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Our working hypothesis is that loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistance tumor cells. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research being performed under this award is focusing on the identification of genes whose loss of function results in the proliferation of malignant epithelial cells that are resistant to the cytotoxic action of taxol, a microtubule formation inhibitor that is used in the treatment of breast cancer. This is being performed with a novel system based on a recently described technique that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistance tumor cells will be critical for the future design of novel therapeutic strategies restoring chemo-sensitivity to chemo-resistant breast tumor cells.

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INTRODUCTION

Circumventing drug resistance is a critical step to increase the treatment success rate for women with breast cancer. The object of this grant is to improve our understanding of cellular genes whose loss of function results in the emergence of chemo-resistant tumor cells. It is becoming apparent that loss of function of checkpoint molecules regulating cell cycle progression and apoptosis are important in both tumor cell evolution and in the emergence of chemo-resistant malignant cells. Of the relatively small number of checkpoint molecules identified at this time, the best understood is the p53 tumor suppressor. Loss of p53 function through targeted deletion (in the case of the p53 knock-out mouse) renders cells deficient in their ability to undergo cell cycle apoptosis upon exposure to DNA damaging agents (1,2). Consistent with these in vitro observations, patients with hematological malignancies (3) and cancers of the stomach (4), lung (5) and breast (6) are less likely to respond successfully to chemotherapeutic agents as compared to patients with wild type p53 alleles. It is however obvious that p53 gene alterations do not explain all cases of chemo-resistance in human malignancies, as well as in breast cancer patients. Also, there is in vitro evidence which shows that breast tumor cells can become resistant to the action of chemotherapeutic drugs in the absence of p53 gene mutations (7).

Our working hypothesis is that loss of function of a number of diverse checkpoint molecules will lead to the proliferation of chemo-resistance tumor cells. Besides the p53 pathway, relatively little is known regarding other checkpoint molecules whose loss of function results in chemo-resistance. This is probably due to the fact that loss of function of these molecules occurs through very subtle genetic alterations which are not easily detected by any current molecular techniques. Research being performed under this award is focusing on the identification of genes whose loss of function results in the proliferation of malignant epithelial cells that are resistant to the cytotoxic action of taxol, a microtubule formation inhibitor that is used in the treatment of breast cancer. This is being performed with a novel system based on a recently described technique (8) that allows for the isolation of genes encoding selectable recessive phenotypes. Identifying molecular pathways that are altered in chemo-resistance tumor cells will be critical for the future design of novel therapeutic strategies restoring chemo-sensitivity to chemo-resistant breast tumor cells.

BODY

Overview of the in vitro knock-out system.

An in vitro knock-out approach is being developed to identify genes that are important for mediating the cytotoxic action of specific chemotherapeutic drugs. The strategy employed (see figure 1) is a modified version of one that was used to identify cellular genes whose loss of function results in the transformation of immortalized NIH3T3 cells (8). This approach relies on a gene search insert and a cell line that contains high levels of the muristerone transcription factor, VgRXR. The gene search insert encodes a G418 resistance/B-galactosidase fusion protein. The fusion cDNA is downstream of a functional splice acceptor (SA) site, but is not downstream of a functional promoter. Therefore, expression of the fusion product is dependent on the integration of the gene search insert into transcribed sequences supplying a splice donor site. The first step in this process is to transfect the gene search insert into a target cell line containing high levels of the muristerone transcription factor, VgRXR. After transfection, cells are cultured in the presence of G418 to select for those cells harboring integration of the gene search insert into expressed cellular sequences. After two weeks of selection, those cells that are G418 resistant are pooled and expanded. Once a library of knock out cells have been generated, $1x10^7$ cells derived from at least 5,000 G418 resistant clones are then exposed to muristerone. Muristerone is an inducer of the VgRXR transcription factor. Treatment of cells with this hormone will induce antisense transcripts complementary to sequences upstream of the gene search vector and in theory, should reduce the expression of protein encoded by the other allele. Twenty-four hours later, these muristerone treated cells will be exposed to a specific concentration of the chemotherapeutic drug taxol that kills 99.999999% of the parental target cell line. Once drug resistant colonies have been isolated and expanded, the subsequent series of experiments will verify that the acquisition of drug resistance is mediated by the antisense transcript produced by the gene search vector, and not by spontaneous acquisition of drug resistance or by direct insertional inactivation by the transfected gene search construct. This will be performed by comparing the sensitivity of cells from the resistant colonies to drug -/+ muristerone. Those cells that remain resistant to the cytotoxic action of these drugs in the presence of muristerone, but not in its absence, will have likely acquired drug resistance through the antisense knock-out procedure. Standard recombinant DNA techniques will then be used to identify the specific cDNA whose loss of function results in the clonal proliferation of chemo-resistant cells. Finally, the isolated cDNA will be subcloned into a eukaryotic expression vector and transfected into chemo-resistant epithelial cells. Those cDNAs that reverse the drug resistance phenotype of the cells will be sequenced and characterized by data base searches.

