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The purpose of this research project was to characterize growth inhibitory effects of interleukin-4 (IL-4) in breast cancer cells and identify key signaling molecules which may be targets for future strategies to enhance the negative growth effects of IL-4. We have reported that IL-4–mediated growth inhibition is associated with increased apoptosis. Furthermore, we have identified that IL-4 activates two important signaling molecules IRS-1 and STAT6. Inhibition of IRS-1 does not block IL-4-mediated growth effects while inhibition of STAT6 decreases IL-4-mediated growth inhibition and apoptosis. Additionally, over-expression of STAT6 mimics the effect of IL-4 by decreasing growth and increasing apoptosis. In addition to these findings, we have also explored several other aspects of growth inhibition and apoptosis in breast cancer cells. We found that IGF-I is an effective mediator of survival following treatment with chemotherapy agents which induce apoptosis. Also, we have shown that the appearance of DNA laddering represents an end-stage processing mechanism that is not an accurate indicator of apoptosis in all strains of MCF-7 cells. Finally, we examined the mechanism of IFN gamma –mediated growth inhibition of breast cancer cells in vitro. In addition to the JAK-STAT pathway, interferon gamma decreases breast cancer cell growth in p21 dependent and independent pathways. In conclusion, our research on the mechanism and signal transduction pathways of IL-4 in human breast cancer cells has resulted in numerous original and significant findings as well as expanded our knowledge of mechanisms of apoptosis and growth inhibition of human breast cancer cells.

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Jennifer L Hooch 7.31.2000

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Introduction

The purpose of this research project was to characterize growth inhibitory effects of interleukin-4 (IL-4) in breast cancer cells and identify key signaling molecules which may be targets for future strategies to enhance the negative growth effects of IL-4. We have reported that IL-4–mediated growth inhibition is associated with increased apoptosis. Furthermore, we have identified that IL-4 activates two important signaling molecules IRS-1 and STAT6. Inhibition of IRS-1 does not block IL-4-mediated growth effects while inhibition of STAT6 decreases IL-4-mediated growth inhibition and apoptosis. Additionally, over-expression of STAT6 mimics the effect of IL-4 by decreasing growth and increasing apoptosis. In addition to these findings, we have also explored several other aspects of growth inhibition and apoptosis in breast cancer cells. We found that IGF-I is an effective mediator of survival following treatment with chemotherapy agents which induce apoptosis. Also, we have shown that the appearance of DNA laddering represents an end-stage processing mechanism that is not an accurate indicator of apoptosis in all strains of MCF-7 cells. Finally, we examined the mechanism of IFN gamma –mediated growth inhibition of breast cancer cells in vitro. In addition to the JAK-STAT pathway, interferon gamma decreases breast cancer cell growth in p21 dependent and independent pathways. In conclusion, our research on the mechanism and signal transduction pathways of IL-4 in human breast cancer cells has resulted in numerous original and significant findings as well as expanded our knowledge of mechanisms of apoptosis and growth inhibition of human breast cancer cells.

Annual Summary

The title of this research grant is "Over-expression of IL-4 signaling pathway to inhibit breast tumor growth". In the annual summary for 1998-1999, we reported significant progress on this project including publication of several key findings. First, we reported that interleukin-4 induces growth inhibition and apoptosis in human breast cancer cells [1]. While it had previously been reported that IL-4 inhibits the growth of some transformed cells, we were the first to describe a possible mechanism, apoptosis, for the growth inhibitory effect. The next step in this research project involved examining the signaling pathway activated by IL-4 in human breast cancer cells in vitro and determining what signaling molecules were required for IL-4-mediated growth effects. We found that IL-4 treatment resulted in the phosphorylation of two important signaling molecules, IRS-1 and STAT6. Using an inducible anti-sense strategy to inhibit IRS-1 expression in MCF-7 breast cancer cells, we showed that IRS-1 is not required for IL-4-mediated growth inhibition and apoptosis. In contrast, MCF-7 cells stably expressing anti-sense IRS-1 were less sensitive to IGF-I-mediated proliferation and protection from apoptosis [2]. As a result, we examined the importance of STAT6 in IL-4-mediated growth effects. Using a dominant-negative STAT6 molecule, we demonstrated that IL-4 failed to inhibit the growth of MCF-7 cells when STAT6 activity was inhibited by over-expression of a dominant negative protein. Furthermore, over-expression of full-length STAT6 resulted in growth inhibition [3]. Therefore, we concluded that while IL-4-mediated growth effects are associated with phosphorylation of IRS-1, STAT6 and not IRS-1 is required for IL-4-induced growth inhibition and apoptosis.

In addition to work directly addressing characterization and over-expression of IL-4 signaling pathway in breast cancer cells, this research project also generated several other publications involving growth inhibition and apoptosis of breast cancer cells. First, we examined the ability of IGF-I to enhance survival of breast cancer cells following treatment with chemotherapy drugs [4]. Next, we examined the effect of strain-specific

differences on the ability of MCF-7 cells to undergo DNA laddering [5] Finally, we showed that IFN gamma inhibits the growth of breast cancer cells in both p21-dependent and independent mechanisms [6].

IL-4 and IGF-I (insulin-like growth factor-I) share common signaling molecules, namely IRS-1. In contrast to IL-4, however, IGF-I induces proliferation and increases survival of breast cancer cells. Although our work eventually demonstrated that IRS-1 is not required for IL-4-mediated growth effects, we were interested to further explore the growth effects mediated by IGF-I via IRS-1. As a result, we examined the ability of IGF-I to increase survival of breast cancer cells following treatment with two common chemotherapy agents, doxorubicin and paclitaxel [4]. Because many breast cancer cells express components of the IGF-I pathway and therefore potentially sensitive to growth effects mediated by IGF-I, it is possible that some tumors are less responsive to chemotherapy agents which rely on induction of apoptosis. In fact, we showed that IGF-I increased survival and decreased the apoptosis of MCF-7 breast cancer cells after treatment with both anti-cancer drugs. Interestingly, we found that IGF-I may have multiple mechanisms for enhancing survival. For example, in the case of doxorubicin-mediated apoptosis, IGF-I treatment resulted in a decrease in apoptotic cells, but no change in cell number or cell cycle distribution. In contrast, following paclitaxel treatment, IGF-I mediated an increase in cell number and a shift of cells into the S-phase of the cell cycle, both indicating proliferation, but did not change the actual number of cells undergoing apoptosis. In support of IGF-I activating two different mechanisms to enhance cell survival, we found that IGF-I's protective effect following doxorubicin-induced cell death required activation of both PI-3 kinase and Erk1/Erk2 MAPK while IGF-I's protective effect following paclitaxel required only Erk1/Erk2 MAPK. As PI-3 kinase has been implicated in inhibition of apoptosis and Erk1/Erk2 is commonly associated with proliferation, it is apparent that IGF-I can both decrease apoptosis and increase proliferation to enhance survival.

The observation that IL-4 induced apoptosis in breast cancer cells was made only after multiple indices for apoptosis were confirmed. These included appearance of pyknotic nuclei, chromosome condensation, cleavage of poly-ADP-ribose polymerase (PARP), appearance of sub-G1 DNA content on FACS sorting, and degradation of DNA into internucleosomal fragments. In the course of these experiments, we learned that internucleosomal fragmentation of DNA into so called DNA ladders was controversial in some cells, including MCF-7 breast cancer cells. While some investigators, including us, have published DNA laddering following various apoptotic stimuli including IL-4, other laboratories have reported that MCF-7 cells do not undergo DNA laddering at all. However, it has previously been reported that MCF-7 cells maintained in different laboratories for extended periods of time exhibit unique characteristics that suggest the development of different strains of MCF-7 cells. Therefore, we hypothesizd that the apparent difference in the ability of MCF-7 cells to undergo DNA laddering might be strain-specific. Accordingly, we found that when a panel of MCF-7 cells from different laboratories was treated with adriamycin, only 3 strains produced ladders while 3 did not [5]. The lack of DNA laddering was not due to a lack of sensitivity to the drug as all cell lines were growth inhibited in a dose-responsive manner. Nor was the absence of DNA ladders due to a failure to undergo apoptosis as a comparable portion of cells from laddering and non-laddering strains had sub-G1 DNA content by FACS sorting. From this work, we were able to conclude that while laddering may be a convenient way to detect apoptosis, it may not be accurate in all cell lines.

The final set of data which stemmed from our work on IL-4 in breast cancer cells came about while we examined IL-4-mediated activation of the JAK-STAT pathway. Other groups have shown that the immunoregulatory cytokine interferon gamma may enhance growth inhibition of breast cancer cells by other cytotoxic agents and has been shown to act via activation of STATs. Therefore, we examined DNA binding of STATs using a specific element from the c-fos gene promoter. While IL-4 failed to activate binding of STATs ,

interferon gamma treatment resulted in a protein-DNA complex containing STAT1 and possibly STAT3. (We later learned that IL-4 activation of STATs was limited to STAT6 and required a very specific DNA binding sequence which was different from the one we used to identify interferon gamma-mediated activation of STAT1.) We then went on to characterize interferon gamma-mediated effects on breast cancer cell lines. [6] We found that interferon gamma inhibited growth of several cell lines and this was associated with changes in the cell cycles mainly an increase of cells in G1. Interestingly, interferon gamma did not inhibit anchorage independent growth of one cell line, MDA-MB-231. We found that this loss of inhibition in soft agar was associated with a lack of the cyclin dependent kinase inhibitor, p21. While p21 mRNA was present, p21 protein was not induced interferon gamma treatment in MDA-MB-231 cells as it was in other breast cancer cell lines. Finally, we found that the loss of p21 protein could be rescued by inhibiting the proteosome, suggesting that degradation of p21 is enhanced in this cell line and may be one mechanism or inactivating this cell cycle regulating protein.

In conclusion, our work on this DOD funded research project has been exciting and fruitful. We have contributed to knowledge about the role of IL-4 and its signaling pathway in breast cancer cells. Additionally, we have pursued related questions as they stemmed from this work. I have now published 4 original papers in reputable journals (with 2 still in submission) and have successfully completed a dissertation titled, "The role of IRS-1 in IGF-I and IL-4 – mediated growth effects in human breast cancer cells." Thank you for your support of this project and of my doctoral taining.

Key Research Accomplishments

1. Interleukin-4 inhibits growth of breast cancer cell lines and induces apoptosis.
2. IL-4 activates IRS and JAK-STAT signaling pathways in breast cancer cell lines.
3. IL-4-mediated growth inhibition and apoptosis occurs even when IRS-1 protein and mRNA are reduced.
4. Inhibition of STAT6 blocks IL-4-mediated growth inhibition and apoptosis and overexpression STAT6 increases IL-4-mediated growth effects.
5. IGF-I is a potent survival factor for MCF-7 breast cancer cells and acts by both increasing proliferation and decreasing apoptosis.
6. The appearance of DNA ladders does not correspond to the induction of apoptosis in different strains of MCF-7 cells.
7. Interferon gamma also inhibits the growth of breast cancer cells and utilizes both p21-dependent and p21-independent pathways.

Reportable Outcomes

Publications:

1. **Gooch JL**, Lee AV, Yee D. Interleukin-4 (IL-4) induces growth inhibition and apoptosis in human breast cancer cells. *Cancer Research*. 58:4199-4205, 1998.

2.Yee D, Jackson JG, Weng C-N, **Gooch JL**, Lee AV. The IGF system in breast cancer. In: K. Takano, Hizuka N, Takahashi S-I (ed.). Molecular Mechanisms to regulate the activities of insulin-like growth factors, pp. 319-325: Elsvier Science B.V., Amsterdam, 1998.

3. Lee AV, Jackson JG, **Gooch JL**, Hilsenbeck SG, Coronado-Heinsohn E, Osborne CK, Yee D. Enhancement of the insulin-like growth factor signaling in human breast cancer: Estrogen regulation of insulin receptor substrate-1 (IRS-1) in vitro and in vivo. *Molecular Endocrinology*, 13(5): 787-796, 1999.

4. **Gooch JL**, Van Den Berg CL, Yee D. Insulin-like growth factor (IGF) -I rescues breast cancer cells from chemotherapy-induced cell death: proliferative and anti-apoptotic effects. *Breast Cancer Research and Treatment*, 56:1-10, 1999.

5. **Gooch JL**, Yee D. Strain-specific differences in the formation of apoptotic DNA ladders in MCF-7 breast cancer cells. *Cancer Letters*, 144:31-37, 1999.

6. Lee AV, **Gooch JL**, Osterreich S, Guler B, Yee D. IGF-I-induced degradation of IRS-1 is mediated by the 26S proteosome and requires PI-3 kinase. *Molecular Cell Biology*, 5:1489-1496 , 2000.

7. **Gooch JL**, Herrera R, Yee D. The role of p21 in IFN-gamma-mediated growth inhibition in human breast cancer cells. *Cell Growth and Differentiation*, 6:335-342, 2000.

8. **Gooch JL**, Lee AV, Christy B, Yee D. The role of insulin receptor substrate-1 (IRS-1) in insulin-like growth factor -I (IGF-I)- and interleukin-4 (IL-4)-mediated growth effects in human breast cancer cells. Submitted, 2000.

9. **Gooch JL**, Christy B, Yee D. STAT6 mediates growth inhibition and apoptosis in human breast cancer cells. Submitted, 2000.

Presentations:

1. **Gooch JL**, Jackson JG, Yee D. Interleukin-4 signaling in human breast cancer cells. Society for Experimental Biology and Medicine, Southwest Section, San Antonio, TX, 1996. *Proc of the Society for Exp Biol and Med*, 1997.

2. **Gooch JL**, Lee AV, Yee D. Interleukin-4 induces growth inhibition and apoptosis in breast cancer cells. Poster and workshop presentation. 7th Annual Symposium on Cancer Research in San Antonio, San Antonio, TX, 1997.

3. **Gooch JL**, Lee AV, Yee D. Ligand dependent degradation of insulin receptor substrate-1 (IRS-1) in human breast cancer. Endocrine Society 80th Annual Meeting, New Orleans, LA, 1998.

4. **Gooch JL**, Lee AV, Yee D. Ligand dependent degradation of insulin receptor substrate-1 (IRS-1) in human breast cancer. Student competition, 8th Annual Symposium on Cancer Research in San Antonio, San Antonio, TX, 1998.

5. **Gooch, JL**, Van Den Berg CL, Yee D. Inhibition of chemotherapy-induced apoptosis by insulin-like growth factor

(IGF) -I involves both proliferative and anti-apoptotic mechanisms. 81st Annual Endocrine Society Meeting, San Diego, CA, 1999.

Dissertation:

Department of Molecular Medicine PhD program, dissertation title, **"Characterization of growth inhibitory and apoptotic signaling by Interleukin- 4 in human breast cancer cells"**. Defended September, 1999.

References:

1. **Gooch JL**, Lee AV, Yee D. Interleukin-4 (IL-4) induces growth inhibition and apoptosis in human breast cancer cells. *Cancer Research*. 58:4199-4205, 1998.

2. **Gooch JL**, Lee AV, Christy B, Yee D. The role of insulin receptor substrate-1 (IRS-1) in insulin-like growth factor -I (IGF-I)- and interleukin-4 (IL-4)-mediated growth effects in human breast cancer cells. Submitted, 2000.

3. **Gooch JL**, Christy B, Yee D. STAT6 mediates growth inhibition and apoptosis in human breast cancer cells. Submitted, 2000.

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6. **Gooch JL**, Herrera R, Yee D. The role of p21 in IFN-gamma-mediated growth inhibition in human breast cancer cells. *Cell Growth and Differentiation*, 6:335-342, 2000.

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Report

Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death – proliferative and anti-apoptotic effects

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Key words: apoptosis, breast cancer, doxorubicin, IGF-I, paclitaxel

Summary

Insulin-like growth factor (IGF)-I protects many cell types from apoptosis. As a result, it is possible that IGF-I-responsive cancer cells may be resistant to apoptosis-inducing chemotherapies. Therefore, we examined the effects of IGF-I on paclitaxel and doxorubicin-induced apoptosis in the IGF-I-responsive breast cancer cell line MCF-7. Both drugs caused DNA laddering in a dose-dependent fashion, and IGF-I reduced the formation of ladders. We next examined the effects of IGF-I and estradiol on cell survival following drug treatment in monolayer culture. IGF-I, but not estradiol, increased survival of MCF-7 cells in the presence of either drug. Cell cycle progression and counting of trypan-blue stained cells showed that IGF-I was inducing proliferation in paclitaxel-treated but not doxorubicin-treated cells. However, IGF-I decreased the fraction of apoptotic cells in doxorubicin- but not paclitaxel-treated cells. Recent work has shown that mitogen-activated protein kinase (MAPK) and phosphotidylinositol-3 (PI-3) kinase are activated by IGF-I in these cells. PI-3 kinase activation has been linked to anti-apoptotic functions while MAPK activation is associated with proliferation. We found that IGF-I rescue of doxorubicin-induced apoptosis required PI-3 kinase but not MAPK function, suggesting that IGF-I inhibited apoptosis. In contrast, IGF-I rescue of paclitaxel-induced apoptosis required both PI-3 kinase and MAPK, suggesting that IGF-I-mediated protection was due to enhancement of proliferation. Therefore, IGF-I attenuated the response of breast cancer cells to doxorubicin and paclitaxel by at least two mechanisms: induction of proliferation and inhibition of apoptosis. Thus, inhibition of IGF-I action could be a useful adjuvant to cytotoxic chemotherapy in breast cancer.

