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A novel adenoviral vector expressing human Fas/CD95/APO-1 enhances p53-mediated apoptosis

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Abstract

Recent evidence suggests an intriguing link between p53 and the Fas pathway. To evaluate this association further, we utilized a recombinant adenoviral vector (AdWTp53) to overexpress wild-type p53 in lung cancer (A549, H23, EKVX and HOP92) and breast cancer (MDA-MB-231 and MCF-7) cell lines and observed an increase in the Fas/CD95/APO-1 protein levels. Furthermore, this increase correlated with the sensitivity of the cell lines to p53-mediated cytotoxicity. To examine the effects of Fas over-expression in cells resistant to p53 over-expression, we constructed AdFas, an adenoviral vector capable of transferring functional human Fas to cancer cells. Interestingly, infection of p53-resistant MCF-7 cells with AdFas sensitized them to p53-mediated apoptosis. These studies indicate that combined over-expression of Fas and wild-type p53 may be an effective cancer gene therapy approach, especially in cells relatively resistant to p53 over-expression.

Keywords: adenovirus; Fas/CD95/APO-1; p53; apoptosis; gene therapy

Abbreviations: DISC, death-inducing signaling complex; IGFBP, insulin-like growth factor binding protein

Introduction

The p53 protein plays a central role in the cellular role response to DNA damaging conditions. Many different types of DNA damage result in a rapid and proportional increase in p53 levels which also probably undergoes activation from a latent inactive form to a transcriptionally active form.[1] Events downstream of p53 lead to two major responses: cell cycle arrest and apoptosis. Cell cycle arrest lends the cell an opportunity for DNA repair, while apoptosis presumably eliminates cells with extensive DNA damage.[2,3]

In recent years, the ability of p53 to induce apoptosis has been exploited for cancer gene therapy. For example plasmid DNAs or adenoviral vectors expressing p53 have been shown to inhibit the tumor growth in a variety of animal models.[4,5] However, a careful investigation in many laboratories has revealed that some cancer types are resistant to killing by overexpression of p53. One such tumor type extensively studied in our laboratory is MCF-7, a breast cancer cell line.[6] Thus, we believe that for further development of cancer gene therapy approaches using p53, it is important to understand the mechanisms of p53-mediated apoptosis. It is particularly important to understand why certain tumors are resistant to p53 overexpression and to investigate ways to circumvent this resistance. One such strategy, based on overexpression of Fas/CD95 in cancer cells, is examined in this report.

Fas (CD95/APO-1), a death-domain bearing member of the tumor necrosis factor receptor super-family, is a transmembrane protein that, on specific engagement with the Fas-ligand or an agonistic antibody conveys an apoptotic signal to the cell.[7] Oligomerization of the Fas receptors following crosslinking with Fas ligand or agonistic antibody leads to recruitment of caspase-8 (FLICE/MACH/Mch5) to the receptor complex and triggers the apoptosis-inducing protease cascade by cleaving the caspase 1 zymogen.[8,9] In recent years, studies have focused on the potential relationship between Fas and p53 expression.[10,11] Up-regulation of Fas following overexpression of p53 by an adenoviral vector as well as following stable transfection by a temperature sensitive p53-mutant has previously been reported in lung cancer cells.[12] Others have reported that exogenous expression of p53 by microinjection can induce functional Fas protein that can be stimulated by the anti-Fas antibody to induce apoptosis.[13] However, these studies do not establish a direct correlation of Fas up-regulation with p53-mediated apoptosis.

In this report, we examine the effects of p53 over-expression, using a recombinant adenoviral vector, on the expression of the Fas receptor in a panel of human tumor cell lines and present data that suggests that p53 mediated induction of Fas protein correlates with p53 toxicity. We also describe the construction of a novel adenoviral vector that can transfer functional Fas to cancer cells. Furthermore, we present evidence that overexpression of Fas can sensitize cells to p53 mediated apoptosis. Based on these results, we believe that an adenoviral vector expressing Fas will be an asset to our future cancer gene therapy approaches.

Results

AdWTp53 infection leads to induction of Fas that is functional and can be utilized to enhance AdWTp53 mediated cytotoxicity

We have previously reported that over-expression of human wild-type p53 by an adenoviral vector (AdWTp53) resulted in significantly greater toxicity to MDA-MB-231 cells in comparison to MCF-7 cells.[6] Since other studies have reported that infection of lung cancer cells with a similar adenoviral vector expressing wild-type p53 results in up-regulation of the Fas receptor in these cells,[12] we decided to examine the effects of wild-type p53 on Fas induction in p53 sensitive as well as resistant cells. We therefore infected MDA-MB-231 and MCF-7 cells with AdWTp53 (10 p.f.u./cell) and analyzed p53, Fas and $p21^{Waf1/Cip1}$ by Western blot. As shown in Figure 1, infection with AdWTp53 resulted in similar levels of p53 protein in both breast cancer cell lines. Furthermore, the p53 protein was functional in both cell lines as it led to induction of similar levels of $p21^{Waf1/Cip1}$, a cyclin dependent kinase inhibitor that is transcriptionally regulated by p53. Of interest was the observation that the level of Fas induced following AdWTp53 infection of MDA-MB-231 cells was significantly greater than that detected in MCF-7 cells.