Summary of work performed in the first and second year of this award.

We were able to meet all technical objectives in the first year of this award (see previous progress report). Specifically, we were able to generate an inducible line (9-8 cells; a derivative of the human epithelial H1299 tumor cell line) and knock out a relatively high number of expressed genes in this line (technical objectives #1 to #3 of the original proposal). The H1299 line was used for these experiments since it can be transfected at very high efficiencies and is derived from the same cell type that can give rise to breast tumors. The main objectives of work performed in the second year of this award was to perform drug survival assays with taxol (see technical objective #4 of the statement of work in the original proposal) and to identify those taxol resistant clones that are less sensitive to taxol in the presence of muristerone than in its absence (see technical objective #5 of the statement of work in the original proposal). Presented in this annual report is the description of experiments relating to the completion of both of these technical objectives.

Technical objective #4: Optimization of drug survival assays and the selection of drug resistant cells harboring the gene search and transactivating vectors: Cells derived from the knock-out library were seeded into 150 x 25 mm dishes and cultured in the presence of zeocin (100 ug/ml), G418 (200 ug/ml) and puromycin (0.5 ug/ml). When cells reached approximately 90% confluency, they were treated with varying concentrations of taxol for 2 hours. After treatment, cells were washed in phosphate-buffered saline (PBS) and re-fed with complete media containing the same concentrations of zeocin, G418 and puromycin. Cells were washed and re-fed every other day for two weeks. After two weeks, the number of taxol resistant colonies were counted (see Table 1). Based on the numbers obtained, we chose the minimal concentration of taxol used (1 ug/ml) that gave a minimum background of resistant colonies to try to identify those cells in the knock-out library that are resistant to the cytotoxic action of this drug in the presence of the functional transactivator.

To identify these cells, cells derived from the knock-out library were seeded into 10 100 x 15 mm dishes and cultured in the presence of zeocin, G418 and puromycin. When cells reached approximately 50% confluency, they were transfected by the calcium phosphate methods with the pVgRXR expression. This was performed to insure maximal expression of the muristerone inducible transactivator. After transfection, cells were seed into 10 150 X 25 mm dishes and cultured in the presence of zeocin, G418 and puromycin for one day. Cells were then pre-treated for 2 hours with 20 uM muristerone, then treated with 1ug/ml of taxol for two-hours in the presence of 20 uM muristerone. Muristerone is used as a hormone to activate the transactivator and increase the levels of antisense RNA that is complementary to sequences where the gene search insert the has integrated. This should reduce the expression of protein encoded by both alleles of the gene containing the B-gal/G418 fusion. After taxol treatment, cells were washed in PBS, re-fed with complete media containing muristerone, zeocin, G418 and puromycin and incubated overnight at 37°C. After the overnight incubation, cells were washed and re-fed with media containing only zeocin, G418 and puromycin. Cells were washed and re-fed with this media every other day for two weeks. After two weeks, 20 colonies were identified to be resistant to taxol in muristerone co-treated cells.

Technical objective #5: To identify transactivator mediated chemo-resistant tumor cells (i.e. those that are more resistant to taxol in the presence of muristerone than in its absence): After completion of technical objective #4, the next goal was to identify those colonies whose resistance to taxol is dependent on the presence of the functional transactivator (i.e. more resistant to taxol in the presence of muristerone than in its absence). Individual colonies were first isolated using cloning cylinders and trypsin and seeded into T75 flasks. Cells were cultured in the presence of zeocin, G418 and puromycin until they reached 50% confluency (this took approximately 1 month). After cells reached 50% confluency, cells were trypsinized and viable frozen cells were prepared from half of the cells while the other half was re-seeded into 100 X 15 mm dishes. Cells were again transfected with the pVgRXR expression construct by the calcium phosphate method. After transfection, cells were split into two dishes. The next day, one of these dishes was treated with muristerone and taxol as described above. The other dish was treated in a similar manner, except that muristerone was not included in the media. Two weeks later, the overall viability of cells were determined in all of the 20 clones -/+ muristerone treatment. The majority of clones were very sensitive to taxol, regardless of whether they were treated with muristerone (see Table 2). There were two clones that were relatively resistant to taxol (clones 12 and 16), but again there were no difference in the sensitivity of these clones -/+ muristerone treatment. We were able to identify two clones (clones 13 and 17) that were much more resistant to the action of taxol in the presence of muristerone than in its absence. These clones may contain an antisense mediated knock-out of a protein that is important in mediating sensitivity to taxol. In the absence of muristerone, this protein is presumably expressed at high enough levels to block tumor cell proliferation in the presence of taxol. However, when the expression of this protein is blocked via

muristerone-induced antisense RNA production, the cells are relatively resistant to this chemotherapeutic drug. Our goal of work in year 3 of this award is to determine the identify of this protein in cell clones 13 and 17 and determine if these proteins can restore taxol sensitivity when overexpressed in the corresponding clones.