Introduction

Insulin-like growth factor (IGF)-I is an important factor for the development, survival, and proliferation of many tissues including neoplastic tissues [1]. IGFs have also been shown to play an important role in proliferation and survival of transformed cells [2], and possibly in transformation itself [3, 4]. The role of IGFs in breast cancer has been studied, and it is clear that IGF-I is a potent mitogen for breast cancer cells *in vitro* and *in vivo* [5, 6].

In addition to its effect on proliferation, IGF-I has been shown to enhance tumor growth through the induction of survival pathways which inhibit apoptosis. *In vitro* experiments have shown that cells which over-express IGF-I receptor (IGF-IR), and are subsequently more responsive to IGF-I, are protected from etoposide-induced apoptosis [7]. The inverse is also true that cells which are deficient in IGF-II (an IGF-IR ligand) production are more sensitive to apoptotic stimuli, including chemotherapy drugs [8]. Finally, targeted disruption of the IGF-IR by expression of either a dominant-negative mutant or a truncated form of the receptor results in the induction of apoptosis [8, 9].

The role of IGF-I as a survival factor as well as a mitogen has also been demonstrated *in vivo*. For example, tumors of a variety of origins including breast, lung, colon, and kidney over-express IGF-IR as well as produce IGF-I and IGF-II, suggesting a possible

autocrine/paracrine activation of the IGF pathway [3]. Also, a decrease in IGF-IR results in the induction of apoptosis in transplantable rodent and human tumors. Conversely, over-expression of IGF-IR protects cells from apoptosis *in vivo* [4]. Interestingly, analyses of the IGF-IR have revealed distinct regions of the cytoplasmic domain of the receptor which are responsible for proliferative and anti-apoptotic signal transduction [10]. However, the specific mechanism whereby IGF-I promotes cell survival when challenged with cytotoxic agents has not been clarified.

Since many chemotherapeutic agents may induce apoptosis, the influence of IGF-I as a survival factor could, in part, determine whether a cytotoxic agent is successful at inhibiting tumor cell growth. In fact, it has been proposed that IGF-I may play a role in the development of multi-drug resistance by increasing proliferation and inhibiting apoptosis of drug-treated cancer cells [11, 12]. Two of the most active agents in breast cancer treatment are doxorubicin and paclitaxel; both have been shown to induce apoptosis [13–15]. Therefore, we were interested to determine if IGF-I treatment could alter proliferation and the induction of apoptosis of breast cancer cells treated with doxorubicin or paclitaxel.

Some molecules activated by IGF-I have been identified, including mitogen-activated protein kinase (MAPK) and phosphotidylinositol-3 (PI-3) kinase. Our laboratory and others have shown that these two molecules are required for IGF-I-induced proliferation [16, 17]. Recent work into IGF-I anti-apoptotic signaling has also demonstrated the importance of PI-3 kinase and its downstream substrate, Akt [18–20]. Further, a direct link between PI-3 kinase and the apoptosis-regulating Bcl family of proteins has been established through Akt phosphorylation of BAD [21, 22]. Therefore, activation of this pathway could also be important for IGF-I-mediated inhibition of chemotherapy-induced cell killing.

Using an IGF-I-responsive breast cancer cell line, MCF-7, we show here that IGF-I reduces apoptosis in doxorubicin- and paclitaxel-treated cells. However, IGF-I did not have any effect on doxorubicin-mediated growth inhibition of an IGF-I non-responsive cell line, MDA-MB-435A. Interestingly, the effects of IGF-I on doxorubicin- and paclitaxel-induced cell death appear to involve at least two mechanisms: induction of proliferation and inhibition of apoptosis. Therefore, we conclude that IGF-I may decrease the responsiveness of breast tumor cells to apoptosis-inducing cytotoxic agents. As a result, inhibition of the IGF-I pathway may be a useful adjunct to cytotoxic therapies.

Materials and methods

Materials

MCF-7 cells were provided by C. Kent Osborne (San Antonio, TX) and were maintained in improved minimal essential medium (IMEM) (Gibco, Bethesda, MD) plus phenol red supplemented with 5% fetal bovine serum (Summit, Ft. Collins, CO). MDA-MB-435A cells were obtained from Nils Brünner (Copenhagen, Denmark) and were maintained in IMEM without phenol red and supplemented with 5% fetal bovine serum. IGF-I was from GroPep (Adelaide, Australia). Doxorubicin was from Sigma (St. Louis, MO) and paclitaxel was from Bristol-Myers (Princeton, NJ). PD098059 was from CalBioChem (Cambridge, MA) and wortmannin was from Sigma.

DNA laddering

MCF-7 cells were plated at 1.0×10^6 cells per 10 cm dish and MDA-MB-435 cells were plated at 0.5×10^6 cells per 10 cm dish. Cells were allowed to recover for 24 h and were then washed once with 1 × PBS alone or with growth factor before drug treatments were added as indicated. After 48 h, the cells were washed with 1 × PBS, harvested with trypsin-EDTA, pelleted (including any cells from the medium), washed with 1 × PBS, pelleted again, and then lysed in 0.15 M NaCl, 10 mM Tris–HCl (pH 7.8), 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.5% NP-40 on ice for 40 min. Lysates were centrifuged at 1000 × *g* for 10 min and pellets were resuspended in 0.35 M NaCl, 10 mM Tris–HCl, 1 mM $MgCl_2$, and 1 mM DTT on ice for 20 min. Lysates were then extracted once with phenol–chloroform and DNA was precipitated with 0.01 M $MgCl_2$ and 2.5 volumes 100% ethanol overnight at −20°C. DNA was pelleted at 14, 000 × *g* for 20 min, resuspended in Tris–EDTA plus 0.1 mg/ml RNase A, and incubated at 37°C for 1 h. 1 mg/ml proteinase K was added followed by incubation at 37°C for an additional hour. DNA was then electrophoresed in 1.5% agarose gels containing ethidium bromide.

Monolayer growth assay

Growth assays were performed by 3-[4,5-dimethylthiazol-2-yl]2,5 diphenyltetrazolium bromide (MTT)

assay as described [23]. MCF-7 cells were plated in quadruplicate at a density of 15,000 cells per well in 48-well cell culture plates. Cells were allowed to adhere overnight and were then washed once with 1 × PBS. Following treatment, 30 µl of MTT (5 mg/ml in PBS) was added to each well for 4 h. Medium and MTT were then removed, DMSO plus 2.5% IMEM was added, and absorbance was measured at 540 nm with a differential filter of 690 nm.

Flow cytometry

MCF-7 cells were plated at 0.5×10^6 cells per 60 mm dish. Cells were allowed to recover for 24 h and were then washed once with 1 × PBS. Serum-free medium was added overnight. The next day, drug treatments were added as indicated. Treatments were continued for 48 h and then cells were washed once with 1 × PBS, harvested with trypsin-EDTA, pelleted, washed once with 1 × PBS, pelleted again, and resuspended with 200 µl of PBS. 200 µl of ice-cold 70% ethanol was added dropwise while vortexing. Cells were fixed overnight at −20°C and then 0.5 ng/ml propidium iodide plus 0.5 mg/ml RNAse A was added. Cells were analyzed using a Facstar Plus (Becton-Dickinson, San Jose, CA) flow cytometer, gated on forward light scatter, pulse height, and pulse width for analysis of cell cycle fractions, and ungated mode for detection of cells with subG1 DNA content. Resulting histograms were evaluated using Modfit LT™ software (Verity House, Topsham, ME).

Results

IGF-I has been reported to enhance tumor growth both by increasing cell proliferation and by inhibiting programmed cell death [4, 5]. Programmed cell death, or apoptosis, is commonly seen after treatment of cells with cytotoxic agents [13]. Therefore, we were interested to find out if IGF-I could alter the response of breast cancer cells to treatment with the chemotherapy agents doxorubicin and paclitaxel. We treated IGF-I-responsive MCF-7 cells with increasing concentrations of doxorubicin and paclitaxel and examined the effect of IGF-I on apoptosis by DNA laddering. Both doxorubicin and paclitaxel treatments resulted in DNA laddering characteristic of apoptosis in MCF-7 breast cancer cells. IGF-I decreased the amount of fragmented DNA induced by doxorubicin and paclitaxel over a range of drug concentrations (Figure 1A). Further, the protective effect of IGF-I was dose-responsive – increased protection was observed with increasing amounts of IGF-I (Figure 1B).

We next examined the effect of IGF-I treatment on doxorubicin- and paclitaxel-induced growth inhibition in monolayer culture. MCF-7 cells were treated with doxorubicin and paclitaxel plus vehicle control (SFM), IGF-I, or estradiol. As estradiol is also a potent mitogen for MCF-7 breast cancer cells, it was included as a control. Figure 2A shows that IGF-I significantly increased survival of both doxorubicin- and paclitaxel-treated cells. In contrast, estradiol had only a slight effect on cell number following drug treatment. We also examined the effect of IGF-I on doxorubicin-induced growth inhibition of an IGF-I non-responsive cell line, MDA-MB-435A. MDA-MB-435A breast cancer cells express IGF-IR but although IGF-I may play a role in invasion and metastasis of this cell line [26], the cells do not proliferate in response to IGF-I. We found that IGF-I did not have any effect on proliferation or survival of doxorubicin-treated MDA-MB-435A cells in monolayer culture (Figure 2B).

We next determined whether the apparent decrease in apoptosis seen with IGF-I treatment was a result of more total cells due to IGF-I-mediated proliferation or a result of fewer dying cells due to IGF-I induction of anti-apoptotic mechanisms. To investigate this we examined the effect of IGF-I on cell cycle progression of doxorubicin- and paclitaxel-treated cells. Flow cytometric sorting of only intact cells was used to determine the fraction of cells in G1/G0, S, and G2/M-phases of the cell cycle; as a result, no sub-G1 peak is visible. Both doxorubicin and paclitaxel treatments resulted in an increased proportion of cells in G2/M compared to SFM treatment. This is consistent with the fact that both doxorubicin and paclitaxel have been described to block cells in G2/M [24, 25]. In cells treated with SFM, IGF-I resulted in a 28% decrease in G0/G1, a 35% increase in S, and a 22% increase in G2/M (Figure 3). All changes were statistically significant (Table 1). Doxorubicin-treated cells showed no significant response in G0/G1 or S-phase due to IGF-I. Interestingly, there was a small but statistically significant increase in G2/M (Table 1). In contrast, paclitaxel-treated cells demonstrated a response to IGF-I that was comparable to control (SFM) with a 46% decrease in G1, a 6% increase in S-phase and a 25% increase in G2/M. Changes in G0/G1 and G2/M due to IGF-I were statistically significant (Table 1). Therefore, paclitaxel-treated cells responded to IGF-I with changes in the cell cycle similar

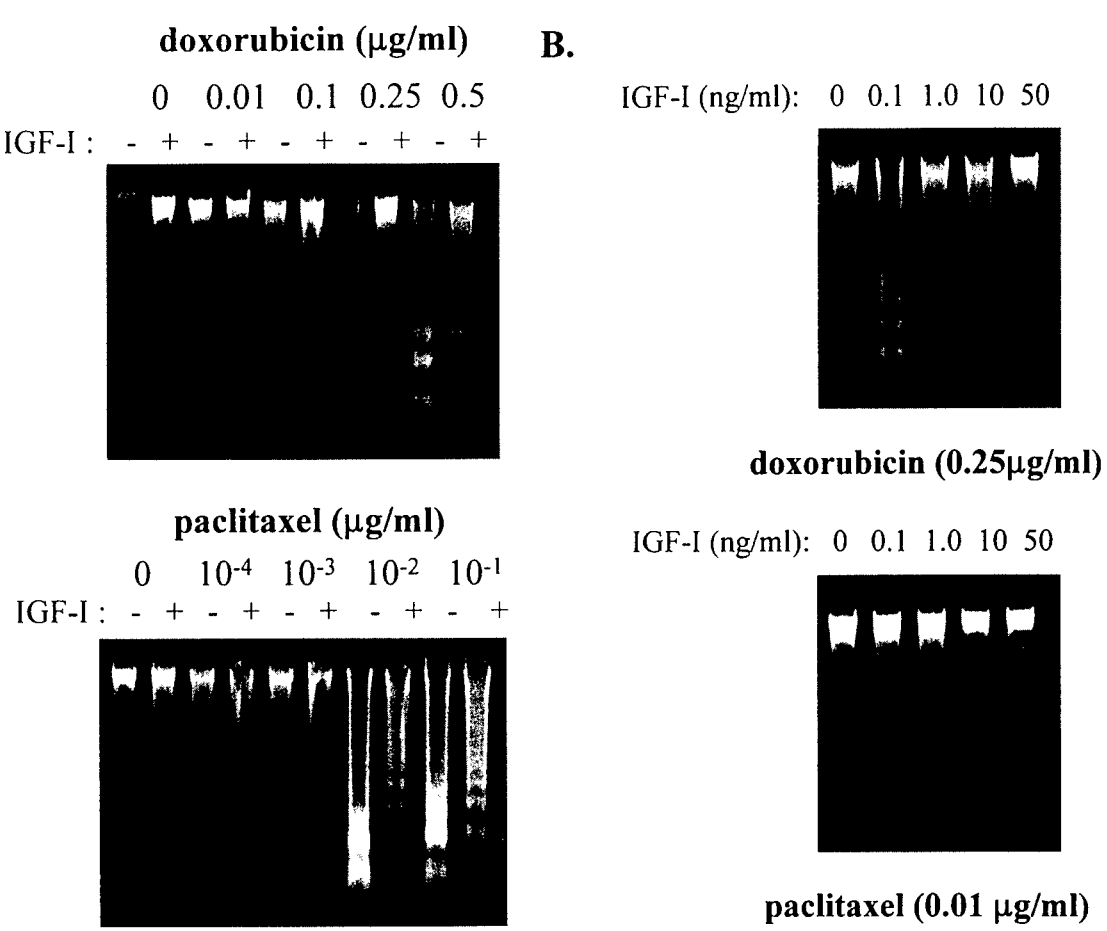

Figure 1. IGF-I reduces DNA laddering induced by doxorubicin and paclitaxel. (A) MCF-7 cells were treated with doxorubicin and paclitaxel at the concentrations indicated either alone or with 50 ng/ml IGF-I. Fragmented DNA was collected and separated by agarose gel electrophoresis. Data shown are representative of three separate experiments. (B) MCF-7 cells were treated with 0.25 μg/ml doxorubicin or 0.01 μg/ml paclitaxel along with increasing amounts of IGF-I. Fragmented DNA was collected and separated by agarose gel electrophoresis. Data shown are representative of three separate experiments.

to control, implying that these cells are proliferating in response to IGF-I. In contrast, doxorubicin-treated cells failed to respond to IGF-I-mediated proliferation.

We next looked at induction of apoptosis as measured by sub-G1 DNA content. Figure 4 shows the effect of IGF-I on the percentage of apoptotic cells in control, doxorubicin- and paclitaxel-treated cells. Flow cytometric sorting of all cells (including non-intact cells) was used to determine the fraction of cells with sub-G1 DNA content. IGF-I significantly ($p < 0.003$) reduced the amount of cells undergoing apoptosis in doxorubicin-treated cells but not paclitaxel-treated cells.

To further clarify the mechanism of IGF-I-mediated inhibition of doxorubicin- and paclitaxel-induced apoptosis, we treated cells with doxorubicin and paclitaxel alone or with IGF-I and then stained cells with trypan-blue to distinguish dead cells from living cells. Figure 5 shows that IGF-I induced a significant increase in total paclitaxel-treated cells ($p < 0.02$) but had no effect on the percentage of dead cells. In contrast, IGF-I induced only a small increase in total cells following doxorubicin treatment that was not significant. However, addition of IGF-I significantly reduced the percentage of dead cells after doxorubicin treatment ($p < 0.04$).

