Ectopic Expression of Interferon Regulatory Factor-1 Promotes Human Breast Cancer Cell Death and Results in Reduced Expression of Survivin

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ABSTRACT

The overexpression of the inhibitor of apoptosis protein, survivin, may provide tumor cells with a distinct survival advantage in situ; hence, therapeutic strategies have been designed to inhibit its expression. In this study, we ectopically expressed the interferon regulatory factor (IRF)-1 protein in the breast carcinoma cell lines MDA-MB-468 and SK-BR-3 using a recombinant adenovirus (Ad-IRF-1). By screening microarray analysis of cDNA from the human breast cancer cell line MDA-MB-468 infected with Ad-IRF-1, we observed a 15-fold down-regulation of the survivin gene when compared with uninfected cells. Consequently, we tested survivin expression in Ad-IRF-1–infected MDA-MB-468 and SK-BR-3 breast cancer cell lines. Immunoblotting analyses supported the contention that ectopic expression of the IRF-1 protein results in down-regulation of survivin protein expression that is independent of p53. In addition, Ad-IRF-1 infection of these human breast cancer cell lines induces the expression of p21. We also report that increased apoptosis is observed in tumor cells infected with Ad-IRF-1 compared with Ad-W5 mock-infected cells and that cell death is further augmented when the IRF-1–infected cells are cultured with Adriamycin. Moreover, in a xenogeneic mouse model of breast carcinoma, in vivo treatment of tumor-bearing mice with intratumoral Ad-IRF-1 injections results in tumor growth inhibition. In resected tumors from mice that had been treated with Ad-IRF-1, tumor cells that express the IRF-1 transgene have a predominant IRF-1–positive, survivin-negative phenotype. Collectively, these observations suggest that therapies designed to enhance IRF-1 expression within tumor cells may represent novel treatment strategies for breast cancer.

INTRODUCTION

The interferon regulatory factor (IRF)-1 transcription factor was originally identified as a regulator of the human interferon (IFN)-β gene (1), and more recently, IRF-1 has been found to be involved in signal transduction of other ligands, including tumor necrosis factor and interleukin-1β (2), interleukin-12 (3), and CD40 ligand (4). In addition, several studies have shown that IRF-1 may function as a potential tumor suppressor. In fact, ectopic expression of IRF-1 can revert oncogene-transformed cultured cell lines to a normal phenotype (5), whereas the loss of IRF-1 contributes to tumor development in conjunction with c-Ha-ras in vivo (6). In breast cancer, IRF-1 expression has been found to be decreased in a significant proportion of cancer cells compared with adjacent normal breast parenchyma (7). More recently, basal IRF-1 mRNA was found to be lower in an antiestrogen-unresponsive, estrogen receptor-positive breast cancer tumor cell line when compared with antiestrogen-sensitive, estrogen receptor-positive (8) cell lines. Although the antiestrogen tested was able to induce IRF-1 mRNA in estrogen receptor-positive antiestrogen-sensitive breast cancer tumor lines, IRF-1 could not be induced in the antiestrogen-resistant estrogen receptor-positive cell line (8). In contrast, down-regulation of target genes by IRF-1 has not been thoroughly investigated. To evaluate potential genes that may be repressed by IRF-1 induction, we screened microarray analysis of cDNA isolated from the human breast cancer cell line MDA-MB-468 infected with Ad-IRF-1 and observed a 15-fold down-regulation of the inhibitor of apoptosis survivin gene that was not seen in uninfected cells. Moreover, the microarray analysis showed no differences in survivin in Ad-W5–infected MDA-MB-468 cells when compared with uninfected cells.