We further examined whether the p53-induced Fas receptor is functional by using an anti-Fas antibody that is known to induce apoptosis in cells expressing the Fas receptor.[14] Using the MTS assay described in Materials and Methods, a fivefold greater toxicity was observed in MDA-MB-231 cells, infected with AdWTp53 (10 p.f.u./cell) and exposed to the anti-Fas antibody compared to infection with AdWTp53 alone (Figure 2). Toxicity was even lower in cells infected with control virus (AdNull) and treated with the antibody or exposed to the antibody alone. These observations indicate that the p53-induced Fas protein expression in MDA-MB-231 cells is functional and can interact with an antibody that serves as a surrogate for the Fas ligand. In contrast, infection of MCF-7 cells with AdWTp53 and exposure to anti-FAS antibody resulted in much lower cytotoxicity compared to that observed in MDA-MB-231.

AdWTp53 toxicity correlates with induction of Fas in multiple cell lines

Comparison of AdWTp53 cytotoxicity of MDA-MB-231 *versus* MCF-7 cells suggests that the induction of Fas protein expression may be a marker of p53-mediated cytotoxicity. We, therefore compared the cytotoxicity of AdWTp53 infection to that produced by a control virus, AdNull, in a panel of human cancer cell lines. Using the 7-day sulforhodamine assay, the IC_{50} of each virus for each cell line was determined as the dose, in p.f.u./cell, that resulted in 50% cell death. The ratio of the IC_{50} of AdNull and AdWTp53, expressed as the 'Relative Cytotoxicity', was used to compare the sensitivity of different cell lines to p53-mediated toxicity (the bigger this ratio, the more sensitive the cell line). Although AdWTp53 was more toxic than AdNull to all cell lines examined, there was a marked difference in the relative sensitivity of the cells to AdWTp53. Three of the cell lines, MDA-MB-231, EKVX and HOP92 were relatively more sensitive to AdWTp53 when compared to A549, MCF-7 and H23 human tumor cell lines (Figure 3).

To explore the relationship of Fas induction to AdWTp53 toxicity, we studied the expression of p53 and Fas/CD95/APO-1 proteins following AdWTp53 infection in these cell lines. Infection with AdWTp53 resulted in comparable levels of p53 by Western blot analysis in all cell lines examined (Figure 3). Therefore, differences in infectability of cells by AdWTp53 could not explain the differences in sensitivity of these cells to p53 overexpression. We next examined Fas/CD95/APO-1 protein expression in AdWTp53 infected cells by Western blot and quantified the levels of Fas induction by densitometric analyses. The 'p53 Inducible Fas' for each

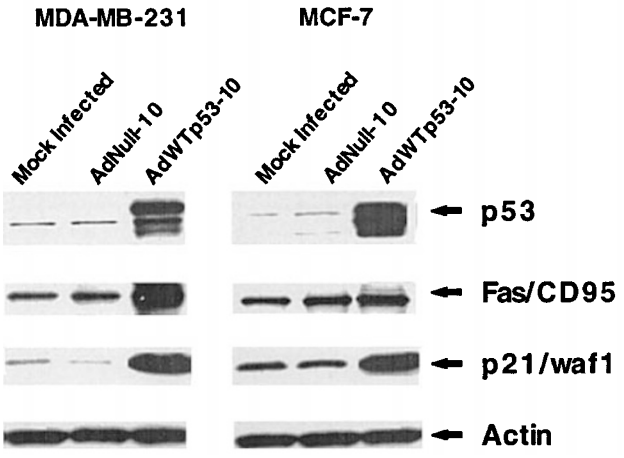

Figure 1 Following AdWTp53 infection, Fas/CD95/APO-1 is induced at higher level in MDA-MB-231 cells compared to MCF-7 cells. Cells (1×10^6) of each cell line were infected with 10 p.f.u./cell of AdWTp53 or AdNull, harvested and subjected to Western blot analysis for p53, Fas/CD95/APO-1 and $p21^{Waf1/Cip1}$ and actin, as described in Materials and Methods

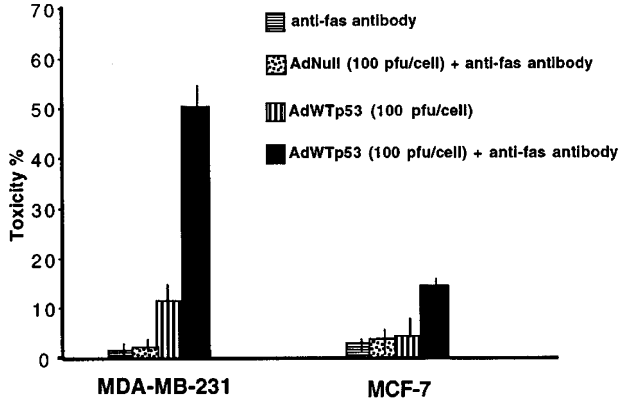

Figure 2 p53 induced Fas is functional and agonistic anti-fas antibody acts synergistically with AdWTp53 in MDA-MB-231 cells. Toxicity to MDA-MB-231 and MCF-7 cells, following overnight infection with AdWTp53 at various doses and exposure to an IgM monoclonal anti-Fas antibody (clone CH 11, MBL Corp.) for 24 h, was determined using an MTS colorimetric assay as described in Materials and Methods