Results of DNA laddering experiments and monolayer growth experiments show that IGF-I reduces the efficacy of doxorubicin and paclitaxel. Flow cytometry analyses and cell counts suggest that IGF-I reduces the efficacy of doxorubicin and paclitaxel by at least two distinct mechanisms. In doxorubicin-treated cells,

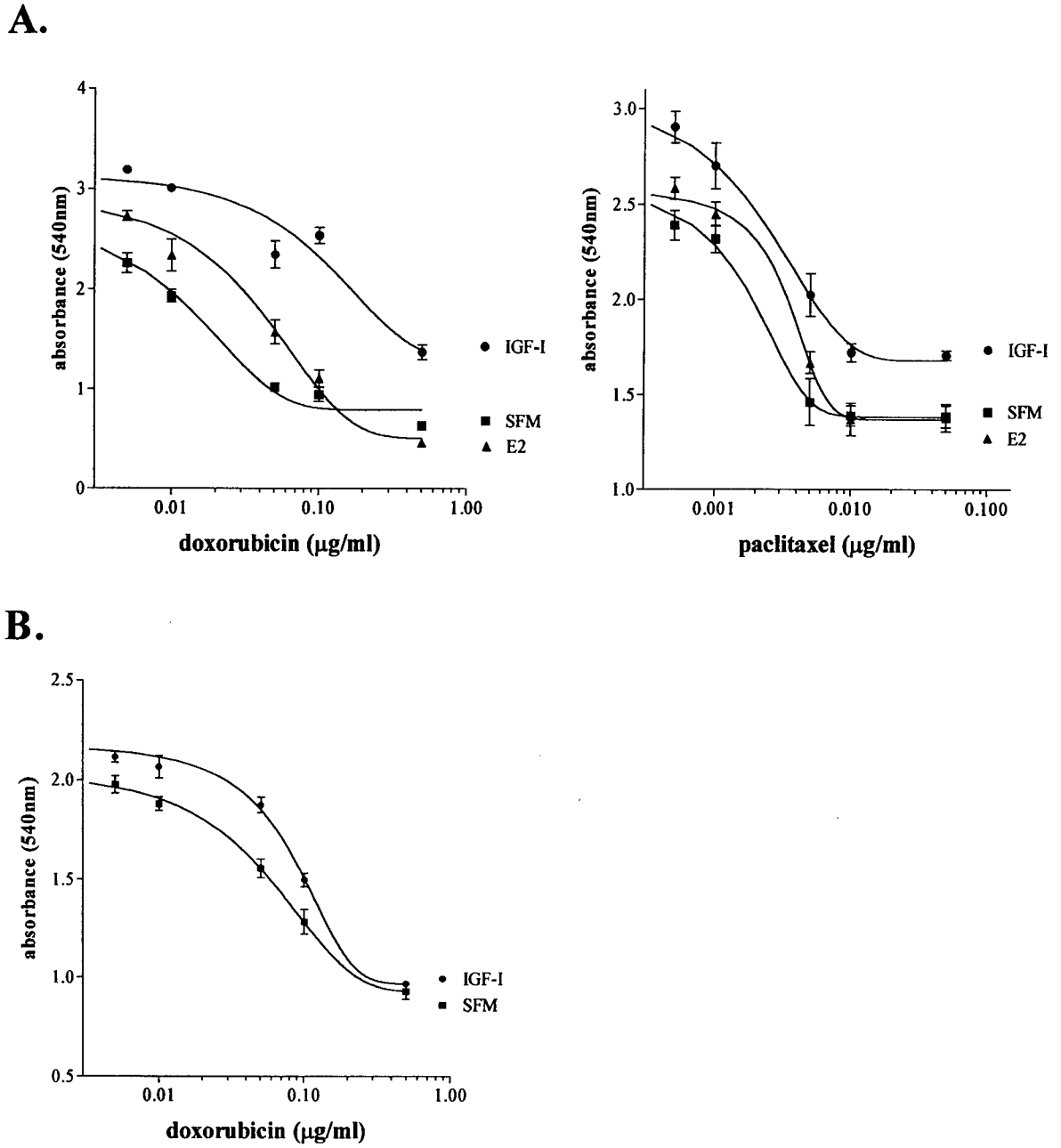

Figure 2. IGF-I increases survival of doxorubicin but not paclitaxel-treated cells. (A) MCF-7 cells were grown in monolayer culture and treated with increasing concentrations of doxorubicin or paclitaxel along with serum-free medium (SFM), 50 ng/ml IGF-I, or 10^{-9} M estradiol. Growth was measured by MTT assay. Data are plotted using absorbance values (540 nm). Error bars represent ± SEM of quadruplicate samples. Data shown are representative of three separate experiments. (B) MDA-MB-435A cells were grown in monolayer culture and treated with increasing concentrations of doxorubicin along with SFM or 50 ng/ml IGF-I. Growth was measured by MTT assay. Data are plotted using absorbance values (540 nm). Error bars represent ± SEM of quadruplicate samples. Data shown are representative of three separate experiments.

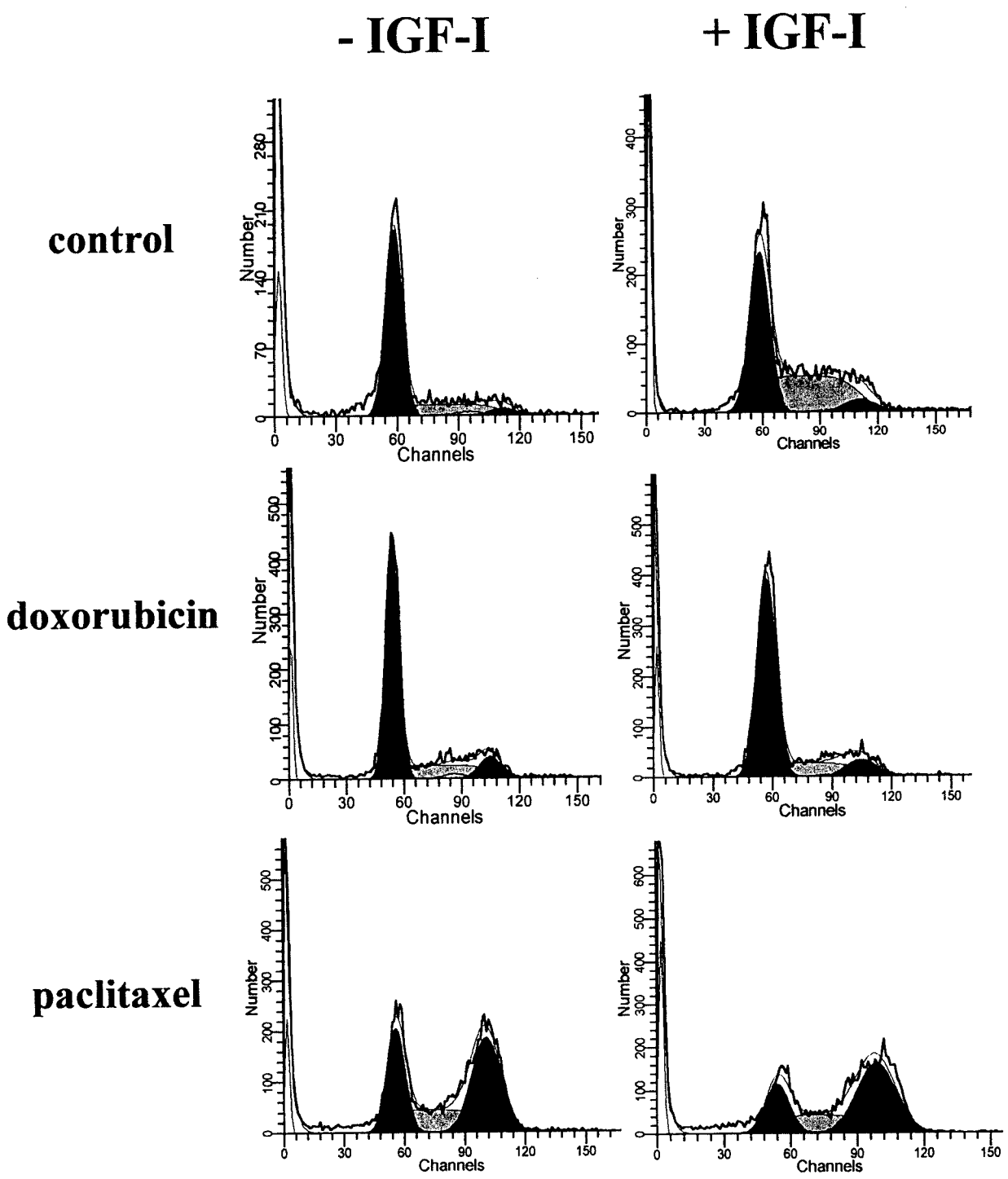

Figure 3. IGF-I stimulates entry into S-phase of paclitaxel-treated cells but not doxorubicin-treated cells. MCF-7 cells were treated with SFM (control), 0.25 μg/ml doxorubicin, or 0.01 μg/ml paclitaxel with and without 50 ng/ml IGF-I. Cells were ethanol fixed, stained with propidium iodide, and sorted by flow cytometry in gated mode to analyze only intact cells for DNA content. Modfit LT^{TM} software was used to determine cell cycle fractions. Representative histograms of triplicate samples are shown for each treatment. Data shown are representative of three separate experiments.

Table 1. IGF-I induces statistically significant changes in the cell cycle of doxorubicin- and paclitaxel-treated cells

IGF-I	G0/G1	*p*	*S*	*p*	G2/M	*p*
Control						
–	69.4 ± 0.8		27.2 ± 0.5		3.5 ± 0.3	
+	46.7 ± 0.4	0.001	47.7 ± 1.2	0.002	5.7 ± 0.7	0.05
Doxorubicin						
–	73.7 ± 2.5		17.0 ± 1.6		9.3 ± 0.9	
+	74.5 ± 0.9	0.77	12.7 ± 1.0	0.09	12.8 ± 0.3	0.02
Paclitaxel						
–	34.5 ± 2.0		22.1 ± 1.1		43.4 ± 2.7	
+	18.7 ± 1.3	0.003	23.5 ± 1.3	0.45	57.8 ± 1.9	0.01

Results of flow cytometry were analyzed using Student's *t*-test (2-tailed, unpaired). Data shown are the mean of triplicate samples ± SEM.

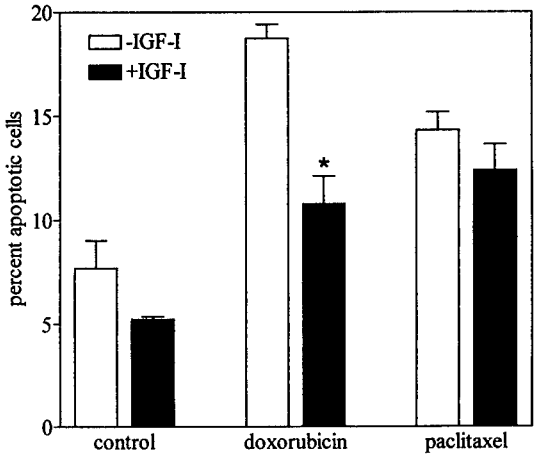

Figure 4. IGF-I reduces apoptotic fraction in doxorubicin-treated cells but not paclitaxel-treated cells. MCF-7 cells were treated with SFM (control), 0.25 μg/ml doxorubicin, or 0.01 μg/ml paclitaxel, with or without 50 ng/ml IGF-I. Cells were ethanol fixed, stained with propidium iodide, and analyzed by flow cytometry using an ungated method to analyze all cells for sub-G1 DNA content. Modfit LT™ software was used to determine the fraction of apoptotic cells in each sample. Asterisk represents a statistically significant difference ($p < 0.003$) between doxorubicin and doxorubicin plus IGF-I. Error bars represent ± SEM of triplicate samples. Data shown are representative of three separate experiments.

an increase in overall cell survival and a reduction of apoptotic fraction is observed although the proliferative response to IGF-I appears less than in controls. In contrast, paclitaxel-treated cells appear to proliferate in response to IGF-I but do not demonstrate increased cell survival or decreased numbers of apoptotic cells. In this case, the reduction of DNA laddering seen with IGF-I treatment was possibly due to an overall increase in cell number corresponding to a decreased apoptotic fraction.

IGF-I has been shown to activate both MAPK and PI-3 kinase in these cells. While both molecules have been implicated in proliferation, PI-3 kinase has also been linked to induction of anti-apoptotic pathways. We have previously shown that IGF-I induction of MAPK and PI-3 kinase can be effectively inhibited by PD090859 and wortmannin, respectively, in these cells [17]. Therefore, we used these inhibitors to determine whether IGF-I depends on either MAPK or PI-3 kinase activation to block apoptosis. We show in Figure 6 that, as before, both doxorubicin and paclitaxel induced DNA laddering, and addition of IGF-I reduced the appearance of ladders. Inhibition of MAPK by PD090859 blocked IGF-I rescue of paclitaxel-induced DNA laddering but had no effect on IGF-I's ability to reduce doxorubicin-induced DNA laddering. Wortmannin, a PI-3 kinase inhibitor, blocked IGF-I rescue of both doxorubicin- and paclitaxel-induced apoptosis. Therefore, IGF-I inhibition of doxorubicin-induced DNA laddering appears to require PI-3 kinase but not MAPK, while IGF-I inhibition of paclitaxel-induced apoptosis requires both PI-3 kinase and MAPK.

Discussion

IGF-I reduces the fraction of cells undergoing apoptosis induced by doxorubicin and paclitaxel treatments, as measured by DNA laddering. Also, IGF-I enhances survival of cells exposed to increasing concentrations of drug in monolayer culture. However,

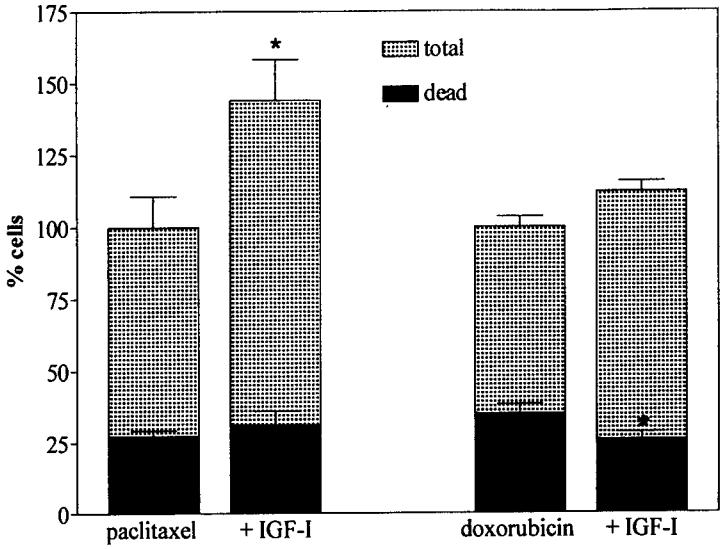

Figure 5. IGF-I increases total cells following paclitaxel treatment but decreases the percentage of dead cells following doxorubicin treatment. MCF-7 cells were treated with 0.25 μg/ml doxorubicin or 0.01 μg/ml paclitaxel, alone or with 50 ng/ml IGF-I. Cells were harvested and stained with 4% trypan-blue to differentiate dead cells from living cells. Total cells as well as dead cells in each treatment group were counted in triplicate. Data are shown as percentage of total cells from doxorubicin or paclitaxel alone treatments. Error bars represent ± SEM of duplicate samples. Asterisks represent statistically significant differences. For the effect of IGF-I on total paclitaxel-treated cells $p < 0.02$ and for the effect of IGF-I on the percentage of dead cells following doxorubicin treatment, $p < 0.04$. Data are representative of two separate experiments.

results from flow cytometry and cell counts of trypan-blue stained cells suggest that IGF-I may use different mechanisms to reduce apparent cell killing due to the two drug treatments – inhibition of apoptosis following doxorubicin treatment and induction of proliferation after paclitaxel treatment.

IGF-I mediates an anti-apoptotic effect following doxorubicin treatment and decreases the number of cells undergoing apoptosis but does not appear to induce proliferation. Analysis of sub-G1-DNA as well as cell counts of trypan-blue stained cells show that the number of apoptotic cells due to doxorubicin treatment is reduced by IGF-I. Moreover, following doxorubicin treatment, the proliferative response to IGF-I appears to be impaired – G0/G1 and S-phases do not change significantly and the number of trypan-blue excluding cells also remains unaffected. Therefore, IGF-I decreases the formation of DNA ladders by decreasing the number of cells undergoing apoptosis.

In contrast, IGF-I does not appear to change the number of apoptotic cells following paclitaxel treatment and instead induces proliferation in the surviving cells. Paclitaxel-treated cells still respond to IGF-I by moving into the cell cycle. Also, cell counts revealed that the total number of cells treated with both

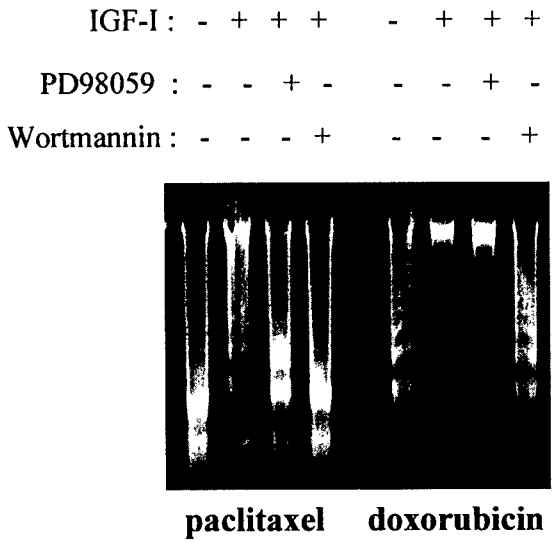

Figure 6. IGF-I inhibition of paclitaxel-mediated apoptosis requires MAPK but not PI-3 kinase; IGF-I inhibition of doxorubicin-mediated apoptosis requires both MAPK and PI-3 kinase. MCF-7 cells were pre-incubated with inhibitors of PI-3 kinase (250 nM wortmannin) or MAPK (25 nM PD098059) and then were treated with 50 ng/ml IGF-I plus 0.25 μg/ml doxorubicin or 50 ng/ml IGF-I plus 0.01 μg/ml paclitaxel. Fragmented DNA was collected and separated by agarose gel electrophoresis.

IGF-I and paclitaxel was greater than paclitaxel alone, indicating that IGF-I was able to induce proliferation. Interestingly, the number of dead cells increased slightly in this experiment. This could be due to the fact that paclitaxel inhibits cell division by impairing microtubule function, so that by inducing cells to divide, IGF-I could push some cells toward cell death. Analysis of sub-G1-DNA and cell counts of dead cells demonstrated that IGF-I did not decrease the number of apoptotic cells. Thus, in these two assays, the absolute number of cells undergoing apoptosis remained constant despite the addition of IGF-I. However, at sub-lethal doses of paclitaxel, IGF-I induced proliferation in the remaining cells. The net effect is an increase in total cell number due to IGF-I. DNA laddering appears to be decreased since the relative proportion of apoptotic cells decreases after the total cell number increases.

