization.

Because cancers acquire multiple mutations and develop heterogeneity that might prevent a complete cure if

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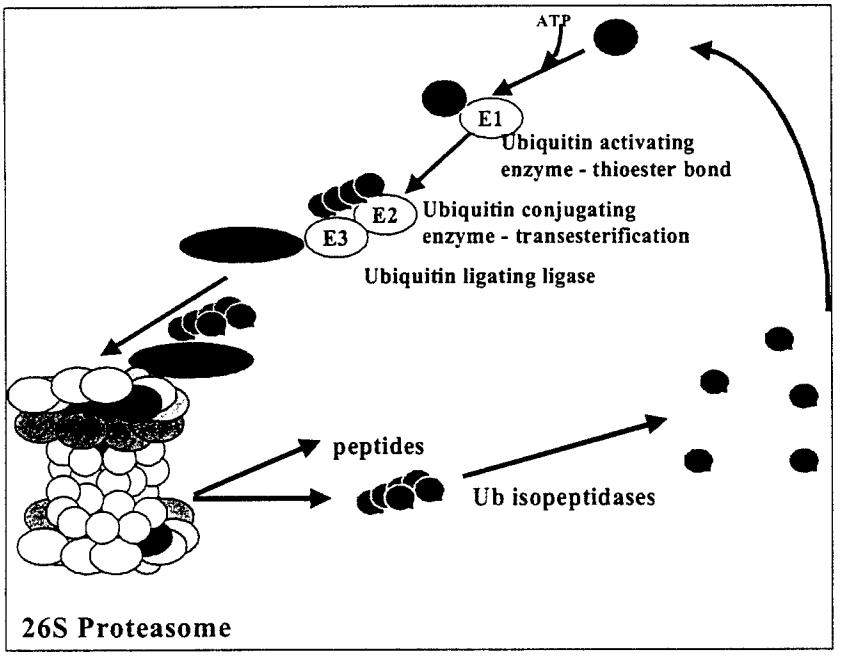

FIG. 1. Ubiquitin/proteasome degradation pathway. Ubiquitin is a 76-residue protein that can be attached by an isopeptide bond between the e-amino group of lysine on the target and the C-terminal glycine of ubiquitin by a series of activating (E1), conjugating (E3), and ligating (E3) enzymes or adaptor molecules. The polyubiquitinated product then becomes the target for 26S proteasome degradation with recycling of ubiquitin by isopeptidases.

single molecules were targeted as a sole therapy, combination of these biologic approaches with cytotoxic therapies, such as radiotherapy, would seem desirable, in particular, because radio- and chemosensitization is a frequent consequence of treatment. In addition, targeting "downstream" nodal points at which multiple pathways converge may be a better therapeutic strategy than targeting a single gene, a single protein, or a single afferent molecular pathway.

One potential new target for cancer therapy that has recently emerged is the ubiquitin/proteasome system (Fig. 1). This pathway is the main posttranscriptional mechanism that simultaneously controls levels of many short-lived proteins involved in regulation of cell cycle progression, DNA transcription, DNA repair, apoptosis, angiogenesis, inflammation, immunity, and cell growth. Some of the proteins regulated by proteasomal degradation that are relevant to radiation include p53,[12–14] mdm2,[15] p21,[16,17] p27,[18] RB,[19] cyclins A, B and E,[20–22] NF-κB/IκB,[23,24] c-Myc,[25–27] c-Jun,[28] c-Fos,[29] HIF-1α,[30,31] DNA-PKcs,[32] rad23,[33] Bcl-2,[34–36] bax,[37] and caspase-3.[38,39] Many of these molecules are radiation inducible through posttranslational, proteasome-dependent mechanisms and modulate cellular responses to irradiation. Indeed, the proteasome itself appears to be a redox-sensitive target for radiation.[40] The ubiquitin/proteasome system promises to yield a large variety of potential anticancer strategies. In the future, many of these may be directed against the E3 ubiquitin ligases that target specific molecules for destruction. The reversible nonspecific inhibitor of proteasome chymotrypsin-like activity, PS-341 is the first agent deliberately targeting this system to enter phase I clinical trials for cancer.[41]

The consequences of inhibiting proteasome activity with reversible and nonreversible drugs have been described in numerous in vitro studies as cell cycle arrest and death by apoptosis.[36,38,42] In addition, we have shown that the reversible proteasome inhibitor MG-132 can radiosensitize cells in vitro.[24] PS-341 has been previously shown to slow tumor growth in vivo,[43] and, in this study, we show that it can affect cellular radiosensitivity in vitro and in vivo.

PROTEASOME INHIBITORS INCREASE RADIOSENSITIVITY OF CANCER CELLS

Our in vitro experiments with proteasome inhibitors MG-132 and PS-341 show synergistic effects of the drugs and ionizing radiation on the survival of TRAMP-C1 mouse prostate carcinoma cells. For these experiments, cells were treated with 100 nmol/l PS-341 and 10 μmol/l MG-132 (50% survival doses) for 3 hours, and the drug was washed out before irradiation. Treatment resulted in dose enhancement ratios of 1.5 and 1.2, respectively (Fig. 2A). To test the dose dependence of the radiosensitization effect, experiments with different doses of PS-341 were performed (Fig. 2B). These revealed that the drug has a radiosensitizing effect at doses as low as 2.5 and 5 nmol/l (dose enhancement ratios of 1.1 and 1.5, respectively), cause only partial inhibition of

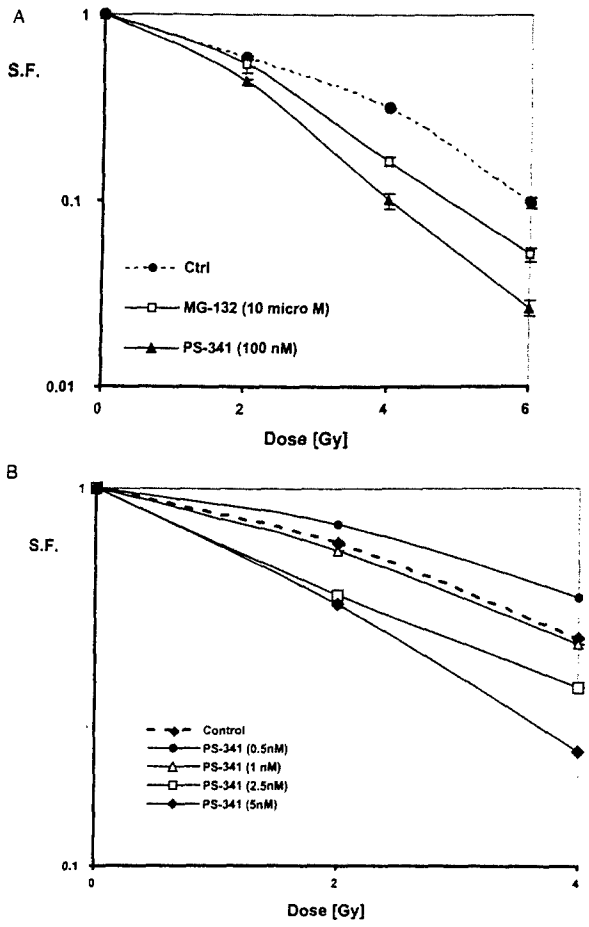

FIG. 2. Clonogenic assay. Murine TRAMP-C1 prostate adenocarcinoma cells were trypsinized, washed, and incubated at 37°C in the presence of (**A**) 100 nmol/l PS-341 or 10 μmol/l MG-132 or (**B**) 0.5, 1, 2.5, and 5 nmol/l PS-341. Three hours later, cells were washed to remove drugs, irradiated with 2, 4, or 6 Gy, and plated in culture dishes. After 14 days cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Survival fraction is expressed as percentage of control.

proteasome activity (30–50%) and are minimally toxic, causing less than 15% cell death. At very low dose levels (<1 nmol/l), PS-341 seems to have no effect on clonogenic cell survival after irradiation, and it may even act to slightly protect cells.

PS-341 has been shown to slow the growth of PC3 tumors in vivo.[43] We observed a similar delay in tumor growth after combined treatment with PS-341, and the effect of irradiation that was at least additive over drug or radiation treatment alone (Fig. 3). Mice treated with 0.3 mg/kg of PS-341 intravenously 3 hours before a single dose of 25-Gy radiation showed a 15-day tumor growth delay versus 6 to 7 days with radiation or drug alone.

DISCUSSION

Targeting individual molecules that are aberrantly expressed in specific cancers can often lead to tumor radiosensitization. This approach has enormous appeal, but may be limited by the heterogeneity and multiple mutations that are associated with the cancerous state. Targeting downstream nodal points involving multiple pathways may be a more robust strategy. Perhaps the ultimate downstream pathway for molecules involved in radiation responses is the proteasome. The proteasome inhibitor PS-341 has entered phase I clinical trials alone and in combination with chemotherapeutic agents (fluorouracil and leucovorin)[41] (Adams J, personal communication, 2000). It appears to be surprisingly well tolerated in mice and humans, provided the intertreatment interval is sufficient to protect against gastrointestinal toxicity.[42] Proteasome inhibitors have been shown to result in apoptosis of almost all cancer cells by mechanisms that have yet to be fully elucidated. Drexler,[44] as well as Soldatenkov and Dritschilo,[45] has suggested that activation of a Bcl-2-sensitive pathway is involved, whereas Hermann and coworkers[46] excluded Bcl-2 and p53 involvement. Normal cells, in contrast, appear to be less affected by treatment, and may even be initially rescued from apoptosis induced by ionizing radiation, glucocorticoids, or phorbol ester inhibitors.[47] Cycling cells are perhaps more sensitive than nonproliferating cells because of the dependency of the cyclin/cdk system on proteasome function.[48] It is also possible that the addiction of cancer cells for various stress proteins gives them greater sensitivity to proteasome inhibition. For example, NF-κB is a survival pathway that is overexpressed in numerous cancers, and activation of NF-κB is tightly controlled through degradation of its inhibitor IκB by the ubiquitin/proteasome system. Proteasome inhibition results in inhibition of NF-κB expression, and, although this does not necessarily result in radiosensitization,[11] cells that are addicted to this pathway may be particularly sensitive. This may explain the promising preliminary responses to PS-341 treatment seen in multiple myeloma (Adams J, personal communication, 2000), where NF-κB expression is probably maintained through the action of interleukin-6 acting in as an autocrine growth factor.

The importance of the ubiquitin/proteasome system for cellular function and responses to therapy has only recently come to the fore. This system integrates with phosphorylation/dephosphorylation as a master controller of intracellular events and is assuming an importance at least as great as these better understood pathways. Expression of many of the molecules critically involved in DNA repair, cell death, and stress reactions are modulated through this pathway. It is redox and radiation sensitive and has proved to be intimately involved in radiation responses at many different levels.[40] Although the extent of the diversity of proteasome structures and ubiquitin-related molecules has yet to be fully appreciated, and their relevance to therapeutic responses yet to

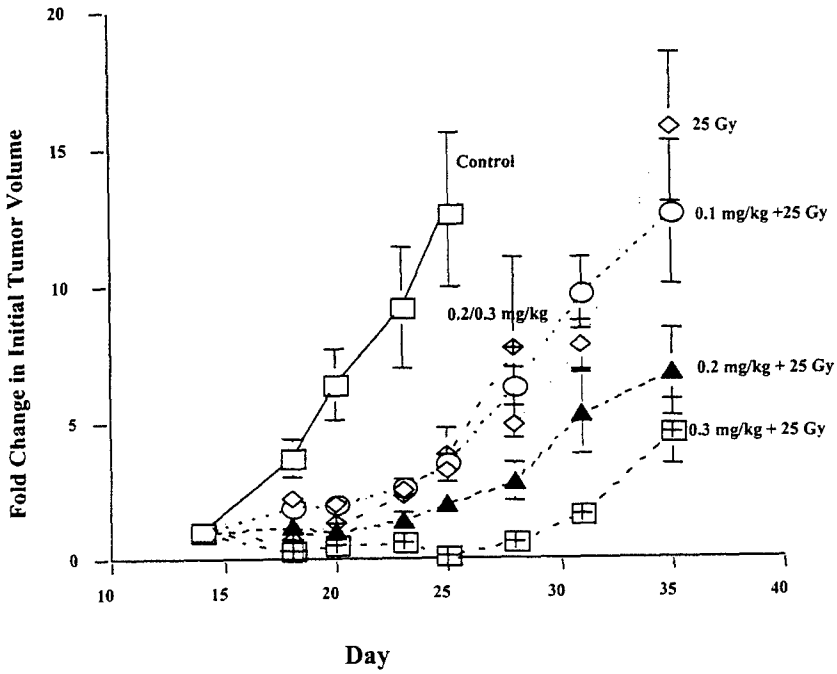

FIG. 3. In vivo tumor growth assay. TRAMP-C1 cells (5×10^5) were implanted subcutaneously in the hind legs of 8-week-old C57/Bl6 mice. When tumors were 6 mm in diameter, animals were treated with different doses of PS-341 (0.1, 0.2, and 0.3 mg/kg intravenously) alone or in combination with 25-Gy local tumor irradiation. Tumor volume was measured until tumors reached 12 mm in diameter.

be elucidated, this system promises to yield strategically important targets for cancer treatment, including improving the therapeutic benefit of radiation therapy.

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the immobilized peptide initiates a signal pathway(s) that either competes or inactivates survival pathways induced by the damaging agents. (Supported in part by CA 75152, CA 56666, ADCRC 9908 and T34GM08718)

#3583 Protein Kinase C Delta Involvement in Radiation-Induced Mammary Tumor Cell Death. M.A. McCracken, R.A. McKay, and J.S. Strobl. *Isis Pharmaceutical, Carlsbad, CA, and West Virginia University, Morgantown, WV.*

Protein kinase C (PKC) delta functions in survival/death signaling pathways in human mammary tumor cells. Inhibition of PKC delta in MCF-7 cells by stable ectopic expression of PKC delta regulatory domain (RD delta) antagonized 12-*O*-tetradecanylphorbol-13-acetate (TPA) induced apoptosis (Kufe et al, 2000). Expression of PKC RD delta in MTLn3 mammary tumor cells decreased anchorage-independent growth (Jaken et al, 1999). Gamma irradiation (IR) of MCF-7 and MDA-MB-231 cells resulted in giant cell formation, impaired clonogenic survival and decreased cell numbers as assessed using the tetrazolium dye metabolism assay (MTS). Apoptotic death of irradiated MCF-7 cells was rare (~.6%) (Strobl, 1998). We hypothesized that inhibition of specific PKC isoforms would enhance radiation-induced cell death. We compared the effects of PKC delta antisense (ISIS#13513) and scrambled PKC delta antisense oligonucleotide (ISIS#13514) on radiation-induced cell death of MCF-7 and MDA-MB-231 cells. Survival was measured 5 or 7 days after antisense + 5.6 Gy IR treatment using the MTS assay. In MCF-7 cells, IR + 200nM ISIS#13513 reduced cell survival 1.4-fold (n=3) compare with IR + ISIS#13514. In MDA-MB-231 cells, IR + 100nM ISIS#13513 reduced cell survival by 1.8-fold (n=4) compared with IR + ISIS#13514. Rottlerin is a selective PKC delta inhibitor (IC50=3-6uM). Rottlerin (6uM) decreased cell survival after 5.6Gy IR by 4.6- and 2.4-fold, respectively, in MCF-7 and MDA-MB-231 cells. These data suggest that PKC delta inhibition causes radiosensitization of human breast tumor cell lines in vitro, and support the development of PKC delta isoform specific inhibitors as radiosensitizing agents. Supported by USARMY DOD Grant#DAMD17-99-1-9449 and The Susan G. Komen Foundation. Jaken S et al (1999) *Can.Res.* 59:3230-38; Kufe D et al (2000) *J.Biol.Chem.* 275:21793-96; Strobl J et al (1998) *B.C.R.T.* 51:83-95.

#3584 Lack of Radiosensitization after Monastrol Treatment of Two Human Carcinoma Cell Lines. Xuexian Yan and Ming Teng. *Vanderbilt University Medical Center, Nashville, TN.*

OBJECTIVE: Monastrol is a newly developed inhibitor of mitotic spindle bipolarity. Specifically, monastrol inhibits the motility of the mitotic kinesin Eg5. This inhibition causes the cells to be arrested in the G2/M phase of the cell cycle. Since several compounds which have similar effect on mitosis have been proved to sensitize ionic radiation, We combined monastrol and radiation on two human carcinoma cell lines, MCF-7 breast cancer cell line and Du-145 prostate cancer cell line, and tried to figure out if monastrol is a potential radiation sensitizer. METHODS: Both cell lines were exposed to 5 achievable monastrol concentrations ranging from 10 uM to 200 uM. Monastrol pretreatment for 24 hours before radiation was tested. The radiation dose ranged from 0 to 7.5 Gy for MCF-7, 0 to 10 Gy for DU-145 delivered in a single fraction. Cellular surviving after treatment with monastrol and /or radiation was determined by colonogenic surviving assay. Cell cycle distribution as determined by flow cytometry was performed after various dose-time combination of monastrol. RESULTS: Flow cytometry studies with monastrol alone demonstrated a high dose-dependent effect of G2/M phase block in both cell lines. Resultant surviving fractions were also time and dose dependent. The interaction between monastrol and radiation was primarily additive in each of the cell lines for all monastrol concentration-radiation dose combinations studied. CONCLUSIONS: Monastrol significantly arrested the cells in G2/M phase. But it lacked a radiosensitizing effect on MCF-7, DU-145 cells in this study.

#3585 Genetic Basis of Clinical Radiation Response in Glioblastoma Multiforme. M. Golubic, O. Chernova, L. Hawthorn, S. Chernova, J. Evans, K. Signorelli, J. Cowell, and G. H. Barnett. *Cleveland Clinic Foundation, Cleveland, OH.*

Glioblastoma multiforme (GBM) is an almost uniformly fatal brain tumor, with patients' median survival time of less than one year in spite of aggressive treatments including surgery, radiation, and chemotherapy. Despite the clear benefits of radiation therapy in prolonging the survival of some patients with GBM, only about one third of patients demonstrate objective radiographic response. To pinpoint genetic difference between GBMs that responded well to radiation treatment and those that did not, we analyzed gene expression profiles of two non-responding (NR), and two responding (R) tumors using Affymetrix Hu6800 oligonucleotide arrays. Comparison between two paired tumor samples (R vs NR) revealed that 423 and 236 genes were differentially expressed (sort score > 0.5). Of those, 33 genes were consistently increased or decreased in their expression in both R tumors compared to NR tumors. These differentially expressed genes are known to regulate cell motility, cellular responses to DNA damage, cell cycle, angiogenesis, and apoptosis. For example, decreased expression of genes known to stimulate tumor cell motility and increased expression of genes that inhibit cell migration was observed in R tumors. The gene expression of six candidate genes was also determined by real time PCR quantitation analysis of four GBM samples. Investigation of these genes should help provide important insights into the biologic mechanisms at work, facilitate identification of tumors that are susceptible or resistant to radiation therapy, and design approaches to specifically enhance radiosensitivity of these deadly neoplasms.