Survivin is a 16,500 intracellular protein (9), characterized by a 70-amino acid motif termed BIR (10), and it has been shown to inhibit cell death induced by various apoptotic stimuli (10–12). In genome-wide searches, survivin constituted the fourth top transcriptome in various cancers, yet its expression was low or undetectable in identical normal tissues (13). Among 60 human tumor cell lines used for the National Cancer Institute’s anticancer drug screening program, the highest relative levels of survivin were present in lung and breast cancer cell lines (10). In primary tissues, survivin mRNA has been detected in 90.2% of cases of breast cancers investigated, whereas only 23% of adjacent noncancerous breast tissues expressed detectable levels of survivin (14). Using an antisurvivin antibody, 70.7% of breast cancer specimens of histologic stages I to III obtained from patients with invasive breast carcinomas who did not receive any form of treatment before surgery expressed cytoplasmic survivin. Adjacent normal tissues did not express the survivin protein (15). Similarly, in other studies, 79.1% of breast carcinomas were positive for survivin expression (16). In addition, the apoptotic index was significantly lower in survivin-positive tumors than in survivin-negative tumors, and the overall 5-year survival for patients with survivin-positive tumors was less than patients with survivin-negative tumors. Taken together, these data suggest that decreased apoptosis that correlated with survivin expression may be a predictive indicator of poorer prognosis in breast carcinoma (15).

Given these correlates, therapeutic strategies have targeted survivin expression. Antisense oligonucleotides and dominant negative survivin mutants that inhibit survivin expression or function have been evaluated (17). In addition, a phosphorylation-defective survivin mutant, Thr56→Ala, has been shown to trigger apoptosis and enhance cell death in both in vitro and in vivo model systems (18). In addition, adenoviruses (Ads) encoding ribozymes were shown to decrease the level of survivin mRNA in MCF-7 breast carcinoma cells and resulted in a 2-fold increase in the number of apoptotic cells (19).

In these current studies, we show a decrease in survivin expression and increased cell death after infecting MDA-MB-468 and SK-BR-3 breast cancer cells to ectopically express the IRF-1 using a recombinant Ad (Ad-IRF-1). These data supported our screening microarray analysis that revealed a 15-fold down-regulation of survivin in Ad-IRF-1–infected MDA-MB-468 cells when compared with uninfected cells.
IRF-1–infected MDA-MB-468 tumor cells compared with uninfected cells. Reduction in survivin expression occurs in a p53-independent manner. Interestingly, p21 was up-regulated after Ad-IRF-1 infection of tumor cells. Increased apoptosis was observed in IRF-1–infected cells, and tumor cell death was enhanced when IRF-1–infected cells were cultured with the chemotherapeutic drug Adriamycin. Lastly, in vivo studies show tumor growth inhibition in mice treated with the Ad-IRF-1 recombinant virus, with resected tumor nodules exhibiting deficient expression of survivin after IRF-1 infection in situ.

MATERIALS AND METHODS

Cell Lines and Culture. The MDA-MB-468, SK-BR-3, and MCF-7 cell lines were purchased from American Type Culture Collection (Manassas, VA). The human breast carcinoma MCF-7 expresses wild-type p53 protein (20). The MDA-MB-468 and SK-BR-3 breast carcinomas harbor mutations in p53. MDA-MB-468 has a codon change at position 273, whereas SK-BR-3 has a codon change at position 175 (21). The MDA-MB-468 cell line has an Arg-His amino acid change that abolishes DNA binding (22), whereas SK-BR-3 harbors an arginine residue to histidine mutation, resulting in the loss of all known p53 functions (23). The MDA-MB-468 tumor cells were propagated in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) and Ham’s F-12 (Invitrogen Life Technologies, Carlsbad, CA) media at a 1:1 ratio. SK-BR-3 cells were grown in RPMI 1640 (BioWhittaker), and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, and 0.01 mg/mL bovine insulin. All cell cultures were maintained in a humidified atmosphere of 5% CO2/95% air at 37°C.

Microarray Analysis by Affymetrix Chip Hybridization. For cDNA preparation, total RNA was extracted and purified from Ad-5, Ad-IRF-1, and uninfected MDA-MB-468 cells with the Qiagen RNeasy kit (Qiagen, San Diego, CA), and the chips were prepared and analyzed as described previously (24).