Also, IGF-I-mediated inhibition of doxorubicin-induced apoptosis requires PI-3 kinase, a molecule which has been identified as an important transducer of anti-apoptotic signals, but not MAPK; this supports the observation that IGF-I effects are primarily due to the inhibition of apoptosis. By contrast, IGF-I inhibition of paclitaxel-induced apoptosis is blocked by inhibitors of both PI-3 kinase and MAPK. As MAPK and PI-3 kinase have been shown to be required for IGF-I-induced proliferation of MCF-7 cells, this supports the requirement for proliferative signaling in IGF-I inhibition of paclitaxel-induced apoptosis. However, the roles of MAPK and PI-3 kinase are complex and involve considerable cross-talk [16]. As a result, it is difficult to completely separate the functions of these two signaling proteins.

The activation of two different mechanisms to reduce cell kill by chemotherapy agents could be related to the mechanisms of action of the drugs. In addition to DNA-damage, doxorubicin has been shown to induce activation of stress-associated signaling molecules such as Jun N-terminal kinase (JNK) [27] and perpetuate a signal cascade that may ultimately result in apoptosis. Paclitaxel has also recently been shown to activate JNK [28], but it is generally believed that paclitaxel induces apoptosis by directly interfering with microtubules, thus blocking DNA strand separation during mitosis [29, 30]. Thus, it seems likely that IGF-I can inhibit doxorubicin-induced apoptosis by blocking the pro-apoptotic signal and activating anti-apoptotic molecules. In the case of paclitaxel-induced apoptosis, however, damage to microtubules cannot be undone; therefore IGF-I increases cell number by inducing proliferation.

IGF-I has long been recognized as an important mitogen and more recently as a survival factor for many cell types. However, its role as a mitogen and survival factor in human breast cancer cells is less well-defined. Also, the consequences of IGF-I responsiveness on chemotherapy-induced cell killing have not previously been evaluated. We have shown that the ability of a tumor cell to respond to IGF-I may have important consequences for the induction of apoptosis by doxorubicin and paclitaxel. IGF-I protects cells from chemotherapy-induced apoptosis by at least two mechanisms: increasing proliferation and directly inhibiting programmed cell death. Importantly, cells which are not responsive to IGF-I-induced proliferation (and are usually estrogen receptor negative) do not derive any protective effect against apoptosis from IGF-I. Therefore, if addition of IGF-I decreases efficacy of chemotherapy drugs in IGF-I responsive cancer cells, the converse may also be true – that is, inhibition of IGF-I could increase effectiveness of apoptosis-inducing therapies.

In conclusion, IGF-I action in breast cancer cells results in multiple growth-related effects including proliferation, co-operative enhancement of estrogen signaling [31], and inhibition of programmed cell death. While inhibition of the IGF-I pathway will undoubtedly have direct consequences on the growth of a tumor, it is likely that there will also be other effects, such as the loss of protection from apoptosis-inducing chemotherapeutic agents. As IGF-I is known to have an even greater effect on cells in suspension than on cells grown in monolayer culture [3], it is possible that the consequences of IGF-I protection from cytotoxic drugs may be more significant *in vivo* than *in vitro*. As such, inhibition of IGF-I action could be a useful adjunct to cytotoxic chemotherapy in patients with breast cancer.

Acknowledgements

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References

1. Kleinberg DL: Role of IGF-I in normal mammary development. Breast Cancer Res Treat 47: 201–208, 1998
2. Werner H, Le Roith D: The insulin-like growth factor-I receptor signaling pathways are important for tumorigenesis and inhibition of apoptosis. Crit Rev Oncol 8: 71–92, 1997
3. Baserga R, Hongo A, Rubini M, Prisco M, Valentinis B: The IGF-I receptor in cell growth, transformation and apoptosis. Biochim Biophys Acta 1332: F105–F126, 1997
4. Resnicoff M, Baserga R: The role of insulin-like growth factor I receptor in transformation and apoptosis. Ann New York Acad Sci 842: 76–81, 1998
5. Yee D, Jackson JG, Weng C-N, Gooch JL, Lee AV: The IGF system in breast cancer. In: Takano K, Hizuka N, Takahashi, S-I (eds), Molecular Mechanisms to Regulate the Activities of Insulin-like Growth Factors, Elsevier, 1998, pp 319–325
6. Cullen KJ, Yee D, Sly WS, Perdue J, Hampton B, Lippman ME, Rosen N: Insulin-like growth factor receptor expression and function in human breast cancer. Cancer Res 50: 48–53, 1990
7. Sell C, Baserga R, Rubin R: Insulin-like growth factor I (IGF-I) and the IGF-I receptor prevent etoposide-induced apoptosis. Cancer Res 55: 303–306, 1995
8. Lamm GM, Christofori G: Impairment of survival factor function potentiates chemotherapy-induced apoptosis in tumor cells. Cancer Res 58: 801–807, 1998
9. Liu Y, Lehar S, Corvi C, Payne G, O'Connor R: Expression of the insulin-like growth factor I receptor C terminus as a myristylated protein leads to induction of apoptosis in tumor cells. Cancer Res 58: 570–576, 1998
10. O'Connor R, Kauffman-Zeh A, Liu Y, Lehar S, Evans GI, Baserga R, Blattler WA: Identification of domains of the IGF-IR that are required for protection from apoptosis. Mol Cell Biol 17, 1997
11. Guo YS, Houston CW, Thompson JC, Townsend CMJ: Insulin-like growth factor-I promotes multidrug resistance in MCLM colon cancer cells. Cell Physiol 175: 141–148, 1998
12. Geier A, Beery R, Haimsohn M, Karasik A: Insulin-like growth factor-1 inhibits cell death induced by anticancer drugs in the MCF-7 cells: involvement of growth factors in drug resistance. Cancer Invest 13: 480–486, 1995
13. Rasbridge SA, Gillett CE, Seymour AM, Patel K, Richards MA, Rubens RD, Millis RR: The effects of chemotherapy on morphology, cellular proliferation, apoptosis and oncoprotein expression in primary breast carcinoma. Br J Cancer 70: 335–341, 1994
14. Saunders D, Lawrence W, Christensen C, Wappler N, Raun H, Deppe G: Paclitaxel-induced apoptosis in MCF-7 breast cancer cells. Int J Cancer 70: 214–220, 1997
15. Torres K, Horwitz SB: Mechanisms of taxol-induced cell death are concentration dependent. Cancer Res 58: 3620–3626, 1998
16. Dufourny B, Alblas J, van Teeffelen HAAM, van Schaik FMA, van der Burgi B, Steenbergh PH, Sussenbach JS: Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. J Biol Chem 272: 31163–31171, 1997
17. Jackson JG, White MB, Yee D: Insulin receptor-substrate-1 (IRS-1) is the predominant signaling molecule activated by insulin-like growth factor-I (IGF-I), insulin, and interleukin-4 (IL-4) in estrogen receptor positive human breast cancer cells. J Biol Chem 273: 9994–10003, 1998
18. Minshall C, Arkins S, Freund GG, Kelley KW: Requirement for phosphatidylinositol 3-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. J Immunol: 939–947, 1996
19. Kulik G, Klippel A, Weber MJ: Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Bol 17: 1595–1606, 1997
20. Franke TF, Yang S-I, Chan TO, Datta K, Kazauskas A, Morrison DK, Kaplan DR, Tsichlis PN: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF activated phosphatidylinositol 3-kinase. Cell 81: 727–736, 1995
21. Datta SR, Didek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME: Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231–241, 1997
22. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ: Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-XI. Cell 87: 619–628, 1996
23. Twentyman PR, Luscombe M: A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br J Cancer 56: 279–285, 1987
24. Schiff PB, Horwitz SB: Taxol stabilizes microtubules in mouse fibroblast cells. PNAS 77, 1980
25. Tewey KM, Rowe TC, Tand L, Halligan BD, Liu LF: Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226, 1984
26. Dunn SW, Ehrlich M, Sharp NJH, Reiss K, Solomon G, Hawkins R, Baserga R, Barrett JC: A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the adhesion, invasion, and metastasis of breast cancer. Cancer Res 58: 3353–3361, 1998
27. Yu R, Shtil AA, Tan T-H, Roninson IB, Kong A-NT: Adriamycin activates c-jun N-terminal kinase in human leukemia cells: a relevance to apoptosis. Cancer Lett 107: 73–81, 1996
28. Wang T-H, Wang H-S, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J: Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both ras and apoptosis signal-regulating kinase pathways. J Biol Chem 273: 4928–4936, 1998
29. Manfredi JJ, Parness J, Horwitz SB: Taxol binds to cellular microtubules. J Cell Biol 94, 1982
30. Horwitz SB, Lothstein L, Manfredi JJ, Mellado W, Parness J, Roy SN, Schiff PB, Sorbara L, Zeheb R: Taxol: mechanisms of action and resistance. Ann New York Acad Sci 466: 733–744, 1986
31. Lee A, Weng C, Jackson J, Yee D: Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. J Endo 152: 39–47, 1997

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Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells

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Abstract

We tested the ability of seven MCF-7 strains to undergo DNA fragmentation, as measured by DNA laddering, following doxorubicin-induced apoptosis. Four strains were found to undergo DNA laddering while three were not. All strains were inhibited by doxorubicin, although sensitivity differed. Finally, we show by detection of sub-G1 DNA that doxorubicin induced the same fold increase in apoptosis in MCF-7-ATCC, which did not ladder, and MCF-7-MG, which did ladder. Therefore, detection of DNA ladders is not an accurate indicator of apoptosis in MCF-7 cells as fragmentation of DNA appears to vary between strains.

Keywords: Apoptosis; DNA laddering; MCF-7; Doxorubicin; Breast cancer

1. Introduction

Apoptosis, or programmed cell death, is a process whereby cells undergo an active process of cell suicide. Apoptosis is induced during normal developmental processes, and by environmental changes such as extracellular calcium and growth factor depletion, DNA-damaging agents such as UV irradiation, and cytotoxic agents such as doxorubicin and paclitaxel (for review, see [1]). Apoptosis has been well characterized and is thought to proceed through a series of conserved steps. Early steps include chromatin condensation, nuclear membrane blebbing, and cytoplasmic shrinkage. Late steps include loss of adherence, degradation of DNA, packaging of compacted organelles, condensed cytoplasm, and nuclear material into membrane-bound apoptotic bodies, and, finally, phagocytosis by neighboring cells [2,3].

Many techniques have been developed to detect programmed cell death. One of the most commonly used techniques for confirmation of apoptosis is identification of DNA ladders. This is based on the observation that cells undergoing apoptosis appear to cleave their DNA into very precise fragments of 180–200 base pairs [4], representing cleavage at internucleosomal junctions. DNA fragmented in this way forms a 'ladder' which can generally be detected by gel agarose electrophoresis of DNA isolated from apoptotic cells. However, identification of apoptosis based on DNA laddering has led to controversy as some cells show apparent morphologic induction of apoptosis in the absence of DNA fragmentation. One such cell line is the breast cancer cell line, MCF-7.

MCF-7 cells were the first human breast cancer

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cells to be permanently maintained in cell culture (for review, see [5]). Over the years, MCF-7 cells have been distributed to laboratories all over the world and have been continually maintained. This has led to the development of apparent 'strains' of MCF-7 cells. In some instances, selection for strain-specific characteristics has been done purposely as in the case of MCF-7-doxorubicin resistant (MCF-7-AdR) cells [6]. Beginning in 1985, researchers began reporting differences in MCF-7 characteristics between cells, which had been maintained in different laboratories, including differential sensitivity to tamoxifen [7], and differences in levels of expression of ER and PR as well as responsiveness to estrogen [8]. More recently, Burrow et al. reported that MCF-7 cells from different laboratories exhibit different susceptibility to tumor necrosis factor α (TNFα)-induced apoptosis [9].

While there are some published reports of MCF-7 cells producing apoptotic DNA ladders, [10,11] the majority of reports state that MCF-7 cells do not undergo apoptotic DNA fragmentation [12–15] leading some investigators to conclude that there are differences in the apoptotic process itself in these cells. Oberhammer et al. [15] examined the relationship between induction of morphologic characteristics of apoptosis including loss of adherence and chromatin condensation in a variety of cells and found that MCF-7 breast cancer cells exhibited changes in nuclear structure consistent with apoptosis but failed to produce DNA ladders. Further, Miller et al. reported that cell death due to serum deprivation, TNFα, or doxorubicin in MCF-7 cells was not associated with degradation of DNA [13]. Several other investigators have reported similar findings [12,14,16]. Due to the apparent conflict surrounding the formation of DNA ladders in MCF-7 cells, we decided to investigate to possibility of strain-specific differences in the ability of MCF-7 cells to undergo DNA fragmentation.

2. Materials and methods

2.1. Materials

The following strains of MCF-7 cells were obtained from various sources and maintained in our laboratory (University of Texas Health Science Center, San Antonio, TX), and for the purposes of these experiments were maintained in improved minimal essential medium (IMEM) plus phenol red supplemented with 5% fetal calf serum (Summit, Ft. Collins, MO): MCF-7-ATCC (passage 128), MCF-7 BK (passage 30, Benita Katzenellenbogen, University of Illinois City, IL), MCF-7-C and MCF-7-AdR (C- passage 125, AdR- passage 30, Ken Cowen, NIH, Bethesda, MD), MCF-7-D (passage 118, Hassadah Degani, Weizmann Institute of Science, Rehovot, Israel), MCF-7-L (passage 70, Marc Lippman, Georgetown University, Washington D.C.), and MCF-7-MG (passage 254, William McGuire, UTHSCSA, San Antonio, TX).

2.2. DNA laddering

Protocol was adapted from Lowe et al. [17]. Various strains of MCF-7 cells were plated at 2.0×10^6 cells per 10-cm dish. Cells were allowed to recover for 24 h and were then washed once with 1 × phosphate-buffered saline (PBS) before drug treatments were added in serum-free medium (SFM). After 48 h, adherent and non-adherent cells were collected, centrifuged at 1200 rev./min for 5 min to pellet all cells, washed with 1 × PBS, pelleted again, and then lysed in cold 0.15 M NaCl, 10 mM Tris–HCl (pH 7.8), 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 0.5% NP-40 on ice for 40 min. Lysates were centrifuged at 1500 rev./min for 10 min and pellets were resuspended in cold 0.35 M NaCl, 10 mM Tris–HCl, 1 mM $MgCl_2$, and 1 mM DTT on ice for 20 min. Lysates were then extracted once with phenol–chloroform and DNA was precipitated with 0.01 M $MgCl_2$ and 2.5 volumes 100% ethanol overnight at −20°C. DNA was collected by centrifugation at 14 000 rev./min for 20 min, resuspended in 10 mM Tris–1 mM EDTA plus 0.1 mg/ml RNase A, and incubated at 37°C for 1 h. Proteinase K (1 mg/ml) was added followed by incubation at 37°C for an additional hour. DNA was then electrophoresed in 1.5% agarose gels containing ethidium bromide.

2.3. Growth assay

Growth assays were performed by 3-[4,5–dimethylthiazol–2–yl]2,5diphenyltetrazolium bromide (MTT) assay as described [18]. MCF-7 cells were plated in quadruplicate at a density of 15 000

cells per well in 48-well cell culture plates. Cells were allowed to adhere overnight and were then washed once with 1 × PBS before increasing concentrations of drug were added in SFM for 48 h. Following treatment, 30 μl of MTT (5 mg/ml in PBS) were added to each well for 4 h. Medium and MTT were then removed, DMSO plus 2.5% IMEM was added, and absorbance was measured at 540 nm with a differential filter of 690 nm.

2.4. Flow cytometry analysis

MCF-7 cells were plated at 2.5×10^5 cells per well in a 6-well plate. Cells were allowed to recover for 24 h and were then washed once with 1 × PBS. The next day, drug treatment was added in SFM. Treatments were continued for 48 h and then adherent and non-adherent cells were collected, centrifuged at 1200 rev./min for 5 min to pellet all cells, washed with 1 × PBS, pelleted again, and then resuspended in 50 μl of PBS. Ice-cold 70% ethanol (100 μl) was added dropwise while vortexing. Cells were fixed overnight at −20°C and then 0.5 ng/ml propidium iodide plus 0.5 mg/ml RNase A were added. Cells were analyzed using a Facstar Plus (Becton-Dickinson, San Jose, CA) flow cytometer for detection of cells with sub-G1 DNA content. We have previously found that sub-G1 DNA content correlates well with other measures of apoptosis in MCF-7 cells [19].

