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Transduction of adenovirus-mediated wild-type p53 after radiotherapy in human cervical cancer cells

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Abstract

Objective. Radiotherapy is the mainstay of treatment for advanced cervical cancer and most cervical cancers have evidence of HPV (human papilloma virus) infection, which inactivates the p53 gene. The goal of this study was to determine the effect of combining radiotherapy and Adp53 infection on the growth of cervical carcinoma cells.

Methods. The silta cervical carcinoma cell line contains wild-type p53 and HPV 16 infection. The C33A cell line has a p53 mutation. The Adp53 recombinant adenovirus contains the cytomegalovirus promoter, wild-type p53 cDNA, and a polyadenylation signal in a minigene cassette inserted into the El-deleted region of a modified adenovirus 5. Transduction efficiency was assessed by using an adenovirus containing the *Escherichia coli* β-galactosidase gene and expression of wild-type p53 in infected cells was evaluated by Western blot analysis. One group of cells was irradiated prior to infection, the other group received no irradiation, but were either infected with virus or mock-infected. Cells were analyzed for viability 1 to 7 days after infection by using the sulforhodamine B assay. The percentage of cells undergoing apoptosis was determined by using a TUNEL assay.

Results. Fifty percent of C33A cells were transduced with 5 muthplicities of infection of virus whereas SiHa cells required 25 multiplicities of infection. Adp53 expression was found 48 h after infection. In the cells treated with both radiation and Adp53 infection growth inhibition was increased compared with inhibition resulting with either treatment alone. The combination treatment also increased the percentage of cells undergoing apoptosis.

Conclusion. Combining radiotherapy with Adp53 infection increases the inhibition of growth of cervical cancer cells in vitro. This combination treatment has the potential of increasing efficacy and of therapy.

Keywords: Apoptosis; Human papilloma virus; Radiosensitivity; Viral transduction

Introduction

Recent studies indicate that the combination of radiotherapy and chemotherapy improve survival of patients with advanced cervical cancer [1–5]. However, radiotherapy remains central to the treatment and ultimate cure of these patients. Unfortunately, in some cases, the ultimate goal of cure may require radiation doses, with or without chemotherapy, that increase the risk of normal tissue injury, leading to morbidity and even mortality. Additionally, some patients will be unable to tolerate the side effects that result from combining two cytotoxic therapies such as radiation and chemotherapy. There also exists a wide variation in a tumor's responsiveness to therapy. The variance in radiosensitivity observed in human cancers may result from the loss of activity of some tumor suppressor genes, such as the *p53* gene [6]. Wild-type p53 regulates cell cycle arrest and controls cellular proliferation by induction of apoptosis. Abrogation of p53 function can lead to increased resistance to ionizing radiation, as observed in tumor cells of the head and neck [7]. This study is important to be certain that

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radiation, the mainstay of therapy, can be used with Ad*p53* in the cervix without dampening the effects of p53 gene therapy or increasing radiation resistance.

Inactivation of the *p53* gene in cervical cancer via point mutation or allelic loss is infrequent, with an overall incidence of 1–6% [8]. However, up to 90% of cervical cancers contain human papilloma virus (HPV[1]) DNA (60% of which is HPV type 16) and express low levels of p53 [9]. The HPV virus contains two genes, *E6* and *E7*, which are capable of transforming normal cells into tumor cells. The *E7* gene product binds to cell cycle control proteins of the retinoblastoma gene product (pRB) and cyclin A, resulting in proliferation and immortalization of cells [10,11]. The E6 protein forms complexes with p53 and promotes its degradation via the ubiquitin-dependent mechanism [10]. The effects of these two HPV genes are thought to contribute to the development of cervical cancer. There are several cervical cell lines that are infected with HPV and do not have *p53* mutations, as well as several that are not infected with HPV and have *p53* mutations that can be used as models for the study of the biology of new treatments.