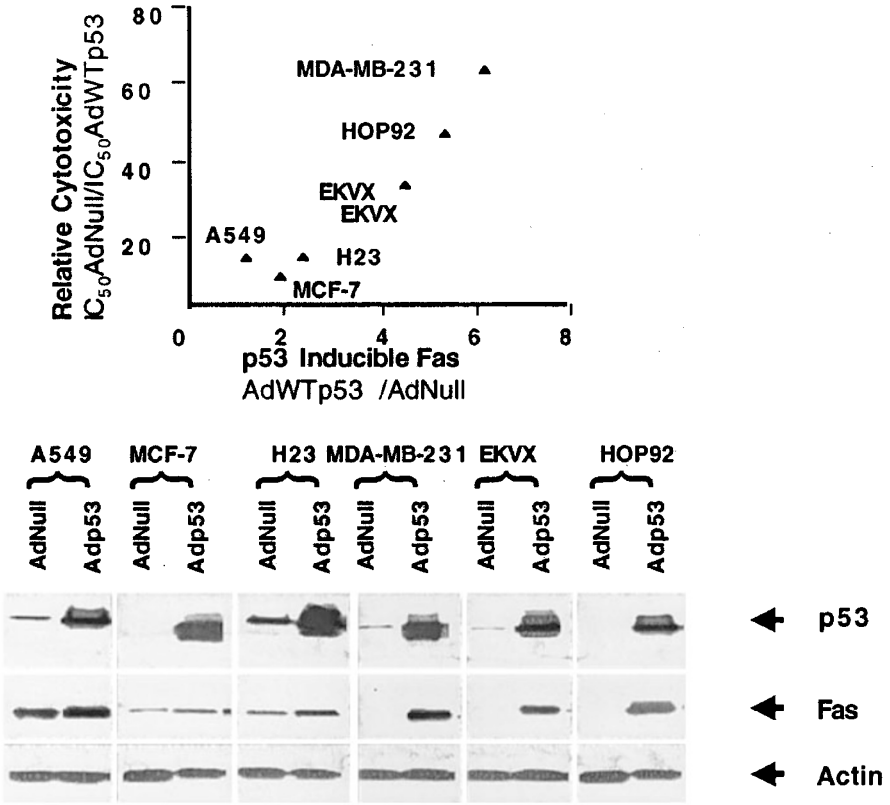

Figure 3 Sensitivity of cell lines to p53-mediated toxicity correlates with Fas induction. Using a sulforhodamine colorimetric assay, the sensitivity of each cell line to AdWTp53 was determined by comparing the IC_{50} (dose in p.f.u./cell of virus that is toxic to 50% of cells, assuming the survival of uninfected cells being 100%) of control virus (AdNull) to the IC_{50} of AdWTp53. Determination of 'p53 inducible Fas' was done by Western blot analysis (shown in the lower panel) and densitometric quantification, using the NIH Image software, of cells infected with AdWTp53 (10 p.f.u./cell) relative to that in cells infected with AdNull (10 p.f.u./cell)

cell line was then determined by dividing the level of Fas protein following AdWTp53 infection by that following AdNull infection. As can be seen in Figure 3, Fas protein levels following p53 overexpression by AdWTp53 varied considerably among the p53 sensitive and p53 resistant cell lines. Cell lines in which AdWTp53 infection was associated with greater toxicity (EKVX, HOP92 and MDA-MB-231) exhibited a greater increase in the degree of Fas induction (4.4, 5.25 and 6.13-fold respectively). In contrast, cell lines that were relatively resistant to AdWTp53 infection (A549, MCF-7 and H23) showed markedly less Fas induction (1.74, 1.34 and 2.3-fold respectively). Thus, differences in sensitivity to p53 overexpression of various cell lines may be related to differences in p53-mediated level of induction of Fas in these cell lines.