3. Results

In order to determine if strain-specific differences affect the ability of MCF-7 cells to undergo DNA laddering, we treated seven different strains of MCF-7 cells with doxorubicin, a cytotoxic agent which is a well-characterized inducer of apoptosis [20,21]. We collected fragmented DNA and visualized laddering by agarose gel electrophoresis (Fig. 1). MCF-7-ATCC and MCF-7-BK did not produce DNA ladders following doxorubicin treatment whereas MCF-7-C, MCF-7-D, MCF-7-L and MCF-7-MG strains did produce DNA ladders. MCF-7-AdR cells, which were selected for resistance to doxorubicin, were included as a negative control. Interestingly, there appeared also to be differences in the amount of DNA laddering formed following doxorubicin treatment. Doxorubicin treatment of

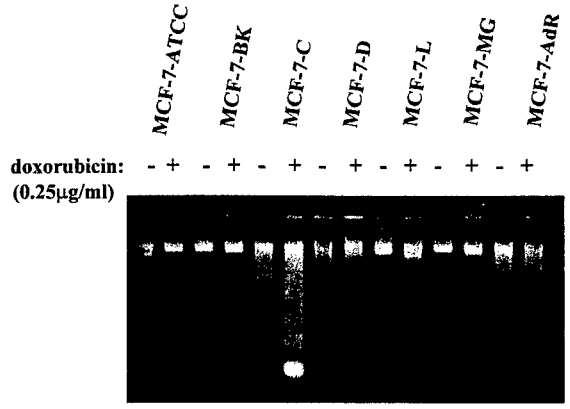

Fig. 1. MCF-7 cells show strain-specific differences in the induction of apoptotic DNA laddering. MCF-7 strains were treated with SFM alone or SFM plus 0.25 μg/ml doxorubicin for 48 h. Fragmented DNA was collected and separated by agarose gel electrophoresis. Data shown are representative of three separate experiments.

MCF-7-C and MCF-7-D produced a large amount of fragmented DNA while smaller amounts were seen in MCF-7-L and MCF-7-MG formed an intermediate amount. Also, MCF-7-C cells seemed to be more sensitive to growth factor withdrawal, as culture in SFM alone stimulated production of DNA laddering. To rule out the possibility that some MCF-7 strains fail to form DNA ladders following doxorubicin treatment alone, we also treated MCF-7 strains with paclitaxel, another potent inducer of apoptosis in human breast cancer cells [22]. MCF-7-ATCC, MCF-7-BK and MCF-7-AdR all failed to produce DNA ladders following paclitaxel treatment (data not shown). MCF-7-AdR cells, which express a multi-drug resistance phenotype, did produce ladders when grown to confluence in serum-starved conditions (data not shown).

MCF-7 cells appeared to have strain-specific differences in their ability to undergo DNA fragmentation following doxorubicin treatment. We, therefore, examined the responsiveness of the various strains to doxorubicin in monolayer culture. We treated the MCF-7 strains with increasing concentrations of doxorubicin and determined the effect of drug treatment on survival (Fig. 2). All strains were growth inhibited by doxorubicin in a dose-responsive manner, except for MCF-7-AdR, which were again included as a negative control. Therefore, the lack of DNA laddering observed in MCF-7-ATCC and MCF-7-

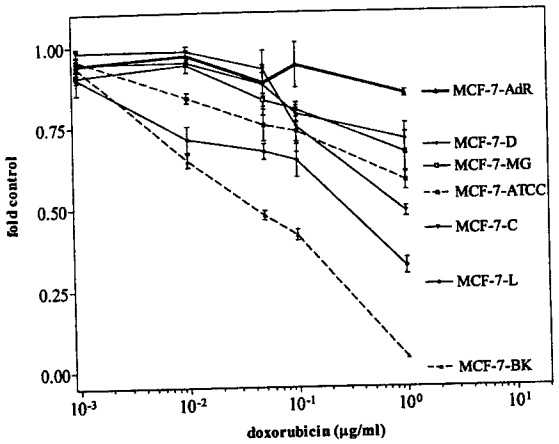

Fig. 2. MCF-7 strains show dose-dependent inhibition to doxorubicin in monolayer culture. MCF-7 strains were plated in quadruplicate in 48-well plates and treated with increasing concentrations of doxorubicin. Growth was determined after 48 h by MTT assay. Error bars represent the mean of quadruplicate samples ± SEM. Data shown are representative of two separate experiments.

BK was not due to a lack of growth inhibition by doxorubicin. In fact, MCF-7-BK cells were the most sensitive strain to doxorubicin-mediated growth inhibition.

Finally, we confirmed that doxorubicin induced apoptosis in the MCF-7 strains by an independent assay for measurement of apoptosis. The presence of sub G-1 DNA has been shown to correlate with apoptosis detected by other means [3,23] and can be used to quantitate the fraction of cells in a population which are undergoing apoptosis. We, therefore, examined sub-G1 DNA content by flow cytometry following doxorubicin treatment. Drug treatment resulted in a 3.2- and 3.7-fold increase of cells with sub-G1 DNA content in MCF-7-ATCC and MCF-7-MG cells, respectively; a small change (1.7-fold) was seen in MCF-7-AdR following doxorubicin treatment, although the absolute level of sub G1-DNA remained much lower than in the doxorubicin-sensitive cells (Fig. 3). Thus, an inability to detect DNA laddering is not associated with a deficiency in the capacity to undergo apoptosis.

4. Discussion

We have shown that MCF-7 strains differ in their ability to undergo apoptotic DNA fragmentation. Two strains, MCF-7-ATCC and MCF-7-BK do not appear to form DNA ladders after doxorubicin treatment while MCF-7-C, MCF-7-D, MCF-7-L, and MCF-7-MG do form DNA ladders. MCF-7-AdR cells were included in these experiments as a negative control for doxorubicin-induced apoptosis as they are resistant to doxorubicin. However, MCF-7-ATCC and MCF-7-BK also failed to undergo DNA ladders when treated with paclitaxel, another potent inducer of apoptosis in human breast cancer cells [22]. We have also shown that failure to exhibit apoptotic DNA ladders is not associated with a lack of response to cytotoxic drug as all breast cancer cell lines (except MCF-7-AdR) were growth-inhibited by doxorubicin in a dose-dependent manner. Furthermore, the lack of DNA laddering was also not associated with a failure to undergo apoptosis as doxorubicin induced the appearance of a sub-G1 peak in both MCF-7-ATCC and MCF-7-MG.

Our data suggest two things. First, DNA laddering represents a late event in the progression through apoptotic cell death and is not related to the ability of MCF-7 cells to undergo apoptosis, or to the amount of induction of apoptosis. Second, the ability of MCF-7 cells to undergo DNA fragmentation appears to be an event which has been either acquired or lost as a result of cell culture in some MCF-7 strains.

Degradation of DNA into internucleosomal fragments may represent an end-stage event. As a result, the exact nature of the DNA degradation may not be as important as the initiating events and thus apoptosis can occur fully in the absence of DNA fragmentation. Several investigators have offered alternative theories to explain the lack of precise packaging of DNA into internucleosomal fragments in some cells. For example, Oberhammer et al. demonstrated the formation of 50- and 300-kb DNA fragments in a variety of cell types including MCF-7 cells. The authors suggested that the large fragments represented cleavage of higher-order chromatin structures prior to and, in some cases, instead of degradation into 180- to 200-bp internucleosomal fragments [15]. This idea is supported by several other investigators [14,24,25]. Therefore, it appears likely that apoptotic degradation of DNA is a multi-step process that begins with cleavage of higher-order chromatin into large fragments followed by, in some cells, cleavage into internucleosomal fragments.

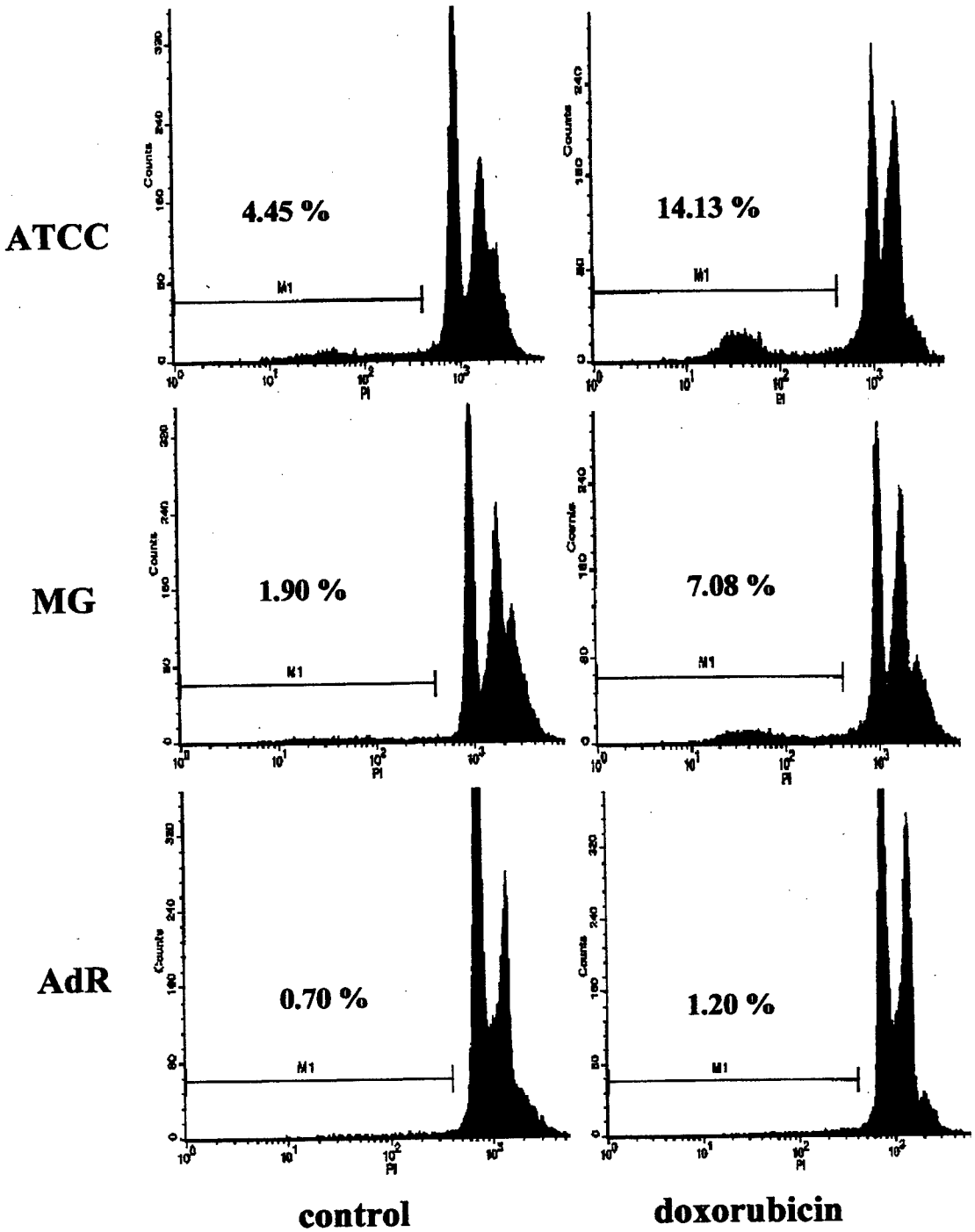

Fig. 3. MCF-7 strains undergo apoptosis irrespective of DNA laddering. MCF-7-ATCC, MCF-7-MG, and MCF-7-AdR were plated in triplicate in 6-well plates and treated with SFM alone or SFM plus 0.5 μg/ml doxorubicin for 48 h. Cells were collected, ethanol-fixed, stained with propidium iodide, and sorted by DNA content. Error bars represent the mean of triplicate samples ± SEM. Data shown are representative of three separate experiments.

It also appears likely that more than one endonucleolitic activity is required for the entire process. The identification of DFF40 by Wang et al. as the endonuclease responsible for targeted cleavage at the nucleosome linker histone H1 [26] begins to complete the story of apoptotic DNA degradation. Nevertheless, there may be other factors which act upstream of DFF40 that function in the early steps of DNA cleavage. Investigators tend to agree, however, that whether cells undergo internucleosomal DNA fragmentation or just higher-order cleavage, processing of DNA is a late event and apoptosis occurs independent of DNA laddering. Recent data from DFF45 (the regulatory subunit of DFF40) knockout mice show that while cells from a variety of tissues from DFF45 −/− mice fail to produce DNA ladders, the mice appear normal and undergo normal immune system development [27]. This further supports the idea that DNA laddering is a processing step that is not required for normal apoptosis.

The ability to undergo DNA fragmentation appears to represent a strain-specific characteristic of cultured MCF-7 cells. It is interesting to speculate that this characteristic is the result of either a gain-of-function or a loss-of-function due to prolonged cell culture. Cells from the original tumor were either capable of undergoing DNA fragmentation and some strains have subsequently lost the ability, or cells from the original tumor did not form DNA ladders and some strains have subsequently gained this function. Either of these possibilities may represent clonal selection. While much has been learned since DNA fragmentation was first reported in 1980 [4], the significance of precise apoptotic DNA fragmentation has yet to be fully defined. While DNA laddering appears to be a poor indicator of apoptosis in MCF-7 cells, these cells may provide a unique opportunity to further explore and perhaps manipulate DNA fragmentation and apoptosis.

Acknowledgements

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References

[1] R.A. Schwartzman, J.A. Cidlowski, Apoptosis: the biochemical and molecular biology of programmed cell death, Endocr. Rev. 14 (1993) 133–151.
[2] L.E. Gerschenson, R.J. Rotello, Apoptosis: a different type of cell death, FASEB J. 6 (1992) 2450–2455.
[3] M.M. Compton, A biochemical hallmark of apoptosis: internucleosomal degradation of the genome, Cancer Metas. Rev. 11 (1992) 105–119.
[4] A.H. Wyllie, Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation, Nature 284 (1980) 555–556.
[5] A.S. Levenson, V.C. Jordan, MCF-7: the first hormone-responsive breast cancer cell line, Cancer Res. 57 (1997) 3071–3078.
[6] P.J. Vickers, R.B. Dickson, R. Shoemaker, K.H. Cowan, A multidrug-resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth in vivo, Mol. Endocrinol. 2 (1988) 886–892.
[7] R. Reddel, L. Murph, R. Hall, R. Sutherland, Differential sensitivity of human breast cancer cell lines to growth-inhibitory effects of tamoxifen, Cancer Res. 45 (1985) 1525–1531.
[8] C.K. Osborne, K. Hobbs, J.M. Trent, Biological differences among MCF-7 human breast cancer cell lines from different laboratories, Breast Cancer Res. Treat. 9 (1987) 111–121.
[9] M.E. Burrow, C.B. Weldon, Y. Tang, G.L. Navar, S. Krajewski, J.C. Reed, T.G. Hammond, S. Clejan, B.S. Beckman, Differences in susceptibility to tumor necrosis factor α-induced apoptosis among MCF-7 breast cancer cell variants, Cancer Res. 58 (1998) 4940–4946.
[10] N. Kyprianou, H.F. English, N.E. Davidson, J.T. Isaacs, Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation, Cancer Res. 51 (1991) 162–166.
[11] J.L. Gooch, C.L. Van Den Berg, D. Yee, Insulin-like growth factor (IGF) -I rescues breast cancer cells from chemotherapy-induced cell death – proliferative and anti-apoptotic effects, Breast Cancer Res. Treat. (1999) in press.
[12] A.M. Warri, R.L. Huovinen, A.M. Laine, P.M. Martikainen, P.L. Harkonen, Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro, J. Natl. Cancer. Inst. 85 (1993) 1412–1418.
[13] T. Miller, L.A. Beausang, M. Meneghini, G. Lidgard, Death-induced changes to the nuclear matrix: the use of anti-nuclear matrix antibodies to study agents of apoptosis, Biotechniques 15 (1993) 1042–1047.
[14] F.A. Fornari Jr, W.D. Jarvis, S. Grant, M.S. Orr, J.K. Randolph, F.K.H. While, V.R. Mumaw, E.T. Lovings, R.H. Freeman, D.A. Gewirtz, Induction of differentiation and growth arrest associated with nascent (nonoligosomal) DNA

fragmentation and reduced c-myc expression in MCF-7 human breast tumor cells after continuous exposure to a sublethal concentration of doxorubicin, Cell Growth Diff. 5 (1994) 723–733.

[15] F. Oberhammer, J.W. Wilson, C. Dive, A.E. Wakeling, P.R. Walker, M. Sikorska, Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation, EMBO J. 12 (1993) 3679–3684.

[16] G.M. Cohen, X.-M. Sun, R.T. Snowden, D. Dinsdale, D.N. Skilleter, Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation, Biochem. J. 286 (1992) 331–334.

[17] S.W. Lowe, H.E. Ruley, T. Jacks, D.E. Housman, p53-dependent apoptosis modulates the cytotoxicity of anticancer agents, Cell 74 (1993) 957–967.

[18] P.R. Twentyman, M. Luscombe, A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity, Br. J. Cancer 56 (1987) 279–285.

[19] J. Gooch, A.V. Lee, D. Yee, Interleukin-4 inhibits growth and induces apoptosis in human breast cancer cells, Cancer Res. 58 (1998) 4199–4205.

[20] A. Skladanowski, J. Konopa, Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumor cells, Biochem. Pharm. 46 (1993) 375–382.

[21] S.A. Rasbridge, C.E. Gillett, A.-M. Seymour, K. Patel, M.A. Richards, R.D. Rubens, R.R. Millis, The effects of chemotherapy on morphology, cellular proliferation, apoptosis and oncoprotein expression in primary breast carcinoma, Br. J. Cancer 70 (1994) 335–341.

[22] D.E. Saunders, W.D. Lawrence, C. Christensen, N.L. Wappler, H. Raun, G. Deppe, Paclitaxel-induced apoptosis in MCF-7 breast cancer cells, Int. J. Cancer 70 (1997) 214–220.