Gu et al. [11] studied the effects of E6 on DNA damage-induced transcriptional activity and reported that HPV 18 E6 inhibits *p53* transcriptional activity following exposure to DNA damaging genotoxic agents, such as ultraviolet radiation. Likewise, HPV 16 E6-expressing cells treated with DNA-damaging agents did not increase p53 protein levels/activity or inhibit DNA synthesis [12]. Cells with normal p53 function respond to radiation-induced damage by exhibiting growth arrest at the G_1-S phase of the cell cycle to allow DNA repair or apoptosis to occur. Transfection of the HPV 16 *E6* gene results in enhanced resistance of fibroblasts to ionizing radiation [13].

Hamada et al. [14] reported growth inhibition of cervical cancer cells in vitro by infecting them with adenovirus-mediated *p53* DNA (Ad*p53*). Although *p53* mutations in cervical cancers are rare (1% to 6% overall incidence), they can occur and cause complete downregulation of p53 function [8]. And as inactivation of *p53* is an important step in the pathogenesis of cervical cancer, we explored the effect of wild-type *p53* transfection on a cell line containing a mutated *p53* gene (C33A), as well as on a cell line infected with HPV 16 (SiHa), which is more common in cervical cancers [9]. We introduced the wild-type *p53* gene into both irradiated and nonirradiated cervical cancer cells to assess the effects on their growth in vitro and to determine whether the mechanism of growth suppression is due to induction of apoptosis.

[1] Abbreviations used: CMV = cytomegalovirus; HPV = human papilloma virus; MOI = multiplicity of infection; PBS = phosphate-buffered saline; RB = retinoblastoma; SRB = sulforhodamine B.

Materials and methods

The squamous carcinoma cell lines SiHa and C33A, derived from human cervical tumors, were kindly provided by Dr. Reuben Lotan (Houston, TX). SiHa is HPV 16 positive and contains wild-type *p53*. C33A carries the mutant *p53*, but does not harbor the HPV virus [15]. Cells were grown as monolayer cultures in Dulbecco's minimal essential medium (DMEM/Ham's F-12), supplemented with heat-inactivated fetal bovine serum, penicillin, and streptomycin.

The recombinant adenovirus (Ad*p53*) contains the cytomegalovirus (CMV) promoter, wild-type p53 cDNA, and a SV40 polyadenylation signal in a minigene cassette inserted into the El-deleted region of modified adenovirus 5 [16]. A similarly modified adenovirus containing the *Escherichia coli β-galactosidase* gene (Ad*CMVβ-gal*) was used to evaluate transduction efficiency and as a control vector. Clones of the Ad*p53* and β-gal viruses were obtained by plaque purification, using the method of Graham and Prevec [17]. The viral stocks were propagated in 293 cells that were derived from primary embryonic kidney cells that had been harvested 36–40 h post infection, pelleted, resuspended in phosphate-buffered saline (PBS), and lysed, with the debris removed using cesium chloride gradient purification to obtain concentrated virus. The viral titers were determined by plaque assays. For viral infection, cell monolayers were infected with the adenovirus at a predetermined multiplicity of infection (MOI), and incubated for 1 h at 37°C with gentle rocking every 15 min. This was followed by the addition of culture medium and continued incubation.

To determine the efficiency of adenoviral transduction, the Ad*CMVβ-gal* construct was used to infect cells and assess β-galactosidase activity. Twenty-four hours after infection with an MOI ranging from 1 to 250, cells were washed with PBS, fixed with 0.5% glutaraldehyde, and incubated in X-gal staining solution (2% X-gal; potassium ferricyanide, $MgCl_2$, NaCl, Tris, pH 7.4). Five hundred cells in two separate quadrants of the culture dish were counted. The percentage of X-gal-positive cells was determined by counting the blue-stained cells and dividing by 500 cells. The MOI resulting in approximately 50% staining for C33A and greater than 50% for SiHa was used for other experiments.