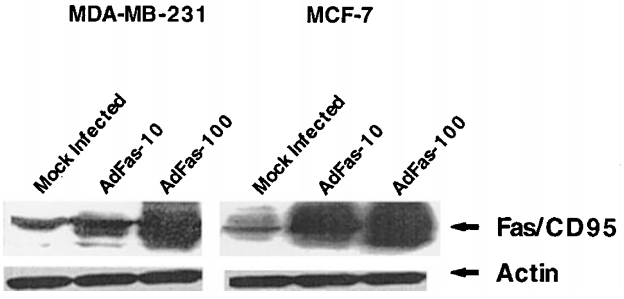

Figure 4 Infection with AdFas leads to a marked increase in Fas protein in MDA-MB-231 as well as MCF-7 cells. Cells were infected with AdFas (10 and 100 p.f.u./cell) or AdControl (100 p.f.u./cell) for 48 h and subjected to Western blot analysis for Fas and actin

AdFas-mediated synthesis of functional Fas/CD95/APO-1 protein

To further examine the role of Fas protein in p53-mediated apoptosis an adenovirus containing Fas cDNA (AdFas) was constructed as described in Materials and Methods. Following infection of two breast cancer cell lines MDA-MB-231 and MCF-7 with 10 p.f.u./cell of AdFas, the Fas protein was detected by Western blot analysis in both cell lines. Furthermore, the amount of Fas protein increased with increasing concentrations of the AdFas vector (Figure 4).

Cross-linking of Fas, either with the Fas ligand or with agonistic antibodies, leads to the formation of a DISC that leads to the recruitment of a cascade of proteases believed to execute the death signal.[8,9] FLICE/Mch 5 is the most

proximal ICE-like protease and proteolytic cleavage of FLICE, resulting in a size 10 kDa fragment, is an obligatory step in the Fas signaling pathway. To investigate whether the Fas protein synthesized as a result of AdFas infection is functional, MDA-MB-231 and MCF-7 cells were either mock infected or infected for 48 h with AdNull or AdFas (both at 100 p.f.u./cell) and then exposed to an agonistic anti-Fas antibody (CH11, MBL Corp.). Following treatment with this antibody for 6 h, the cells were harvested and subjected to Western blot analysis for p10 (the active FLICE cleavage product). As shown in Figure 5, in MDA-MB-231 cells, the cleavage product could only be detected in the AdFas infected cells exposed to the anti-Fas antibody and not in mock infected or AdNull infected cells that were similarly treated. In MCF-7 cells, the cleavage product was detectable in antibody treated cells that were mock infected or infected with AdNull. However, the amount of detectable p10 increased substantially when AdFas infected MCF-7 cells were exposed to the anti-Fas antibody. Thus, in both cell lines examined, infection with AdFas led to synthesis of the Fas protein that, on exposure to an agonistic anti-Fas antibody, resulted in proteolytic activation of FLICE/Mch 5.

Co-infection with AdFas sensitizes MCF-7 cells to AdWTp53-mediated apoptosis

We have previously observed that AdWTp53 infection induces apoptosis, as detected by DNA laddering, in MDA-MB-231 and not in MCF-7 cells.[6,15] To quantitate the induction of apoptosis following AdWTp53 infection in these cell lines, both cell lines we re-infected with AdWTp53 and AdNull (10 p.f.u./cell) for 48 h and then subjected to flowcytometric analysis for determination of the percentage undergoing apoptosis (cells with subdiploid DNA content). As shown in Figure 6, in MDA-MB-231 cells the sub-G1 population increased from 3.8 and 5% in mock infected and AdNull infected cells respectively, to 28% in cells infected with AdWTp53. In contrast, in MCF-7 cells no such increase was observed with the sub-G1 population being 2.1% in mock infected cells, 2.3% in AdNull infected cells and 3.4% in AdWTp53 infected cells. Following AdWTp53 infection, an increase in the G2/M population was observed in both cell lines.

As the toxicity of p53 over-expression in multiple cell lines seems to correlate with the Fas protein levels, we also investigated whether increasing the Fas levels exogenously would sensitize a resistant cell line to AdWTp53 infection. To examine this, MCF-7 cells were infected for 48 h with AdFas alone at 50 p.f.u./cell, a combination of AdWTp53 (10 p.f.u./cell) and AdNull (50 p.f.u./cell) or a combination of AdWTp53 (10 p.f.u./cell) and AdFas (50 p.f.u./cell) and then subjected to flowcytometric analysis for apoptosis. The results shown in Figure 7(a) indicate that infection with AdFas alone was relatively non-toxic with only 3.8% of cells revealing a sub diploid DNA content. Following infection with the combination of AdWTp53 and AdNull, an additive effect was seen as the sub-G1 population increased to 6.7%. However, the combination of AdWTp53 and AdFas was remarkably more toxic as the sub-G1 population increased to 21.7%. The effect of combination of AdWTp53 and AdFas on Apoptosis was further evaluated by DAPI staining, which brightly stains the nuclei of the cells undergoing apoptosis; and by CPP32 cleavage, which represents activation of pro-caspase 3, a hall mark of apoptosis. Results of DAPI staining showed that combination samples exhibited bright and condensed nuclei after 48 h infection (Figure 7b). Moreover, Western blot analysis also showed CPP 32 activation in cells which were co-infected with AdFas and AdWTp53 (Figure 7c). These observations suggest p53 and Fas proteins co-operate to induce apoptosis.