[23] I. Nicoletti, G. Migliorati, M.C. Pagliacci, F. Grignani, C. Riccardi, A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry, J. Immunol. Methods 139 (1991) 271–279.

[24] L.D. Tomei, J.P. Shapiro, F.O. Cope, Apoptosis in C3H/10T 1/2 mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double-strand cleavage, Proc. Natl. Acad. Sci. USA 90 (1993) 853–857.

[25] D.G. Brown, X.-M. Sun, G.M. Cohen, Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation, J. Biol. Chem. 268 (1992) 3037–3039.

[26] X. Liu, P. Li, P. Widlak, H. Zou, X. Luo, W.T. Garrard, The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis, Proc. Natl. Acad. Sci. USA 95 (1998) 8461–8466.

[27] J. Zhang, X. Liu, D.C. Scherer, L. van Kaer, X. Wang, M. Xu, Resistance to DNA fragmentation and chromatin condensation in mice lacking DNA fragmentation factor 45, Proc. Natl. Acad. Sci. USA 95 (1998) 12480–12485.

The Role of p21 in Interferon γ-mediated Growth Inhibition of Human Breast Cancer Cells[1]

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Abstract

IFN-γ-mediated growth inhibition requires signal transducers and activators of transcription (STAT)-1 activation and may require induction of the cyclin-dependent kinase inhibitor p21. Using an electrophoretic mobility shift assay, we identified STAT1 activation after IFN-γ treatment in breast cancer cell lines. Accordingly, IFN-γ inhibited proliferation of monolayer cultured MCF-7 and MDA-MB-231 breast cancer cells. Interestingly, IFN-γ inhibited anchorage-independent growth of MCF-7 cells but had no effect on MDA-MB-231colony formation. Because p21 has been shown to play a role in anchorage-independent growth and is a transcriptional target of STAT1, we examined the effect of IFN-γ on p21 mRNA. We found that IFN-γ induced p21 mRNA in MCF-7 cells but not in MDA-MB-231 cells. Furthermore, IFN-γ induced activation of a p21 promoter-luciferase reporter construct that contained the STAT1-inducible element in MCF-7 cells, but not in MDA-MB-231 cells. IFN-γ treatment resulted in increased p21 protein in MCF-7 cells, whereas MDA-MB-231 cells did not appear to express detectable p21, even after IFN-γ treatment. However, in MDA-MB-231 cells, p21 protein was detected only after proteosome inhibition, suggesting that degradation may be responsible for the undetectable level of p21 in these cells, despite the abundant mRNA levels. Finally, focus formation of MDA-MB-231 cells was inhibited by overexpression of p21. In conclusion, STAT1 activation does not appear to be sufficient for IFN-γ-mediated growth inhibition. Furthermore, the role of p21 appears to be complex because monolayer growth inhibition occurs in the absence of p21, but anchorage-independent growth inhibition may require p21. Breast cancer cells may provide a unique model for further study of IFN-γ signaling.

Introduction

IFN-γ is a pleiotropic immunoregulatory cytokine that functions to enhance cellular immune response by increasing T-cell cytotoxicity and natural killer cell activity. Furthermore, IFN-γ increases expression of HLA genes and up-regulates expression of HLA class I and II molecules in untransformed as well as transformed cells (1, 2). In addition to its role as a immunoregulatory factor, IFN-γ inhibits the growth of a number of nonhematopoietic cell types, including tumor cells (3–7). In fact, IFN-γ has been considered as an antitumor therapeutic and has been tested in the treatment of human cancer (8–11). In metastatic breast cancer, IFN-γ has been shown to enhance the growth-inhibitory effect of tamoxifen (12, 13).

IFN-γ action begins with binding of the cytokine to a heterodimeric receptor that induces activation of the JAK[3]/STAT pathway (14, 15). JAK proteins are tyrosine kinases that associate with non-tyrosine kinase receptors and phosphorylate the receptor, other JAK proteins, and downstream signaling molecules such as STAT proteins. In the case of IFN-γ signaling, JAK1 and JAK2 associate with the receptor and then recruit and phosphorylate STAT1. After phosphorylation, STAT1 dimerizes and then translocates to the nucleus to activate transcription (16). Several cell cycle-regulatory proteins have been shown to be modulated by IFN-γ treatment, including CDK inhibitor p21 (17, 18), retinoblastoma protein (19), and CDK2 (17).

STAT1 is activated by a number of ligands besides IFN-γ including prolactin, platelet-derived growth factor, colony-stimulating factor 1, EGF, interleukin 10, and IFN-α (20–22). It has been shown that EGF- and IFN-γ-mediated growth inhibition requires activation of STAT1. EGF-mediated growth inhibition of A431 cells is abrogated by the expression of dominant negative STAT1 (23). Also, the STAT1-deficient cell line U3A was found to be unaffected by IFN-γ (24, 25). When STAT1 was transfected into this cell line, IFN-γ-mediated growth inhibition was restored (18, 26). Chin *et al.* (18) demonstrated that after EGF stimulation, STAT1 and STAT3 molecules can bind to SIEs within the p21 promoter. Furthermore, up-regulation of p21 mRNA and protein has been shown to be associated with IFN-γ-mediated growth inhibition (18, 27). As a result, it has been proposed

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[3] The abbreviations used are: JAK, Janus-activated kinase; STAT, signal transducers and activators of transcription; CDK, cyclin-dependent kinase; EGF, epidermal growth factor; SIE, c-*sis*-inducible element; β-gal, β-galactosidase; IMEM, improved MEM; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SFM, serum-free medium.

that the mechanism of IFN-γ-induced growth inhibition is STAT1-mediated transactivation of p21.

Whereas IFN-γ signaling has been characterized in some systems, less is known about IFN-γ-mediated growth inhibition and signal transduction in breast cancer cells. Harvat and Jetten (3) showed that IFN-γ significantly inhibited the growth of normal mammary epithelial cells and induced a G_1 arrest but only slightly inhibited the growth of breast cancer cells. The authors proposed that the difference in response to IFN-γ was due to defects in the IFN-γ signal transduction pathway in breast cancer cells. Therefore, we examined the effect of IFN-γ on the growth of breast cancer cell lines and investigated IFN-γ activation of STAT1 and its putative transcriptional target, p21.

We show that two breast cancer cell lines activate STAT1 in response to IFN-γ. In addition, IFN-γ inhibits monolayer growth of MCF-7 and MDA-MB-231 breast cancer cell lines, but anchorage-independent growth is inhibited only in MCF-7 cells. Whereas MCF-7 cells up-regulate p21 mRNA and protein levels after IFN-γ treatment, MDA-MB-231 cells failed to up-regulate p21 mRNA in response to IFN-γ. MDA-MB-231 cells do not express detectable p21 protein, except in the presence of specific inhibitors of the proteosome. Therefore, degradation of p21, in addition to transcriptional regulation, may be a mechanism for controlling expression. As a result, the role of p21 in IFN-γ-mediated growth inhibition appears to be complex; inhibition of growth in monolayer culture occurs in the absence of p21, but inhibition of anchorage-independent growth appears to require p21. Breast cancer cell lines may provide a unique model system for further elucidation of the role of p21 in IFN-γ-mediated signal transduction.

Results

MCF-7 and MDA-MB-231 Cells Activate STAT1 in Response to IFN-γ. IFN-γ-induced growth inhibition has been shown to be dependent on activation of the transcription factor STAT1 (18, 28). We examined IFN-γ induction of activated STAT1 in MCF-7 and MDA-MB-231 cells by electrophoretic mobility shift assay using the high affinity SIE site from the c-*fos* gene as a probe. The fibroblast cell line A431 has been previously shown to activate STAT1 and STAT3 after stimulation with EGF (29, 30) and was therefore included as a positive control for STAT1 activation. After IFN-γ treatment, extracts from both MCF-7 and MDA-MB-231 cells formed DNA-protein complexes that appeared to contain STAT1 (Fig. 1*A*). We confirmed the identity of the DNA-protein complex by incubating MCF-7 and MDA-MB-231 protein extracts with an antibody to STAT1. We observed a supershifted complex in both cell lines, whereas the addition of STAT2 antibody had no effect on the mobility of the complex (Fig. 1*B*).

IFN-γ Inhibits the Growth of Breast Cancer Cell Lines in Monolayer Culture and Increases the G_0-G_1 Fraction of the Cell Cycle. IFN-γ has been reported to inhibit the growth of a number of different cell types, including normal mammary epithelial cells and breast cancer cell lines (3, 4, 7, 8). We first examined the monolayer growth of two breast cancer cell lines treated with IFN-γ over a period of 4 days

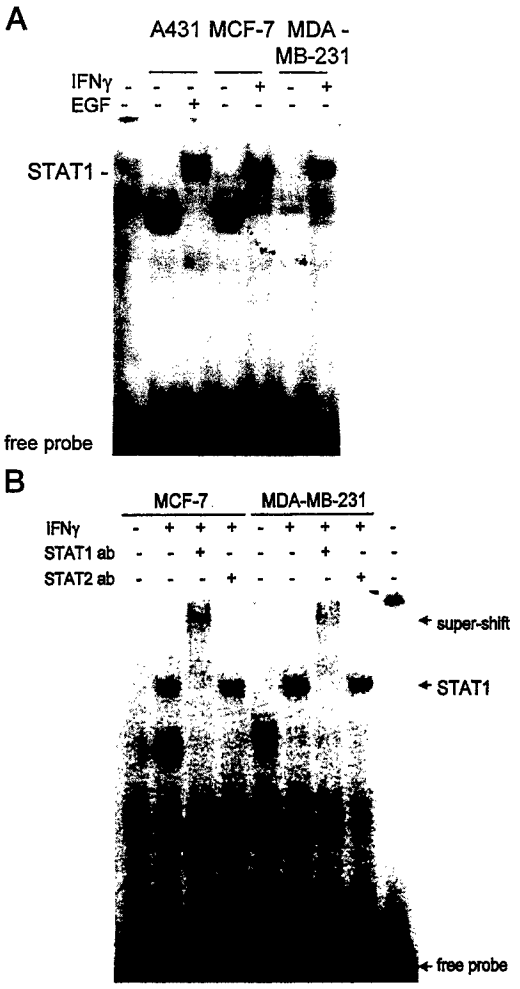

Fig. 1. STAT1 DNA binding is activated by IFN-γ in both MCF-7 and MDA-MB-231 cells. *A*, MCF-7 and MDA-MB-231 whole cell lysates were treated with and without 10 ng/ml IFN-γ and incubated with the labeled probe representing a STAT1 consensus binding site from the c-fos promoter. A431 cells treated with 30 ng/ml EGF were included as a positive control for STAT1 and STAT3 binding. Complexes were resolved by PAGE and autoradiography. *B*, MCF-7 and MDA-MB-231 whole cell lysates were incubated with labeled probe, and antibodies to STAT1 or STAT2 were added. Complexes were resolved by PAGE and visualized by autoradiography. Only STAT1 antibodies resulted in a supershift of the complex.

and found that growth of MCF-7 cells and MDA-MB-231 cells was significantly inhibited by IFN-γ (Fig. 2; $P < 0.002$ and $P < 0.001$ for MCF-7 and MDA-MB-231 cells, respectively). However, MCF-7 cells, which are hormone and growth factor dependent, experienced a lag in proliferation when incubated with IFN-γ. This inhibition was seen only in the presence of a low amount of serum (0.25%) or estradiol (data not shown) and not when cells were cultured in the absence of mitogenic stimulation. Thus, cell proliferation was required to see the inhibitory effects of IFN-γ, although the effect was weak because IFN-γ was unable to inhibit the growth effects of 5% serum. This supports previous findings that IFN-γ-mediated growth inhibition is related to control of the cell cycle.

IFN-γ-mediated growth inhibition has previously been attributed to an arrest of the cell cycle in G_1 (17, 19). Therefore,

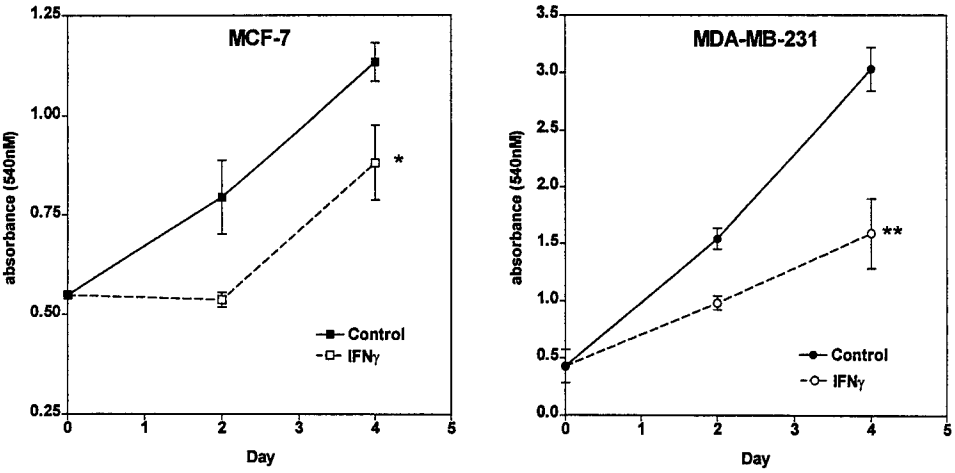

Fig. 2. IFN-γ inhibits the growth of MCF-7 and MDA-MB-231 cells in monolayer culture. MCF-7 cells were cultured in SFM plus 0.25% serum, and MDA-MB-231 cells were cultured in SFM alone. IFN-γ (10 ng/ml) was added, and growth was determined by MTT assay at days 0, 2, and 4. Each *point* represents the mean of triplicate samples ± SE. *Asterisks* represent statistically significant differences due to IFN-γ. *, $P < 0.002$; **, $P < 0.001$.

we examined the cell cycle distribution of MCF-7 and MDA-MB-231 cells after IFN-γ treatment by flow cytometry. Table 1 shows cell cycle distributions of MCF-7 and MDA-MB-231 cells treated with IFN-γ. Both cell lines demonstrated a statistically significant increase in G_0-G_1 cells ($P < 0.05$ for MCF-7 cells and $P < 0.04$ for MDA-MB-231 cells) and decreases in S-phase and G_2-M-phase cells after IFN-γ treatment, consistent with the decrease observed in monolayer growth.

Because growth in soft agar is one hallmark of the transformed phenotype, we also examined the response of breast cancer cell lines to IFN-γ in an anchorage-independent growth assay. Colony formation of MCF-7 cells in soft agar was significantly inhibited by the addition of IFN-γ ($P < 0.007$). However, IFN-γ had no effect on the colony formation of MDA-MB-231 cells (Fig. 3). It has been reported previously that growth in soft agar has different requirements than proliferation in monolayer culture. Growth under anchorage-independent conditions has been associated with changes in cell cycle-regulatory proteins (31, 32). Furthermore, anchorage-independent growth can be inhibited by overexpression of p21 (33, 34). Because p21 is also a putative STAT1 transcriptional target, we next examined the effect of IFN-γ on p21 protein levels.

MCF-7 Cells, but not MDA-MB-231 Cells, Up-Regulate p21 mRNA in Response to IFN-γ. It has been reported that IFN-γ may induce growth inhibition by increasing transcription of the CDK inhibitor p21 (17, 35). It has also been reported that p21 may be transcriptionally up-regulated by STAT binding to a SIE within the p21 promoter (18). Moreover, IFN-γ-mediated up-regulation of p21 appears to be concomitant with activation of STAT1 DNA binding (27). Using a RNase protection assay, we examined the effect of IFN-γ on p21 mRNA levels in MCF-7 and MDA-MB-231 cells. RNA from A431 cells treated with EGF was included as a positive control for p21 mRNA up-regulation. Eight h after exposure, the levels of p21 were quantitated by densitometric analysis and then normalized by the levels of 36B4 (included as a loading control). In MCF-7 cells, a modest increase in p21 mRNA was detected after IFN-γ treatment. Whereas p21 mRNA was detectable in MDA-MB-231 cells, IFN-γ treatment did not appear to increase the level of mRNA (Fig. 4). Also, labeled probe hybridized with tRNA, which did not result in a protected band, was included as a negative control (data not shown).

Table 1 IFN-γ-mediated growth inhibition is associated with an increase in G_0-G_1 and a decrease in S phase and G_2-M phase of the cell cycle

MCF-7 and MDA-MB-231 cells were cultured in IMEM plus 5% FCS and treated with 10 ng/ml IFN-γ for 24 h. Cells were fixed in ethanol, stained with propidium iodide, and sorted by DNA content. Data shown are the mean of duplicate samples ± SD. Data shown are representative of three separate experiments.

	G_0-G_1	*P*	S phase	*P*	G_2-M phase	*P*
MCF-7						
Control	44.4 ± 3.1		37.7 ± 3.1		17.9 ± 0.2	
IFN-γ	53.2 ± 1.0[a]	0.05	31.9 ± 0.5	0.10	14.5 ± 1.5	0.17
MDA-MB-231						
Control	51.2 ± 3.8		33.4 ± 2.3		15.3 ± 1.6	
IFN-γ	66.9 ± 1.7[a]	0.04	25.9 ± 0.4[a]	0.04	7.1 ± 1.5[a]	0.03

[a] $P \leq 0.05$.