Prior to infection, cell cultures in asynchronous growth and 70–80% confluency were trypsinized, washed, and resuspended in medium. Cells were then transferred into 15- or 50-mL centrifuge tubes of nonattachable polypropylene and laid flat in a ^{137}Cs irradiator emitting γ-rays at 4.0 Gy/min. Cells in single suspension received 4.0 Gy of radiation at room temperature, under aerobic conditions. Immediately after irradiation, 10^4 cells/well were placed in 96-well plates in quadruplicate for the growth studies or $1.5–2.5 \times 10^6$ cells were placed in 100-mm culture plates for apoptosis studies. Cells were allowed to attach to the plastic for 4–12 h and then infected with Ad*p53*, Ad*CMVβ-gal*, or mock-infected.

To assess the levels of p53 protein expressed in cells infected with Ad*p53*, Western blot analysis was performed. Cells were removed from plates by gentle scraping 48 and 72 h after mock-infection, infection with Ad*p53* or Ad*CMVβ-gal*, with or without prior radiation. Cells were pelleted, rinsed in cold Phosphate Buffered Saline (PBS), and lysed in 1 × Radio Immuno Precipitation Assay Buffer (RIPA) (Phenylmethylsufonyl Floride, $_dH_2O$, 2× RIPA, and Aprotinin) and a sonicator. Cells were then centrifuged at 13,200 rpm, and the supernatant was removed. The Bio-Rad protein assay (Dynatech MR 500, Carson City, NE) was performed to determine protein concentration. Twenty-five micrograms of protein was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Hybond-ECL membrane (Amersham, Arlington Heights, IL). The membrane was blocked with 5% nonfat dry milk and 0.1% Tween 20, probed with primary mouse monoclonal antibody (Oncogene Science, Cambridge, MA) and mouse anti-human β-actin moncloncal antibody (Amersham), and incubated over night at 4°C. Membranes were then incubated with secondary peroxidase-labeled goat anti-mouse immunoglobulin antibody (Boehringer-Mannheim, Indianapolis, IN). Anti-α actin was used as a control to establish protein equality. The membrane was processed and developed according to the manufacturer's suggestions. Densitometry was performed to determine relative p53 protein expression.

The sulforhodamine B (SRB) assay was performed according to the method of Skehan et al. [18], with some modifications. Briefly, cells plated in 96-well plates were fixed with trichloroacetic acid on selected days and stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. Thereafter, the SRB dye was discarded and cells were washed in 1.0% acetic acid to remove unbound dye. Cells were left to air dry until no moisture was visible. Unbuffered Tris saline base (10 mM) was added to cells, and plates were then shaken for 5 min to solubilize bound dye for reading. The optical densities of each culture were read on an automated spectrophotometric plate reader at a wavelength of 490 nm (Dynotech MR 500). Cells were either untreated, Ad*CMVβ-gal*-infected, Ad*p53*-infected, irradiated only, irradiated and then infected with Ad*CMVβ-gal*, or irradiated and then infected with Ad*p53*. Cells were plated at 10^4 cells/well in 96-well plates, with or without previous irradiation as described. Cells were allowed to attach for 4–8 h, and were then infected with Ad*p53*, Ad*CMVβ-gal*, or mock-infected as previously described, then incubated for varying times. C33A cells were infected with an MOI of 5, SiHa cells were transfected with an MOI of 25. These MOI were chosen because transduction experiments showed approximately 50% transduction for C33A and greater than 50% transduction for SiHa at these viral doses. Cells were fixed for the SRB assay 1, 3, 5, and 7 days after infection. The mean absorption reading of triplicate samples for each day was compared between groups. All experiments were repeated at least three times.

To determine the percentage of cells undergoing apoptosis, cells from each of the six treatment conditions were collected 72 h after infection and fixed in 4% paraformaldehyde (pH 7.4) at room temperature. Cells were washed with PBS and then permeabilized in a solution of 0.1% Triton X-100 in 0.1% sodium citrate. After two more washings in PBS, the cells were resuspended in TdT-mediated duTp Nick End Labeling (TUNEL) reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Boehringer-Mannheim), and one sample was suspended in Label solution as a negative control. The cells were incubated for at least 1 h and then analyzed by using fluorescence-activated cell sorting (Becton Dickinson, Franklin Lakes, NJ).