Discussion

Apoptosis mediated by p53 as a response to extensive DNA damage is a well documented phenomenon. However, the molecular and biochemical events associated with p53-mediated apoptosis have yet to be well defined. Both transcriptional regulation of cellular genes and direct protein-protein signaling have been postulated to be mechanisms underlying p53 mediated apoptosis.[16] Bax and IGF-BP3 are genes that have been shown to be regulated by p53 and the proteins encoded by these genes are known to

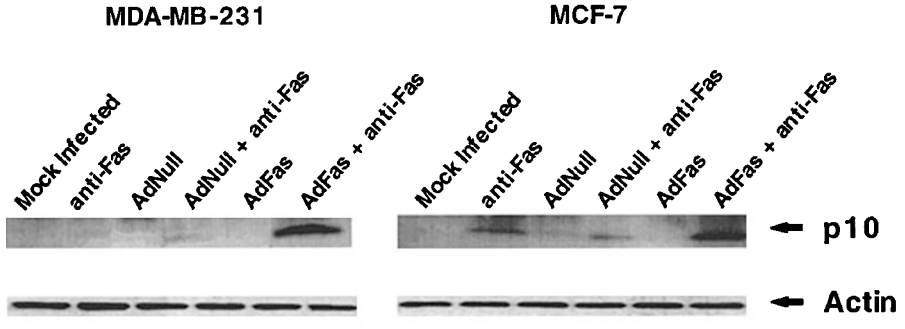

Figure 5 Fas protein produced after AdFas infection is functional. Cells were mock infected or infected for 24 h with AdNull or AdFas (both 100 p.f.u./cell), exposed to the anti-Fas antibody (clone CH 11, MBL Corp.) for 12 h and subjected to Western blot analysis for the presence of the 10 kDa proteolytic cleavage product (p10) of FLICE/Mch 5. Infection with AdFas followed by exposure to the anti-Fas antibody resulted in the appearance of the p10 cleavage product in both MDA-MB-231 and MCF-7 cells. Actin levels are shown as loading control

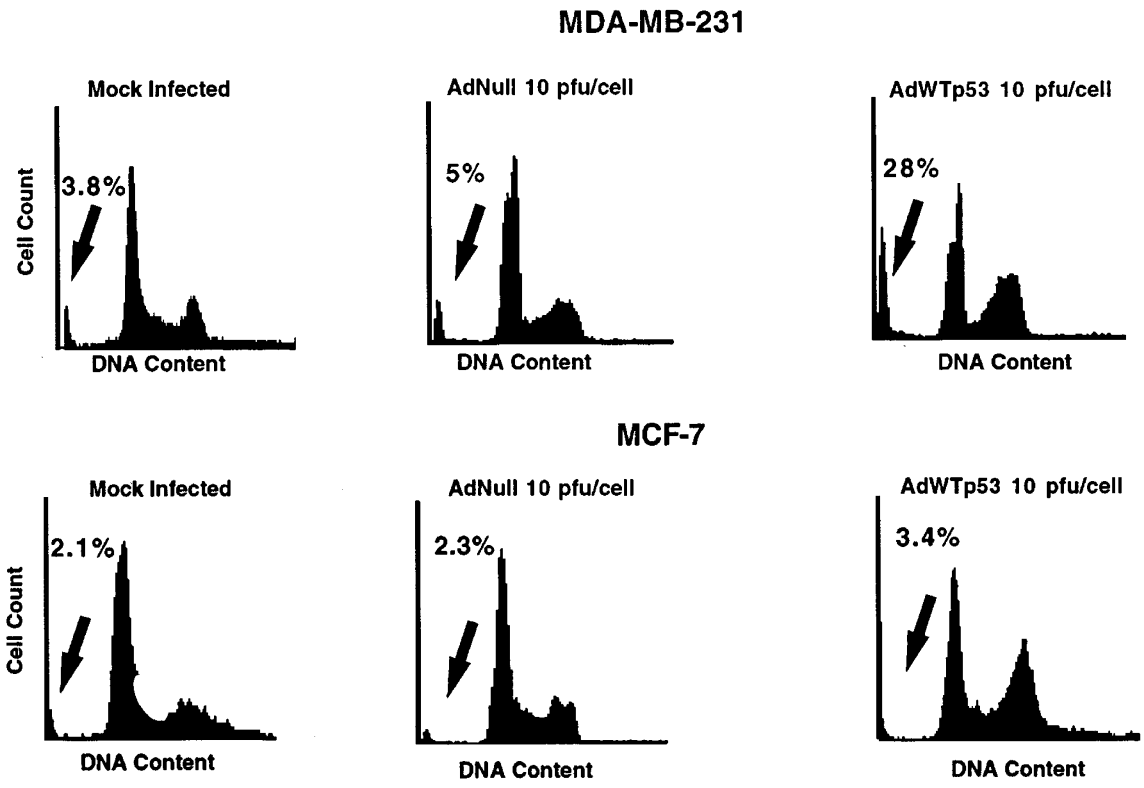

Figure 6 p53 overexpression leads to apoptosis of MDA-MB-231 but not MCF-7 cells. Propidiumm iodide stained DNA of ethanol fixed MDA-MB-231 and MCF-7 cells, following mock infection or infection with AdWTp53 and AdNull (both at a dose of 10 p.f.u./cell for 48 h) was analyzed for apoptosis as represented by the appearance of a sub-G1 population