A Region of the p21 Promoter Containing the STAT-inducible Element Is Required for IFN-γ-mediated Transcriptional Activation. We next examined IFN-γ-mediated transcriptional activity of a p21 promoter-luciferase construct. It has been shown that STAT1 binds to a SIE element located at −603 bp and induces transcription of the *p21* gene (18). Therefore, we looked at activation of the p21 promoter by IFN-γ in MCF-7 and MDA-MB-231 cells using a series of deletion constructs. MCF-7 cells showed significant ($P < 0.05$) inducible activation of the −837-bp promoter fragment when treated with IFN-γ. When MCF-7 cells were transfected with a promoter fragment that was −567 bp in length and therefore lacked the SIE, no induction by IFN-γ was seen (Fig. 5). Also, activity on a promoter fragment that was −143 bp in length was comparable with activation of vector alone. In contrast, when compared with raw luciferase values in MCF-7 cells, IFN-γ treatment resulted in only minimal induction of the −843-bp fragment in MDA-MB-231 cells. β-gal values, determined as a control for transfection efficiency, were similar in MCF-7 and MDA-MB-231 cells and

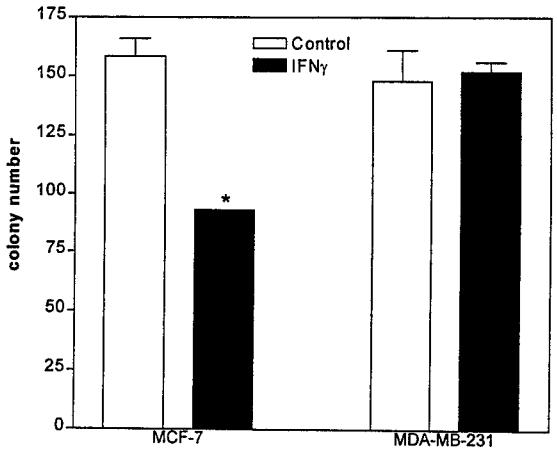

Fig. 3. IFN-γ inhibits anchorage-independent growth of MCF-7 cells but not MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were plated in soft agar with or without 10 ng/ml IFN-γ. Colonies were allowed to form and were counted after 10 days. *Error bars*, the mean of duplicate samples ± SE.

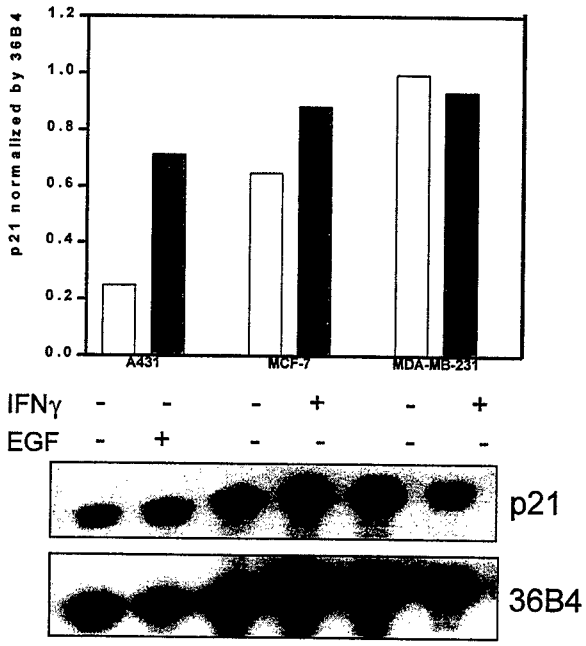

Fig. 4. IFN-γ induces increased p21 transcription in MCF-7 cells but not in MDA-MB-231 cells. RNA from MCF-7 and MDA-MB-231 cells treated with and without 10 ng/ml IFN-γ was collected, and p21 mRNA expression was detected by RNase protection assay. RNA from A431 cells treated with and without 30 ng/ml EGF was included as a positive control. Hybridization to labeled probe representing 36B4 was used as a loading control. p21 message was quantitated, and values for p21 divided by 36B4 are depicted as a bar graph. Data shown are representative of three independent experiments.

did not change with transfection of different constructs or IFN-γ treatment (data not shown). Therefore, IFN-γ appears to induce activation of the *p21* gene primarily through a region containing the SIE in MCF-7 cells, but in MDA-MB-231 cells, IFN-γ did not significantly induce the activity of any p21 promoter fragments.

IFN-γ Treatment Results in Increased p21 Protein in MCF-7 Cells; no p21 Protein Is Detectable in MDA-MB-231 Cells, Even after IFN-γ Treatment. We next determined whether the increase in p21 mRNA due to IFN-γ resulted in increased p21 protein in MCF-7 cells. The fibroblast cell line A431 which has also been shown to up-regulate p21 in response to EGF, was included as a positive control. After 8 h of treatment, both A431 and MCF-7 cells responded with an increase in p21 protein. Interestingly, MDA-MB-231 cells did not appear to express detectable p21 protein, even after IFN-γ treatment (Fig. 6).

We considered that the absence of p21 protein in the presence of detectable p21 mRNA could be due to a mutation in the coding region of the gene. Although mutations of p21 are rare, several have been described previously (36, 37). After sequencing the complete coding region of the *p21* gene in MDA-MB-231 cells, we determined that the gene contained a previously described Ser-Arg polymorphism at codon 31 (38), but no mutations (data not shown). An alternative explanation for the lack of detectable protein could therefore be constitutive degradation of p21 protein in MDA-MB-231 cells. Therefore, we treated MDA-MB-231 and MCF-7 cells with lactacystin, an inhibitor of the p26 subunit of the proteosome (39, 40), for increasing lengths of time and examined p21 protein expression. In Fig. 7, we show that lactacystin treatment increased p21 protein levels in MCF-7 cells after about 4 h. Interestingly, we also found that inhibition of the proteosome resulted in rescue of p21 protein in MDA-MB-231 cells as well.

To further investigate the loss of p21 protein in MDA-MB-231 cells and the role of p21 in growth inhibition, we transiently transfected MDA-MB-231 cells with p21 and then examined the ability of MDA-MB-231 cells to form foci. MCF-7 cells were also transfected as a positive control. Fig. 8*A* shows that p21 protein was expressed in pooled MDA-MB-231 cells transiently transfected with a copy of the *p21* gene, but not when cells were transfected with vector alone. Colony formation was significantly ($P < 0.05$) inhibited in both MCF-7 and MDA-MB-231 cells after transfection with p21 (Fig. 8*B*).

Discussion

In MCF-7 cells, IFN-γ treatment results in the activation of STAT1, up-regulation of p21 mRNA and protein, and activation of a p21 promoter construct containing a SIE. These data, along with the finding that IFN-γ inhibits the growth of MCF-7 cells in both monolayer and anchorage-independent growth, strongly support the idea that IFN-γ inhibits growth via activation of STAT1, which subsequently transcriptionally up-regulates p21. MDA-MB-231 cells, however, are inhibited in monolayer culture by IFN-γ and show a statistically significant increase in G_0-G_1 phase but do not up-regulate p21 mRNA in response to IFN-γ or express detectable p21 protein. This suggests that IFN-γ may inhibit monolayer growth via a p21-independent signaling mechanism. Interestingly, MDA-MB-231 cells were not inhibited by IFN-γ in an anchorage-independent growth assay, but foci formation is inhibited when p21 is ectopically expressed, suggesting a possible role for p21 in IFN-γ-mediated inhibition of colony formation.

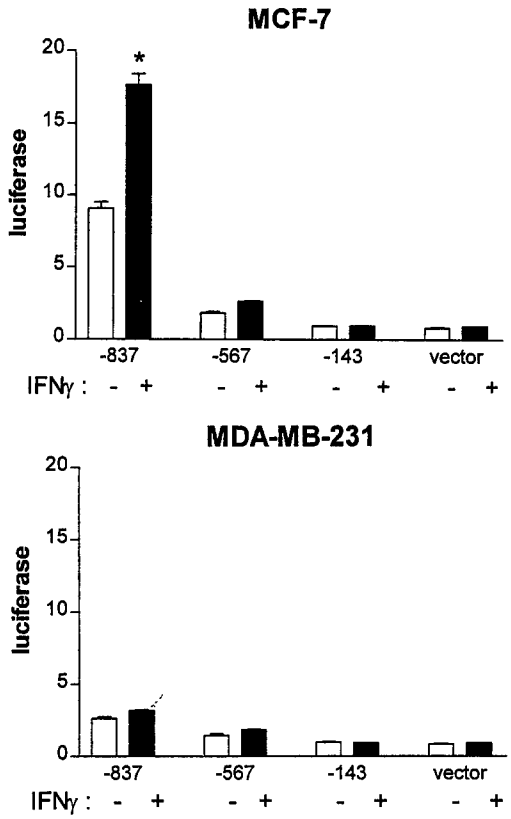

Fig. 5. STAT1 transactivates a p21 promoter-luciferase reporter construct in MCF-7 cells but not in MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were transfected with constructs containing a luciferase gene downstream of various p21 promoter constructs. The cells were treated with 10 ng/ml IFN-γ for 24 h, and then luciferase was measured. *Error bars*, the mean of triplicate samples ± SE. The *asterisk* represents a statistically significant difference between control and IFN-γ ($P < 0.05$). Data shown are representative of three separate experiments.

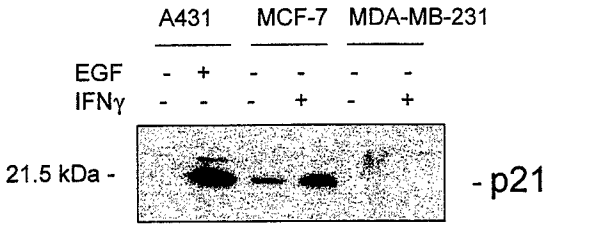

Fig. 6. p21 protein is induced by IFN-γ in MCF-7 cells but not in MDA-MB-231 cells. Total protein was collected from MCF-7 and MDA-MB-231 cells treated with and without 10 ng/ml IFN-γ. Lysates were then separated by SDS-PAGE and immunoblotted with p21 antibody. Total protein from A431 cells treated with EGF was included as a positive control for the induction of p21 protein.

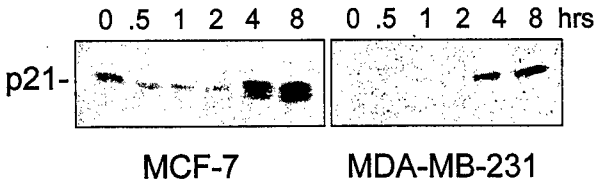

Fig. 7. Inhibition of the proteosome rescues p21 protein in MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with lactacystin (10 μM), a specific inhibitor of the proteosome, for increasing lengths of time. p21 protein was then detected by Western blotting.

Our data show that the inability of IFN-γ to induce p21 is due to at least two defects in MDA-MB-231 cells. First, whereas STAT1 is activated after IFN-γ treatment and can bind to a SIE construct, no increase in p21 mRNA or promoter activity can be detected. The lack of p21 mRNA induction by IFN-γ and the lack of promoter activation associated with STAT1 suggest that there may be transcriptional defects in this pathway in MDA-MB-231 cells. Recent work by Zhang *et al.* (41) suggests that STAT1 may cooperate with other transcription factors to activate transcription at discrete sites within a promoter. In MDA-MB-231 cells, STAT1 activation alone is insufficient to enhance transcription of STAT1-regulated genes.

Second, the lack of detectable protein despite the production of mRNA suggests that regulation of p21 expression in MDA-MB-231 cells could also occur posttranscriptionally. Our data indicated that p21 was regulated by degradation of the protein. Inhibition of the proteosome with lactacystin restored a detectable level of p21 in MDA-MB-231 cells. The p21 protein level could also be enhanced by inhibition of degradation in MCF-7 cells, indicating that degradation may be a common means of regulating p21 protein. In addition to degradation of the protein, it is also possible that p21 is regulated by other posttranscriptional mechanisms. Esposito *et al.* (42) reported that induction of p21 protein in cells exposed to oxidative stress (a p53-independent mechanism) occurs via a posttranscriptional mechanism of mRNA stabilization. The authors suggest that manipulation of mRNA stability might be a way to rapidly control levels of p21 protein in the absence of p53. In the case of MDA-MB-231 cells, it is interesting to speculate that in addition to mutation of the *p53* gene (43), the hormone- and growth factor-independent cell line has developed a mechanism that enhances protein degradation in addition to a means that prevents the transcriptional induction of p21 by inhibitory cytokines such as IFN-γ.

Our data show that whereas IFN-γ inhibits the growth of both MCF-7 and MDA-MB-231 cells in monolayer culture, only MCF-7 cells are inhibited in anchorage-independent growth. There is ample evidence in the literature to suggest that there are different requirements for adherent growth such as monolayer culture and anchorage-independent growth in soft agar (31, 32). Transformation releases cells from dependence on an adherent matrix for growth and allows the formation of colonies in soft agar. This release from anchorage dependence has been characterized by alterations in cell cycle proteins and activities. Therefore, it is particularly interesting that IFN-γ-mediated growth inhibition of MCF-7 cells represents a reversion of the transformed phenotype, as evidenced by decreased growth in soft agar, whereas IFN-γ fails to change MDA-MB-231 anchorage-independent growth. One explanation for this difference may be the lack of p21 expression by MDA-MB-231 cells because increased p21 expression has been shown to be associated with decreased colony formation in soft agar (33, 34).

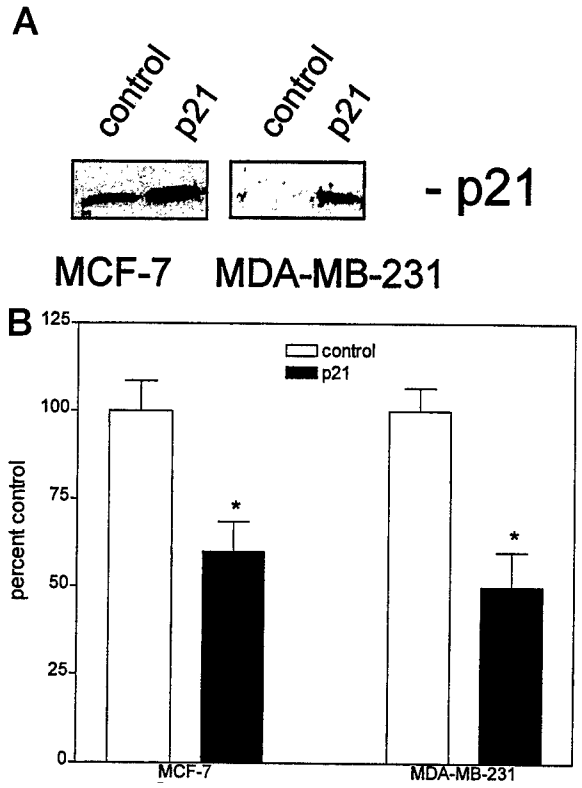

Fig. 8. Transfected p21 is expressed in MDA-MB-231 cells and inhibits foci formation. *A*, MDA-MB-231 and MCF-7 cells were transfected with p21 or vector alone (pCDNA3.1), and lysates were collected, separated by SDS-PAGE, and immunoblotted for p21. *B*, MDA-MB-231 and MCF-7 cells were transfected with p21 or vector alone and then selected with neomycin for 3 weeks. The resulting foci were stained with 1% crystal violet and counted. *Asterisks* represent statistically significant differences due to p21 ($P < 0.05$).

Breast cancer cells suggest a complex role for p21 in IFN-γ-mediated growth inhibition. Monolayer growth of breast cancer cells is inhibited by IFN-γ, although p21 protein is not detectable in MDA-MB-231 cells, suggesting that IFN-γ-mediated growth inhibition may be p21 independent. However, anchorage-independent growth of MDA-MB-231 cells is not inhibited by IFN-γ, and this is associated with a lack of p21 protein. Therefore, whereas monolayer growth inhibition by IFN-γ may occur in the absence of p21, anchorage-independent growth appears to require p21 expression. The inability of IFN-γ to inhibit tumor growth could be due to multiple post receptor defects, and human breast cancer cell lines appear to provide a unique model system for further investigation.

Materials and Methods

Materials

MCF-7 cells were provided by C. Kent Osborne (University of Texas Health Science Center, San Antonio, TX) and maintained in IMEM (Life Technologies, Inc., Bethesda, MD) plus phenol red supplemented with 5% fetal bovine serum (Summit, Ft. Collins, CO). MDA-MB-231 and A431 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in IMEM without phenol red plus 5% fetal bovine serum. Insulin-like growth factor I was obtained from GroPep (Adelaide, Australia), and IFN-γ was obtained from Sigma (St. Louis, MO). STAT1, STAT2, and p21 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoretic Mobility Shift Assay

STAT1 was detected with the high-affinity STAT-binding site from the c-*fos* gene promoter (GTGACATTTCCCGTAAATC; Ref. 44). Extracts were made as follows: cells were treated with 10 ng/ml IFN-γ for 45 min and then washed once with 1× PBS and harvested with trypsin-EDTA. Cells were centrifuged, and pellets were resuspended in high-salt homogenization buffer [20 mM Tris-HCl (pH 7.5), 2 mM DTT, 20% glycerol, 0.4 M KCl, 10 mg/ml pepstatin, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml antipain, and 100 mM phenylmethylsulfonyl fluoride]. Protein (10 μg) was incubated with end-labeled probe at room temperature for 30 min. For the supershift reaction, 1 μg of antibody was added to protein extracts and incubated at room temperature for 30 min before the addition of labeled probe. Reactions were run on nondenaturing 4% acrylamide, 0.5% Tris-borate EDTA gels. Gels were dried and exposed to film. Data shown are representative of three separate experiments.