Progress in relation to the statement of work proposed in the initial application.

We have completed all technical objectives outlined in the initial proposal for years 1 and 2 of this award. We are now ready to determine the identify of cDNAs in clones 13 and 17 that are linked to the B-gal/G418^r fusion and determine if any identified cDNAs alter taxol sensitivity.

RESEARCH ACCOMPLISHMENTS

1) Generation of an inducible cell line.

2) Generation of a knock-out cell library in this inducible line.

3) Identification of clones derived from this knock-out cell library that are resistant to taxol in the presence of muristerone, but not in its absence.

REPORTABLE OUTCOMES

Manuscripts/Abstracts: In the course of these studies, we noticed that the half-life of the MDM2 onco-protein was longer in the 9-8 cells than what has been reported for MDM2 in other non-transformed human cell lines. This result prompted us to determine if there is any evidence for the de-regulation of MDM2 degradation in human tumor cells. Because we have a relatively large bank of human leukemic cell lines where we have already characterized p53 and mdm2 expression status, we have looked first for alterations in these types of tumor cells. Interestingly, we have found for the first time that the pathway regulating MDM2 protein degradation can be altered in human tumor cells. We are currently investigating the frequency by which this pathway is altered in human breast cancer cell lines. This work has led to a publication in Cancer Research (manuscript included in appendix) and was presented at the American Association for Cancer Research Meeting in Philadelphia.

Development of cell lines: We have generated a very good inducible cell line and a knock-out cell library that we will make available to other investigators upon request after we have published a manuscript that describes these items.

Employment opportunities: The P.I.was awarded a grant to pursue these studies while working as an Assistant Professor at Allegheny University of the Health Sciences. After the declaration of bankruptcy at Allegheny, it became very difficult to perform research at this Institution. Because of the amount of funding that had been obtained by the P.I. (including for this project), the P.I. was able to move his laboratory to a more respected Institution that possesses far better resources. Our move to the Fels Institute for Cancer Research and Molecular Biology at Temple University would probably not have been possible without the support of the US army and should enable the P.I. to become a more productive tumor biologist in the future.

CONCLUSIONS

We have made substantial progress in the first and second year of this proposal in developing a novel in vitro knock-out approach that will allow us to identify genes that mediate chemo-sensitivity to chemotherapeutic drugs in human epithelial cells. Although this project is high risk, it has enormous potential to identify factors that are important in mediating the cytotoxic action of chemotherapeutic drugs. If successful, this project will lead to long term studies that will try to dissect the function of identified proteins and work that will examine the possibility that these factors may be altered in chemo-resistant mammary tumors. These studies will hopefully lead to novel therapeutic strategies that will restore chemo-sensitivity to chemo-resistant tumor cells in women with breast cancer.

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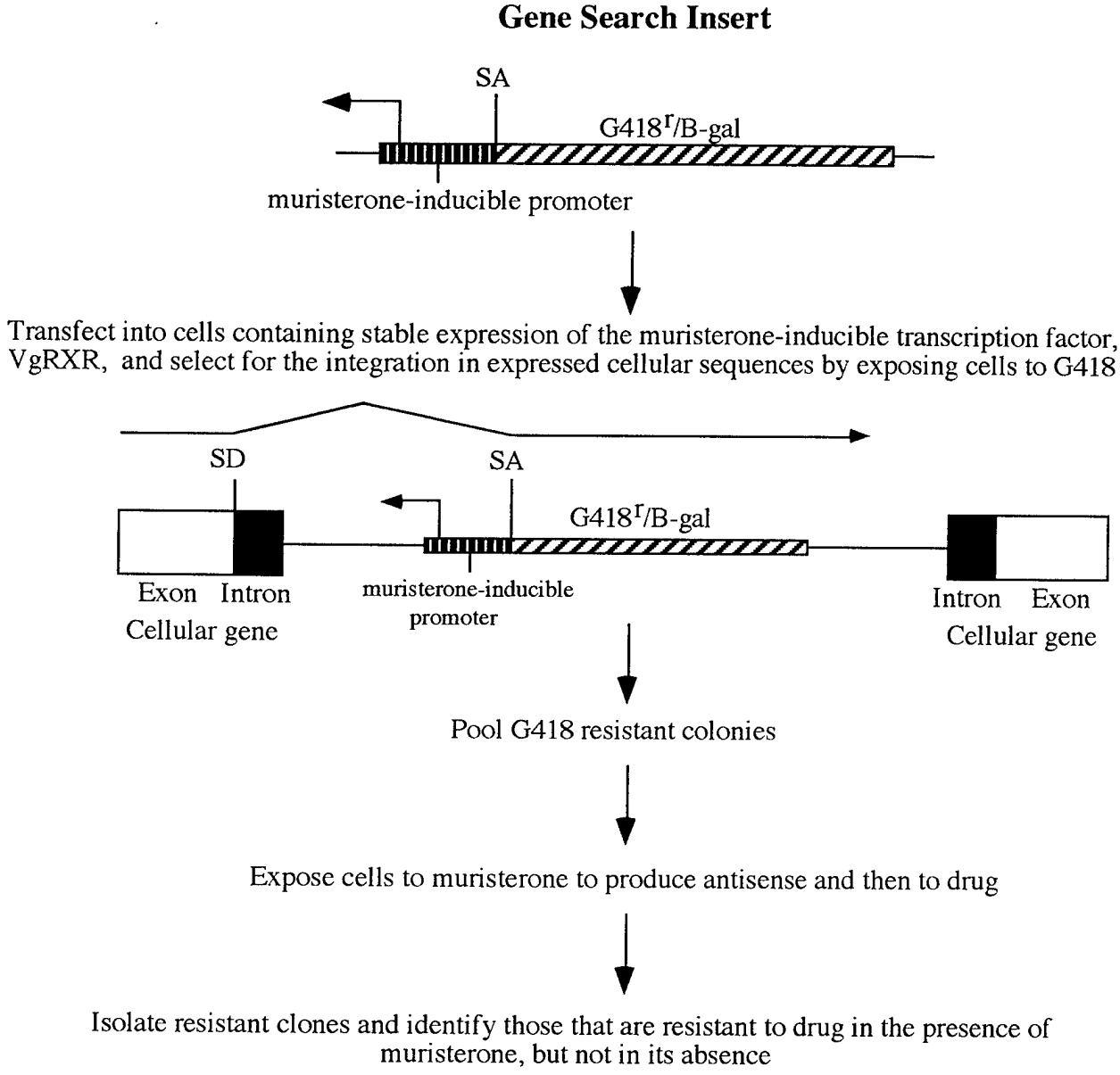
APPENDICES