#3586 TPA Radiosensitizes LNCaP Cells by Down Regulation of ATM. Adriana Haimovitz-Friedman, Maureen McLoughlin, Desiree Ehleiter, Wen-Chieh Liao, Richard Kolesnick, and Zvi Fuks. *Memorial Sloan-Kettering Cancer Center, New York, NY.*

We reported recently that treatment of LNCaP human prostate cancer cells with TPA and radiation initiated an apoptotic response via activation of the enzyme ceramide synthase (CS) and *de novo* synthesis of the sphingolipid ceramide (Garzotto M et. al. 1999), while radiation alone failed to induce apoptosis in LNCaP cells. Recent studies in our laboratory showed that the CS pathway is activated in response to DNA damage and that this activation is regulated by the Ataxia Telangiectasia-Mutated (ATM) gene product (Liao W-C et. al. 1999). Functional loss of the ATM protein, mutated in the human genetic disorder of ataxia-telangiectasia (A-T), leads to a pleiotropic phenotype, with radiation hypersensitivity as one of the most widely studied. In this study we demonstrated that TPA reduced the amount of ATM protein by 70% in LNCaP cells. Radiation alone had no significant effect on the ATM protein levels and together with TPA there was no significant further reduction in ATM protein levels. TPA-induced reduction in the levels of ATM protein correlated with increased apoptosis in LNCaP cells. Employing quantitative RT-PCR we showed a 50% reduction of ATM mRNA between 8-16hr of treatment with TPA. Using gel-shift analysis we showed a significant reduction in the amount of Sp-1 binding in extracts from LNCaP cells, as early as 1hr post TPA treatment, peaking at 2hr and increasing back close to control levels by 4hr. These data demonstrates that ATM can be altered at the transcriptional level by incubation with TPA over a 16 h period. Under these conditions TPA-treated LNCaP cells became sensitive to ionizing radiation. These investigations define a new approach to overcome radiation resistance in human prostate cancer cells.

#3587 Gemcitabine Cytotoxicity and Radiation Sensitivity in Mismatch Repair Proficient and Deficient HCT116 Cells. Blaine W. Robinson, Mats Ljungman, and Donna S. Shewach. *University of Michigan, Ann Arbor, MI.*

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; dFdCyd) is a potent ionizing radiation sensitizer in solid tumor cell lines *in vitro* and *in vivo*. Previous work has suggested that radiosensitization (RS) by dFdCyd requires cells to accumulate in S-phase during drug treatment and progress through the cell cycle after subsequent irradiation. While neither dFdCyd triphosphate nor dFdCyd in DNA predicts RS, depletion of dATP due to dFdCyd diphosphate-mediated inhibition of ribonucleotide reductase strongly correlated with RS. This led us to hypothesize that incorporation of an incorrect nucleotide for the missing dATP was important for RS with dFdCyd, and therefore cells deficient in mismatch repair (MMR) would exhibit greater RS. We tested this hypothesis by evaluating the ability of HCT116 colon carcinoma cell lines, which differ in MMR proficiency, to be radiosensitized by dFdCyd. HCT116 + ch2 cells are MMR-deficient due to the lack of hMLH1, and insertion of chromosome 2 provided a second copy of the MMR protein hMSH2. HCT116 + ch3 cells are MMR-proficient with insertion of chromosome 3 containing hMLH1. The MMR-proficient cell line was more sensitive to dFdCyd alone than the MMR-deficient line, with IC_{50} values of 40 and 90 nM, respectively. Interestingly, these cells could not be radiosensitized at concentrations of dFdCyd $\leq IC_{50}$ although extremely high concentrations of dFdCyd ($\geq IC_{90}$) enhanced cell killing with radiation. In contrast, the MMR-deficient cell line showed enhanced cell killing with radiation at the IC_{50} and IC_{90} of dFdCyd, with radiation enhancement ratios of ~1.5. Cell cycle analysis using dual parameter flow cytometry demonstrated that both cell lines accumulated in S-phase following dFdCyd treatment at the IC_{10} and IC_{50}, and shortly after irradiation a prominent but transient G$_2$/M block was observed. In the MMR-deficient cell line, the IC_{10} and IC_{50} of dFdCyd produced a ≥90% decrease in dATP within 4 hours after drug addition, and this low dATP level was maintained for another 4-20 hours. In contrast, the IC_{50} of dFdCyd was unable to sustain a >80% decrease in the dATP level in the MMR-proficient cells. These results suggest that low levels of dFdCTP and/or transient decreases in dATP may activate the MMR pathway in the MMR-proficient cells, ultimately leading to cell death, whereas similar effects in the MMR-deficient cells are not cytotoxic but may cause errors of replication which, if left unrepaired, promote cell death by radiation.

#3588 The Proteasome Inhibitor PS-341 Is a Potential Radiosensitizer. Milena Pervan, Frank Pajonk, Ji-Rong Sun, Rodney H. Withers, and William H. McBride. *Amgen, Thousand Oaks, CA, and University of California, Los Angeles, CA.*

The ubiquitin/proteasome pathway is the main mechanism for degradation of short- lived proteins involved in regulation of cell cycle progression, DNA repair, and apoptosis. It is, therefore, reasonable to expect that modifications in proteasomal activity would affect cellular responses to stress signals, such as ionizing radiation. Several studies have shown that proteasome-specific drugs cause apoptosis of tumor cells in vitro. PS-341, a reversible inhibitor of proteasome chymotrypsin-like activity, has been shown to slow tumor growth in vivo and is currently in Phase I clinical trials. We had previously shown that the less specific, reversible proteasome inhibitor MG-132 can radiosensitize cells in vitro and there is a preliminary report to suggest that PS-341 can do the same. To further test the

hypothesis that proteasomal function can dictate the cellular response to radiation, TRAMP-C1, were pre-incubated with 100nM PS-341 or 10mM MG-132 (50% survival doses) for 3 hours, rinsed to remove drugs and irradiated with 2, 4 and 6 Gy. The surviving fraction was measured by MTT and clonogenic assay. The dose enhancement ratios (DERs) for MG-132 and PS-341 were 1.2 and 1.5 respectively. Experiments using different doses of PS-341 (1, 2.5 and 5nM) showed that PS-341 has a radiosensitizing effect (DERs of 1.1, 1.1 and 1.5, respectively) at doses that cause only partial inhibition of proteasome activity and are minimally cytotoxic. To examine the effect of PS-341 combined with ionizing radiation in vivo, TRAMP-C1 tumors were established in the hind legs of C57B/6 mice. Tumors (6mm in diameter) were treated with different doses of PS-341 (0.1, 0.2 and 0.3 mg/kg) two hours before 25 Gy local irradiation. Tumor volumes were measured for 35 days. This treatment had an at least additive effect over drug or radiation alone. Taken together, these data strengthen the contention that modification of proteasome activity can affect cellular radiosensitivity.

#3589 Redox Properties of Motexafin Gadolinium (Gd-Tex): A Tumor Selective Radiation Enhancer. John E. Biaglow, Richard Miller, and Darren Magda. *Pharmacyclics, Inc., Sunnyvale, CA, and University of Pennsylvania, Philadelphia, PA.*

Motexafin gadolinium (Gd-Tex) is a tumor selective agent that has been shown to enhance the efficacy of radiation in animal tumor models and is currently in Phase III clinical development as an adjuvant to radiation therapy. It was the purpose of this study to investigate Gd-Tex effects on cellular redox reactions involving oxygen consumption, bioreduction of disulfides and reaction with dihydrolipoate and ascorbate. These studies are necessary to define the optimal radiosensitization conditions for Gd-Tex. Gd-Tex does not alter oxygen consumption of the A549 human carcinoma cells. However, it stimulates oxygen consumption in cyanide inhibited cells. The latter effect is typical for many radiosensitizing drugs such as misonidazole and tirapazamine. The stimulation of oxygen consumption occurs when Gd-Tex accepts an electron producing an oxygen reactive radical. This results in superoxide radical anion and peroxide formation. We also tested the effect of Gd-Tex on bioreduction of disulfides (Biaglow, et al., Analytical Biochem. 281, 77-86, 2000). Gd-Tex inhibited the reduction of lipoate to dihydrolipoate, a thioredoxin/thioredoxin reductase linked reaction. It had no effect on glutathione-linked hydroxyethyldisulfide reduction. Lipoate, while not reduced, enhanced the uptake of the green colored Gd-Tex into a number of cell lines. At the present time we do not know if the inhibition of lipoate reduction is due to altered enzyme activity or a rapid reaction of dihydrolipoate with Gd-Tex producing oxygen reactive intermediates. Such a reaction would prevent accumulation of reduced lipoate. Gd-Tex reacts chemically with both dihydrolipoate and ascorbate producing oxygen reactive intermediates. In the presence of cells, incubated with Gd-Tex and ascorbate, oxygen is consumed rapidly producing hypoxic conditions and the accumulation of a new intracellular absorbing species (red). It is obvious from the above information that Gd-Tex is extremely reactive with reducing species and its radiosensitizing effects may depend on these reactions both extracellularly and intracellularly.

#3590 Redox Cycling of Motexafin Gadolinium Leads to Radiation Sensitization in Vitro. Darren Magda, Cheryl Lepp, Nikolay Gerasimchuk, Intae Lee, and Richard Miller. *Pharmacyclics, Inc., Sunnyvale, CA.*

Motexafin gadolinium, (Xcytrin™, Gd-Tex) has been shown to enhance the efficacy of radiation in animal tumor models and is currently in Phase III clinical development as an adjuvant to radiation therapy. Gd-Tex is electron affinic, with a first reduction potential near -50 mV (NHE). In order to understand better the mechanism of its action as a radiation enhancer, the chemical and biochemical properties of this agent were examined in vitro. GdTex was found to catalyze the oxidation of NADPH, ascorbate, and other reducing metabolites, leading to the formation of superoxide anion and hydrogen peroxide. Decreased cell viability correlated strongly with the presence of reducing metabolites, especially ascorbate, in the culture medium. This effect was cell line dependent: For example, E89, a CHO cell variant deficient in pentose phosphate pathway activity, was found to be more sensitive to Gd-Tex than the wildtype cell line K1 under all conditions tested. Incubation of Gd-Tex in the presence of ascorbate also enhanced cell uptake. Treatment of MES-SA (human uterine cancer line) cells with Gd-Tex in conjunction with L-buthionine-[S,R]-sulfoximine (BSO), diamide, or antimycin A resulted in a cooperative inhibition of cell proliferation, as measured by formazan reduction. Incubation of MES-SA cells with BSO (100 μM) and Gd-Tex (50 μM) for 24 h prior to ionizing radiation reduced clonogenic survival (sensitization enhancement ratio at surviving fraction 0.1 = ca. 1.8) relative to treatment with BSO alone. Exposure of B-human lymphatic cells (LYAS) to Gd-Tex (without BSO) enhanced radiation response (SER at SF 0.1 = ca. 1.4); however, no sensitization was observed under these conditions in an apoptosis-resistant variant of this line (LYAR). These findings lead us to suggest that Gd-Tex sensitizes cells to ionizing radiation through a novel mechanism of action, whereby the catalytic oxidation of endogenous reducing metabolites leads to the formation of reactive oxygen species. Radiation enhancement may involve inhibition of DNA repair or downstream processes such as apoptosis.

#3591 Regulator of Interferon-induced Death-2 Sensitizes Ovarian Carcinoma Cells to γ-irradiation. Bei H. Morrison, Joseph A. Bauer, and Daniel J. Lindner. *Cleveland Clinic Foundation, Cleveland, OH.*

Previously, we have identified several novel Regulators of Interferon-induced Death (RID) genes. RID-2 is a growth suppressive and apoptosis-enhancing gene identified by an antisense technical knockout technique. RID-2 is a 50 kDa protein that contains a putative kinase domain. Its substrate has not yet been identified. Over expression of RID-2 in sense orientation conferred enhanced sensitivity to IFN-β induced death. ID50 decreased from 175 IU/ml (vector alone) to 125 IU/ml (sense RID-2). Treatment of NIH-OVCAR-3 human ovarian carcinoma cells with IFN-β (100 IU/ml) causes apoptosis, detectable as early as 16 hr. Overexpression of RID-2 enhances IFN-beta-induced apoptosis in ovarian carcinoma cells. In this study, we determined whether RID-2 expression also modulated the sensitivity of ovarian carcinoma cells to γ-irradiation. NIH-OVCAR-3 cells were stably transfected with pCXN2 vector alone, or with RID-2. Cells received 0, 200, or 400 Rad, plated at a density of 10,000 cells/10 cm dish, and grown for 20 days. Untreated RID-2 cells formed fewer and smaller colonies compared to untreated vector expressing cells. Colonies from untreated RID-2 cells were 67% the area of colonies from untreated vector expressing cells ($p=0.003$). RID-2 overexpression caused increased sensitivity to γ-irradiation, resulting in decreased colony forming units (CFU). After 200 Rad, the number of RID-2 CFU were 80% less than vector CFU ($p=0.001$). Following 400 Rad, the number of RID-2 CFU were 87% less than vector CFU ($p=0.0001$). Over expression of RID-2 enhanced sensitivity to γ-irradiation as well as to IFN-beta-induced growth suppression and apoptosis. Radiation injury and IFN-beta-induced apoptosis are both enhanced by RID-2 expression.

#3592 Antisense of Human Peroxiredoxin II Enhances Radiation-induced Cell Death. Young Min Chung, Sun-Hee Park, Jong Kuk Park, Young-Sik Lee, Hyung Jung Kim, and Young Do Yoo. *Korea Cancer Center Hospital, Seoul, South Korea, and Yonsei University College of Medicine, Seoul, South Korea.*

Human peroxiredoxin II has been known to function as an antioxidant enzyme in cells. Employing head and neck cancer cell lines, we investigated whether expression of Prx II is related to the resistance of cells to radiation therapy in vivo and in vitro, and also whether a Prx II antisense serves as a radiosensitizer. Increased expression of Prx II was observed in tissues isolated from the patients who did not respond to radiation therapy, whereas Prx II expression was weak in tissues from the patients with regressed tumors. Enhanced expression of Prx II in UMSCC-11A (11A) cells was also observed after treatment with γ-radiation. This increased expression conferred cancer cells to radiation resistance, because overexpression of Prx II protected 11A cells from radiation-induced cell death, suggesting that blocking Prx II expression could enhance radiation sensitivity. Treatment of 11A cells with a Prx II antisense decreased induction of Prx II, resulting in enhancing the radiation sensitivity. From these results, we suggest that stress-induced overexpression of Prx II involves in radiation resistance via protection of cancer cells from radiation-induced oxidative cytolysis and a Prx II antisense can be utilized as a radiosensitizer.

#3593 Radiosensitization of Human Glioma Cells *in Vitro* and *in Vivo* with Acyclovir and Mutant HSV-TK Expressed from Adenovirus. Elizabeth A. Rosenberg, William Hawkins, Rupert K. Schmidt-Ullrich, Peck-Sun Lin, and Kristoffer C. Valerie. *Virginia Commonwealth University, Richmond, VA.*

Recently, we demonstrated that an adenovirus (AdCMV-TK75) expressing mutant HSV-TK (HSVTK-75) radiosensitized rat glioma cells *in vitro* and suppressed growth of intracerebrally implanted tumors in syngeneic rats in combination with low concentrations of acyclovir (ACV) much more effectively than a virus expressing wild type HSV-TK. ACV has better pharmacological properties than ganciclovir for treating brain tumors and using a virus expressing a more drug-sensitive HSV-TK, such as HSV-TK75, may further improve radiosensitization of brain tumors. To determine whether human cells also demonstrate improved radiosensitization similar to that seen with rat cells and tumors, we transduced U87 glioma cells with either AdCMV-TK75, AdCMV-TK, expressing wild type HSV-TK, or Adβgal (control) and then treated the cells with 3 μM of ACV for 24 h prior to irradiation. Cells transduced with AdCMV-TK75 were significantly more radiosensitive (SER: 2.1) than cells transduced with either AdCMV-TK or Adβgal by colony-forming survival assay. Furthermore, we found that U87 xenografts grown in nu/nu mice infused with AdCMV-TK75 (2×10^9 pfu) were more sensitive to fractionated irradiation after administration of ACV than tumors infused with Adβgal. Tumors infused with Adβgal, exposed to ACV and then irradiated were ~40% smaller than tumors that were not irradiated. Tumors treated with AdCMV-TK75 and ACV were reduced in size to ~25% of tumors treated with Adβgal and ACV, and irradiation completely abrogated tumor growth. Altogether, these results demonstrate that transduction of human U87 glioma cells *in vitro* and U87 xenografts *in vivo* with AdCMV-TK75 and treatment with ACV produce similar radiosensitization as those with rat glioma cells and syngeneic tumors grown in rats. These results suggest that AdCMV-TK75 would be far more effective than AdCMV-TK expressing wild type HSV-TK for radiosensitizing human gliomas with low concentrations of ACV. Supported by P01CA72955.

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Comment on double-strand break repair and rapamycin treatment

Sir,

Recently, Sikpi and Wang (2000) reported that rapamycin enhanced radiation-induced double-strand break (DSB) repair in ataxia telangiectasia lymphoblasts. They describe two properties of the immunosuppressive drug rapamycin; G_1-arrest in normal and AT lymphoblasts and enhanced DSB repair after irradiation of AT lymphoblasts, with no enhancement of repair in normal cells. They have proposed that radiation-induced DSB repair fidelity and capacity is modulated via signalling pathways that are abnormal in AT cells and sensitive to the effects of rapamycin.

A major, often ignored, aspect of the action of

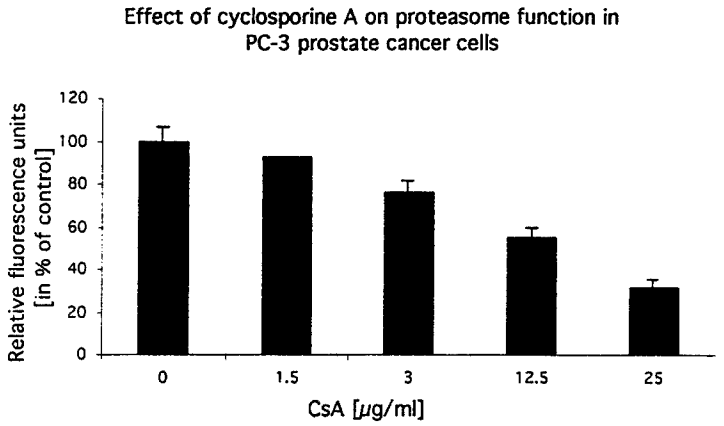

Figure 1. Cyclosporin-A (CsA) is an inhibitor of 26s proteasome function. PC-3 prostate cancer cells were treated with different concentrations of CsA for 30 min. Total cellular protein was extracted and proteolytic activity measured using the fluorogenic proteasome substrate SucLLVY-7-amido-4-methylcoumarin (chymotrypsin-like; Sigma) as described by Glas *et al.* (1998). Pretreatment with CsA caused a dose-dependent inhibition of 26s proteasome function in two independent experiments.

rapamycin on mammalian cells is its inhibitory effect on 26s proteasome function (Wang *et al.* 1997), which is independent of its effects on S6 ribosomal protein kinase p70S6 kinase. Similar effects are seen for the immunosuppressive drug cyclosporin-A (Meyer *et al.* 1997) (figure 1) and the HIV-I protease inhibitor ritonavir (Schmidtke *et al.* 1999). The 26s proteasome is responsible for the degradation of all short-lived and 70–90% (Ciechanover 1994, Lee and Goldberg 1996) of all long-lived proteins. Its activity controls cells cycle progression and various signal transduction pathways (Rolfe *et al.* 1997).