Results

Transduction efficiency was assessed by determining the fraction of blue cells present 24 h after infection with Ad*CMVβ-gal*. MOI describes the ratio of the number of virions per target cell at the time the virus is added to culture, and in our studies, we achieved approximately 50% transduction efficiency with an MOI of 5 for the C33A cells and over 50% transduction with an MOI of 25 for the SiHa cells. Irradiation given 6–8 h before infection resulted in no effect on transduction efficiency (Fig. 1A and B).

p53 expression in cells for each treatment condition was evaluated by Western blot analysis. The C33A cell line, which has a *p53* mutation, had p53 expression, which was measurable even in control cells; however, samples from cells infected with Ad*p53* exhibited higher levels of p53 protein (Fig. 2A). Irradiation prior to Ad*p53* infection had no effect on the level of p53 protein expression. In the SiHa cell line, which has wild-type *p53* but HPV 16 infection, only samples infected with Ad*p53* expressed detectable p53 protein. Similar to C33A, irradiation had no effect on p53 protein levels (Fig. 2B). Thus, radiation prior to Ad*p53* infection does not appear to have influenced p53 expression.

Cellular growth over a period of 7 days after infection was studied by using the SRB assay, which measures cellular protein levels. Although cell survival after irradiation has been traditionally measured by clonogenic assay, studies suggest that this assay is just as effective in measuring in vitro radiosensitivity and has better reproducibility than the clonogenic assay [18–20]. The combination of radiotherapy and Ad*p53* infection increased growth inhibition in both cell lines compared with either treatment alone. In C33A cells, a significant reduction in survival was initially observed for cells treated with combined therapy 3 days after Ad*p53* infection, such that growth of these cells was less than 15% of untreated control cells (Fig. 3A). By 7 days after infection, radiotherapy had inhibited growth of C33A cells 38%, Ad*p53* had inhibited growth 62%, and the combination of these treatments had inhibited growth by 90%. The SiHa cell line showed similar responses, with the greatest growth inhibition occurring for cells receiving radiotherapy fol-