Figure 1. Generation of an in vitro cell knock-out library



Legend. Method for using a random knock-out procedure to identify genes that mediate the cytotoxic action of chemotherapeutic drugs.

Table 1. Sensitivity of cells from the knock-out library to taxol

Taxol Concentration (ug/ml)	# colonies
30	2
10	1
3.0	2
1.0	3
0.3	>100

Legend. 9-8 cells were treated with varying concentrations of taxol for 2 hours. After treatment, cells were washed and re-fed every other day with media containing zeocin, G418 and puromycin. The number of colonies were then determined two weeks later.

Table 2. Sensitivity of cells derived from the 20 independent clones to taxol -/+ muristerone treatment.

	# of colonies two weeks after taxol exposure	
Clone #	**+ muristerone**	**- muristerone**
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	>500	> 500
13	350	50
14	1	5
15	0	0
16	>500	>500
17	130	22
18	0	0
19	0	0
20	0	0

Legend. Taxol resistant clones were transfected with the pVgRXR expression plasmid and seeded into two dishes as described in the Body Section of this report. One of these dishes was treated with taxol and muristerone and one was treated with taxol alone. Two weeks later, the number of taxol resistant colonies were determined.

[CANCER RESEARCH 59, 2064–2067, May 1, 1999]

Advances in Brief

The Pathway Regulating MDM2 Protein Degradation Can Be Altered in Human Leukemic Cells[1]

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Abstract

The MDM2 protein regulates the functional activity of the p53 tumor suppressor through direct physical association. Signals that control MDM2 expression are poorly understood but are likely to play an important role in the regulation of p53 activity. We show here that the half-life of MDM2 protein is shorter in proliferating than in quiescent peripheral blood mononuclear cells. We also demonstrate that MDM2 protein half-life is extended in some, but not all, p53 mutant human leukemic cell lines. In at least one of these p53 mutant lines, increased MDM2 protein stability is associated with higher amounts of MDM2 protein. Moreover, we demonstrate that MDM2 protein accumulates to a much greater extent in proteasome inhibitor-treated cells containing unstable MDM2 than in cells possessing stable MDM2. These results demonstrate that MDM2 expression is regulated by events that control the stability of the protein and suggest that the normal regulation of MDM2 turnover can be altered in tumor cell lines.

Introduction

The transforming potential of the MDM2 oncoprotein is activated by overexpression (1, 2), and high levels of MDM2 protein are present in some human tumor cells (3). The primary mechanism by which MDM2 overexpression is thought to induce cellular transformation is through its ability to bind to the p53 tumor suppressor and block p53 activity (4). MDM2 can inhibit p53 activity by binding to the acidic activation domain of p53 (5) and by targeting p53 for degradation via the proteasome (6, 7). Because the transforming activity of MDM2 has been attributed to the overproduction of protein (1, 2), it is important to understand the mechanisms that regulate MDM2 protein expression and how these mechanisms may be altered in human tumor cells. It is well established that p53 is itself a key regulator of *MDM2* transcription (8–11). Besides those signals that induce p53 transcriptional activity (*e.g.*, DNA damage), very little is known about other cellular signals that may regulate MDM2 protein expression. In the work presented here, we have investigated how MDM2 protein stability may be differentially regulated in quiescent and growth stimulated human PBMCs.[4] We have also examined the possibility that the regulation of MDM2 protein stability may be altered in human leukemic cell lines.