Thus, the transition of cells from G_1- into S-phase depends on the controlled removal of cyclins by the 26s proteasome. Blockage of this pathway by rapamycin could account for the observed G_1 arrest in wild-type and AT lymphoblasts. Furthermore, one of the major signal transduction factors recognized to be under proteasome control is NF-κB, which is constitutively active in AT cells (Jung *et al.* 1998). The major pathway for activation of NF-κB is by proteolytic degradation of IκB by the 26s proteasome which frees NF-κB and allows its nuclear translocation. Jung *et al.* (1995) showed that enhanced radiosensitivity of AT cells is normalized by introduction of an IκB super-repressor. Blockage of the proteasome by rapamycin would be expected to have the same effect. The link between NF-κB-dependent target genes and DSB repair pathways has yet to be firmly established (Pahl 1999), as has a direct role for this pathway in radiosensitivity of AT cells. However, our observation that AT fibroblasts have high constitutive caspase-3 activity compared with normal fibroblasts (figure 2) presents one hypothesis. One of the substrates of caspase-3 is DNA-PKcs, the catalytic subunit of DNA-PK, an enzyme responsible for DNA

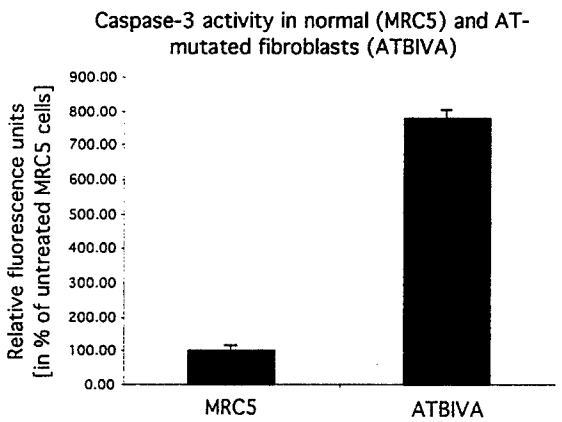

Figure 2. Constitutive caspase-3 activity is high in AT fibroblasts. Exponentially growing normal (MRC5) fibroblast and fibroblast from a patient with ataxia telangiektasia (ATBIVA) were lysed. Caspase-3 activity was assessed using the fluorogenic substrate DEVD-7-amido-4-methylcoumarin (1 µM). AT fibroblasts showed an 8-fold higher constitutive caspase-3 activity when compared with normal fibroblasts. There is some evidence that anti-apoptotic effects of NF-κB are mediated through caspase inhibition resulting from induction of TRAF1 and TRAF2 (Wang *et al.* 1998).

DSB repair. Activation of caspase-3 requires calpain function (McGinnis *et al.* 1999, Wolf *et al.* 1999), that can be inhibited by proteasome inhibitors such as MG-132 (Tsubuki *et al.* 1996). If rapamycin was to have similar effects, it might down-regulate constitutive caspase-3 activity, allowing an increase in DNA DSB repair capacity in AT cells. Caspase 3-mediated cleavage of IkBκ might also be involved in radiation-induced apoptosis of AT cells (Jung *et al.* 1998).

It is relevant that proteasome inhibitors have a significant impact on the survival (Drexler 1997,

Herrmann *et al.* 1998) and radioresistance of cancer cells (Pajonk *et al.* 1999, 2000), including the highly specific inhibitor PS341 that is now in clinical trials (Adams *et al.* 1999, Teicher *et al.* 1999). Rapamycin, cyclosporin-A or ritonavir may have similar effects. Irrespective of whether the hypothesis outlined above is correct in detail, the role of the proteasome in the effects noted by Sikpi and Wang (2000) might be worth further investigation.

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subjects had been treated with stavudine in previous regimens, but only 56% of individuals in the control group. Didanosine had been used in 62.5% of patients with gynaecomastia compared with 44.3% of individuals without disease. Nelfinavir was given to 62.5% of the individuals with gynaecomastia, but was included in the regimen of only 25% of non-affected subjects.

Gynaecomastia caused by HAART has been reported for several drugs, e.g. efavirenz, didanosine, stavudine and various protease inhibitors [1–4]. Brinkmann and colleagues [8–10] postulated that nucleoside reverse transcriptase inhibitor drugs induced mitochondrial toxicity using an accumulative pathway, as damaging mitochondrial γ polymerase and several side-effects of HAART are attributed to this mechanism [11]. Data obtained have shown a possible association between gynaecomastia and antiretroviral drugs; especially nelfinavir, didanosine and stavudine. It was noted that all patients with gynaecomastia had been treated with stavudine in their previous history, and 80% of individuals had stavudine in their existing therapy regimen as gynaecomastia occurred. It must therefore be asked whether there is an association of gynaecomastia with a combination or equilibrium of antiretroviral drugs in the treatment regimen, and if it is related to mitochondrial toxicity. The fact that lactate-dehydrogenase, triglyceride and blood glucose levels were increased in affected patients compared with subjects with no signs might be of importance, because an elevation of these parameters is often suggested to be caused by mitochondrial toxicity or lipodystrophy [7–10]. However, the data presented suggest that an elevation of these parameters do not predict which patients might possibly develop gynaecomastia. Further studies are necessary, with more subjects suffering from gynaecomastia, involving additional laboratory tests (i.e. drug level-monitoring), and calling for subcellular methods in order to clarify further associations.

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Survival of AIDS patients with primary central nervous system lymphoma may be improved by the radiosensitizing effects of highly active antiretroviral therapy

In a recent issue of *AIDS* Hoffmann *et al.* [1] reported dramatically improved survival rates for AIDS patients suffering from primary central nervous system lymphoma (PCNSL) treated with highly active antiretroviral therapy (HAART) and cranial irradiation [1]. In this retrospective analysis the authors showed excellent results of combined treatment (HAART plus cranial irradiation) when compared with cranial irradiation treatment alone or no cancer-specific treatment. The authors concluded that HAART leads to a dramatic improvement of survival in these patients by enhancing immunity. Given that an impaired immune system gives rise to this tumour entity, it is tempting to assume that an enhanced immune system might grant remission from PCNSL in patients in whom the tumour burden is severely reduced by radiation therapy. However, in the multivariate analysis the CD4 cell counts failed to predict outcome, whereas cranial irradiation and HAART were independent variables for survival.

An alternative explanation is that HAART may radiosensitize to cranial irradiation. Part of the HAART protocol involves the use of protease inhibitors initially designed to target only the HIV-1 protease [2]. However, recent studies from our laboratory and another laboratory showed that the HIV-1 protease inhibitors ritonavir [3] and saquinavir (Pajonk *et al.*, submitted for publication) have inhibitory effects on the 26s multicatalytic protease called the proteasome [4]. Protease activity is increased in malignant cells [5], the subcellular distribution of proteasomes changes during the cell cycle [6], and tumour cells show a different pattern

of proteasome sub-unit expression [7], making the proteasome a target for cancer therapy that might be exploited with therapeutic benefit [8]. We and other authors have already shown that the inhibition of this protease using specific inhibitors such as MG-132 or PS-341 induces apoptosis in human tumour cells [9,10]. In addition, short-time inhibition *in vitro* at concentrations sub-optimal to kill tumour cells *in vivo* sensitized malignant cells to ionizing radiation [10,11]. Recent studies from our laboratory revealed comparable effects for saquinavir on human prostate cancer and glioma cell lines (Pajonk *et al.*, submitted for publication). This offers an entirely new explanation for the promising results of Hoffmann and colleagues [1], identifying HIV-1 protease inhibitors as radiosensitizing agents in patients with HIV-related PCNSL. It may be possible to re-analyse the data from this study for the use of HIV-1 protease inhibitors. If the patients with prolonged survival received HIV-1 protease inhibitors, a prospective clinical trial testing HIV-1 protease inhibitors containing HAART regimens versus HAART regimens without these inhibitors, both combined with cranial irradiation, should be considered. Given the expectation of increased radiation neurotoxicity, which was also indicated in the data from Hoffman *et al.* [1], a dose escalation trial may be needed to maximize the therapeutic benefit to be derived from this combined modality treatment. It is worth noting that better responses have been noted to chemotherapy in AIDS patients with non-Hodgkin's lymphoma who receive concurrent HAART [12], possibly because of the same mechanism.

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Intracellular carbovir triphosphate levels in patients taking abacavir once a day

Relatively long intracellular half-lives of active triphosphate metabolites support the once daily dosing of nucleoside analogues such as didanosine and lamivudine [1–4]. The guanosine analogue abacavir is phosphorylated intracellularly into carbovir triphosphate [5]. It is not known whether daily abacavir doses of 600 mg produce levels of intracellular carbovir triphosphate similar to those achieved with the standard abacavir dose of 300 mg twice a day. We examined the intracellular levels of the active nucleotide carbovir triphosphate in HIV-positive patients taking abacavir 600 mg once a day, with the aim of determining whether this drug has the potential to be dosed on a once daily basis.

Five HIV-positive adults (four men, one woman) taking abacavir 600 mg once a day for 5–17 months as a component of multiple drug rescue therapy including two or three other nucleosides, one non-nucleoside, and one or two protease inhibitors consented to participate in this pharmacokinetic study. Blood samples were drawn into cell preparation tubes (Vacutainer CPT, Becton Dickinson, Franklin Lakes, NJ, USA) at time 0 (24 h after the previous abacavir dose) and 1, 12, 14–16, 18, 20, 22, and 24 h after an observed 600 mg abacavir dose. Peripheral blood mononuclear cells were isolated and phosphates extracted using 60% methanol. The methanol extracts were then dried and stored at −70°C. After a second extraction with perchloric acid, endogenous deoxyguanosine triphosphate (dGTP) levels were determined by a DNA polymerase assay. For the measurement of carbovir triphosphate, reaction mixtures included ^{3}H-dGTP, template primer and reverse transcriptase in a total

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The Human Immunodeficiency Virus (HIV)-1 Protease Inhibitor Saquinavir Inhibits Proteasome Function and Causes Apoptosis and Radiosensitization in Non-HIV-associated Human Cancer Cells[1]

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ABSTRACT

Cancer cells frequently show high constitutive activity of the antiapoptotic transcription factor nuclear factor κB (NF-κB), which results in their enhanced survival. Activation of NF-κB classically depends on degradation of its inhibitor IκBα by the 26s proteasome. Specific proteasome inhibitors induce apoptosis in cancer cells and, at nonlethal concentrations, sensitize cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs. Recently, the protease coded by the HIV-I virus has been shown to share cleavage activities with the proteasome. For this reason, we investigated whether the HIV-I protease inhibitor saquinavir can inhibit NF-κB activation, block 26s proteasome activity in prostate cancer cells, and promote their apoptosis. The effect of saquinavir on LPS/IFN-γ-induced activation of NF-κB was assessed by gel-shift assays and by Western analysis of corresponding IκBα-levels. Its effect on 20s and 26s proteasome activity was analyzed with a fluorogenic peptide assay using whole cell lysates from LnCaP, DU-145, and PC-3 prostate cancer cells pretreated with saquinavir for 9 h. Proteasome inhibition in living cells was assessed using ECV 304 cells stably transfected with an expression plasmid for an ubiquitin/green fluorescence protein fusion protein (ECV 304/10). Apoptosis was monitored morphologically and by flow cytometry. Saquinavir treatment prevented LPS/IFN-γ-induced activation of NF-κB in RAW cells and stabilized expression of IκBα. It inhibited 20s and 26s proteasome activity in lysates from LnCaP, DU-145, and PC-3 prostate cancer cells with an IC_{50} of 10 μM and caused the accumulation of an ubiquitin/green fluorescence protein fusion protein in living ECV 304/10 cells. Incubation of PC-3 and DU-145 prostate cancer, U373 glioblastoma, and K562 and Jurkat leukemia cells with saquinavir caused a concentration-dependent induction of apoptosis. In the case of PC-3 and DU-145, saquinavir sensitized the surviving cells to ionizing radiation. We conclude that saquinavir inhibits proteasome activity in mammalian cells as well as acting on the HIV-I protease. Because saquinavir induced apoptosis in human cancer cells, HIV-I protease inhibitors might become a new class of cytotoxic drugs, alone or in combination with radiation or chemotherapy.

INTRODUCTION

HIV-I encodes for a protease required for the cleavage of the viral gag-pol polyprotein, and its inhibition leads to the release of noninfectious virus particles (1). The development of specific HIV-I PIs[3] has revolutionized HIV therapy. At present, five different HIV-I PIs (ritonavir, saquinavir, nelfinavir, indinavir, and amprenavir) are clinically used (2). Bioavailability of at least ritonavir, saquinavir, and indinavir is limited by the fact that they are substrates, and in part inhibitors (3), of the same multidrug resistance gene product (*mdr-1*), P-glycoprotein (4, 5), that is a common cause for failure of chemotherapy in cancer patients.

The cleavage sites of action for HIV-I protease were once thought to be unique and distinct from those of mammalian proteases. However, recently, the 20s proteasome has been shown to cleave the same sites (1). This led us to investigate whether saquinavir is an inhibitor of the 20s proteasome, as was previously reported for ritonavir (6). Proteasome inhibition may contribute to some of the effects of PIs that seem to be independent of virus inhibition, such as its immunemodulatory properties in HIV patients and its antitumoral action on HIV-associated Kaposi-sarcoma (6). We also examined whether saquinavir exhibits antitumoral effects in non-HIV-associated cancer of the prostate.

MATERIALS AND METHODS

Cell Culture. Cultures of PC-3, LnCaP, and DU-145 human prostate carcinoma and U373 glioblastoma cells and K562 erythroleukemia and Jurkat T-cell leukemia cells (American Type Culture Collection, Rockville, MD), RAW 264.7 murine macrophages (a gift of Dr. G. Hildebrandt, Department of Radiation Oncology, University Clinic Leipzig, Leipzig, Germany), and ECV 304 human bladder carcinoma cells (DSMZ, Braunschweig) were grown in 75-cm^2 flasks (Greiner) at 37°C in a humidified atmosphere of 5% CO_2/95% air. DMEM (Cell Concepts, Freiburg, Germany) and RPMI 1640 (Cell Concepts) were supplemented with 10% FCS and 1% penicillin/streptomycin (Life Technologies, Inc.) before use. Saquinavir (a generous gift of Dr. Christiane Moecklinghoff, Hoffmann La-Roche, Grenzach, Germany) was solubilized in ethanol/H_2O at a concentration 10 mM and used at concentrations of 0, 20, 50, 60, 80, and 100 μM. Controls received solvent only.

Irradiation. PC-3 and DU-145 cells were trypsinated, counted, and diluted to a concentration of 10^6 cells/ml. The cell suspension was immediately irradiated at room temperature using a ^{137}Cs-laboratory irradiator (IBL 637; CIS bio international) at a dose rate of 0.78 Gy/min). Corresponding controls were sham irradiated.

Clonogenic Survival. Colony-forming assays were performed immediately after irradiation by plating an appropriate number of cells (2×10^3 to 2×10^4) into Petri dishes, in triplicate. After 14-days culture, cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Resulting survival plots were fitted using a linear-quadratic model.

Transfection. ECV 304 cells were maintained in DMEM (10% FCS, 1% penicillin/streptomycin). Twelve h before transfection, cells were trypsinized and plated at a density of 250,000 cells/well into six-well plates. Cells were transfected with 5 μg of a plasmid (pEGFP-N1; Clontech) coding for an Ub-R-GFP fusion protein under control of a cytomegalovirus promoter (7; a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10% FSC, 1% penicillin/streptomycin) supplemented with 500 μg/ml G418 (Sigma), and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-Hl FACSCalibur, Becton Dickinson) using CellQuest Software before and after treatment with the proteasome inhibitor MG-132 (50 μM, Calbiochem) for 10 h at 37°C. Clone 10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

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[3] The abbreviations used are: PI, protease inhibitor; NF-κB, nuclear factor κB; GFP, green fluorescence protein; PMSF, phenylmethylsulfonyl fluoride; AMC, 7-amido-4-methylcoumarin; Ub, ubiquitin; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

Cell Extracts and Electrophoretic Mobility Shift Assays. Cellular extracts were prepared from normal and saquinavir-treated cells by dislodging the cells mechanically, washing with ice-cold PBS, and lysing them in TOTEX buffer [20 mM HEPES (pH 7.9), 0.35 mM NaCl, 20% glycerol, 1% NP40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 50 μM PMSF, and 90 trypsin inhibitor units/ml aprotinin] for 30 min on ice. The lysate was centrifuged at 12.000 × *g* for 5 min. Protein concentration in the supernatant was determined by the Micro BCA method (Pierce). Fifteen μg of protein were incubated for 25 min at room temperature with 2 μl of BSA (10 μg/μl), 2 μl of dIdC (1 μg/μl), 4 μl of Ficoll buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, and 0.1 mM PMSF), 2 μl of buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40, 2 mM DTT, and 0.1 mM PMSF), and 1 μl of the [γ-^{32}P]ATP-labeled oligonucleotide (Promega; NF-κB, AGTTGAGGGGACTTTCCCAGG). A negative control was prepared for one sample by adding unlabeled oligonucleotide in 50-fold excess. Electrophoresis was carried out in native 4% polyacrylamide/0.5-fold Tris/boric acid/EDTA gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphorimager (IPR 1500; Fuji).

Proteasome Function Assays. Proteasome function was measured as described previously (8) with some minor modifications. Briefly, cells were washed with PBS, then with buffer I [50 mM Tris (pH 7.4), 2 mM DTT, 5 mM $MgCl_2$, and 2 mM ATP], and pelleted by centrifugation. Glass beads and homogenization buffer [50 mM Tris (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, and 250 mM sucrose] were added and cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1,000 × *g* for 5 min, and the supernatant was further clarified by spinning at 10,000 × *g* for 20 min. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Twenty μg of protein from each sample was diluted with buffer I to a final volume of 200 μl. To assess 26s function, fluorogenic proteasome substrate SucLLVY-AMC (chymotrypsin-like; Sigma) was dissolved in DMSO and added in a final concentration of 80 μM (in 0.8% DMSO). To assess 20s function, buffer I was replaced by buffer containing SDS [20 mM HEPES (pH 7.8), 0.5 mM EDTA, and 0.03% SDS (9)]. Proteolytic activity was monitored continuously using a fluorescence plate reader (Spectra Max Gemini XS; Molecular Devices; 37°C) at 380/460 nm by release of the fluorescent group AMC.

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP40, SDS, 10 mM PMSF, aprotinin, and sodium vanadate]. Protein concentrations were determined using the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Ten μg of protein were separated on SDS gel (0.1% SDS/12% polyacrylamide) and blotted to polyvinylidene difluoride membranes at 4°C. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, and 5% skim milk) for 1 h at room temperature, the membranes were incubated with a polyclonal antibody against IκBα (0.5 μg/ml; BD PharMingen) for 1 h at room temperature. A secondary horseradish peroxidase-conjugated antibody and the ECLplus system (Amersham) were used for visualization.

Flow Cytometry. For assessment of Ub-R-GFP expression, cells were trypsinized, pelleted (5 min, 500 × *g*) and washed twice in PBS. GFP content was analyzed by flow cytometry using the CellQuest Software (FL1-H, FACSCalibur; Becton Dickinson). The DNA profiles of adherent and nonadherent cells in drug-treated populations were analyzed by flow cytometry using CellQuest Software (FL2-A, FACSCalibur; Becton Dickinson). Cells were washed in PBS and fixed overnight in ice-cold 75% ethanol. The next day, cells were washed in PBS. The cell pellet was incubated with 50 μg/ml RNase A and 1 mg/ml propidium iodide for 10 min and washed in PBS before examination. TUNEL assay was performed using the FlowTACS *in situ* kit (R&D Systems GmbH, Wiesbaden, Germany) following the manufacturer's instructions.

RESULTS

Saquinavir Prevents Activation of Transcription Factor NF-κB. NF-κB signaling is normally dependent on proteasomal degradation of the binding inhibitor IκBα and is prevented by treatment of cells with proteasome inhibitors (10). Murine RAW 264.7 macrophages respond strongly with NF-κB activation to treatment with LPS (0.1 μg/ml) and IFN-γ (100 units/ml) for 6 h. This model was, therefore, used to determine whether simultaneous addition of different concentrations of saquinavir (0, 6.25, 12.5, 25, and 50 μM) affected the activation process. When total cellular lysates were analyzed at 6 h, we found a concentration-dependent inhibitory effect of saquinavir on constitutive and LPS/IFN-γ-induced increase of NF-κB DNA-binding activity (Fig. 1*A*). This inhibition coincided with an accumulation of IκBα, which indicated involvement of the classical activation pathway of NF-κB by proteasome-dependent degradation of IκB (Fig. 1*B*). Similar results were observed for human PC-3 prostate cancer cells using 25-μM concentrations of saquinavir (data not shown).