Ad-IRF-1 Construction and Characterization. The construction of these viruses has been described previously (24). Briefly, a recombinant E1- and E3-deleted Ad containing the mouse IRF-1 gene under a constitutive cytomegalovirus promoter (Ad-IRF-1) was constructed through Cre-lox recombinase as described previously (25). Briefly, the mouse IRF-1 gene was inserted into the shuttle vector pAdlux to create Ad-IRF-1. Recombinant Ad-IRF-1 was generated by cotransfection of SfiI-digested pAdlux-mIRF-1 and V5 helper virus DNA into the Ad packaging cell line CRE8, which expresses Cre recombinase. Recombinant viruses were propagated on CRE8 or 293 cells and purified by cesium chloride density gradient centrifugation and subsequent dialysis. Titers of viral particles were determined by optical density at 550 nm. Ad-IRF-1 was a kind gift from Dr. S. Hardy (Somatix, Alameda, CA). Confirmation of high efficiency adenoviral infection was performed by infection with the enhanced green fluorescent protein. Cells were infected with increasing multiplicities of infection (MOIs), and infected cells were identified by fluorescent microscopy. The functionality of the recombinant Ad-IRF-1 construct had been confirmed by immunofluorescence staining showing increased expression of major histocompatibility complex (MHC) class I proteins (26, 27) in TS/A and C3L5 murine mammary adenocarcinomas and up-regulation of MHC class I in MDA-MB-468, SK-BR-3, MCF-7, and other human breast cell lines infected with Ad-IRF-1. In addition, the growth inhibitory response of MDA-MB-468 and SK-BR-3 cells infected with Ad-IRF-1 was confirmed via the reduction of the yellow tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay and compared to Ad-5 control-infected cells. Cytokines well known to be up-regulated by IRF-1, including IFN-α and IFN-β (28), have also been demonstrated in high levels in the media of all Ad-IRF-1–infected breast cancer cell lines versus controls by ELISA. Moreover, we performed liposome transfection of the MDA-MB-468 cell line with a plasmid encoding the human IRF-1 gene, and we observed identical down-regulation of survivin in transfected cells versus controls, but only at one time point because of the transient and less efficient nature of liposome transfection (data not shown).

Ad-IRF-1 Infection. MDA-MB-468 cells plated in 10-cm plates were washed once in PBS, and serum-free Opti-MEM (Invitrogen Life Technologies) media were added. For adenoviral infection, recombinant Ad at a MOI of 10 was added. Cells were subsequently incubated for 4 hours at 37°C at 5% CO2. After 4 hours, the serum-free media were aspirated, and fresh culture media were added. For the SK-BR-3 cells, a MOI of 25 was used. The MCF-7 cells were infected at a MOI of 50. Previous experiments were conducted to determine the MOIs necessary to achieve equal IRF-1 protein expression in each cell line.

Western Blots. Immunoblotting was performed as described previously (24). Anti-IRF antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti survivin (1:1,000; R&D Systems, Inc., Minneapolis, MN), anti-p53 (1:250; BD PharMingen, San Diego, CA), anti-human Bel-2 (BD Transduction Laboratories, San Jose, CA), and antihuman p21 (1:250; BD PharMingen) were added for 1 hour at room temperature. Equal loading of protein was assessed by using β-actin (1:1,000; Abcam, Inc., Cambridge, MA) as a control. Horseradish peroxidase-conjugated goat antimouse or antirabbit at a concentration of 1:10,000 was added. Protein bands were visualized with Supersignal (West Pico Chemiluminescent Substrate; Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer’s instructions. The bands were exposed on Kodak film (Eastman Kodak, Rochester, NY) to detect the chemiluminescence signals. Densitometric visualization was performed using the White/UV Transilluminator (UVP Products, Upland, CA), and analysis was performed using Labworks (UVP Products) software.

Apoptosis Assay. To evaluate the induction of apoptosis, MDA-MB-468 cells were either uninfected (Opti-MEM alone) or infected with Ad-5 or Ad-IRF-1. After 24 hours, Adriamycin (500 nmol/L; Sigma-Aldrich Corp., St. Louis, MO) was added to the uninfected or infected cultures. Twenty-four hours after Adriamycin addition, apoptosis of tumor cells was measured by staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide using the annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions (BD PharMingen). Cell apoptosis was analyzed by flow cytometry using a Beckman Coulter cytometer (Fullerton, CA).