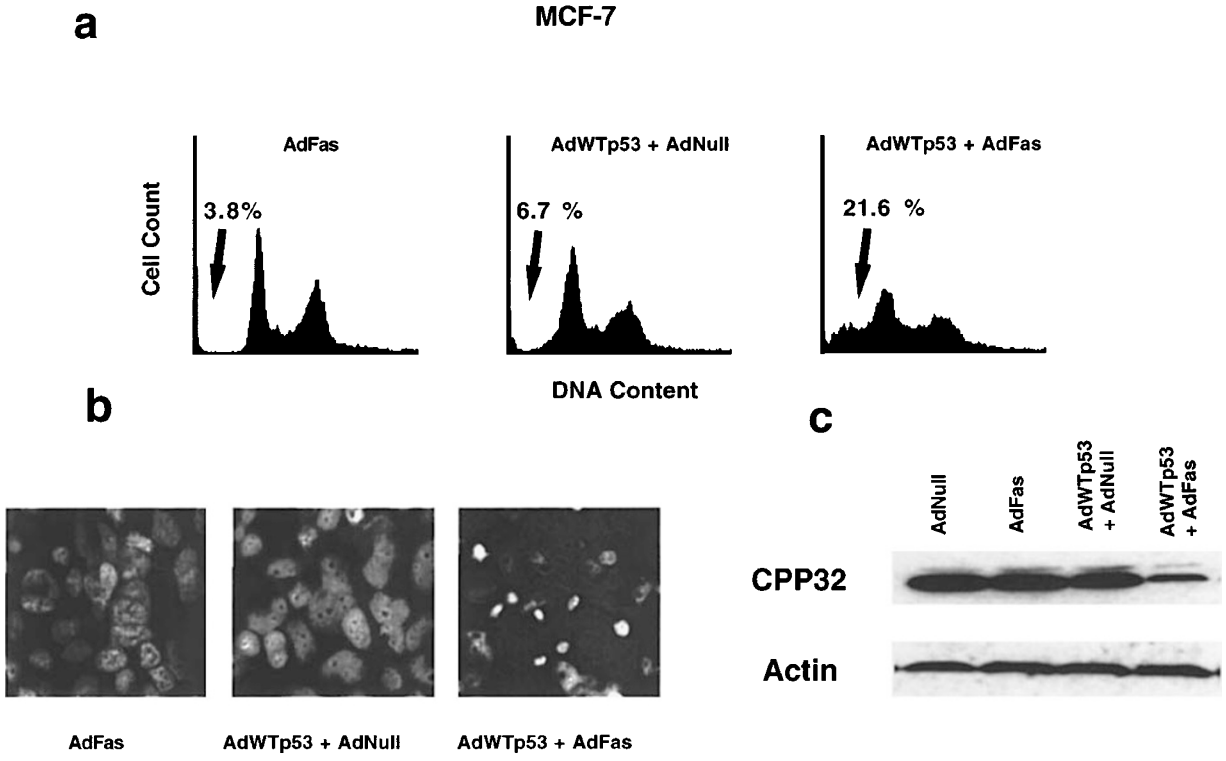

Figure 7 Increased toxicity is seen in MCF-7 cells following co-infection with AdWTp53 and AdFas. MCF-7 cells were infected with AdFas alone (50 p.f.u./cell), a combination of AdWTp53 (10 p.f.u./cell) and AdNull (50 p.f.u./cell) or a combination of AdWTp53 (10 p.f.u./cell) and AdFas (50 p.f.u./cell) for 48 h and analyzed for appearance of a sub-G1 population (**a**), DAPI staining (**b**), and CPP 32 activation (**c**)

play important roles in the apoptotic response and growth regulation.[17,18] However, other p53 inducible genes may be involved in the triggering of apoptosis. One such candidate gene examined in this report, is the Fas gene, the protein product of which is known to transduce a signal for apoptosis.

Fas, a death-domain bearing member of the tumor necrosis factor receptor super-family, is a transmembrane protein that, on specific engagement with the Fas ligand conveys a signal to the cell to undergo apoptosis.[19] The potential relationship between Fas and p53 expression was first reported by Owen-Schaub and co-workers, who observed up-regulation of Fas following overexpression of p53 by an adenoviral vector as well as following stable transfection by a temperature sensitive p53-mutant.[12] Others have reported that exogenous expression of p53 by microinjection can induce functional Fas protein that can be stimulated by anti-Fas antibody to induce apoptosis.[13] However, only a limited number of cell lines were examined for p53 mediated enhancement of Fas and a direct correlation of this up-regulation with p53-mediated apoptosis could not be established from these studies. Since our previous studies indicated that p53 overexpression leads to apoptosis in many tumor cell lines,[6,15] we compared the effect of p53 expression on Fas induction in a variety of cell lines. The experiments described in this report demonstrate that overexpression of wild-type p53 results in induction of the Fas protein in a broad spectrum of cell lines. More importantly, we found a correlation between the level of inducibility of Fas and the sensitivity to p53-mediated toxicity in the cell lines examined. It is interesting, however, that Fas can be induced even in the relatively p53 resistant cells, albeit to a lower degree. It should be noted however, that besides transcriptional regulation of p53-inducible Fas, the Fas protein can be also potentially up-regulated by indirect mechanisms; and a host of factors, including interaction with other cellular proteins which have an anti-apoptotic effect and downstream defects in signal transduction pathways may also be responsible for the variable sensitivity observed in this study.