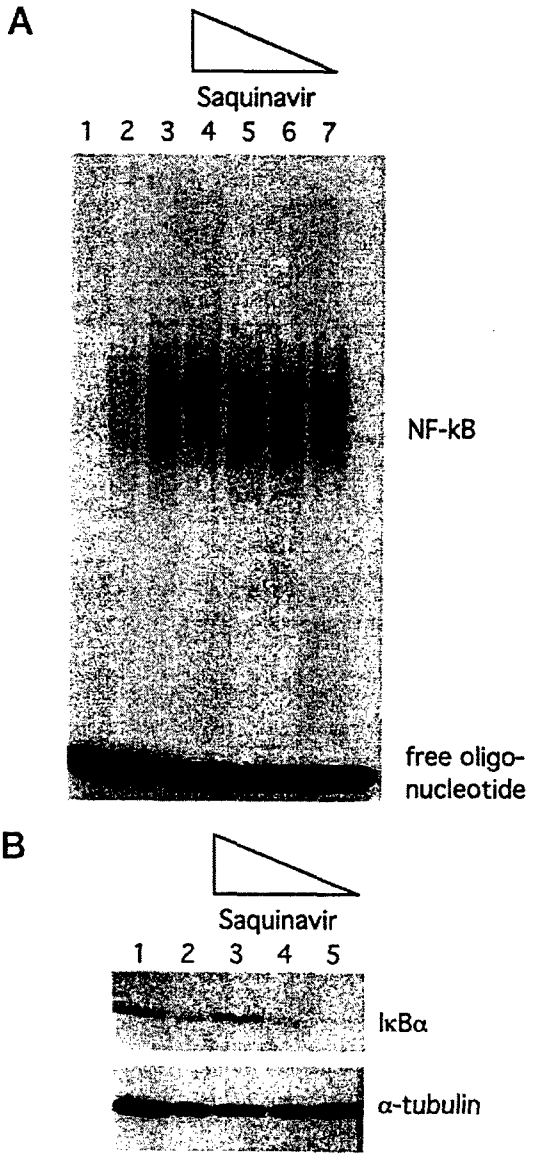

Fig. 1. Saquinavir inhibits activation of NF-κB. In *A*, RAW 264.7 macrophages were stimulated with LPS (0.1 μg/ml) and IFN-γ (100 units/ml) for 6 h in the presence or absence of different concentrations of saquinavir, and cell extracts were analyzed for NF-κB expression by electrophoretic mobility shift assay. *Lane 1*, negative control with unlabeled oligonucleotide added in 50-fold molar excess; *Lane 2*, control nonstimulated cells; *Lane 3*, cells stimulated with LPS/IFN-γ in the absence of saquinavir; *Lanes 4–7*, cells stimulated with LPS/IFN-γ in the presence of saquinavir (*Lane 4*, 50 μM; *Lane 5*, 25 μM; *Lane 6*, 12.5 μM; *Lane 7*, 6.25 μM). *B*, Western blot analysis for IκBα in cell extracts from RAW 264.7 macrophages stimulated with LPS (0.1 μg/ml) and IFN-γ (100 units/ml) for 6 h in the presence or absence of different concentrations of saquinavir (*Lane 1*, untreated controls; *Lane 2*, LPS/IFN-γ, 0 μM saquinavir; *Lane 3*, LPS/IFN-γ, 100 μM saquinavir; *Lane 4*, LPS/IFN-γ, 50 μM saquinavir; *Lane 5*, LPS/IFN-γ, 25 μM saquinavir).

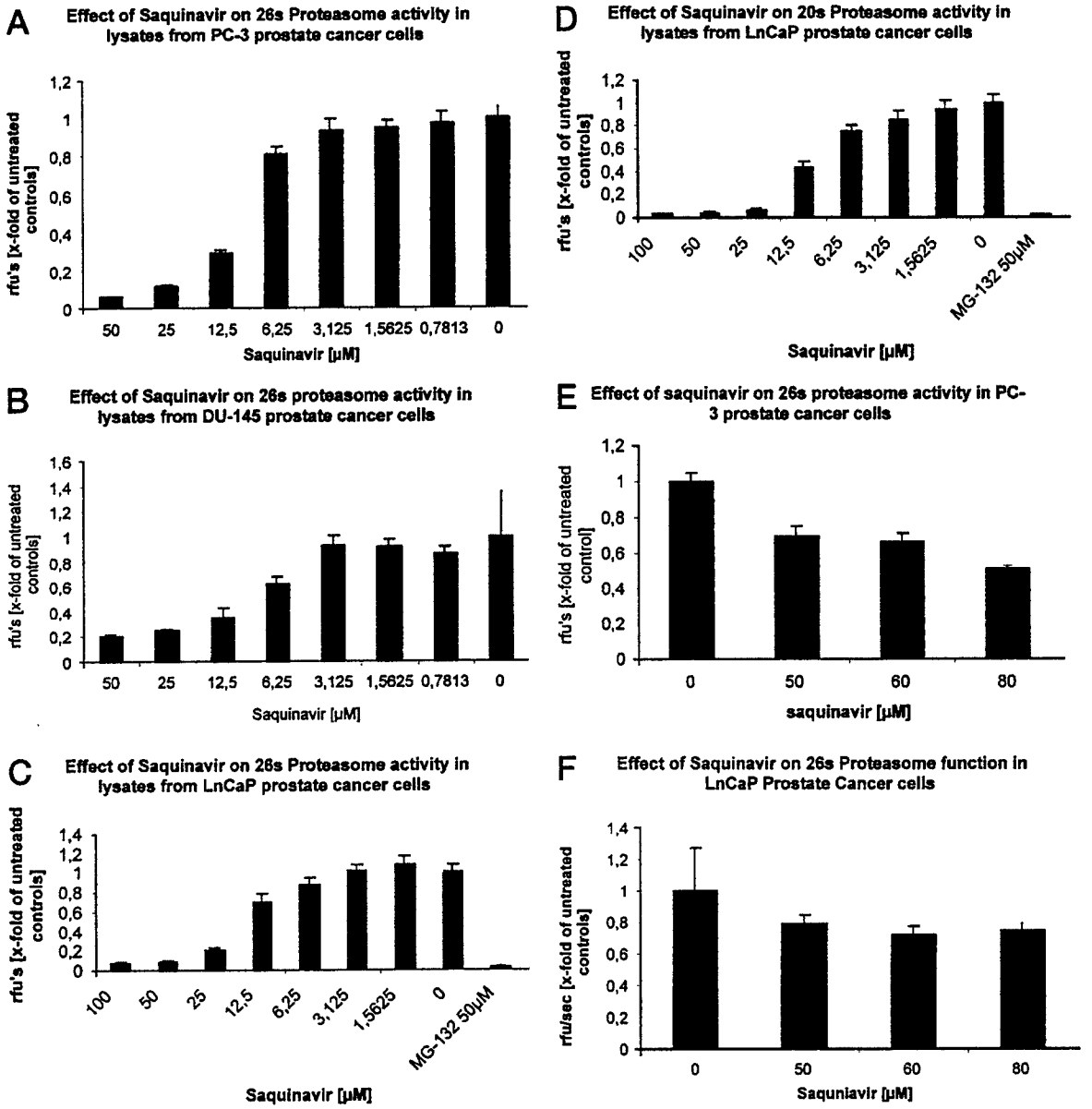

Fig. 2. Saquinavir is a 20s proteasome inhibitor. Chymotryptic 26s and 20s proteasome activity in lysates from PC-3, DU-145, and LnCaP human prostate cancer cells. Cleavage activity was monitored for 30 min and expressed as relative fluorescence units (*rfu's*) expressed as *x*-fold activity of untreated controls. Cellular extracts from PC-3 (*A*), DU-145 (*B*), and LnCaP (*C*) cells were incubated with different concentrations of saquinavir. Chymotryptic 26s proteasome activity was assessed using a fluorogenic peptide assay with the specific proteasome substrate SucLLVY-AMC. Release of the fluorogenic compound AMC was monitored continuously in a fluorescence plate reader (excitation, 380 nm; emission, 460 nm). The specificity of the cleavage reaction was demonstrated by the addition of the proteasome inhibitor MG-132 (*C*, 50 µM). Saquinavir inhibited chymotryptic 26s proteasome activity in all three of the cell lines in a concentration-dependent manner. Saquinavir also inhibited chymotryptic 20s proteasome activity in LNCaP cells (*D*), indicating an effect on the 20s core unit rather than on the 19s regulatory unit. The inhibitory effect was also observed when cells, rather than extracts, were incubated in growth media supplemented with different concentrations of saquinavir for 45 min (*E* and *F*), which indicated that saquinavir enters the cells, although the concentration needed of the drug was higher.

Saquinavir Is an Inhibitor of the 26s Proteasome. We have previously shown that prostate cancer cells express high constitutive levels of NF-κB that can be blocked by treatment with proteasome inhibitors (11). The ability of saquinavir to inhibit proteasome function was examined by incubating cellular extracts of PC-3, DU-145, and LnCaP prostate cancer cells with different concentrations of the drug (100, 50, 25, 12.5, 6.25, 3.125, 1.6, and 0 µM). Chymotryptic, tryptic, and peptidyl-glytamyl 20s and 26s proteasome activities were continuously monitored for 30 min by the release of AMC from the fluorogenic proteasome substrates SucLLVY-AMC, Z-AAR-AMC, and Z-LLE-AMC. Saquinavir inhibited the function of both chymotryptic 26s (Fig. 2, *A*, *B*, and *C*) and 20s (Fig. 2*D*) and peptidyl-glutamyl (data not shown) proteasome activity in a concentration-dependent fashion. The IC_{50} for the chymotryptic 26s proteasome activity was 10 µM, 1.8 µM for the chymotryptic 20s proteasome activity, 5.2 µM for the peptidyl-glutamyl 26s proteasome activity, and 1.6 µM for the peptidyl-glutamyl 20s proteasome activity. We could not show any effect on tryptic 20s or 26s proteasome activity. In contrast, when PC-3 and LnCaP cells were treated with saquinavir supplemented media for 45 min and the extracts were then tested for proteasome activity, the IC_{50} was about 80 µM (Fig. 2, *E* and *F*). The difference in the ability of saquinavir to inhibit proteasome activity in whole cells and in extracts is probably attributable to its bioavailability, which may be adversely affected by saquinavir being a substrate of the *mdr-1* gene product P-glycoprotein, which is expressed at high levels in prostate cancer cells (12).

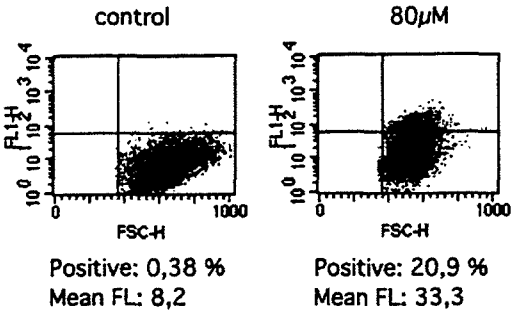

Fig. 3. Saquinavir causes accumulation of the Ub-R-GFP reporter of proteasomal function. Flow cytometric analysis of ECV 304 cells stably transfected with Ub-R-GFP reporter of proteasomal function. Incubation with saquinavir (80 μM) for 9 h caused an accumulation of Ub-R-GFP in cells. Positive cells were detected in the upper right quadrant. *Positive*, the number of positive cells; *Mean FL*, their average fluorescence.

To confirm that saquinavir can inhibit proteasome activity in cells, ECV 304 cells, transfected with Ub-R-GFP fusion protein (clone 10), were treated for 12 h with media supplemented with different concentrations of saquinavir and incubated for an additional 9 h. Subsequent flow cytometric analysis revealed a concentration-dependent increase in GFP-positive cells from, initially, 0.38% (0 μM) to 20.9% (80 μM; Fig. 3).

Saquinavir Induces Apoptosis in Non-HIV-associated Human Cancer Cells. One of the many consequences of proteasome inhibition is induction of apoptosis (13). The ability of saquinavir treatment to achieve this end point was, therefore, tested. In PC-3, cells, 100 μM induced apoptosis starting within 60 min (Fig. 4*A*). Comparable results were obtained in DU-145 (data not shown), U373 (data not shown), and K562 and Jurkat cells (Fig. 4*B*). By 24 h, all of the cell lines showed typical morphological criteria of apoptosis. Most PC-3 cells tolerated up to 50 μM for over 24 h, but 60 μM induced considerable apoptosis by this time point. By 48 h, PC-3 cells showed an increase of the apoptotic (sub-G_1) fraction from 10.4% (0 μM) to 48.2% (50 μM), 55.7% (60 μM) and 78.2% (80 μM; Fig. 4*C*). The induction of apoptosis was confirmed by TUNEL-staining (Fig. 4*D*).

Saquinavir Sensitizes PC-3 and DU-145 Prostate Cancer Cells to Ionizing Radiation. Transient inhibition of proteasome function has been shown to sensitize tumor cells to ionizing radiation (14). To test a possible effect of saquinavir on the clonogenic survival of PC-3 cells after radiation therapy, clonogenic assays were performed. Two-h pretreatment of PC-3 or 3-h pretreatment of DU-145 cells with

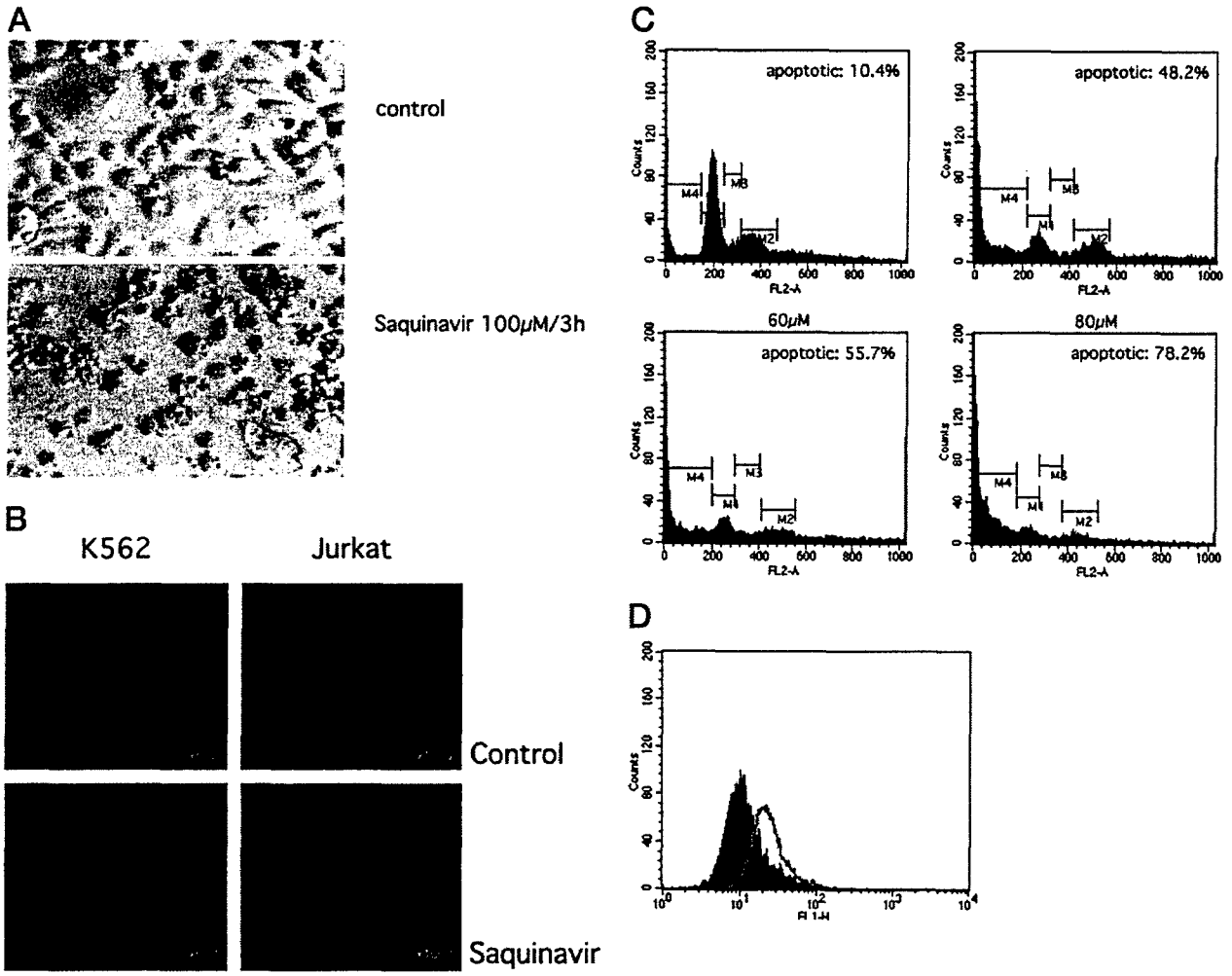

Fig. 4. Saquinavir induces apoptosis in human prostate cancer cells. In *A*, incubation of PC-3 cells with 100 μM saquinavir caused a rapid induction of apoptosis within hours. Cells showed membrane blebbing and chromatin condensation as early as 3 h after the start of incubation. B, propidium iodide staining. Treatment of K562 and Jurkat leukemia cells with saquinavir caused membrane blebbing and chromatin condensation 24 h after the start of treatment. *C*, DNA content of PC-3 prostate cancer cells incubated for 48 h with different concentrations of saquinavir as determined by propidium iodide staining of ethanol-fixed cells. Incubation with saquinavir caused concentration-dependent induction of apoptosis. The percentages of apoptotic cells in the pro-G_1 peaks are indicated. In *D*, TUNEL staining of PC-3 cells 24 h after the start of incubation with 0 μM (*filled histogram*) or 100 μM Saquinavir (*open histogram*) detected TUNEL-positive cells with a shift in mean fluorescence from 13.5 to 25.

50 μM and 60 μM concentrations of saquinavir did not significantly alter the plating efficiency of PC-3 cells (control cells, 52.1 ± 4.2%; saquinavir-treated cells, 47.3 ± 1.7%) and DU-145 cells (control cells, 23.1 ± 2.5%; saquinavir-treated cells, 26.0 ± 2.6%). However, pretreatment with saquinavir sensitized the surviving cells to ionizing radiation (Fig. 5; PC-3: control cells, $\alpha = 0.35$, $\beta = 0.045$, $\alpha/\beta = 7.8$; saquinavir-treated cells: $\alpha = 0.334$, $\beta = 0.069$, $\alpha/\beta = 4.8$; DU-145 cells: control cells, $\alpha = 0.35$, $\beta = 0.034$, $\alpha/\beta = 10.3$; saquinavir-treated cells, $\alpha = 0.36$, $\beta = 0.075$, $\alpha/\beta = 4.8$).

DISCUSSION

The Ub/26s proteasome pathway is the major non-lysosomal proteolytic pathway in mammalian cells. It is responsible for the degradation of short-lived (15), and 70–90% of all long-lived proteins (15, 16). Inhibition of proteasome function has been shown to induce apoptosis in cancer cells (11, 17–22), and partial inhibition sensitizes surviving cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs (11, 23). Proteolysis by the Ub/26s proteasome pathway is an important component of regulation of cellular functions like signal transduction, cell cycle control, and immune responses (24). Interestingly, the 20s core unit of the proteasome is the only mammalian protease known, thus far, to share specific cleavage action sites with the HIV-I protease, which may be a pathogenic mechanism adopted by the virus. A recent report indicated that the HIV-I PI ritonavir inhibits 20s proteasome function (25). Here, we investigated the effect of the HIV-I PI saquinavir, which is clinically less toxic (1), on 20s and 26s proteasome function and the possible physiological consequences of such an inhibition in human cancer cells.