In vivo Treatment of Tumor-Bearing Mice. All animal studies were conducted in accordance with the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council’s Guide for the Care and Use of Laboratory Animals. Female Nude nu/nu-nu (4–6 weeks old) or Fox Chase severe combined immunodeficient (SCID) mice (29–35 days old) were purchased from Charles River Laboratories International (Wilmington, MA).

MDA-MB-468 cells growing in log phase were suspended in Hanks’ balanced salt solution, and 6 × 106 cells were inoculated subcutaneously along the mammary line of female SCID mice. When tumors reached 30 to 35 mm3 by perpendicular measurements, mice were randomized (n = 5–8 mice per group) and received intratumoral injection with 4 × 106 plaque-forming units (pfu) of Ad-IRF-1, Ad-5, or saline in a total volume of 30 μL. Treatment was repeated approximately every 12 days, with a total of five treatments. Tumors were measured by serial measurements of perpendicular diameters using digital calipers. Tumor volumes were calculated according to the formula 0.5 × (width)2 × length (29). Animals that were moribund or had tumors greater than approximately 4,189 mm3 in volume were sacrificed.

Immunohistochemistry of Resected Tumor Nodules. Nude mice were inoculated with 1 × 107 MDA-MB-468 cells resuspended in Hanks’ balanced salt solution. When tumors reached 4 × 4 × 4 mm by perpendicular measurements, mice were treated intratumorally with 4 × 106 pfu of Ad-IRF-1 or Ad-5 in a total of 30 μL. Treated tumors were excised from nude mice 24 hours after treatment and placed in 2% paraformaldehyde. Immunohistochemistry was performed to evaluate IRF-1 and survivin protein expression. The C-20 rabbit polyclonal antibody purchased from Santa Cruz Biotechnology was used to detect IRF-1, and a polyclonal antibody that detects survivin purchased from PharMingen (Transduction Laboratories, San Jose, CA) was used as a control. Horseradish peroxidase-conjugated goat antimouse or antirabbit at a concentration of 1:10,000 was added. Protein bands were visualized with Supersignal (West Pico Chemiluminescent Substrate; Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer’s instructions. The bands were exposed on Kodak film (Eastman Kodak, Rochester, NY) to detect the chemiluminescence signals. Densitometric visualization was performed using the White/UV Transilluminator (UVP Products, Upland, CA), and analysis was performed using Labworks (UVP Products) software.

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was added for 30 seconds, and samples were washed in PBS and then mounted. Several photos (×10 magnification) of the whole tumor were taken with an Olympus BX51 fluorescent microscope (Olympus America, Melville, NY).

Statistical Analyses. Murine tumor dimensions were measured three times a week from the 3rd through the 10th week. Tumor volumes were calculated and natural log-transformed to stabilize daily variances. Tumor growth rate was described with random coefficient polynomial regression models. Tests of treatment group differences were conducted for 1 day each week by comparing predicted (population marginal) means and their SE. P values were adjusted by simulated data from the null multivariate t distribution with the same covariance as the observed data. Differences in tumor growth with adjusted P < 0.05 were considered statistically significant. For apoptosis studies, data were transformed to improve normality and stabilize variances by taking the square root of the percentage of cells that were positive for annexin V. The data were then analyzed in a two-way factorial design that tested Adriamycin at two treatment group differences were conducted for 1 day each week by comparing predicted (population marginal) means and their SE. P values were adjusted by simulated data from the null multivariate t distribution with the same covariance as the observed data. Differences in tumor growth with adjusted P < 0.05 were considered statistically significant. For apoptosis studies, data were transformed to improve normality and stabilize variances by taking the square root of the percentage of cells that were positive for annexin V. The data were then analyzed in a two-way factorial design that tested Adriamycin at two

RESULTS

Decreased Expression of Survivin in Cells Infected with Ad-IRF-1. Our initial screening of microarray analysis of cDNA from the human breast cancer cell line MDA-MB-468 infected with Ad-IRF-1 showed a 15-fold down-regulation of survivin gene expression compared with uninfected cells. Moreover, the microarray analysis showed no differences in survivin expression in Ad-Ψ5-infected MDA-MB-468 cells when compared with uninfected cells. To confirm these data, we infected MDA-MB-468 and SK-BR-3 breast cancer cell lines with Ad-IRF-1 or the Ad-Ψ5 vector control at a MOI of 10 or 25, respectively, and harvested cells at 12-hour intervals for Western blotting analysis.