Cell Growth Assays

All growth assays were performed at least three separate times.

Monolayer Growth. Growth assays were performed by MTT assay as described previously (45). MCF-7 cells were plated in triplicate at a density of 18,000 cells/well, and MDA-MB-231 cells were plated at 12,000 cells/well in 24-well cell culture plates. Cells were allowed to adhere overnight and then washed once in 1× PBS; culture medium was replaced with SFM overnight. Cells were then treated with IFN-γ in SFM supplemented with 1% FCS. After treatment, 60 μl of MTT (5 mg/ml in PBS) were added to the medium for 4 h. Medium and MTT were then removed, DMSO and 2.5% DMEM were added, and absorbance was measured at 540 nm.

Anchorage-independent Growth. MCF-7 cells were plated at a density of 15,000 cells/plate, and MDA-MB-231 cells were plated at a density of 7,500 cells/plate. Cultures were prepared with a base layer of IMEM supplemented with 20% FCS and containing 0.5% low-melting point agarose (Sea Plaque; FMC Bioproducts, Rockland ME). Cells were then plated over the base layer in duplicate in IMEM supplemented with 10% FCS and containing 0.5% low-melting point agarose. IFN-γ was added at a concentration of 10 ng/ml, and cells were allowed to grow for 7–10 days before colonies of at least 20 cells were counted.

Flow Cytometry. MCF-7 and MDA-MB-231 cells were plated at 0.5×10^6 cells/60-mm dish. Cells were allowed to adhere overnight and then washed once in 1× PBS; culture medium was replaced with SFM overnight. Cells were then treated with IFN-γ in SFM supplemented with 1% FCS. Treatments continued for 48 h, and cells were then washed with 1× PBS, harvested with trypsin-EDTA, pelleted, washed with 1× PBS, pelleted again, and resuspended in 100 μl of PBS. Ice-cold 70% ethanol (200 μl) was added dropwise while vortexing. Cells were fixed overnight at −20°C, and then 0.5 ng/ml propidium iodide and 0.5 mg/ml RNase A were added. Cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) and gated on forward light scatter, pulse height, and pulse width for analysis of cell cycle fractions. Resulting histograms were evaluated using Modfit LT software (Verity House, Topsham, ME).

Western Blots

Cells were treated with 30 ng/ml EGF or 10 ng/ml IFN-γ for 8 h, harvested with trypsin-EDTA, pelleted, and washed with 1× PBS. Protein was extracted using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1% NP40, 100 mM NaCl, 100 mM sodium orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotinin, and 10^{-7} M phenylmethylsulfonyl fluoride. Protein (50 μg) was analyzed by 12% SDS-PAGE, and after transfer of the proteins to nitrocellulose, the membrane was incubated in 5% milk-Tris-buffered saline Tween 20 [.15 M NaCl, .01 Tris-HCl (pH 7.4), .05% Tween 20] and then immunoblotted with a 1:1000 dilution of anti-p21 antibody. Horseradish peroxidase-conjugated goat antimouse secondary antibody was added at a 1:2000 dilution, and proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Data shown are representative of repeated experiments.

RNase Protection Assay

RNA from A431, MCF-7, and MDA-MB-231 cells treated with SFM or SFM plus 10 ng/ml IFN-γ for 8 h were isolated using the guanidinium thiocyanate method (46), measured by spectrophotometry, and checked for integrity by separation on a 1% formaldehyde-agarose gel. RNase protection was performed according to our previously published method (47), and RNA loading was corrected with the ribosomal protein 36B4 (48). Briefly, 20 μg of RNA were hybridized with radiolabeled antisense complementary RNAs (cRNAs) transcribed from p21 and 36B4 cDNAs. The p21 RNase protection probe was generated by *Pst*I restriction digestion. The resulting 300-bp fragment was subcloned into pGEM4Z. pGEM4Z-p21 was linearized with Xho, and transcription with T7 RNA polymerase was carried out in the presence of [^{32}P]UTP to produce labeled antisense cRNA. For 36B4, a 145-bp *Pst*I-*Pst*I fragment was cloned into pGEM4Z, linearized with *Eco*RI, and transcribed with T7 RNA polymerase. After hybridization of RNA with radiolabeled probe, single-stranded RNA was digested with RNase A, and samples were separated on 8 M urea/6% SDS-PAGE. tRNA was hybridized as a negative control. The gel was dried and exposed to X-ray film. Data shown are representative of repeated experiments.

Promoter Assays

p21 promoter-luciferase constructs were made as described previously (49) and were a gift from Dr. L. P. Freedman (Memorial Sloan Kettering Cancer Center, New York, NY). Cells (2.5×10^5) were plated in triplicate in DMEM + 5% FCS in 6-well plates and transfected the next day. Briefly, cells were washed once with PBS and transiently cotransfected with 1.0 μg of each promoter construct plus 0.1 μg of pSVβ-gal using Lipofectin transfection reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Transfected DNA was left on the cells overnight. The next morning, media were changed to control or 10 ng/ml IFN-γ for 24 h. Cells were harvested, and luciferase was measured using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. β-Gal activity was measured as described by Rouet *et al.* (50). Luciferase values were divided by the appropriate β-gal value. Resulting values for each p21-promoter-luciferase construct were then normalized by values for vector alone and are therefore expressed as arbitrary units. Data shown are representative of repeated experiments.

Statistics

Statistical analyses were calculated on representative experiments. For MCF-7 and MDA-MB-231 monolayer growth, two-way ANOVA was used. For analysis of anchorage-independent growth, reporter assays, and foci formation assays, Student's *t* test was used.

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References

1. Min, W., Pober, J., and Johnson, D. Kinetically coordinated induction of TAP1 and HLA class I by IFN-γ: the rapid induction of of TAP1 by IFN-γ is mediated by STAT1 α. J. Immunol., *156:* 3174–3183, 1996.

2. Mach, B., Steimle, V., Martinez-Soria, E., and Reith, W. Regulation of MCH class II genes: lessons from a disease. Annu. Rev. Immunol., *14:* 301–303, 1996.

3. Harvat, B., and Jetten, A. γ-Interferon induces an irreversible growth arrest in mid-G_1 in mammary epithelial cells which correlates with a block in hyperphosphorylation of retinoblastoma. Cell Growth Differ., *7:* 289–300, 1996.

4. Mueller, H., Flury, N., Liu, R., Scheidegger, S., and Eppenberger, U. Tumour necrosis factor and interferon are selectively cytostatic *in vitro* for hormone-dependent and hormone-independent human breast cancer cells. Eur. J. Cancer, *32A:* 2312–2318, 1996.

5. Supriya, P., Joshi, C., and Ajitkumar, P. IFN-γ inhibits growth of WISH cells in a cell cycle phase-specific manner. J. Interferon Cytokine Res., *18:* 215–217, 1998.

6. Buard, A., Vivo, C., Monnet, I., Boutin, C., Pilatte, Y., and Jaurand, M. C. Human malignant mesothelioma cell growth: activation of Janus kinase 2 and signal transducer and activator of transcription 1α for inhibition by interferon-γ. Cancer Res., *58:* 840–847, 1998.

7. Fujishima, H., Nakano, S., Tatsumoto, T., Masumoto, N., and Niho, Y. Interferon-α and -γ inhibit the growth and neoplastic potential of v-src-transformed human epithelial cells by reducing Src tyrosine kinase activity. Int. J. Cancer, *76:* 423–429, 1998.

8. Platanias, L. Interferons: laboratory to clinic investigations. Curr. Opin. Oncol., *7:* 560–565, 1995.

9. Friedman, R. M. Antitumor effects of interferons. J. Exp. Pathol., *3:* 203–222, 1987.

10. Gleave, M., Mostafa, E., Fradet, Y., Davis, P., Saad, F., Klotz, L., Moore, M., Paton, V., and Bajamonde, A. Interferon γ-1b compared with placebo in metastatic renal-cell carcinoma. N. Engl. J. Med., *338:* 1265–1271, 1998.

11. Baron, S., Tyring, S. K., Fleischmann, W. R., Coppenhaver, D. H., Niesel, D. M., Klimpel, G. R., Stanton, G. J., and Hughes, T. K. The interferons: mechanisms of action and clinical applications. J. Am. Med. Assoc., *266:* 1378–1383, 1991.

12. Seymour, L., and Bezwoda, E. C. Interferon plus tamoxifen treatment for advanced breast cancer: *in vivo* biological effects of two growth modulators. Br. J. Cancer, *68:* 352–356, 1993.

13. Macheledt, J., Budzar, A., Hortobagyi, G., Frye, D., Gutterman, J., and Holmes, F. Phase II evaluation of interferon added to tamoxifen in the treatment of metastatic breast cancer. Breast Cancer Res. Treat., *18:* 165–170, 1991.

14. Darnell, J. STATs and gene regulation. Science (Washington DC), *277:* 1630–1635, 1997.

15. Greenlund, A. C., Farrar, M. A., Viviano, B. L., and Schreiber, R. D. Ligand-induced IFN γ receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). EMBO J., *13:* 1591–1600, 1994.

16. Shui, K. Interferon-activated signal transduction to the nucleus. Curr. Opin. Cell Biol., *6:* 253–259, 1994.

17. Subramaniam, P., and Johnson, H. A role for the cyclin-dependent kinase inhibitor p21 in the G_1 cell cycle arrest mediated by type I interferons. J. Interferon Cytokine Res., *17:* 11–15, 1997.

18. Chin, Y. E., Kitahawa, M., Su, W-C. S., You, Z-H., Iwamoto, Y., and Fu, X-Y. Cell growth arrest and induction of cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$ mediated by STAT1. Science (Washington DC), *272:* 719–722, 1996.

19. Resnitzky, D., Tiefenbrun, T., Berissi, H., and Kimchi, A. Interferons and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietic cells. Proc. Natl. Acad. Sci. USA, *89:* 402–406, 1992.

20. Schaber, J., Fang, H., Xu, J., Grimley, P., and Rui, H. Prolactin activates stat1 but noes not antagonize stat1 activation and growth inhibition by type I interferons in human breast cancer cells. Cancer Res., *58:* 1914–1919, 1998.

21. Larner, A., David, M., Feldman, G., Igarashi, K-i., Hackett, R., Webb, D., Sweitzer, S., Petrocoin, E., III, and Finblo, D. Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. Science (Washington DC), *261:* 1730–1733, 1993.

22. Jamison-Ruff, S., Chen, K., and Cohen, S. Induction by EGF and interferon-γ of tyrosine phosphorylated DNA binding proteins in mouse liver nuclei. Science (Washington DC), *261:* 1733–1736, 1993.

23. Bromberg, J. F., Fan, Z., Brown, C., Mendelsohn, J., and Darnell, J. E. Epidermal growth factor-induced growth inhibition requires stat1 activation. Cell Growth Differ., *9:* 505–512, 1998.

24. McKendry, R., John, J., Flavell, D., Mueller, M., Kerr, I., and Stark, G. High-frequency mutagenesis of human cells and characterization of mutant unresponsive to both α andγ interferons. Proc. Natl. Acad. Sci. USA, *88:* 11455–11459, 1991.

25. Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improta, T., Darnell, J. E., Jr., Stark, G. R., and Kerr, I. M. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon α and γ signal transduction pathways. EMBO J., *12:* 4221–4228, 1993.

26. Bromberg, J., Horvath, C. M., Wen, Z., Schreiber, R. D., and Darnell, J. E., Jr. Transcriptionally active stat1 is required for the antiproliferative effects of both interferon α and interferon-γ. Proc. Natl. Acad. Sci. USA, *93:* 7673–7678, 1996.

27. Su, W-C., Kitagawa, M., Xue, N., Xie, B., Garofalo, S., Cho, J., Deng, C., Horton, W., and Fu, X-Y. Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. Nature (Lond.), *386:* 288–292, 1997.

28. Xu, X., Fu, X-Y., Plate, J., and Chong, A-F. IFN-γ induces cell growth inhibition by fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of fas and fasL expression. Cancer Res., *58:* 2832–2837, 1998.

29. Zhong, Z., Wen, Z., and Darnell, J. Stat3: a stat family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science (Washington DC), *264:* 95–98, 1994.

30. Sadowski, H., and Gilman, M. Cell-free activation of a DNA-binding protein by epidermal growth factor. Nature (Lond.), *362:* 79–83, 1993.

31. Resnitzky, D. Ectopic expression of cyclin D1 but not E induces anchorage-independent cell cycle progression. Mol. Cell. Biol., *17:* 5640–5647, 1997.

32. Kang, J., and Krauss, R. Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. Mol. Cell. Biol., *16:* 3370–3380, 1996.

33. Chen, J., Willingham, T., Shuford, M., Bruce, D., Rushing, E., Smith, Y., and Nisen, P. D. Effects of ectopic overexpression of $p21^{WAF1/CIP1}$ on aneuploidy and the malignant phenotype of human brain tumor cells. Oncogene, *13:* 1395–1403, 1996.

34. Michieli, P., Li, W., Lorenzi, M., Miki, T., Zakut, R., Givol, D., and Pierce, J. Inhibition of oncogene-mediated transformation by ectopic expression of $p21^{Waf1}$ in NIH3T3 cells. Oncogene, *12:* 775–784, 1996.

35. Chang, E. H., Ridge, J., Black, R., Zou, Z. Q., Masnyk, T., Noguchi, P., and Harford, J. B. Interferon-γ induces altered oncogene expression and terminal differentiation in A431 cells. Proc. Soc. Exp. Biol. Med., *186:* 319–326, 1987.

36. Kawasaki, T., Tomita, Y., Bilim, V., Takeda, M., Takahashi, K., and Kumanishi, T. Abrogation of apoptosis induced by DNA-damaging agents in human bladder-cancer cell lines with p21/WAF1/CIP1 and/or p53 gene alterations. Int. J. Cancer, *68:* 501–505, 1996.

37. Bhatia, K., Fan, S., Spangler, G., Weintraub, M., O'Connor, P., Judde, J-G., and Magrath, I. A mutant p21 cyclin-dependent kinase inhibitor isolated from a Burkitt's lymphoma. Cancer Res., *55:* 1431–1435, 1995.

38. Chedid, M., Michieli, P., Lengel, C., Huppi, K., and Givol, D. A single nucleotide substitution at codon 31 (Ser/Arg) defines a polymorphism in a highly conserved region of the p53-inducible gene WAF1/CIP1. Oncogene, *9:* 3021–3024, 1994.

39. Dick, L., Cruikshank, A., Grenier, L., Melandri, F., Nunes, S., and Stein, R. Mechanistic studies on the inactivation of the proteosome by lactacystin: a central role for clasto-lactacystin β-lactone. J. Biol. Chem., *271:* 7273–7276, 1996.

40. Dick, L., Cruikshank, A., Destree, A., Grenier, L., McCormack, T., Melandri, F., Nunes, S., Palombells, V., Parent, L., Plamondon, L., and Stein, R. Mechanistic studies on the inactivation of the proteosome by lactacystin in cultured cells. J. Biol. Chem., *272:* 182–188, 1997.

41. Zhang, J., Vinkemeier, Y., Gu, W., Chakravarti, D., Horvath, C., and Darnell, J. J. Two contact regions between Stat1 and CBP/p300 in interferon γ signaling. Proc. Natl. Acad. Sci. USA, *93:* 15092–15096, 1996.

42. Esposito, F., Cuccovillo, F., Vanoni, M., Cimino, F., Anderson, C. W., Apella, E., and Russo, T. Redox-mediated regulation of $p21^{waf1/cip1}$ expression involves a post-transcriptional mechanism and activation of the mitogen-activated protein kinase pathway. Eur. J. Biochem., *245:* 730–737, 1997.

43. Thor, A. D., Moore, D. H., III, Edgerton, S. M., Kawasaki, E. S., Reihsaus, E., Lynch, H. T., Marcus, J. N., Schwartz, L., Chen, L. C., Mayall, B. H., and Smith, H. S. Acculumation of p53 tumor supressor gene protein: an independent marker of prognosis in breast cancers. J. Natl. Cancer Inst., *84:* 845–855, 1992.

44. Wagner, B. J., Hayes, T. E., Hoban, C. J., and Cochran, B. H. The SIF binding element confers sis/PDGF inducibility onto the c-fos promoter. EMBO J., *9:* 4477–4484, 1990.

45. Twentyman, P. R., and Luscombe, M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. Br. J. Cancer, *56:* 279–285, 1987.

46. Chirgwin, J. M., Przybyla, A. I., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry, *18:* 5294–5299, 1979.

47. Figueroa, J. A., Lee, A. V., Jackson, J. G., and Yee, D. Proliferation of cultured human prostate cancer cells is inhibited by insulin-like growth factor binding protein-1: evidence for an IGF-II autocrine loop. J. Clin. Endocrinol. Metab., *80:* 3476–3482, 1995.

48. Laborda, J. 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. Nucleic Acids Res., *19:* 3998, 1991.

49. Liu, M., Ivarone, A., and Freedman, L. P. Transcriptional activation of the human $p21^{WAF1CIP1}$ gene by retinoic acid receptor. Correlation with retinoid induction of U397 cell differentiation. J. Biol. Chem., *271:* 31723–31728, 1996.

50. Rouet, P., Ranguenez, G., and Salier, J-P. Optimized assays for quantifying transient expression of co-transfected β-galactosidase and CAT-reporter genes. Biotechniques, *13:* 700–701, 1992.