Prostate cancer cells in general show elevated constitutive DNA-binding activity of the antiapoptotic transcription factor NF-κB (26, 27), and we, and others, have demonstrated that the inhibition of NF-κB induces apoptosis in cancer cells (17, 20, 26, 28). NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the IκB family. Activation of this pathway is normally achieved by phosphorylation, polyubiquitination, and subsequent degradation by the 26s proteasome of one of its most important inhibitors, IκBα. Degradation of IκBα frees NF-κB for translocation to the nucleus and activation of its target genetic programs (reviewed in Ref. 29). We have shown that the HIV-I PI saquinavir blocks NF-κB activation in the murine RAW macrophage and human PC-3 prostate cancer cell lines and stabilizes IκBα in a concentration-dependent fashion. Because activation of NF-κB is an important precondition for replication and persistence of HIV (30), this suggests a pathway for action of saquinavir that is independent of direct viral protease inhibition.

Saquinavir, like ritonavir, was shown to directly inhibit 20s and 26s proteasome function *in vitro*. Because the inhibition of both 26s and 20s function showed similar drug concentration dependency, we conclude that it acts on the 20s core unit of the proteasome. Treatment of cells with saquinavir also inhibited proteasome function, although the IC_{50} was markedly higher, perhaps because saquinavir is a substrate for the multidrug resistance (*mdr-1*) gene product P-glycoprotein, which is highly expressed in PC-3 human prostate cancer cells (12). Physiological inhibition of proteasome function by saquinavir was demonstrated by the finding of an accumulation of the Ub-R-GFP reporter of proteasome function (7) in living ECV 304 cells stably transfected with this construct.

A physiological consequence of the saquinavir treatment of PC-3 and DU-145 prostate cancer, U373 glioblastoma, and K562 and Jurkat leukemia cells was apoptosis, which occurred at concentrations that were similar to those needed to inhibit proteasome function. These data are consistent with the hypothesis that saquinavir-induced apoptosis is the result of the inhibition of proteasome function and the blocking of NF-κB activation. We have previously shown that the inhibition of NF-κB activation in these cell lines by transduction with an IκB super-repressor gene also results in apoptosis (11).

The Ub/26s proteasome has recently been identified as a novel target for cancer therapy (11, 21, 31, 32). In this study, short-time preincubation with saquinavir clearly sensitized PC-3 and DU-145 prostate cancer cells to ionizing radiation, which resulted in a change

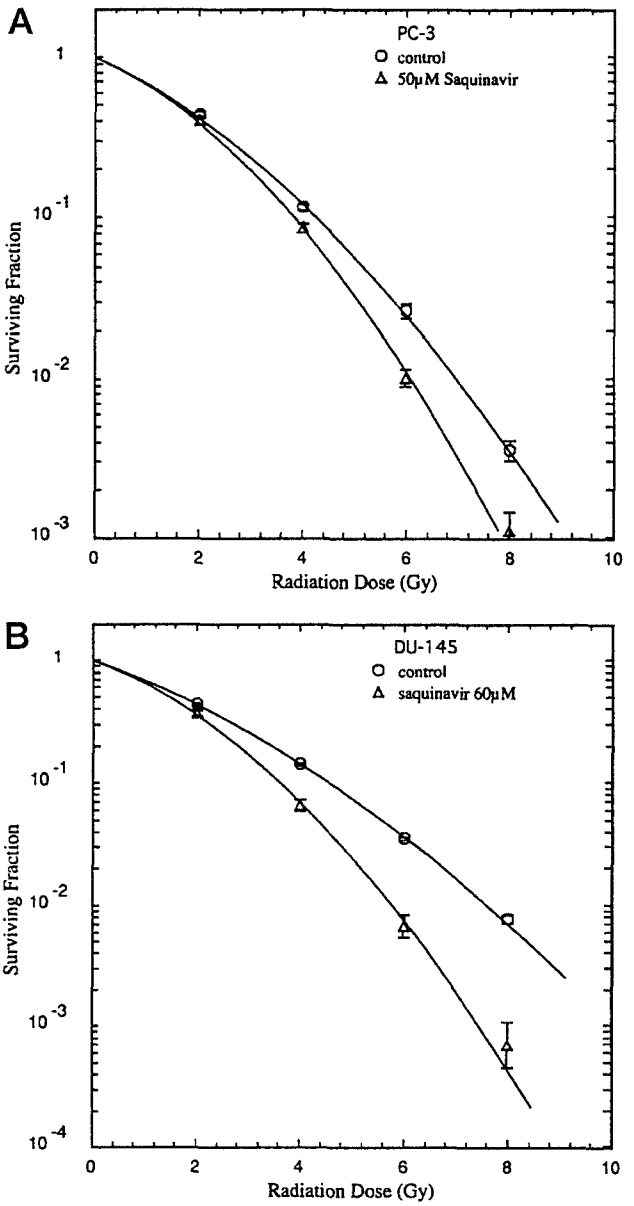

Fig. 5. Saquinavir sensitizes human prostate cancer cells to ionizing radiation. (*A*) PC-3 prostate cancer cells were incubated with 50 μM concentrations and (*B*) DU145 prostate cancer cells were incubated with 60 μM concentrations of saquinavir for one h. Cells were washed twice with PBS, trypsinized and irradiated with 0, 2, 4, 6 or 8 Gy. Cells were plated into Petri dishes in triplicate. After 14 days cells were fixed with 75% ethanol, stained with crystal violet and colonies that consisted of more than 50 cells were counted. The number of colonies of each dose point was normalized to the number of colonies of the corresponding unirradiated control. Resulting survival curves were fitted using a linear quadratic model. Pretreatment with saquinavir sensitized the surviving cells to ionizing radiation (PC-3: control cells: $\alpha = 0.35$, $\beta = 0.045$, $\alpha/\beta = 7.8$; saquinavir-treated cells: $\alpha = 0.334$, $\beta = 0.069$, $\alpha/\beta = 4.8$; DU145: control cells: $\alpha = 0.35$, $\beta = 0.034$, $\alpha/\beta = 10.3$; saquinavir-treated cells: $\alpha = 0.36$, $\beta = 0.075$, $\alpha/\beta = 4.8$).

of the α:β ratio from 7.8 and 10.3, respectively, in control cells to 4.8 in saquinavir-treated cells. The clinical significance of these observations is supported by a recent report showing dramatically improved survival for AIDS patients suffering from HIV-related primary central nervous system lymphoma (PCNSL) treated with highly active antiretroviral therapy (HAART) and cranial irradiation when compared with cranial irradiation or HAART treatment alone (33). Results from two recent reports indicate that this effect is independent of the recovery of the immune system (34, 35). Because the inhibition of proteasome function, in general, sensitizes tumor cells to ionizing radiation (11, 23) and HIV-1 PIs have been shown to inhibit P-glycoprotein-mediated multidrug resistance (3), HIV-1 PIs may become a new class of chemotherapeutic agents in radiochemotherapy. As in HIV therapy, the use of radiation therapy combined with saquinavir and "baby-concentrations" of ritonavir may overcome the problem of low bioavailability of the drug (36).

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The Proteasome in Cancer Biology and Therapy

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Summary:

Proteasomes vary in their composition and in their location within cells, and their baseline activity varies widely between cancer cell lines. Additionally, proteasomal activity can be altered by exposure of cells to various stimuli. We have shown that proteasome activity in both cytosolic and non-cytosolic cellular fractions decreases rapidly following radiation exposure and this post-transcriptional mechanism enables a rapid response to radiation damage. Because modifications of proteasomal activity may increase the benefit of radiation as well as other forms of cancer therapy, further investigation into this area is needed.

Introduction:

In eukaryotic cells, the turnover rate of most cellular proteins is dictated by proteolysis through the proteasome (1). Destruction is not limited to misfolded or damaged proteins, but extends to selective removal of undamaged proteins, which may be triggered by phosphorylation, dephosphorylation, or other modifications. In other words, degradation is selective and specific and controlled at multiple levels. Because of its position in determining levels of protein expression, the proteasome plays a critical role in numerous biologically important processes, including cell cycle progression and arrest, signal transduction leading to cell survival and apoptosis, DNA repair and damage response, and development and function of the immune system. In addition, we have recently shown that proteasome function can be modulated by therapeutic interventions, including radiation therapy (2).

The proteasome can exist in several forms. The core 20S proteasome is a barrel-shaped structure with multiple interior catalytic sites (3). Because the active sites are topologically sequestered, the 20S proteasome is inherently inactive. Activation is achieved by attachment of 19S regulatory particles at both ends to form the 26S proteasome (4). This ATP-dependent protease has a molecular mass of about 2 million Da and comprises about 1% of total cellular protein. Recognition and degradation of proteins by the 26S proteasome normally requires ligation to a ubiquitin (Ub) chain, but recognition through other ubiquitin-like moieties is also possible. The 19S regulator unfolds multi-Ub conjugated proteins and feeds them into the chamber for digestion (5). Alternative activators are 11S (PA28) molecules in place of 19S regulators, which form ATP-ase and Ub-independent proteasomes. Expression of PA28 is up-regulated by interferon and other inflammatory cytokines, which also cause replacement of constitutively expressed catalytic components in the 20S core, resulting in the "immunoproteasome" (6). Nearly all proteins are digested to amino acids by the proteasome, but a few peptides escape and are presented to the immune system on major histocompatibility complex class I molecules for recognition by cytolytic T cells. The immunoproteasome generates antigenic epitopes that are different from those generated by the standard 26S proteasome. The difference may result in less presentation of "self" epitopes by powerful mature antigen presenting cells. Hybrid proteasomes that contain

both a 19S regulator and an 11S activator molecule also exist (7) and these may be particularly important in antigen processing.

Distinct proteasomes degrade particular types of substrates. For the 26S proteasome, substrate specificity is provided by E3 ligases, which catalyse the final step in ubiquitination, and by deubiquitinating enzymes. The immunoproteasome, on the other hand, is less able to degrade ubiquitinated proteins, but has a preference for peptides and damaged proteins (8). Although little is known about this pathway, it is critical in preventing accumulation of protein aggregates, which can disrupt cell function, as is observed in many neurodegenerative diseases. Interactions between proteasome components, regulators, activators, and inhibitors are also regulated spatially (9). Proteasomes can be nuclear or cytoplasmic, membrane-bound or free in the cytoplasm. Their location is dynamically regulated and could define their role. This may be particularly important in the formation of centromeres and kinetochores (10), in antigen processing, and in the nucleus, where poly-ADP ribose polymerase (PARP) can activate the 20S proteasome (11) and facilitate removal of damaged histones that could compromise chromatin integrity.

Materials and Methods:

Cells were grown in 75-cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2/95% air. Dulbecco's Modified Eagle's Medium or RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco BRL)

26S proteasome function was measured in crude cellular extracts prepared as described previously (2). Cytosolic fractions were supernatents from spins at 10,000g for 30 mins and non-cytosolic fractions were the pellets, excluding highly insoluble material. The rate of proteolysis was assessed using the fluorogenic proteasome substrate SucLLVY-MCA to measure the chymotrypsin-like cleavage activity of proteasomes. Release of the fluorescent group 7-amido-4-methylcoumarin (AMC) from the substrate was measured over a 30 minute period in a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, 37° C) at 380/460 nm..

Results:

Recently, we showed that chymotrypsin-like activity of the 26s proteasome in cells was inhibited by up to 50% shortly after low- and high-dose radiation exposure (2). The response was very rapid, occurring within 15 minutes following irradiation. Irradiation of purified proteasomes gave a similar result, indicating that proteasomes are direct radiation targets. The consequences of proteasome inhibition were linked to inhibition of NF-κB expression at low radiation doses. It seems highly likely that expression of other molecules that are regulated by the ubiquitin/proteasome pathway and have been implicated in biological pathways relevant to radioresponses, such as p53, mdm2, p21, are also directly affected by this mechanism.

Since proteasomes exist in different compartments in the cell and have different functions in these sites, we investigated whether radiation inhibited both cytosolic and non-cytosolic activity. PC-3 human prostate cancer cells were irradiated with 10 Gy and soluble and non-soluble cell extracts were prepared. Most proteasome activity was in the cytosol but irradiation decreased activity in both compartments equally (Fig 1). We have noticed that proteasome activity varies between cell lines, suggesting intrinsic factors

determine the level of activity. A comparison of 9 cell lines showed that the rate of degradation of fluorogenic substrate by extracts of various cancer cells varied over two fold (Fig 2). One hypothesis that could explain such variation is that proteasome activity is determined, at least in part, by signal transduction pathways. To test this hypothesis, we used 3 cell lines transduced to express the interleukin-3 (IL-3) gene. We had already shown that this leads to increased expression of major histocompatibility and other cell adhesion molecules (12), through autocrine action. Consistent with the hypothesis, IL-3 gene expression enhanced degradation of fluorogenic substrates through the 26S proteasome in at least 2 cases (Fig 3). Since IL-3 gene expression did not alter intrinsic radiosensitivity of the cell lines (12), we can conclude that 26S proteasome activity per se does not correlate directly with radiosensitivity.

Discussion

Division of labor between 20S and 26S proteasomes, between constitutive and immunoproteasomes, and between proteasomes in different sites exists through substrate selectivity (8). We have presented evidence that the proteasome is a direct sensor of radiation and other stress-related damage. Shortly after exposure even to low radiation doses proteasome activity is inhibited by up to 50%. More complete inhibition by drugs results in cell death by apoptosis and proteasome inhibitors radiosensitize tumor cells in vitro and in vivo (13). The consequences of partial proteasome inhibition following irradiation may depend upon which activities are most affected. In our studies, both cytosolic and non-cytosolic cellular compartments were inhibited, although selective inhibition may exist at more subtle levels. It seems highly likely that the rapid molecular responses that cells make to irradiation are due to this mechanism and involve both the nucleus and cytoplasm. If clearance of damaged proteins is also affected, genomic instability (11), centromere formation, nuclear inclusions, antigen presentation, and other consequences are possible.

Considerable variation in proteasome activity was observed between cancer cell lines, which raises the question of how this variation might impact on cancer behavior and response to therapy. A potential source of such variation is the signaling pathways that are dysregulated by carcinogenesis. Apart from immunoproteasome induction by pro-inflammatory cytokines, little is known about the impact of signal transduction on proteasome expression and function, but this could be crucial to expression of cell regulatory molecules and antigen expression. At the same time, tumor suppressor and oncogene mutations frequently affect processing of these molecules through the Ub-proteasome system (14). Our results with IL-3 gene transduction suggest that there is unlikely to be any simple correlation between proteasome activity and response to radiation therapy, but the level of activity and its nature may still affect outcome. The proteasome therefore represents a valid target for therapeutic modification of cancer treatment.

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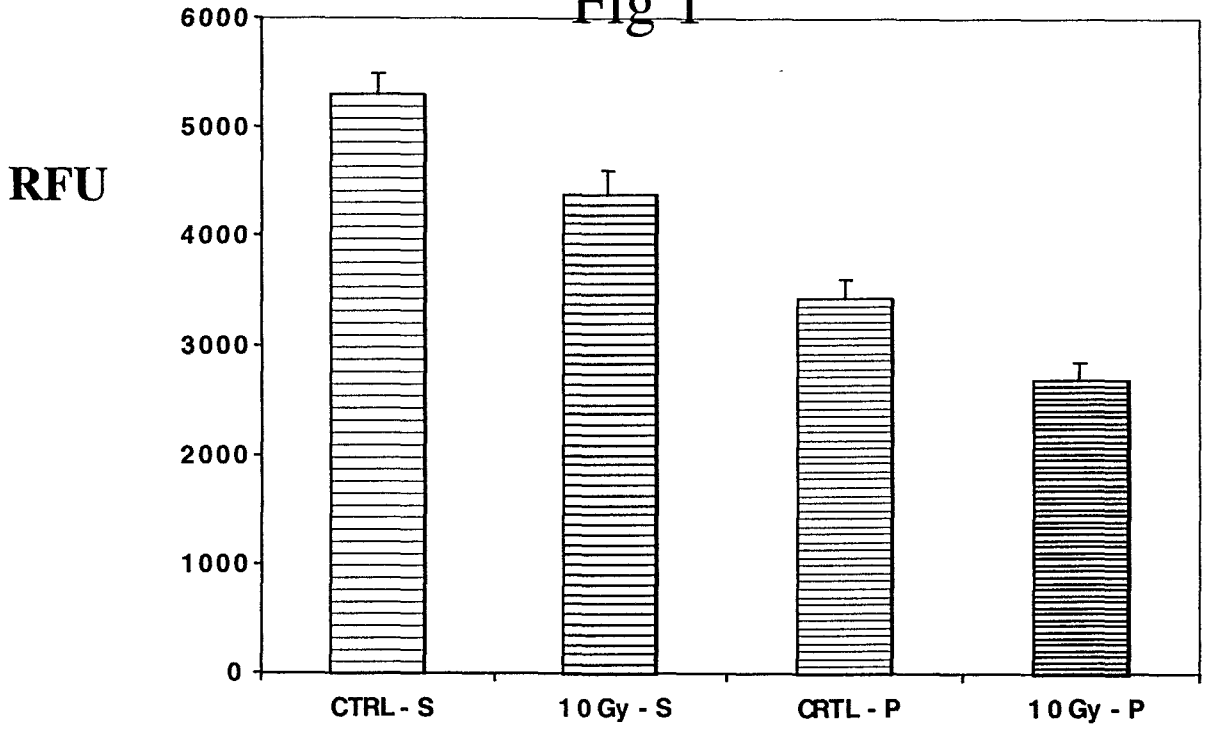
Legends:

Figure 1: Radiation affects the rate of proteolysis by the cytosolic and non-cytosolic 26S proteasome fractions equally.

Figure 2: Rate of proteolysis by 26S proteasome extrcats varies with the cell line.

Figure 3: IL-3 gene transduction increases the rate of proteasome-associated proteolysis.