In previous studies, we had confirmed the functionality of the Ad-IRF-1 virus in these and other breast cancer cell lines by confirming the expression of MHC class I (26, 27, 30) and the secretion of IFN-α and IFN-β known to be up-regulated by IRF-1 in all Ad-IRF-1-infected breast cancer cell lines versus the control treatments (refs. 26 and 28; data not shown).

Ad-IRF-1–infected MDA-MB-468 and SK-BR-3 cells expressed IRF-1 by 12 hours after infection with an apparent peak at 24 hours (Fig. 1). Expression was absent in the Ad-Ψ5 control-infected cells. The expression of survivin was also investigated in these infected cell lines and in uninfected cells. In support of our microarray analyses, the MDA-MB-468 Ad-IRF-1–infected cells expressed reduced levels of the survivin protein (Fig. 2). At 48 hours after infection, p21 expression was observed at 36 hours after infection, and the most profound decrease in survivin expression was observed at 48 hours after infection (Fig. 2A). Survivin expression was also decreased by ~2-fold in the SK-BR-3 Ad-IRF-1–infected cells at the 36 hour time point, with an additional reduction observed at the 48 hour time point (Fig. 2B). Changes in survivin expression were not observed in either the MDA-MB-468 or SK-BR-3 cell lines infected with the Ad-Ψ5 vector.

Induction of p21 in Ad-IRF-1–Infected Cells. The p21/WAF1/CP1 cyclin-dependent kinase inhibitor is essential for DNA damage-induced cell cycle arrest, and its transcription is regulated by p53 (31, 32). Direct binding of IRF-1 to the IRF-E binding site within the human p21 gene promoter has also been shown (33). More recently, colon cancer cells transduced with a p21-adenoviral vector was sufficient to repress survivin mRNA levels (34). To investigate whether ectopic expression of IRF-1 results in the expression of p21, MDA-MB-468 and SK-BR-3 cells were infected, and cells were harvested at 12-hour intervals for immunoblotting. P21 was induced in Ad-IRF-1–infected MDA-MB-468 cells by 24 hours, whereas p21 expression was induced between 24 and 36 hours after IRF-1 infection in the SK-BR-3 cell line (Fig. 3). At 48 hours after infection, p21 expression was decreased in the infected SK-BR-3 cell line. We also examined the expression of the antiapoptotic protein Bcl-2 and the human oncoprotein, MDM2 known to play a role in tumor growth via both p53-dependent and p53-independent mechanisms. MDA-MB-468 tumor cells express both Bcl-2 and MDM2, and expression levels did not change on infection with either the adenoviral control or IRF-1 Ad (data not shown). In contrast, although the SK-BR-3 cell line expressed MDM2, we did not detect a strong Bcl-2 band by immunoblotting. Moreover, infection did not alter the expression of MDM2 or induce the protein expression of Bcl-2 in the SK-BR-3 cell line on infection (data not shown). Similarly, by screening of our microarray analysis, changes in Bcl-2 or MDM2 were not observed in the MDA-MB-468 tumor cell line (data not shown).

The MCF-7 breast carcinoma cell line expresses wild-type p53 and...
with either Ad-IRF-1 or the Ad-IRF-1 would enhance apoptosis, MDA-MB-468 cells were infected Enhanced Cell Death.