Oligomerization of the Fas receptors following cross-linking with Fas ligand or agonistic Fas antibody leads to recruitment of caspase-8 (FLICE/MACH/Mch5) to the receptor complex and triggers the apoptosis inducing protease cascade by cleaving the caspase 1 zymogen.[8,9] Initially, peripheral elimination of chronically activated lymphocytes was postulated to be the main function of the Fas-Fas ligand mediated programmed cell death.[7] Recently, however, many reports have focused on the important role played by this system in the biology of epithelial cancers. Several normal and malignant non-hematopoietic tissues have been shown to express functional Fas that can mediate an apoptotic signal.[20] Interestingly, malignant transformation of epithelial cells has been found to be associated with decreased levels of expression of Fas. For instance, it has been reported that whereas Fas-negative sub-populations are rarely detected by immunohistological examination of colon adenomas, as many as 48% of colon carcinomas are Fas-negative and an additional 39% express Fas at sub-normal levels.[21] Similar observations have also been made in breast cancer cell lines.[22] This down-modulation of Fas has been postulated to be a mechanism of escaping the normal anti-cancer immune surveillance by Fas-ligand expressing T lymphocytes. Other mechanisms of resistance to Fas-mediated apoptosis including defects in transfer of the Fas receptor to the cell surface, abnormal function of the receptor and activation of anti-apoptotic (life sustaining) programs have also been described in cancer cells.[23,24] Thus, strategies to bypass Fas resistance in cancer cells may prove to have therapeutic value. One such approach, i.e. adenoviral over-expression of Fas in cancer cells is described in this report.

Since p53-mediated cytotoxicity correlated with p53-mediated Fas induction, we examined the effects of Fas over-expression in p53 resistant cells. Interestingly, even though adenoviral transfer of Fas to the AdWTp53 resistant MCF-7 cells was non-toxic *per se*, it sensitized the cells to p53 over-expression. As this phenomenon was observed in the absence of an induction of the Fas ligand (data not shown) or exogenously administered anti-Fas antibody, it suggests that Fas induction may be a rate-limiting step in p53-mediated apoptosis or that a protein-protein interaction may exist between p53 and Fas.

Adenoviral vectors leading to p53 overexpression have been shown to be cytotoxic by leading to apoptosis *in vivo* in animal models and are currently being evaluated in Phase I clinical trials.[25] However, p53-mediated apoptosis is not observed in all cancer cell lines and response to p53 over-expression may be determined by a number of variables, which may include the ability to up-regulate Fas as a result of p53 over-expression. Our results indicate that a combination approach utilizing over-expression of p53 and Fas may be effective, especially in p53 resistant situations.

Materials and Methods

Cell culture

MCF-7 (kindly provided by R. Buick, University of Toronto) and MDA-MB-231 (ATCC HTB26) human breast cancer cells, and 293 (ATCC CRL 1573) cells, were cultured as described previously.[26] Lung cancer cells A549, H23, HOP92 and EKVX were kindly provided by Dr. Michael Kelley (Lung Cancer Biology Section, Medicine Branch, National Cancer Institute, Bethesda, Maryland, USA) and cultured in Improved Minimal Essential Medium (IMEM) and 10% FBS (Gibco BRL, Gaithersburg, MD, USA).

Construction of adenoviral vectors and infection procedure

A replication deficient recombinant adenovirus in which the E1a region has been replaced by the Fas expression cassette (AdFas) was constructed by co-transfection of the shuttle vector pAR1 containing the Fas/CD95/APO-1 cDNA expression cassette and Cla1 cut genomic adenoviral DNA using the procedures described previously.[15] The shuttle vector pAR1 was constructed by inserting the human cytomegalovirus (CMV) immediate early promoter and enhancer, and a 2.2 kilobase fragment of Fas (kindly provided by Peter Krammer, German Cancer Research Center, Heidelberg,

Germany) into a null shuttle vector as described previously.[6,15] Following cotransfection of both DNAs into the adenovirus transformed human embryonic kidney cell line 293 (ATCC CRL1573) by calcium phosphate mediated gene transfer technique (Gibco BRL, Gaithersburg, MD, USA), plaques were screened for the presence of Fas sequences by polymerase chain reactions (PCR) using a set of primers which were 5′-CAAGTGACTGACATCAACTCC-3′ and 5′-CCTTGGTTTTCCTTTCTGTGC-3′. A viral plaque containing Fas sequence and devoid of adenovirus E1a sequence was grown in 293 cells (adenovirus transformed human embryonic kidney cells (ATCC CRL1573)), and purified by double cesium chloride gradient.