Fig 1



% of total activity	CTRL	10 Gy
in the supernatant (cytosolic)	0.61	0.62
in the pellet (non-cytosolic)	0.39	0.38

Fig 2

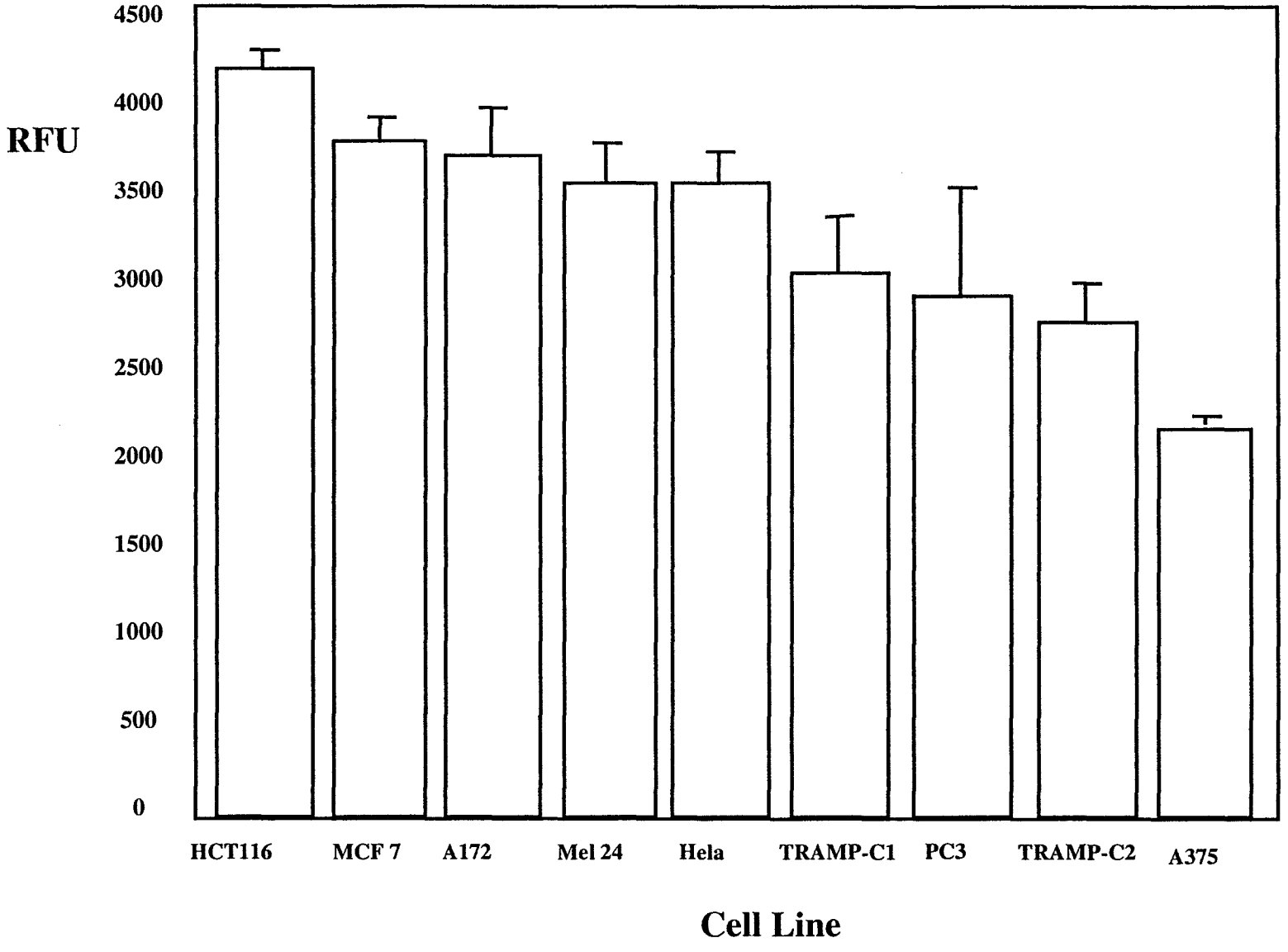
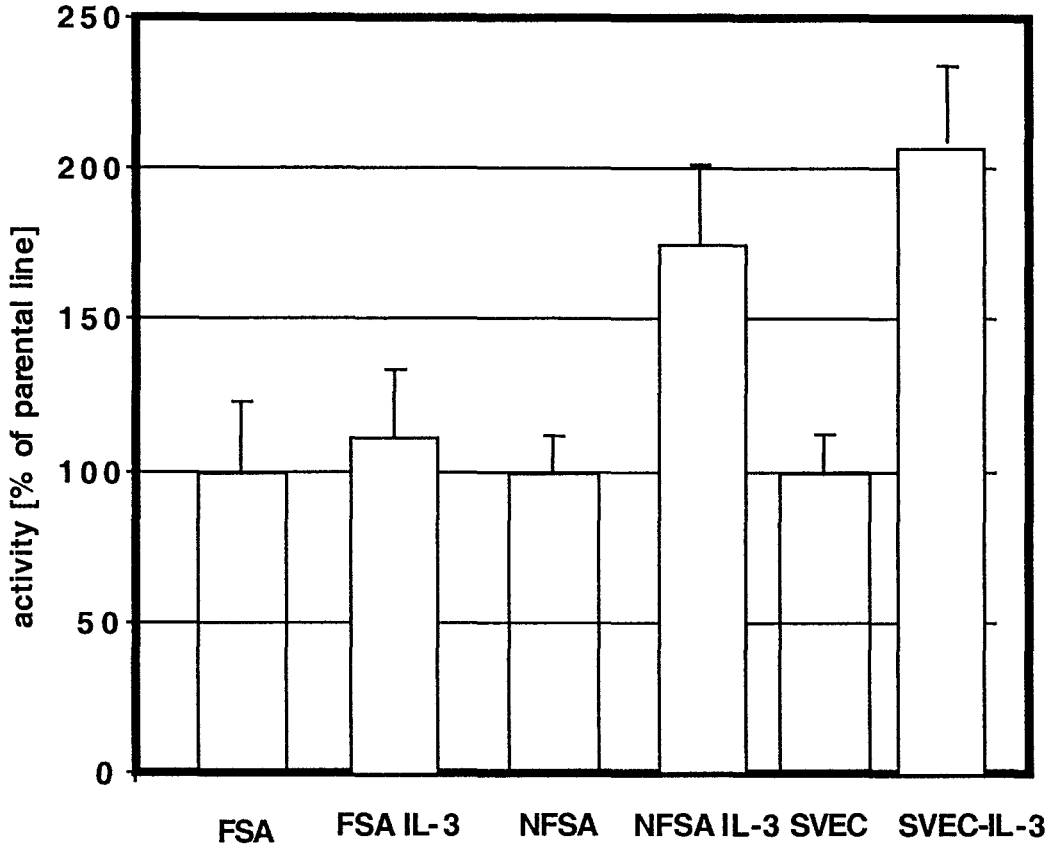

RFU
4500
4000
3500
3000
2500
2000
1500
1000
500
0
HCT116
MCF 7
A172
Mel 24
Hela
TRAMP-C1
PC3
TRAMP-C2
A375
Cell Line

Fig 3


250
200
150
100
50
0
activity [% of parental line]
FSA
FSA IL-3
NFSA
NFSA IL-3
SVEC
SVEC-IL-3

Anthracyclins, proteasome activity and inhibition of P-gp-mediated multi-drug-resistance

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Abstract

Background:

The multi-drug resistance gene 1 codes for the transmembrane efflux pump P-glycoprotein, responsible for an ATP-dependent export of structural unrelated compounds including many chemotherapeutic drugs. P-glycoprotein mediated multi-drug resistance is a common cause of chemotherapy failure. Drugs like cyclosporin A and verapamil can inhibit the efflux of chemotherapeutics, mediated by P-glycoprotein. However, since serum concentrations of these drugs, necessary to inhibit P-glycoprotein function, have severe side effects, they are still not part of standard chemotherapy regimens. Thus, there is an ongoing search for specific inhibitors of P-glycoprotein. Observations that cyclosporin A is also a proteasome inhibitor, led us to investigate a possible cross substrate specificity of P-glycoprotein and the 26s proteasome.

Material&Methods: Primary porcine heart fibroblasts were incubated with different doses of verapamil for 16 hours. VEGF mRNA expression was accessed by RT-PCR. Lysates from ECV 304 cells were incubated with different doses of verapamil, doxorubicin, daunorubicin, idarubicin, epirubicin, dactinomycin, topotecan, mitomycin C and gemcitabine. Chymotryptic 26s and 20s proteasome activity was measured using a fluorogenic peptide assay. Effects of doxorubicin on proteasome function in living cells was monitored in ECV304 cells, stable transfected with ubiquitin/green fluorescent protein fusion protein. Accumulation of daunorubicin in p-glycoprotein highly positive KB 8-5

cells was monitored in the presence of different doses of the proteasome inhibitor MG-132.

Results: Incubation of porcine heart fibroblast with verapamil caused a dose-dependent induction of VEGF mRNA expression. Incubation of crude cellular extracts of ECV 304 cells with Verapamil, doxorubicin, idarubicin, epirubicin, and dactinomycin led to a dose-dependent inhibition of 26s and 20s proteasome function. Incubation of crude cellular extracts of ECV 304 cells with daunorubicin inhibited 26s proteasome function in a dose-dependent manner but did not alter 20s proteasome activity. In contrast, incubation of crude cellular extracts of ECV 304 cells with topotecan, mitomycin C and gemcitabine left 26s and 20s proteasome activity unchanged. Incubation of KB 8-5 cells with different doses of MG-132 caused a dose-dependent accumulation of daunorubicin.

Conclusions: Our data indicates, that the 26s proteasome and P-glycoprotein have overlapping substrate specificities. Use of proteasome inhibitors in cancer therapy might not only increase radiosensitivity but could also sensitize tumors to standard chemotherapy protocols.

Introduction

Multi-drug-resistance (MDR) is a common reason for chemotherapy treatment failure in breast cancer, leukemia and non-Hodgkin lymphoma patients. Multi-drug-resistance is thereby often based on overexpression of the mdr1 gene. This gene codes for P-glycoprotein (P–gp) a 1280 amino acid transmembrane phosphoglycoprotein that functions as an ATP-dependent efflux pump. Numerous pre-clinical and clinical studies have been undertaken to overcome multi-drug-resistance and several substances have been identified, able to revert multi-drug-resistance *in-vitro* (reviewed in (1)). However, so far serum-concentrations of most MDR-modulating drugs required to revert multi-drug-resistance have unacceptable toxicity in-vivo. Therefore, combination protocols using cytotoxic drugs and P-gp inhibitors did not enter standard chemotherapy regimens. Insights into the mechanisms of interaction of these compounds with P-gp could be the basis for the development of more specific inhibitors.

Two of the most commonly used MDR-modulating substances are verapamil, cyclosporine A (CsA) and their derivates. It is remarkable that cyclosporine A has been recently identified as an inhibitor of the 26s proteasome (2). The 26s proteasome is a highly conserved multicatalytic protease responsible for ATP- and ubiquitin-dependent degradation of all short-lived and 70-90% of all long lived proteins including cyclin A, B and E, p21 and p27, p53, cJun, cFos, and IκB. As such a central protease the 26s proteasome controls the cell cycle, signal transduction pathways, apoptosis and major functions of the immune system.

The fact that CsA is a proteasome inhibitor gives rise to the assumption that most of the immunosuppressive properties of CsA are based on this inhibitory effect, causing a decrease of the diversity of MHC-I molecules on the cell surface of target cells (3) as well as apoptotic death of lymphocytes caused by inhibition of the transcription factor NF-κB (4).

Recently, N-benzyloxycarbonyl-L-leucyl-L-leucinal (zLLal), a calpain inhibitor, was reported to cause accumulation of ubiquitinated P-gp in K562 erythroleukemia cells while lactacystin, a potent a highly selective proteasome inhibitor had no effect, suggesting that calpain is involved in P-gp-mediated drug efflux in mdr1 positive cells. However, ubiquitination is usually restricted to 26s proteasome-mediated proteolysis. This led us to investigate a possible link between this protease and multi-drug-resistance.

Material and Methods

Cell culture

Primary porcine cardiac fibroblasts were obtained from fresh perivascular connective tissue from a porcine heart. The samples were minced and single pieces placed into 8.7cm^2 cell culture dishes. The flakes were squeezed under a glass cover slip, and supplemented with Dulbecco's modified eagle medium (DMEM; Cell Concepts, Germany) with 10% fetal calf serum (FCS; Life Technologies), L-Glutamine 200mM (Cell Concepts), 200 IU/ml Penicillin/Streptomycin (Cell Concepts), 10 µg/ml Minocycline (ICN), and 10 µg/ml Amphotericin B (Bristol-Myers Squibb). Fibroblasts started to grow out of the tissue on the second day. Media was changed regularly 3 times a week. To increase the yield, some cover slips were taken off after 2 weeks and placed into new dishes. After reaching confluency, cells were trypsinized, transferred into cell culture flasks, and subsequently fed with DMEM, 10% FCS, 200 mM L-Glutamine, and 100 IU/ml Penicillin/Streptomycin. Cells up to the 12th passage were used for experiments. KB 8.5 human epitheloid carcinoma cells were a generous gift from Dr. Peter Hafkemeyer (University Clinic Freiburg, Germany). EVC 304 human bladder carcinoma cells were obtained from the German Microorganism And Tissue Culture Collection (DSMZ, Braunschweig). Cells were grown in 75 cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2. The medium used was DMEM medium (Sigma) supplemented with 10 % heat inactivated FCS (Sigma) and 1 % penicillin/streptomycin (Gibco BRL). Every 21 days P-gp-positive KB 8.5 cells were selected by addition of colchicin (10ng/ml,

Sigma). 24 hours before drug treatment cells were plated into 6-well plates (Costar) at a density of 10e6 cells/well.

Drug treatment

Stock solutions of all cytotoxic drugs were obtained from the hospital pharmacy, University Clinic Freiburg. MG-132 (Calbiochem) was dissolved in DMSO (10 mM), Lactacystin (Calbiochem) was dissolved in DMSO (1 mM) and small aliquots (10-30 µl) were stored at –20° C. At indicated times cells were washed twice with PBS and the growth medium was replaced by PBS containing Daunorubincin (16-2 µM), MG-132 (0.5-50 µM, 0.5% DMSO) or Lactacystin (5-2 µM) respectively. Control cells for MG-132 and Lactacystin treatment were subjected to DMSO treatment alone (0.5 %).

Proteasome function assays

Proteasome function was measured as described previously (20). Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1,000g for 5 minutes and 10,000g for 20 minutes. Protein concentration was determined by the BCA protocol (Pierce). 100µg protein of each sample was diluted with buffer I to a final volume of 1000µl. The fluorogenic proteasome substrate SucLLVY-7-amido-4-

methylcoumarin (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 80 µM in 1% DMSO. Cleavage activity was monitored continuously by detection of free 7-amido-4-methylcoumarin using a fluorescence plate reader (Gemini, Molecular Devices) at 380/460 nm and 37° C. Incubation of 7-amido-4-methylcoumarin (AMC, 2 µM) with cytotoxic drugs was carried out in buffer I for each sample in parallel and measurements of proteasome function were corrected when necessary.

Drug accumulation assay

Determination of total cellular daunorubicin content was carried out as described elsewhere with some minor modifications: Growth medium was replaced by PBS and cells were incubated at 37° C for 40 minutes. PBS was replaced by fresh PBS containing daunorubicin and MG-132 or daunorubicin alone. In some experiments cells were washed with PBS after daunorubicin treatment and incubated in PBS containing MG-132 for additional 40 minutes at 37° C. After drug treatment cells were washed twice with PBS, re-suspended in 400 µl 50/50 vol% ethanol (100%) / HCl (1M), vortexed and diluted with water to a final volume of 1.4 ml. Fluorescence was measured in quadruplicates of 200µl using a fluorescence plate reader (Gemini, Molecular Devices) at 480/575 nm.

Transfection

ECV 304 cells were maintained in DMEM (10 % FSC, 1% penicillin/streptomycin). 12 hours before transfection cells were trypsinized and

plated at a density of 250.000 cells/well into six-well plates. Cells were transfected with 5µg of a plasmid (pEGFP-N1, Clontech) coding for an ubiquitin-R-GFP fusion protein under control of a CMV promoter (5) (a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10% FSC, 1% penicillin/streptomycin) supplemented with 500µg/ml G418 (Sigma) and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-H, *FACSCalibur*, Becton Dickinson) using *CellQuest* Software before and after treatment with the proteasome inhibitor MG-132 (50µM, Calbiochem) for 10 hours at 37° C. Clone #10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

RT-PCR

First-strand cDNA was synthesized from 1 _g RNA by using Superscript II reverse transcriptase (Gibco BRL) and random hexamers (Pharmacia, Freiburg, Germany) as primers. Polymerase chain reaction (PCR) was carried out in an automatic thermal cycler (PTC 200, MJ Research). 1 _l (5%) of resulting cDNA was amplified in 25 _l PCR-reaction-mixtures using specific primers for porcine VEGF: VEGF forward: 5'-AGG AGA CCA GAA ACC CCA CG-3'; VEGF reverse: 5'-CTC AGT GGG CAC ACA CTC C-3'; ß-actin forward: 5'-GTC CCC ATC TAC GAG G-3'; ß-actin reverse: 5'-GCT CGT AGC TCT TCT CC-3'; (Genescan, Germany). PCR-conditions for VEGF: 94_ C – 53,4_ C - 72_ C, 18 cycles. PCR-

conditions for ß-actin: 18 cycles, 94_ C - 42_ C - 72_ C. All PCR-reactions were in linear range. The amplified products were analyzed on 0.7% agarose gels containing 1% ethidium bromide. Photographs were digitized using a flatbed scanner (CanoScan N650U, Canon). Densitometry was carried out using the NIH-Image Software for Macintosh Computers and the internal gel-macro2. Semi quantitative estimation was done by comparing VEGF to ß-actin mRNA expression.

Results

Verapamil is an inhibitor of 20s and 26s proteasome function

A report showing a pro-angiogenic effect of verapamil in a rat model (6) and the clinical observation from the second *Danish Verapamil Infarction Trial II* (DAVIT II) on 1775 patients, indicating that verapamil significantly reduced mortality and re-infarction rate (7), led us investigate a possible link between verapamil and the proteasome. In fact, cultures of primary porcine heart fibroblasts exhibited a dose-dependent 14fold increase of VEGF mRNA when treated with verapamil (0 to 200 μM) for 16 hours (Fig. 1). As gene expression of VEGF in general is dependent on stabilization of HIF-1α and in turn stabilization of HIF-1α usually occurs after inhibition of proteasome function, we decided to test if verapamil is a direct inhibitor of the 26s proteasome. Incubation of crude extracts of ECV304 cells with different concentrations of verapamil (0, 50, 100 and 200 μM) detected a dose-dependent inhibition of chymotryptic MG-132-sensitive 20s and 26s proteasome function, consistent with a direct inhibitory effect of verapamil on the proteasome (Fig. 2).

Anthracyclins inhibit 20s and 26s proteasome function in a dose-dependent manner

Vincristine, vinblastine and anthracyclins are classical substrates of P-gp (1). It has be recently shown, that vinblastine is a proteasome inhibitor (8). Since two of the most commonly used P-gp inhibitors cyclosporine A and verapamil (9) also

exhibited an inhibitory effect on 20s and 26s proteasome function we addressed the question if anthracyclins in general have an inhibitory effect on this protease. When crude extracts of ECV 304 cells were incubated with different doses of doxorubicin (Fig. 3A/B, 100 - 0 µM), daunorubicin (Fig. 3C/D, 100 - 0 µM), idarubicin (Fig. 3E/F, 100 - 0 µM), epirubicin (Fig. 3G/H, 100 - 0 µM) and dactinomycin (Fig. 3I/J, 10 – 0 µM) we observed a dose dependent inhibition of 26s proteasome function with IC_{50} values of 65.5 µM for doxorubicin, 13.7 µM for daunorubicin, 38.6 µM for idarubicin, 29.2 µM for epirubicin and 26 µM for dactinomycin. In contast, topotecan, mitomycin C and gemcitabine had no measurable effect on 26s proteasome function (data not shown). 20s proteasome function was inhibited by doxorubicin (IC_{50} 5.8 µM), idarubicin (IC_{50} 92 µM), epirubicin (IC_{50} 12.5 µM) and dactinomycin (IC_{50} 19.9 µM) but not by daunorubicin. In order to demonstrate the significance of this finding in living cells we incubated ECV304/10 cells, stable transfected with an expression plasmid for an ubiquitin-GFP fusion protein with doxorubicin (100 µM) for 12 hours. When analyzed by fluorescence microscopy the cells showed an accumulation of doxorubicin in a perinuclear structure while GFP accumulated in the whole cytoplasm in a dose-dependent manner, indicating an inhibition of proteasome function by doxorubicin in living cells (Fig. 3K).

MG-132 treatment reverts multi-drug-resistance in P-gp expressing KB 8-5 cells

The human epitheloid carcinoma cell line KB 8-5 is a well-characterized tumor cell line exhibiting multi-drug-resistance on the basis of P-gp expression. In our

initial experiments we observed that treatment of KB 8.5 cells with MG-132 (3.125 to 50μM) caused induction of apoptosis within 24 hours, indicating that MG-132 enters KB 8-5 cells and is not eliminated by P-gp function. This observation was in accordance with numerous studies reporting induction of apoptosis in cancer cells by proteasome inhibitors (10)(11-14). When KB 8-5 cells were treated with different doses of MG-132 and daunorubicin (10 μM) for 45 minutes we found a dose-dependent MG-132-induced cytoplasmic accumulation of daunorubicin (4-fold increase at 50μM MG-132, Fig. 4). However, treatment of KB 8-5 cells with the more specific proteasome inhibitor lactacystin failed to alter daunorubicin accumulation (data not shown). To exclude the possibility that lactacystin is a substrate for P-gp and is eliminated from cell by P-gp function we treated KB 8-5 cells with MG-132 or lactacystin for 3 hours and accessed the inhibitory effect of both drugs on chymotryptic 26s proteasome function by a fluorogenic peptide assay in crude cellular extracts. As expected, both inhibitors abolished 26s proteasome function (data not shown).