Materials and Methods

Cell Culture. All lines were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Inc.). The M8166 (T-cell) line possesses wt *p53* alleles, whereas the CEM (T-cell), HEL (erythroleukemia), Jurkat (T-cell), MOLT4 (T-cell), and Raji (B-cell) lines possess mutant *p53* alleles (12). PBMCs were isolated with Ficoll-Paque (Pharmacia) according to the manufacturer's instructions. Quiescent PBMCs were cultured in complete RPMI 1640 and used for studies immediately after isolation from normal donors. To generate a population of actively proliferating T cells, quiescent PBMCs were seeded at 2×10^6 cells/ml in complete RPMI 1640 supplemented with 1% PHA-M (Life Technologies, Inc.). After 3 days, recombinant IL-2 (Sigma) was added into the media to a final concentration of 1 unit/ml. Cells were counted every other day and maintained at 1×10^6 cells/ml in IL-2-containing media. Once cells possessed a doubling time of ~48 h, they were used for the appropriate studies.

Cycloheximide and MG115 Treatment of Cells. Cells were seeded at 2×10^6 cells/ml in their respective culture medium containing 75 μg/ml of cycloheximide or 10 μM MG115. Cells were harvested at different time points after cycloheximide treatment and pelleted by centrifugation. For Western analysis, cell pellets were stored at −80°C prior to extraction of protein.

Protein Extraction and Western Analysis. Cells were lysed in TENN buffer [50 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 0.5% NP40, and 150 mM NaCl] supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml soy trypsin inhibitor, and 1 μg/ml pepstatin A. Lysates were clarified by centrifugation, and the protein concentration was determined by the Bradford method (Bio-Rad). Samples were then mixed with 4× SDS-PAGE sample loading buffer, boiled, separated on SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes. Blots were probed with 0.5 μg/ml p53 (DO-1; Calbiochem), 3 μg/ml MDM2 (IF2; Calbiochem), and actin (0.1 μg/ml, Sigma) antibodies as described previously (12). Signals were visualized by using horseradish peroxidase-conjugated antibodies (sheep anti-mouse for MDM2 and p53, Amersham; goat-anti rabbit for actin, Boehringer Mannheim) and enhanced chemiluminescence (DuPont NEN).

RNA Isolation and RNase Protection. Total RNA was isolated using RNazol B (Tel-Test, Inc.) as per manufacturer's instructions. RNase protection using an MDM2 probe that measures the levels of MDM2-P1 and MDM2-P2 transcripts was performed as described previously (12), except that 7.5 μg of total RNA were used in hybridization reactions.

Results

MDM2 and p53 Half-Life Analysis in Quiescent and Growth-stimulated PBMCs. To determine whether the stability of MDM2 protein may be differentially regulated in quiescent *versus* proliferating cells, the half-life of MDM2 protein was measured in both untreated and PHA/IL-2-treated PBMCs. Freshly isolated PBMCs from normal individuals are comprised predominantly of quiescent T cells (~70%), whereas the treatment of PBMCs with PHA and the culturing of these cells with IL-2 will generate a relatively pure population of cycling T cells. MDM2 protein half-life was measured by determining the level of protein at various time points after treatment with the protein synthesis inhibitor cycloheximide. Interestingly, Fig. 1*a* shows that the level of MDM2 protein declined much more rapidly in cycloheximide-treated, growth-stimulated cells than in unstimulated cells. The half-life of MDM2 in growth-stimulated

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[4] The abbreviations used are: PBMC, peripheral blood mononuclear cell; wt, wild type; PHA, phytohemagglutin; IL, interleukin.

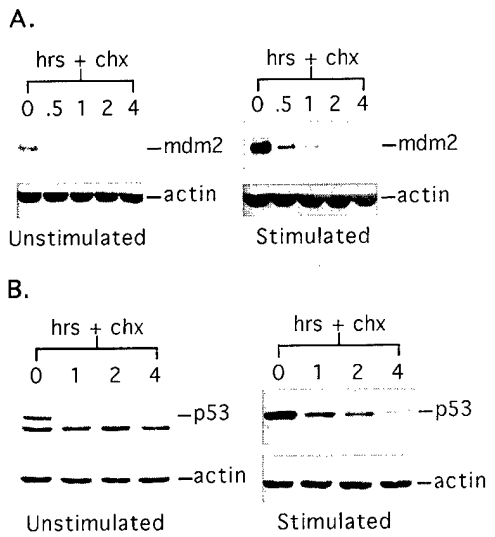

Fig. 1. MDM2 (*A*) and p53 (*B*) half-life in untreated and growth-stimulated PBMCs. PBMCs were obtained from healthy normal individuals and isolated by Ficoll gradients. To generate an active cycling lymphocyte population, PBMCs were stimulated with PHA and then grown in the presence of IL-2 for at least a week until the cells possessed a doubling time of ~48 h. Then, freshly isolated PBMCs and stimulated PBMCs were treated with the protein synthesis inhibitor cycloheximide (at 75 μg/ml) for the indicated period of time. Cells were harvested, extracts were prepared, and the level of either MDM2 (*A*) or p53 (*B*) protein was measured by Western blot analysis as described in "Materials and Methods." Blots were also probed with polyclonal antibodies against actin to show equivalent loading of protein.