AdWTp53 was constructed by co-transfection of the shuttle vector pDK10 containing the p53 expression cassette and pJM17 plasmid DNA by methods previously described.[6] AdNull is an adenoviral vector which contains no insert and is replication deficient.

Infection with recombinant viruses was accomplished by exposing cells to different doses of adenovirus in serum-free IMEM for 2 h followed by addition of serum containing medium for 1–7 days.

Assays for cell death

Cytotoxicity of adenovirus vectors, AdWTp53 and AdNull, was assessed using the two colorimetric assays: the 7 day sulforhodamine assay and the 36 h MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxypheny)-2-(4-sulfophenyl)-2H-tetrazolium, Promega) assay.[26] For the 7 day assay, 500 cells were plated in each well of 96 plates and incubated for 24 h. Cells were then exposed to the appropriate cell growth medium except that the concentration of the serum was reduced to 2%. Different doses of adenovirus vectors were included in the incubation medium (several fivefold dilutions). After 2 h incubation at 37°C, the serum concentration was increased to 10% and the cells were incubated for 7 days at 37°C. Cells were then fixed by the addition of ice-cold 50% trichloroacetic acid (TCA) (added onto the top of the medium in each well to a final concentration of 10%), incubated at 4°C for 1 h, washed five times with water and then air dried. TCA-fixed cells were stained for 20 min with 0.4% (wt/vol) sulforhodamine B (Sigma, St. Louis, MO, USA) dissolved in 1% acetic acid followed by rinsing four times with 1% acetic acid. An $O.D._{564}$ was obtained using a Bio Kinetics Reader EL340 (Bio-Tek Instruments) and was used as a measure of cell number. Toxicity in percentage, was calculated from the surviving cells, assuming 100% survival for uninfected cells. In the 7 day sulforhodamine assay, the IC_{50} for each virus was determined as the dose, in p.f.u./cell, that resulted in 50% cell death. The ratio of the IC_{50} of AdNull and AdWTp53, expressed as the 'Relative Cytotoxicity', was used to compare the sensitivity of different cell lines to p53-mediated toxicity (the higher this ratio, the more sensitive the cell line).

For the MTS assay, cells were similarly treated and infected with adenoviral vectors. Following 24 h infection with AdWTp53, the cells were exposed to anti-Fas antibody (CH-11, MBL Corp.) At a final concentration of 1 μg/ml. Twelve hours later, the MTS reagent was added per the manufacturer's directions and the O.D. read on the Bio Kinetics Reader (Bio-Tek Instruments). Toxicity in percentage, was calculated from the surviving cells assuming 100% survival for uninfected cells.

Apoptosis was quantified by analyzing the sub-G1 (sub-diploid) population by measuring the fluorescence activity of propidium iodide stained DNA of permeabilized and fixed cells on the FacsCalibur instrument (Becton Dickinson) using the previously published protocol.[27]

$$\%\ \text{sub} - \text{G1 population} = \frac{\text{number of cells with flourescence intensity below G1}/ \times 100}{\text{total number analyzed}}$$

Western blot analysis

Cells (1×10^6) were plated in 10 cm dishes, incubated with adenovirus vectors for 24 h, scraped and cell lysates subjected to analysis as previously described.[26] Following antibodies were used: anti-p53 (AB-2, Oncogene Research Products, Cambridge, MA, USA), anti-p21 (AB-1, Oncogene Research Products, Cambridge, MA, USA), anti-Fas (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FLICE/Mch5 p10 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti cpp32(AB-1, Oncogene Research Products, Cambridge, MA, USA) and anti-actin (I-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To quantify levels of Fas/CD95, Western blot images were scanned and subjected to densitometric analyses using the NIH Image software.

DAPI staining

Cells were incubated with adenovirus vectors for 48 h, scraped and cell pellets were subjected to DAPI staining analysis as previously described.[27,28] At the desired time interval, the cultured dishes were washed with phosphate buffered saline (PBS) and harvested by trypsin. Fifty thousand cells were resuspended in 50 μl of PBS. Ten μl of 22% bovine serum albumin (BSA) was added to sample and cell/BSA suspension was pipetted into the bottom of a cytofunnel mounted with a microscope slide into the Cytocentrifuge and spun at 500 r.p.m. for 5 min. Slides were dried for 30 min at room temperature and washed with PBS twice. Then 200 μl of DAPI 2.5 μg/ml in 0.05 M phosphate buffer (pH 7.2) was added and allowed to incubate for 30 min at room temperature. The cells were washed twice with PBS. The DAPI solution and any samples stained with were stored from light. The DAPI solution was stored at 4°C.

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