Apoptosis was not observed in MCF-7 Ad-IRF-1–infected cells (data not provided Fig. 4). Interestingly, even at MOIs as high as 200, enhanced apoptosis remained comparable at all time points evaluated. The expression of p53 and p21 also remained constant over the 48-hour time interval (Fig. 4). The IRF-1 transgene was expressed as early as 12 hours after infection. Unlike the MDA-MB-468 and SK-BR-3 cell lines, however, survivin expression was also investigated for differential survivin expression upon infection with the adenoviral vector control or IRF-1 (Fig. 4). In parallel, cells were harvested, and cell lysates were prepared for Western blotting analysis. Similar to previous results (Fig. 2), a decrease in survivin expression was observed in Ad-IRF-1–infected cells in the absence and presence of Adriamycin treatment (Fig. 6). There appeared to be an increase in survivin expression in the Ad-PS5–infected cells. Although a slight increase in survivin expression was observed in IRF-1–infected cells treated with Adriamycin, the expression of survivin was much less than that of uninfected or Ad-PS5–infected MDA-MB-468 tumor cells.

**DISCUSSION**

The range of tumor cell types expressing elevated levels of survivin supports the contention that this gene product may represent a *bona fide* target for novel anticancer therapeutics. In this study, we have ectopically expressed the transcription factor IRF-1 using an Ad delivery system. From our previously performed screening of cDNA microarray analysis of Ad-IRF-1–infected versus control MDA-MB-468 breast cancer cells, we observed a 15-fold down-regulation of the survivin mRNA compared with uninfected cells. Our current *in vitro* studies confirmed that survivin protein expression was similarly de-

In *vivo* Treatment of Tumor-Bearing Mice. To investigate the effects of Ad-IRF-1 on tumor growth in *vivo*, tumor-bearing female SCID mice were treated intratumorally with $4 \times 10^5$ pfu of Ad-PS5 or Ad-IRF-1. Treatments were repeated at ~12-day intervals. Tumor size was monitored, and tumor volumes were calculated. Although tumors continued to grow in both the saline-treated and Ad-PS5–treated cohorts, tumor growth was inhibited in mice treated with Ad-IRF-1 (Fig. 7). The tumor volumes were statistically less in IRF-1–treated mice between days 56 and 70 inclusive (all $P$ values < 0.05). The tumor volumes of mice in the vector control-treated cohort were not significantly different from those of the saline-treated group. The final Ad-IRF-1–treatment occurred at 68 days after tumor inoculation, and after the 70 day time point, inhibition of tumor growth in treated mice was no longer significant.

Survivin Expression Is Absent in Interferon Regulatory Factor-1–infected Cells. To investigate survivin and IRF-1 expression in *vivo*, nude mice were inoculated with $1 \times 10^7$ MDA-MB-468 tumor cells. When these mice developed palpable tumors, mice were treated intratumorally with $4 \times 10^5$ pfu of Ad-IRF-1 or Ad-PS5 vector control. Twenty-four hours after treatment, tumors were excised and stained for IRF-1 and survivin. Tumors excised from the Ad-PS5 vector control–treated cohort expressed survivin throughout the tumor tissue; however, IRF-1 was not expressed (Fig. 8A). In contrast, tumors excised from mice treated with Ad-IRF-1 displayed a predominant IRF-1-positive, survivin-negative phenotype in *vivo* (Fig. 8B).

![Fig. 3. Ad-IRF-1 infection promotes cancer cell expression of p21. MDA-MB-468 (A) and SK-BR-3 (B) cells infected with Ad-PS5 vector control or Ad-IRF-1. A MOI of 10 was used for the MDA-MB-468 cell line, and a MOI of 25 was used for the SK-BR-3 cell line. Infected cells were harvested at the indicated time points after infection, and immunoblotting was performed as described in Materials and Methods. NI, uninfected cells.](image-url)

![Fig. 4. MCF-7 cells are refractory to Ad-IRF-1–induced modulation of survivin and p21. MCF-7 cells were infected with Ad-PS5 vector control or Ad-IRF-1 at a MOI of 50. Cells were harvested at the indicated time points and prepared as described in Materials and Methods. NI, uninfected cells.](image-url)
creased in MDA-MB-468 and SK-BR-3 breast carcinoma cell lines after infection with Ad-IRF-1.

Our results further suggest that the down-regulation of survivin is independent of wild-type p53 because both the MDA-MB-468 and SK-BR-3 cell lines harbor mutations in p53. Our results are in contrast to other studies that show a reduction in survivin mRNA levels when human cell lines are infected with wild-type p53 (35). Moreover, studies using the H1299 (p53−/−) human non–small-cell lung cancer cell line stably transfected with a temperature-sensitive p53 allele displayed significant decreases in survivin RNA levels in the presence of wild-type p53 (36).