cells measured by the method used here (<30 min) is consistent with previous studies that have used radioactive protein labeling methods to measure MDM2 half-life in cell lines (2). Because cycloheximide may alter the expression of other factors that may regulate MDM2 protein stability, we did attempt to measure MDM2 protein stability by radiolabeling and immunoprecipitation as well. Although we were able to measure MDM2 stability in cycling cells by pulse-chase experiments (the half-life of MDM2 protein was found to be very similar to that measured with cycloheximide in these cells), we could not detect MDM2 protein in quiescent cells after radiolabeling and immunoprecipitation (data not shown). This is most likely due to a very low rate of MDM2 synthesis in quiescent cells (12). Therefore, we cannot rule out the possibility at this time that MDM2 half-life is also short in quiescent cells and that cycloheximide is altering the expression of factors that may regulate MDM2 stability in these cells.

MDM2 can regulate the stability of the p53 tumor suppressor through direct physical association (6, 7). To determine whether growth stimulation also modulates p53 protein stability, p53 half-life analysis was performed on untreated and PHA/IL-2-treated PBMCs. Fig. 1*b* shows that there is not a dramatic difference in the half-life of p53 protein measured in stimulated and unstimulated cells. In fact, p53 stability in cycling T cells may even be slightly longer than that measured in quiescent PBMCs. This result significantly reduces the concern that the difference in MDM2 half-life measured in cycling *versus* quiescent PBMCs using cycloheximide is due to a difference in the ability of cycloheximide to block protein synthesis in these two cell populations. It also suggests that the rate of p53 protein degradation is not directly coupled to the rate of MDM2 turnover.

The Half-Life of MDM2 Protein Is Extended in Human Leukemic Cells. The results presented above suggest that MDM2 expression can be regulated by mechanisms controlling MDM2 protein stability. Because MDM2 has oncogenic properties when overexpressed and MDM2 protein levels have been shown to be elevated in a number of different cancers in the absence of increased RNA levels (including in hematological malignancies; Ref. 13), we investigated the possibility that alterations in the regulation of MDM2 protein stability may contribute to high levels of MDM2 protein in human tumor cells. We first performed MDM2 Western blot analysis on a series of leukemic cell lines. Although no lines expressed a high level of MDM2 protein (>10-fold above the level of MDM2 protein measured in PHA/IL-2-stimulated PBMCs), we were surprised to find similar amounts of MDM2 protein in wt and mutant p53-containing lines and that the p53 mutant Jurkat line expressed the highest amount of MDM2 protein (Fig. 2*a*). This line expressed six times more MDM2 protein (as determined by densitometric analysis) than that measured in PHA/IL-2-stimulated PBMCs.

The expression profile of MDM2 in leukemic lines was not expected, considering that the majority of these lines possess mutant p53 and should possess low amounts of MDM2-P2 transcripts (12); p53-regulated transcripts that have been shown to be translated at high efficiencies *in vitro* and in cells in culture (14, 15). To verify the absence of these transcripts in mutant p53 lines, RNase protection analysis was performed. As expected, those lines with mutant p53 did not possess MDM2-P2 transcripts (Fig. 2*b*). Fig. 2*b* also shows that all lines possessed similar amounts of MDM2-P1 transcripts; MDM2 transcripts that are regulated by the p53-independent promoter of the *MDM2* gene. These results raise the possibility that MDM2-P2 transcripts are not translated more efficiently in lymphoid cells. However, it is also conceivable that the half-life of MDM2 protein is longer in some of the lines with undetectable amounts of MDM2-P2 transcripts, but expressing higher or similar level of MDM2 protein as wt p53-containing lines. To address this, the half-life of MDM2 protein was measured in several mutant p53 lines. All lines analyzed possessed similar doubling times, and half-life studies were performed with cells in their logarithmic stage of growth. Fig. 3 shows that the half-life of MDM2 protein was clearly longer in the HEL, CEM, Jurkat, and MOLT4 lines than in the Raji line or in PHA/IL-2-stimulated PBMCs. It is unlikely that the difference in MDM2 half-life measured in the lines is do to an unequal inhibition of protein synthesis by cycloheximide because this compound was found to inhibit incorporation of