Discussion

P-gp mediated multi-drug-resistance is one of the most common causes of chemotherapy failure in cancer patients. Numerous *in-vitro* and *in-vivo* studies have been undertaken to circumvent MDR pharmacologically using P-gp modulating compounds like verapamil, cyclosporine A, reserpine, staurosporine, propafenone, phenoxazine, chloroquine, phenothiazine and their derivates (reviewed in (1)). However, non-P-gp-related site effects usually limit the clinical usage of these drugs in standard chemotherapy regimens. Insights into the mechanisms of MDR-modulation by these compounds could offer the basis for the development of specific P-gp inhibitors. Our observation that verapamil has inhibitory effects on the cleavage activity of the 26s proteasome and a recent report showing a comparable effect for vinbastine (8) led us investigate the effects of anthracyclins on the activity of this protease. We found a dose-dependent inhibitory effect on 26s proteasome function for all five anthracyclins tested. Additionally, except of daunorubicin, anthracyclins also inhibited 20s chymotryptic function in a dose-dependent manner.

A direct inhibitory effect of this class of chemotherapeutics on the proteasome is especially remarkable as tumor cells in general exhibit different expression pattern of proteasome subunits and a different distribution pattern of the proteasomes between cytoplasm and nucleus when compared with normal tissue cells (15-17). Further, anthracyclins are co-transported into the nucleus along with proteasomes (18, 19) and inhibition of proteasome function in tumor cells in general induces apoptosis (10) (11-14) and sensitizes the surviving cells to

ionizing radiation (20, 21). Direct inhibition of proteasome function might therefore be a major mechanism of this class of cytotoxic drugs.

Mechanistically, P-gp modulating drugs are either high-affinity substrates or inhibitors of ATP-dependent transport by P-gp (1). Our observation, that inhibitors of the 26s proteasome, the central protease of the major eukaryotic ATP-dependent protein degradation pathway, have inhibitory effects on P-gp function, could indicate that P-gp and the 26s proteasome have overlapping substrate specificities. Differences in proteasome subunit expression and patterns of proteasome distribution between malignant and normal cells can explain why specific proteasome inhibitors like PS-341 are clinically well tolerated (15-17). Thus, with proteasome inhibitors entering first clinical trials (22, 23), this class of substances might offer a new strategy to overcome P-gp-related MDR, combined with direct cytotoxic and radiosensitizing effects on tumors cells.

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Figures

Fig. 1

Verapamil induces expression of VEGF mRNA in porcine heart fibroblasts. Treatment of fibroblasts with 0, 50, 100 and 200µM verapamil for 16 hours caused a dose-dependent increase in VEGF mRNA expression (14fold at 200µM).

Fig. 2

Verapamil is an inhibitor of 26s proteasome function

Incubation of crude cellular extracts of ECV 304 cells with different doses of verapamil (50, 60, 80, 100, 200 µM) inhibited proteolysis of SucLLVY-AMC in a dose-dependent manner, indicating an inhibition of 26s proteasome function.

Fig. 3

Anthracyclins are inhibitors of proteasome function

(A-J) Incubation of crude cellular extracts of ECV 304 cells with different doses of doxorubicin (Fig. 3A/B, 100 - 0 µM), daunorubicin (Fig. 3C/D, 100 - 0 µM), idarubicin (Fig. 3E/F, 100 - 0 µM), epirubicin (Fig. 3G/H, 100 - 0 µM) and dactinomycin (Fig. 3I/J, 10 – 0 µM) caused an inhibition of 26s proteasome function. 20s proteasome was inhibited in case of doxorubicin, idarubicin, epirubicin, dactinomycin but not daunorubicin. (K) Incubation of

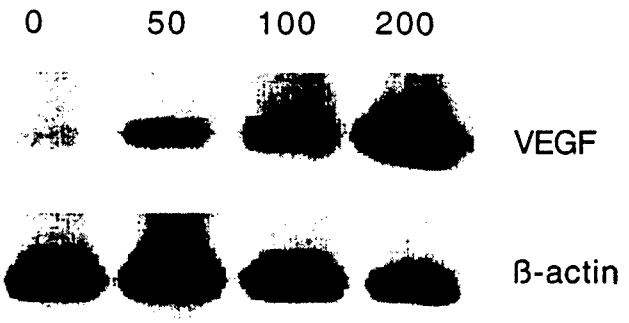
ECV 304 cells with doxorubicin (100μM, 16 h), stable transfected with an ubiquitin-GFP fusion protein, caused accumulation of GFP, indicating an inhibition of GFP in living cells.

Fig. 4

MG-132 treatment inhibits P-gp function

Incubation of P-gp expression KB 8-5 cells with increasing doses of MG-132 (50, 25, 12.5, 6.25, 0μM) caused a dose-dependent accumulation of daunorubicin, indicating inhibition of P-gp function by MG-132.

Fig. 1


0
50
100
200
VEGF
ß-actin

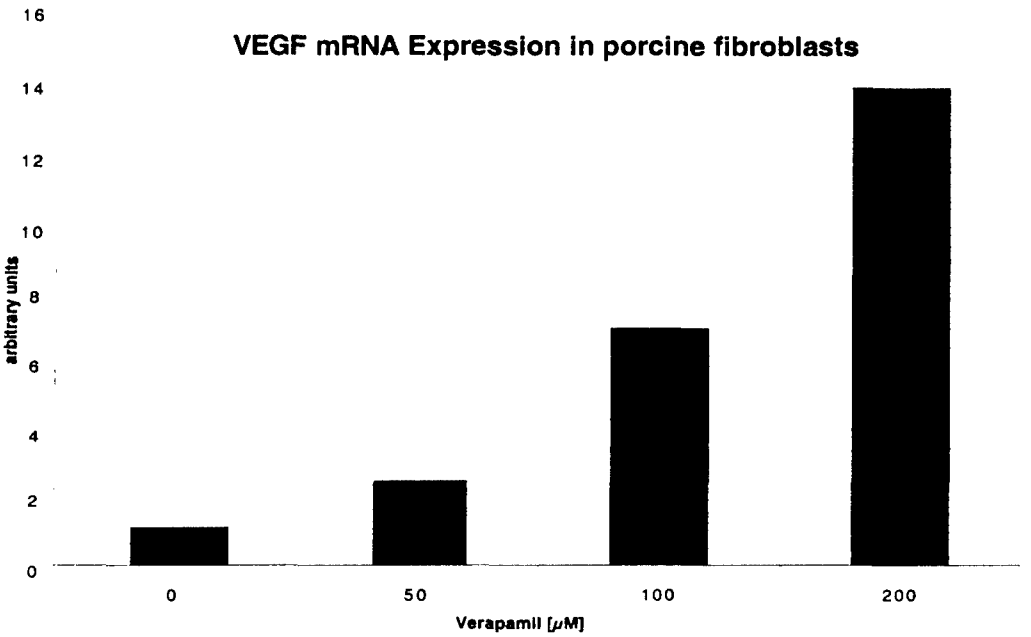

VEGF mRNA Expression in porcine fibroblasts
arbitrary units
16
14
12
10
8
6
4
2
0
0
50
100
200
Verapamil [µM]

Figure 2

Effect of Verapamil on 26 S Proteasome Function (ECV304 cells)

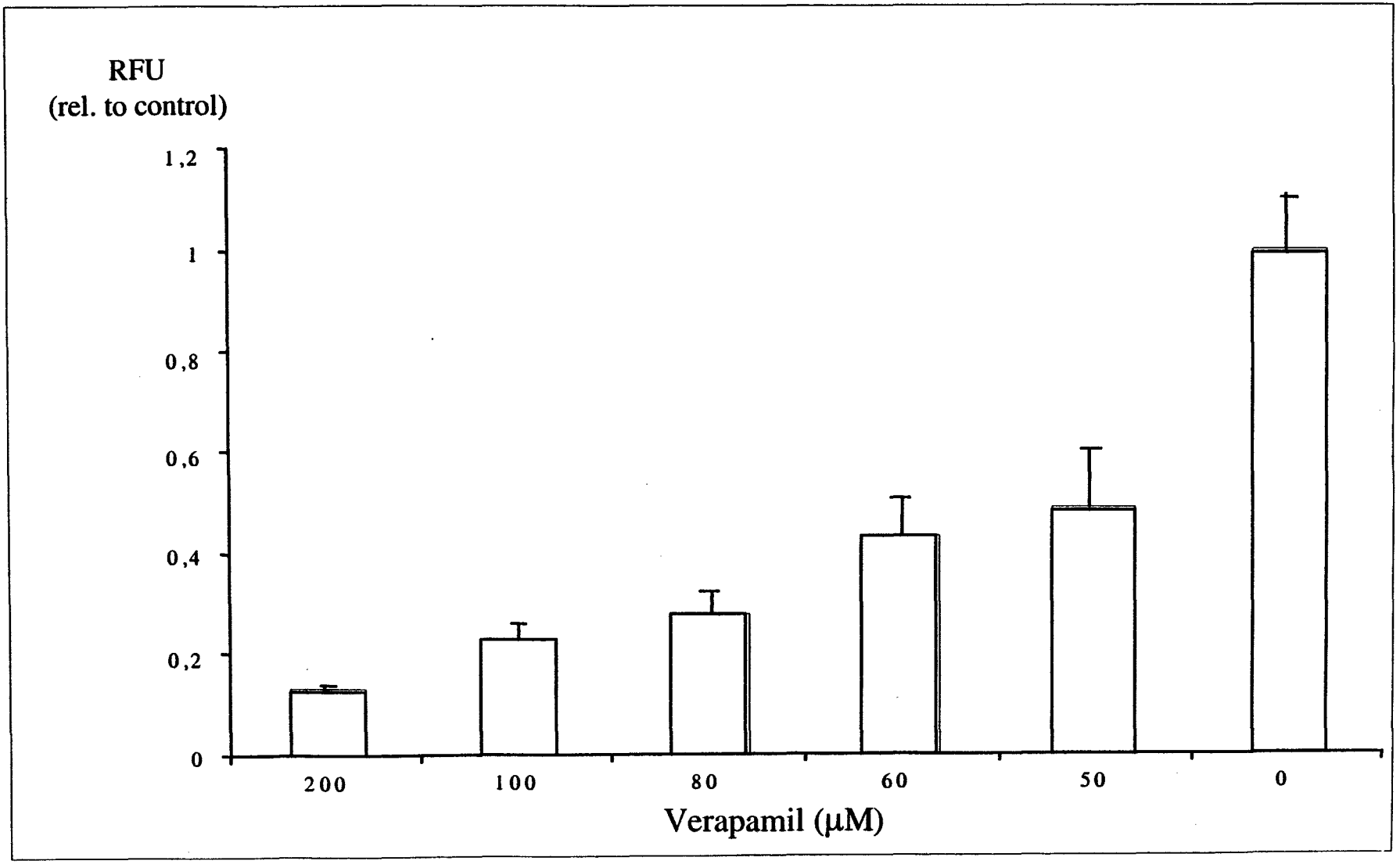
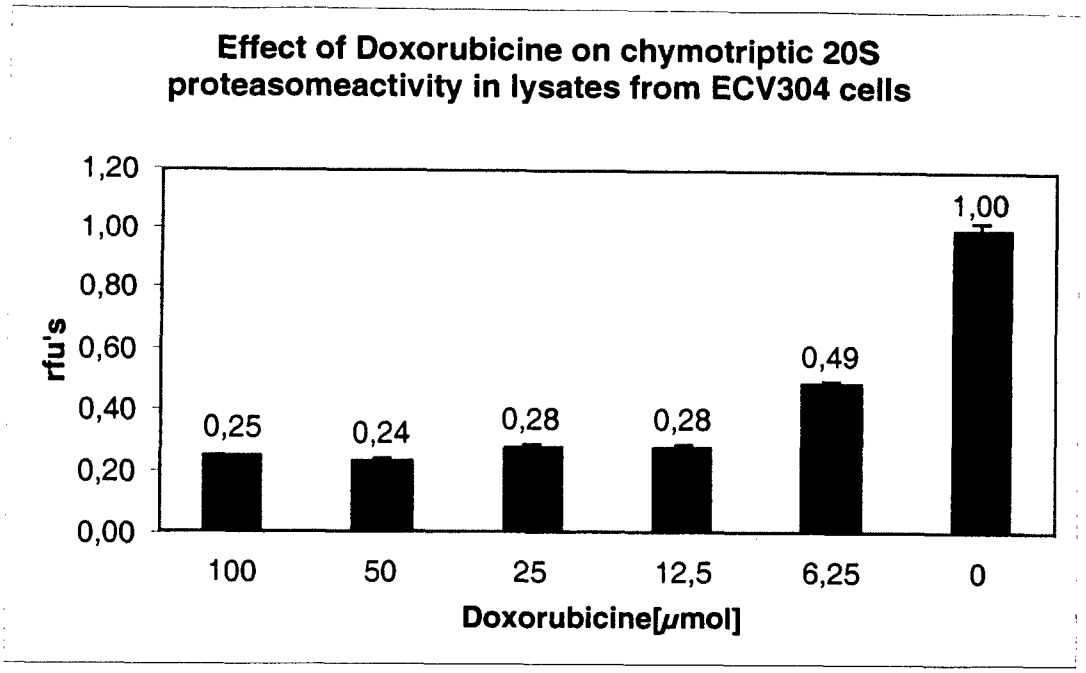
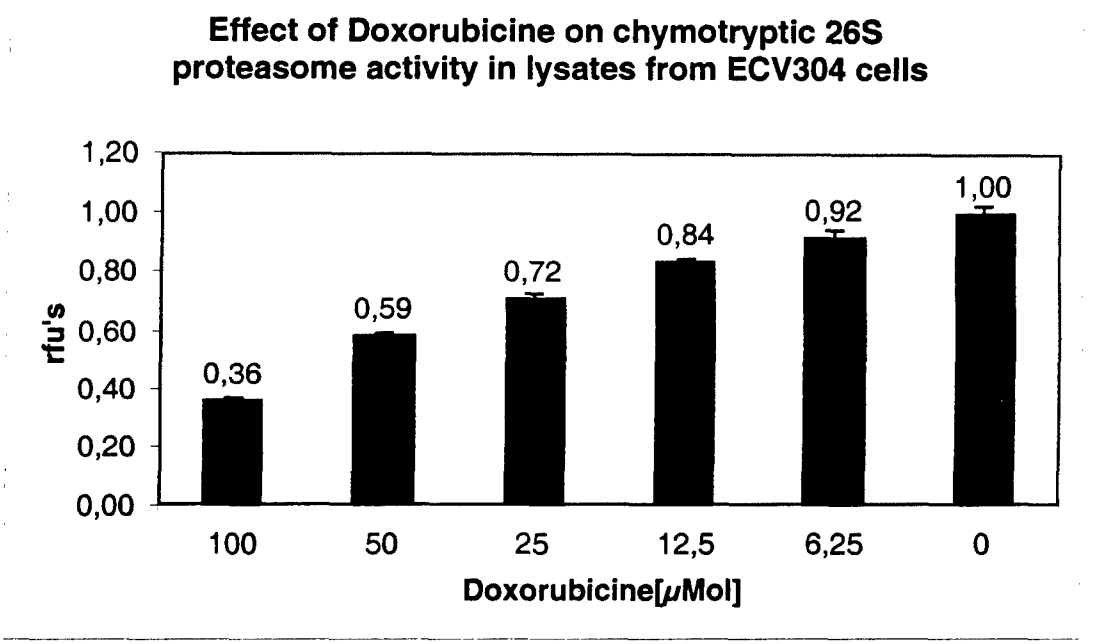


Fig 3

A


Effect of Doxorubicine on chymotriptic 20S proteasomeactivity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,25
0,24
0,28
0,28
0,49
1,00
100
50
25
12,5
6,25
0
Doxorubicine[μmol]

B


Effect of Doxorubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
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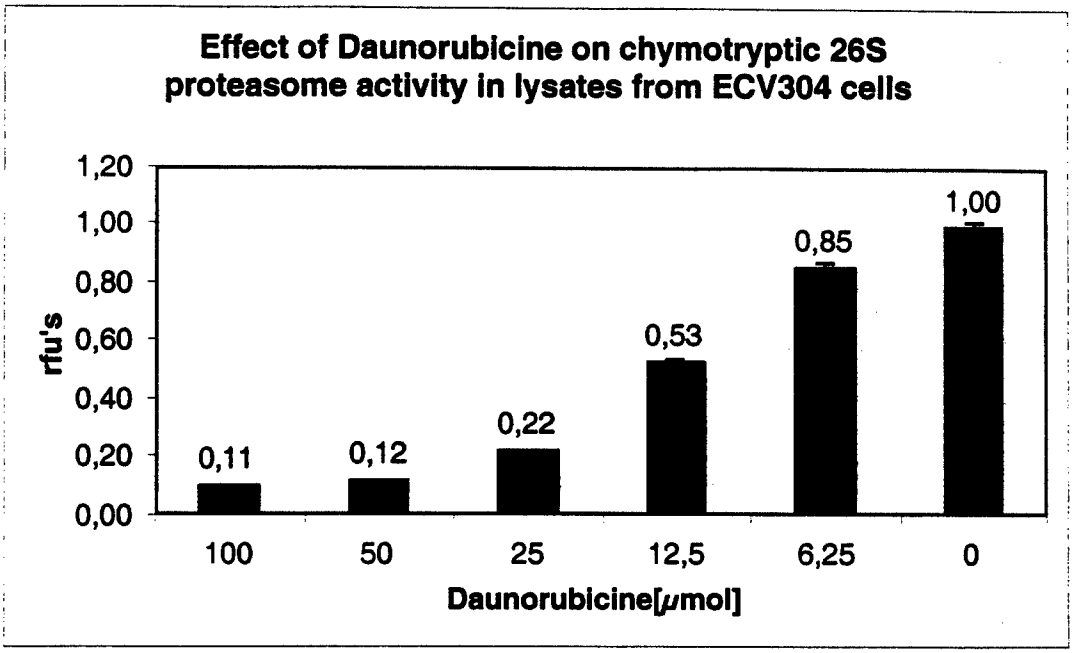
1,00
0,80
0,60
0,40
0,20
0,00
0,36
0,59
0,72
0,84
0,92
1,00
100
50
25
12,5
6,25
0
Doxorubicine[μMol]

Fig. 3

C


Effect of Daunorubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
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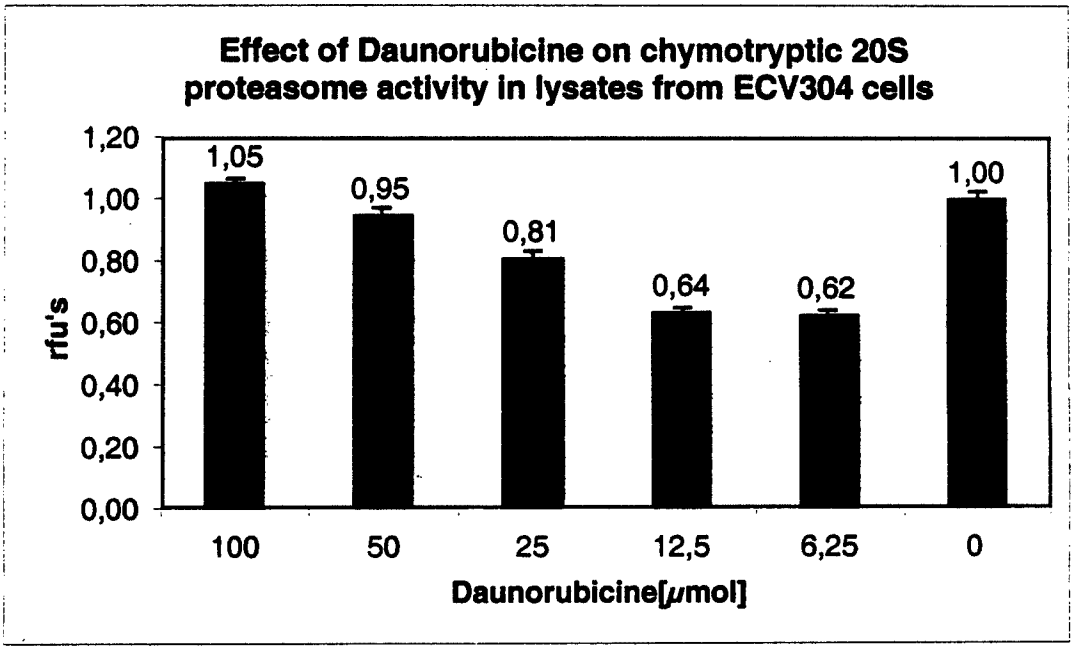
0,60
0,40
0,20
0,00
0,11
0,12
0,22
0,53
0,85
1,00
100
50
25
12,5
6,25
0
Daunorubicine[μmol]