To date, the mechanism by which wild-type p53 may mediate the repression of survivin is unclear. Wild-type p53 was shown to directly bind to the p53-binding site of the survivin promoter, and this consensus sequence was necessary to mediate repression (36). On the other hand, recent studies have also shown that p53-dependent repression remained the same in H1299 (p53−/−) cells transfected with p53 expression plasmids and survivin luciferase reporter constructs containing mutations in the p53-binding site within the promoter (34). Studies have also suggested that wild-type p53 may recruit histone deacetylase complexes that modify the local chromatin structure, leading to repressed survivin transcription (35). In our studies, the breast cancer cell line MCF-7 that harbors a wild-type p53 gene did not exhibit lower levels of survivin protein expression. In fact, even in the presence of ectopic IRF-1, survivin expression did not change in this cell line.

Although wild-type p53 appeared not to be required for IRF-1 modulation of survivin expression, p21, a known p53 target gene, was up-regulated in IRF-1–infected MDA-MB-468 and SK-BR-3 cells. p21 interacts with cyclin/cyclin-dependent kinase complexes and may inhibit their activity (37, 38), resulting in cell cycle arrest in G1 or G2 (39, 40). Several studies have shown that the induction of p21 transcription is p53 dependent (39, 41, 42), and the p21 promoter contains two conserved p53-binding sites (32).

There are also several p53-independent mechanisms that result in...
p21 activation. These include binding of the transcription factor Sp1 (43), tumor suppressor protein BRCA1 (44), and activator protein 2 (45) to the p21 promoter. In our studies, although p53 was mutated, p21 was up-regulated in IRF-1–infected MDA-MB-468 and SK-BR-3 cells. Recently, direct binding of IRF-1 to the IRF-E binding site within the human p21 gene promoter has been demonstrated (33). Similarly, during DNA damage, IRF-1 activates the p21 promoter independent of p53 (46). HCT116 human colon cancer cells that have deleted both p53 alleles also up-regulate p21 when infected with Ad-IRF-1, whereas enhancement of p21 expression was not observed in HCT116 cells transfected with wild-type p53. Moreover, greater growth inhibition was observed in IRF-1–infected HCT116 cells lacking p53 than in wild-type cells (47). These data support the contention that in the absence of wild-type p53, the introduction of IRF-1 can result in p21 up-regulation and down-regulation of survivin. Similarly, recent studies have shown that p53-deficient H1299 human non–small-cell lung cancer cells transduced with a p21-adenoviral vector alone is sufficient to repress survivin mRNA levels (34). Collectively, these studies suggest that p21 is sufficient for mediating the negative regulation of survivin gene expression.

In addition, our studies demonstrated an additive effect of Ad-IRF-1 infection and chemotherapy in promoting cell death. MDA-MB-468 cells were infected with Ad-IRF-1 and either left untreated or treated with Adriamycin. There was an increase in the number of apoptotic and necrotic cells in Ad-IRF-1–infected MDA-MB-468 cells compared with cells treated with Adriamycin alone. Our statistical analyses on three independent experiments demonstrated that the effect of the combination of IRF-1 and Adriamycin on cell death was additive. Moreover, we observed a slight increase in survivin expression in cells infected with IRF-1 and treated with Adriamycin. Despite this minimal increase in survivin expression, cell death was enhanced when these Ad-IRF-1–infected cells were treated with Adriamycin. It is possible that this observed increase in survivin expression in IRF-1/Adriamycin–treated cells is biologically insignificant. In addition, it is possible that the mechanisms that mediate enhanced apoptosis in Ad-IRF-1/Adriamycin–treated cells may differ from cells infected with Ad-IRF-1 or treated solely with Adriamycin. Future studies will address these mechanisms. It should be noted that an increase in both survivin mRNA and protein expression has been shown in an Adriamycin--
DOWN-REGULATION OF SURVIVIN BY ECTOPIC EXPRESSION OF IRF-1


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