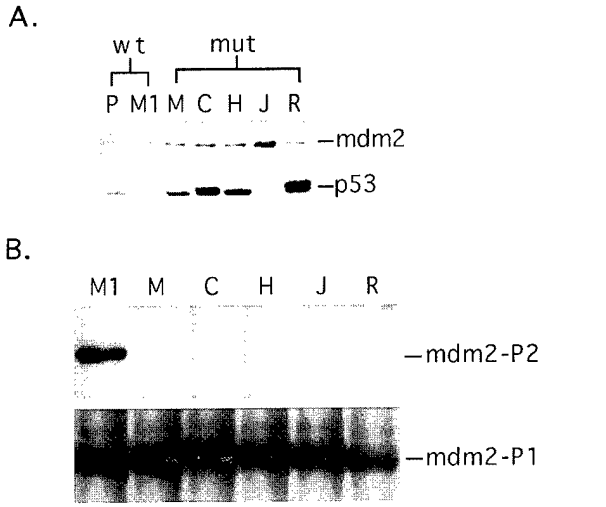

Fig. 2. MDM2 protein and MDM2-P2 transcript levels in human leukemic cell lines. MDM2 and p53 protein levels were measured as described in "Materials and Methods" from the following cell lines: *Lane P,* PHA/IL-2-stimulated PBMCs; *Lane M1,* M8166 T-cell line; *Lane M,* MOLT4 T-cell line; *Lane C,* CEM T-cell line; *Lane H,* HEL erythroleukemia line; *Lane J,* Jurkat T-cell line; and *Lane R,* Raji B-cell line. Ponceau S staining of membranes was performed to verify equivalent loading of protein. High levels of p53 protein were measured in lines possessing missense p53 mutations, whereas undetectable levels of p53 protein were present in the Jurkat line harboring a frameshift p53 mutation. *B,* the levels of MDM2-P1 and MDM2-P2 transcripts was measured in these same lines by RNase protection as described in "Materials and Methods."

Fig. 3. MDM2 protein stability in human leukemic cell lines. Human leukemic cell lines were treated with the protein synthesis inhibitor cycloheximide (at 75 μg/ml) for the indicated period of time. Cells were then harvested, extracts were prepared, and the level of MDM2 protein in these extracts was subsequently measured by Western blot analysis. Abbreviations used are the same as those used in the legend of Fig. 4. Ponceau S staining of membranes was performed to verify equivalent loading of protein.

[^{35}S]methionine to a similar degree in all lines tested (data not shown).

MDM2 protein has been shown to be degraded via the proteasome (16). The longer half-life of MDM2 protein measured in some of these leukemic lines may be attributed to differences in the way MDM2 protein is degraded by the proteasome. To address this hypothesis, the Jurkat (stable MDM2), MOLT4 (stable MDM2), and Raji (unstable MDM2) lines were treated with the proteasome inhibitor MG115 for varying periods of time, and the level of MDM2 protein was measured after treatment. Fig. 4 demonstrates a greater fold increase in MDM2 protein levels in the Raji line (~5-fold at the 2-h time point as determined by densitometric analysis) than in either the Jurkat or MOLT4 cell lines (both displaying a 2-fold induction of protein at the 2-h time point). This result supports those presented in Fig. 3 that show that the half-life of MDM2 protein is longer in the Jurkat and MOLT4 lines than in the Raji line and suggests that the normal regulation of proteasome-mediated degradation of MDM2 protein can be altered in human leukemic cells.

Discussion

p53 and MDM2 expression is regulated at multiple levels by mitogenic signals. Growth stimulation of human lymphocytes has previously been shown to induce an overall increase in both p53 RNA and p53 protein (17, 18). MDM2 RNA (specifically, those transcripts that are regulated by p53) and MDM2 protein have also been shown to be up-regulated in growth-stimulated cells (12). We show here that the stability of MDM2 protein is regulated by growth stimulation signals as well. Interestingly, we show that MDM2 protein has a shorter half-life in growth-stimulated PBMCs than in quiescent PBMCs. This may appear to be inconsistent with an overall increase in the amount of MDM2 protein. However, considering that MDM2 expression can be regulated at multiple levels, the increase in MDM2 protein measured in stimulated cells is probably due to the fact that growth stimulation induces a greater fold increase in MDM2 transcription and/or translation than turnover of MDM2 protein.

What could be the functional significance for the differential regulation of p53 and MDM2 expression in quiescent *versus* proliferating cells? It is probably not a high priority for a nondividing cell to expend energy required for either the synthesis or degradation of proteins involved in controlling damaged-induced cell cycle arrest. However, an actively proliferating cell needs to possess an increased capacity to respond to the potentially harmful effects of cellular damage. One way to increase the capacity for a p53 response is to increase the amount of latent p53. We propose that this is accomplished by events that regulate p53 and MDM2 RNA production (12, 17, 18), as well as MDM2 degradation (see Fig. 1). The various mechanisms controlling p53 and MDM2 expression are likely to play an important role in generating an optimal ratio of MDM2 and p53 proteins that is needed to maintain high levels of latent p53 protein in actively proliferating cells.