D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rfu's
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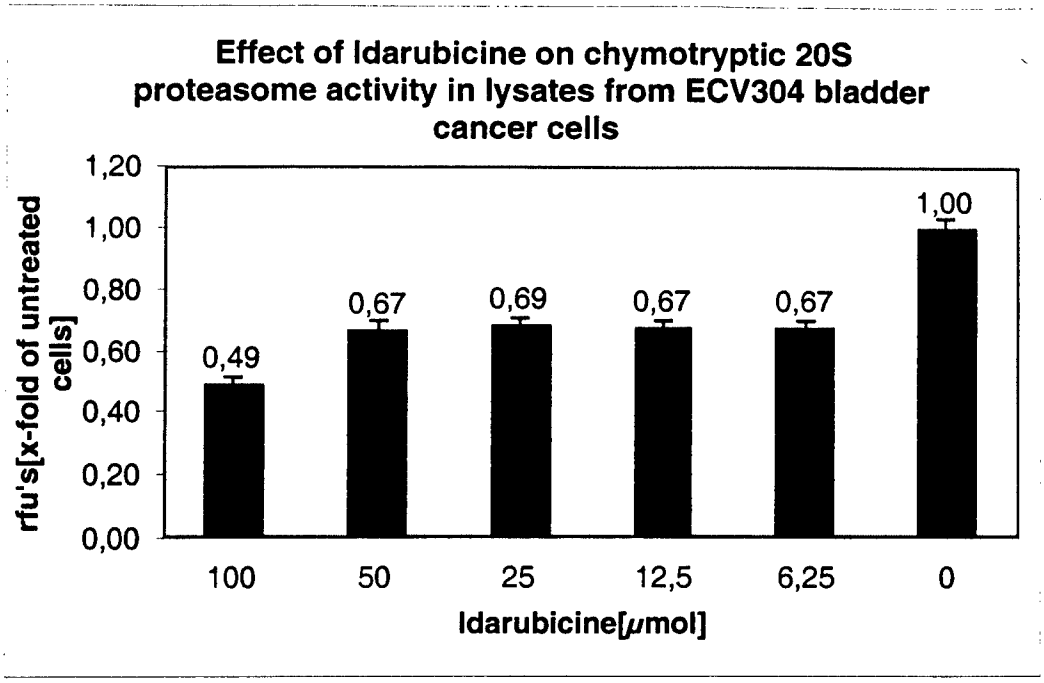
1,00
0,80
0,60
0,40
0,20
0,00
1,05
0,95
0,81
0,64
0,62
1,00
100
50
25
12,5
6,25
0
Daunorubicine[μmol]

Fig. 3

E


Effect of Idarubicine on chymotryptic 20S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's[x-fold of untreated cells]
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,49
0,67
0,69
0,67
0,67
1,00
100
50
25
12,5
6,25
0
Idarubicine[µmol]

F

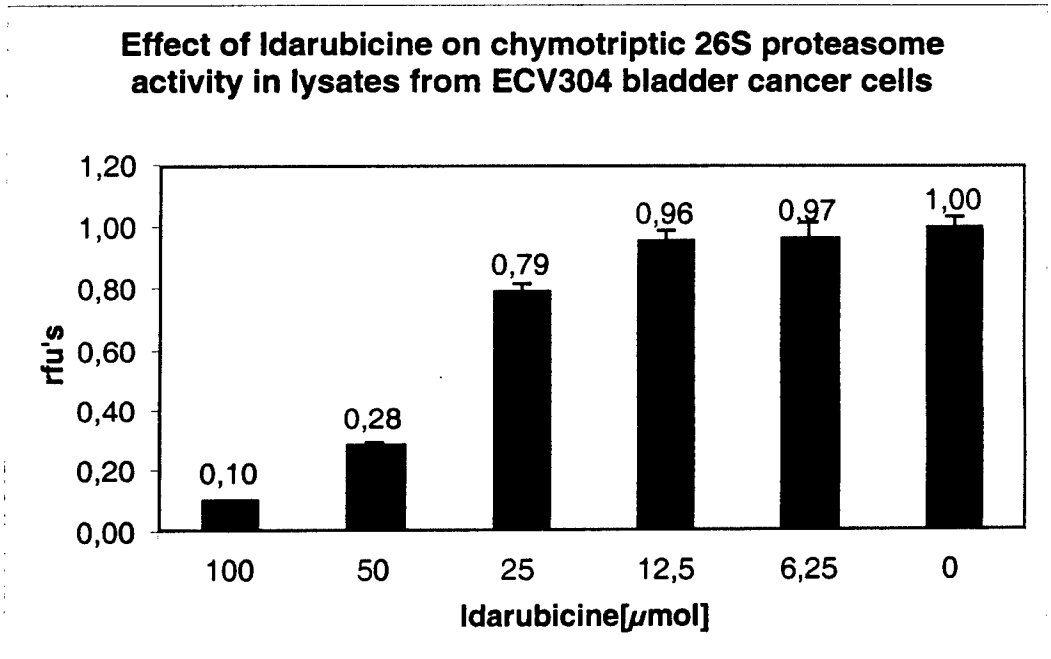
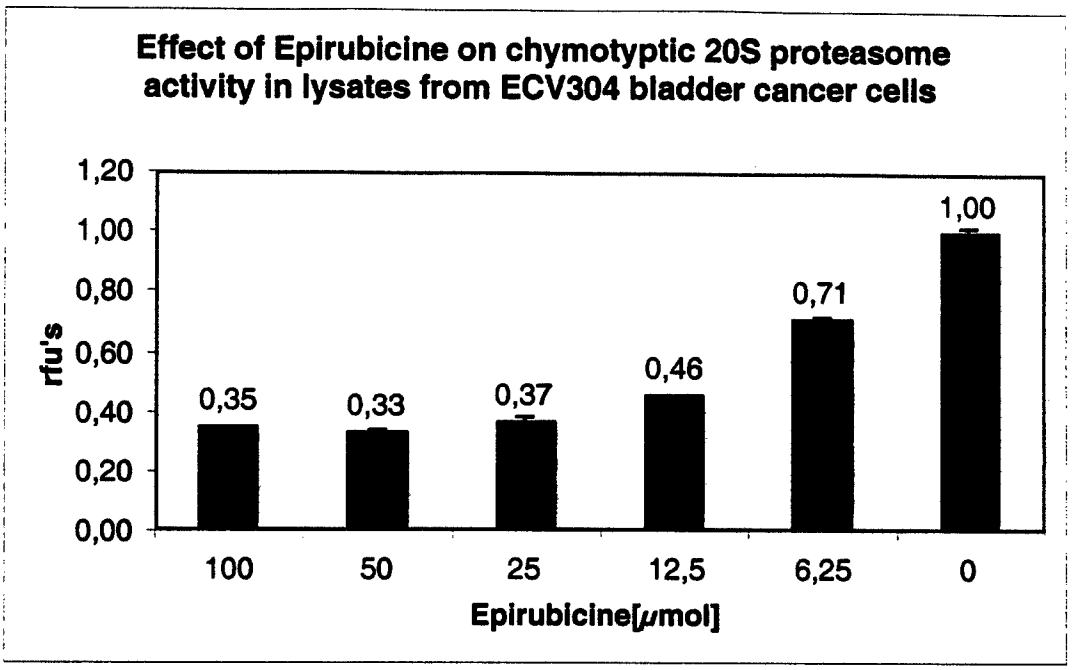

Effect of Idarubicine on chymotriptic 26S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,10
0,28
0,79
0,96
0,97
1,00
100
50
25
12,5
6,25
0
Idarubicine[µmol]

Fig. 3

G


Effect of Epirubicine on chymotyptic 20S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,35
0,33
0,37
0,46
0,71
1,00
100
50
25
12,5
6,25
0
Epirubicine[µmol]

H

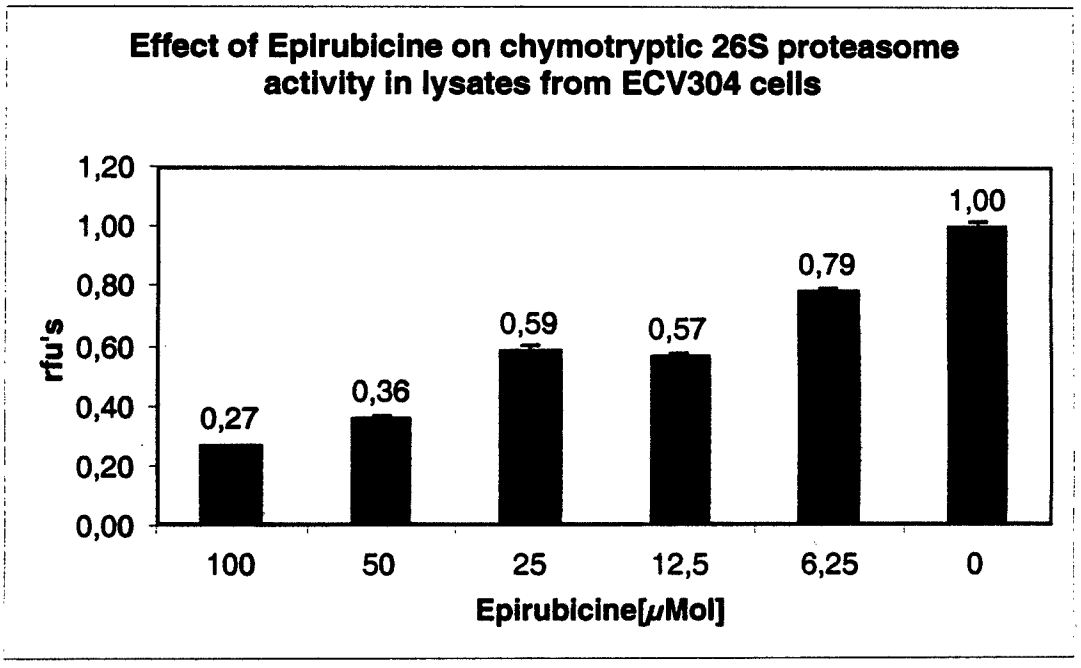
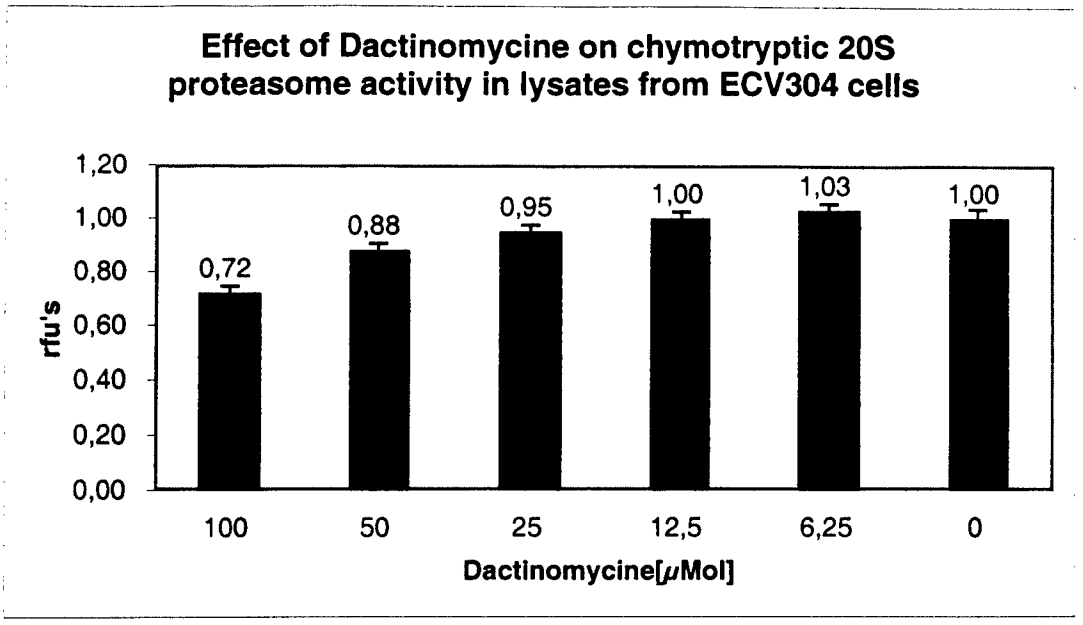

Effect of Epirubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,27
0,36
0,59
0,57
0,79
1,00
100
50
25
12,5
6,25
0
Epirubicine[µMol]

Fig. 3

I


Effect of Dactinomycine on chymotryptic 20S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,72
0,88
0,95
1,00
1,03
1,00
100
50
25
12,5
6,25
0
Dactinomycine[µMol]

J

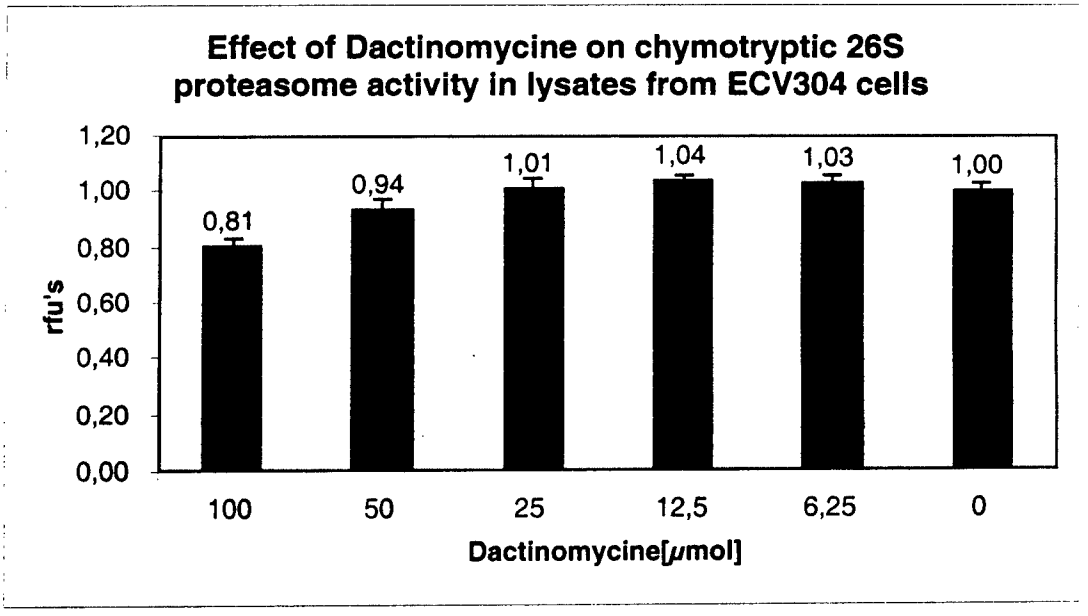
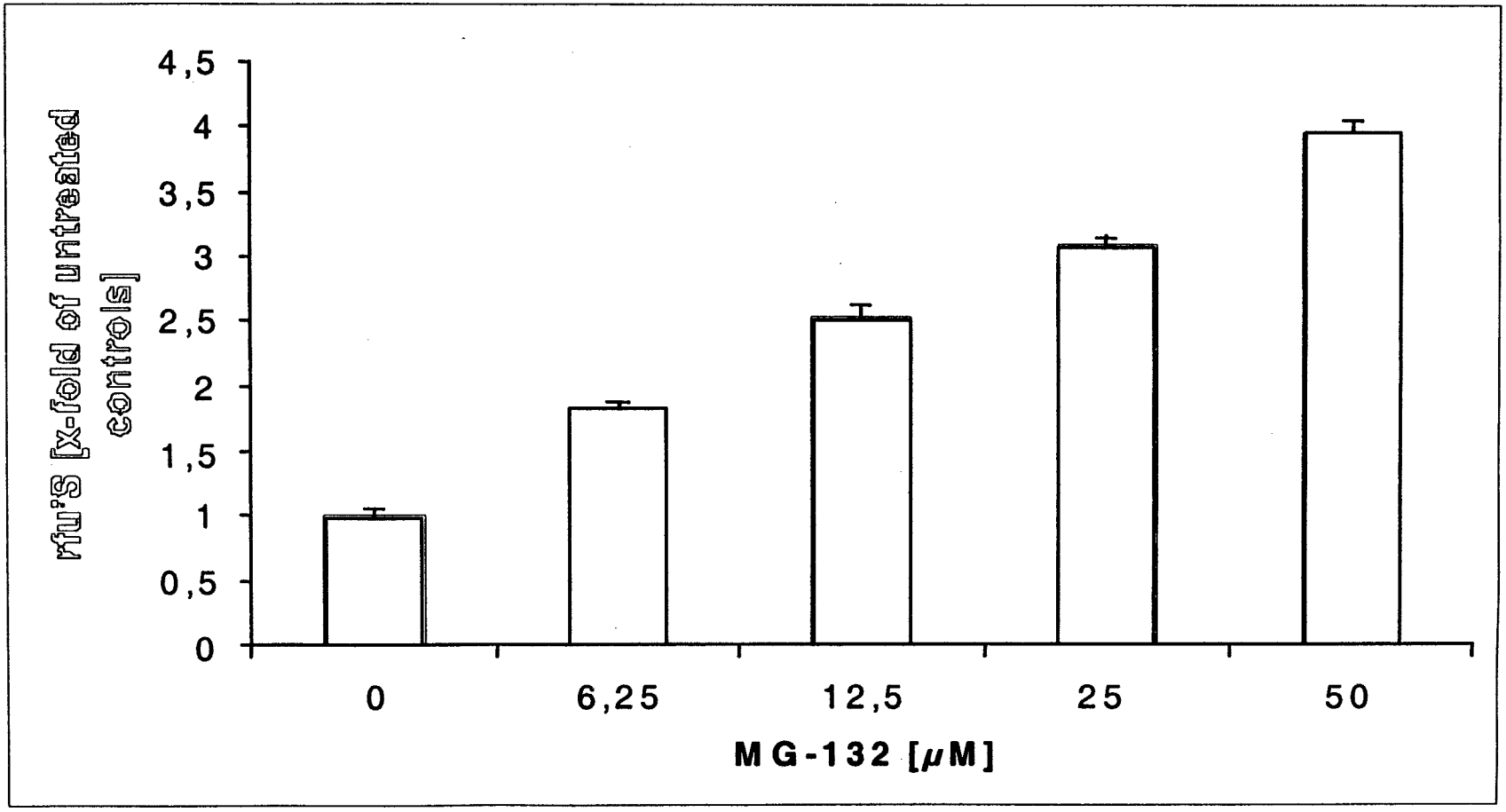

Effect of Dactinomycine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,81
0,94
1,01
1,04
1,03
1,00
100
50
25
12,5
6,25
0
Dactinomycine[µmol]

FIGURE 4

Accumulation of Daunorubicin in KB 8.5 Cells



KB 8.5 cells that overexpress P-gp cause dose dependent accumulation of drug after MG-132 treatment as measured by fluorescence