It appears that there are multiple mechanisms that can lead to the overproduction of MDM2 protein in human tumor cells, including *MDM2* gene amplification (3) and enhanced translation of MDM2-P2 transcripts (15). Besides presenting data that suggest that MDM2 protein stability is differentially regulated in quiescent *versus* active cycling cells, we also present data that suggest that the normal regulation of MDM2 protein turnover mediated by the proteasome can be altered in human tumor cells. The half-life of MDM2 protein was found to be extended in a number of leukemic cell lines, and MDM2 accumulated to a much lesser degree in proteasome inhibitor-treated cells harboring stable MDM2 than in treated cells possessing unstable MDM2. In at least one of these lines (the Jurkat line), the increased stability of MDM2 protein appears to contribute to a high amount of protein (a 6-fold greater amount of protein than that measured in normal cycling lymphocytes). It is therefore possible that alterations in the regulation of MDM2 turnover via the proteasome may be another mechanism that can contribute to the overproduction of MDM2 protein in human tumor cells. It is, however, unlikely that stabilized MDM2 is sufficient for the inhibition of p53 function in the cell lines analyzed here because the *p53* gene is mutated in all lines possessing MDM2 with a long half-life. Alternatively spliced variants of MDM2 lacking the p53 binding region have been shown to transform NIH3T3 cells (19), and overexpression of MDM2 has been documented to alter cell cycle control pathways in p53 null cells *in vivo* (20). Recently, it has also been documented that MDM2 overexpression can block the growth-inhibitory activities of TGF-β1 via a p53-independent mechanism (21) and that p53 null transgenic mice that overexpress MDM2 develop a different spectrum of tumors than mice that are only null for p53 (22). Moreover, although rare, alterations in both MDM2 and p53 expression have been detected in primary human tumor cells (23). Therefore, it is conceivable that stabilized MDM2 may provide a selective growth advantage in p53 mutant cells by altering p53-independent growth control pathways. It is, however, possible that alterations in MDM2 turnover do not contribute at all to the transformation process but are a consequence of alterations in other critical growth control molecules that are important in the regulation of MDM2 stability. It has been reported recently that the alternative translation product of the human *CDKN2A* locus (p14^{arf}) is regulated by p53 and can regulate MDM2 stability (24). Thus, MDM2 protein stability may be associated with either p53 or p14^{arf} expression status. Interestingly, both the Jurkat (stable MDM2) and Raji (unstable MDM2) lines possess undetectable levels of p14^{arf} protein (25) and harbor mutant *p53* alleles. It is therefore likely that the stability of MDM2 will not be associated

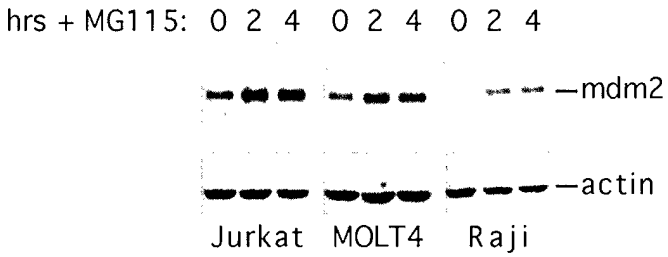

Fig. 4. MDM2 protein levels in MG115-treated cells harboring stable or unstable MDM2. Cells were treated with the proteasome inhibitor MG115 (final concentration, 10 μm) for the indicated period of time. Western blots using either a monoclonal antibody against MDM2 or polyclonal antibodies against actin were performed on protein extracts prepared from untreated and treated cells as described in "Materials and Methods."

simply with p53 or $p14^{arf}$ expression status, and that there are other unknown growth control molecules that are important in the regulation of MDM2 stability that are altered in human leukemic cells.

In summary, our results suggest that MDM2 protein expression is regulated by mechanisms controlling the stability of the protein. It will be of interest for future studies to determine whether MDM2 stability is regulated by signals that not only induce proliferation but also inhibit cell growth (*i.e.,* signals that promote senescence, differentiation, and others). Our data also suggest that mechanisms regulating MDM2 stability can be altered in human tumor cell lines. We did perform half-life studies using primary leukemic cells and found that some patients harbor cells possessing MDM2 with a long half-life (data not shown). However, because we could not control for the proliferative rate of tumor cells derived from the various patients (unlike in cell lines), we do not know if the differences in MDM2 stability measured in primary tumors are due to difference in the proliferative rate of the cells or due to alterations in pathways regulating MDM2 stability. More work will obviously be needed to identify the various factors that play a role in the regulation of MDM2 stability, how alterations in these pathways lead to MDM2 stabilization in cell lines, and whether alterations in the regulation of MDM2 stability occur in primary human tumors.

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