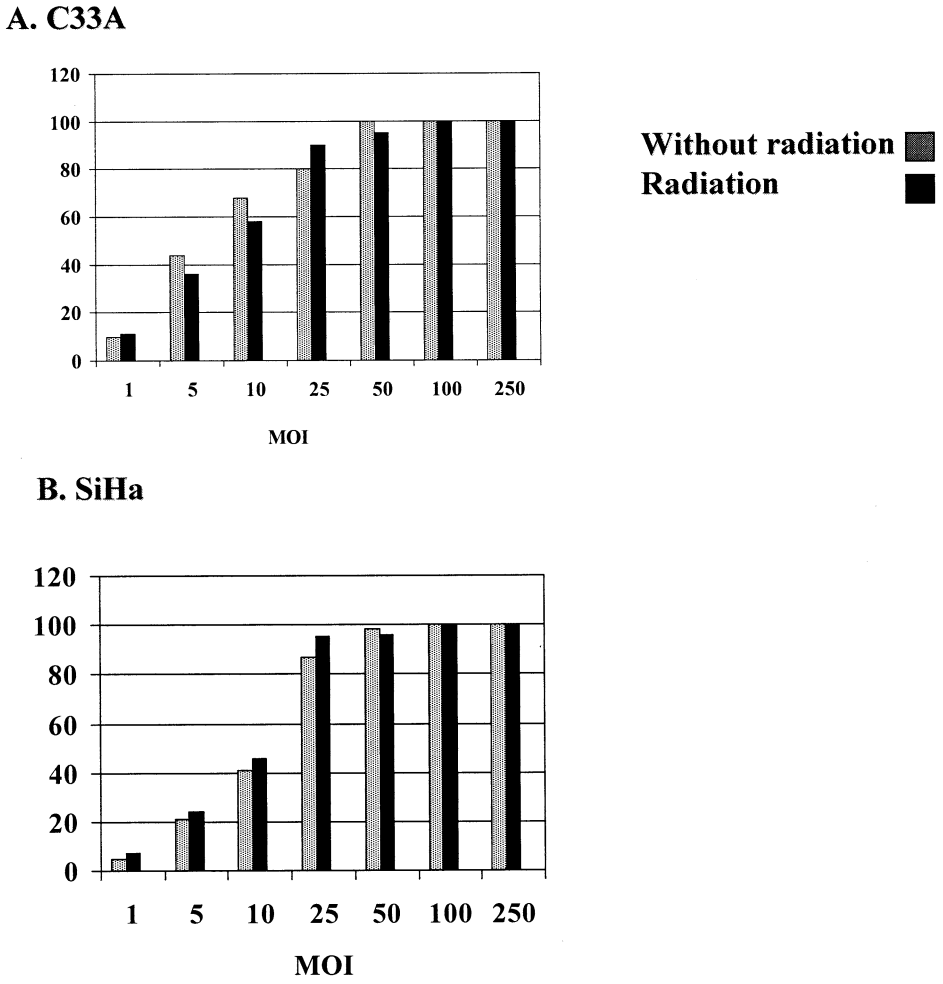

Fig. 1. Cells were infected with varying doses of Ad*CMVβ-gal* and 24 h later, stained with X-gal; 500 cells in two separate quadrants of the culture dish were counted. The percentage of X-gal-positive positive cells was determined by counting the blue-stained cells and dividing by 500 cells. No difference in transduction efficiency was found between cells that were irradiated (black columns) or not irradiated (shaded columns) prior to infection. (A) C33A cells, (B) SiHa cells. MOI = multiplicity of infection.

lowed by Ad*p53* infection (Fig. 3B). On day 7 after infection, radiotherapy inhibited cell growth by 40%, Ad*p53* by 69%, and the combination by 94%.

TUNEL analysis was done to determine whether apoptosis was occurring in these cells. This assay preferentially labels apoptotic-induced DNA strand breaks on the 3′-OH side with the enzymes terminal deoxynucleosidyl transferase (tdt) and DNA polymerase, distinguishing them from necrotic cells or cells with primary DNA strand breaks induced by radiation [21]. Although one of the benefits of the TUNEL assay is its specificity, a disadvantage is test interference, which can result in false negative or positive results. DNA cleavage can be absent or incomplete in some forms of apoptotic cell death, leading to false negative results [22]. Conversely, extensive DNA fragmentation may occur in late stages of necrosis, making it more difficult for the assay to distinguish apoptotic cells from necrotic cells, causing false positive results [23,24]. To avoid this problem, apoptotic mode of cell death was confirmed by examining the morphology of the cervical carcinoma cells, identifying patterns characteristic of apoptosis such as cellular shrinkage and formation of apoptotic bodies. As suggested by Darzynkiewicz et al. [25], we included cells floating in the media in our study sample as a method of ensuring the inclusion of all apoptotic cells. In both cell lines, Ad*p53* alone increased the percentage of cells undergoing apoptosis (17% of C33A cells and 22% of SiHa cells, as the mean percentage). Minimal, if any, apoptosis was found with 4.0-Gy irradiation alone. Both cell lines, when treated with radiotherapy prior to Ad*p53* infection, had significantly increased apoptosis, although the effect was more prominent in the C33A cells (Fig. 4). The mean percentage of C33A and SiHa cells treated with radiotherapy and Ad*p53* infection that underwent apoptosis was 91% and 38%, respectively.

Discussion

In most cervical cancers p53 function is abrogated. Although *p53* mutations in cervical cancers are rare (1% to 6%

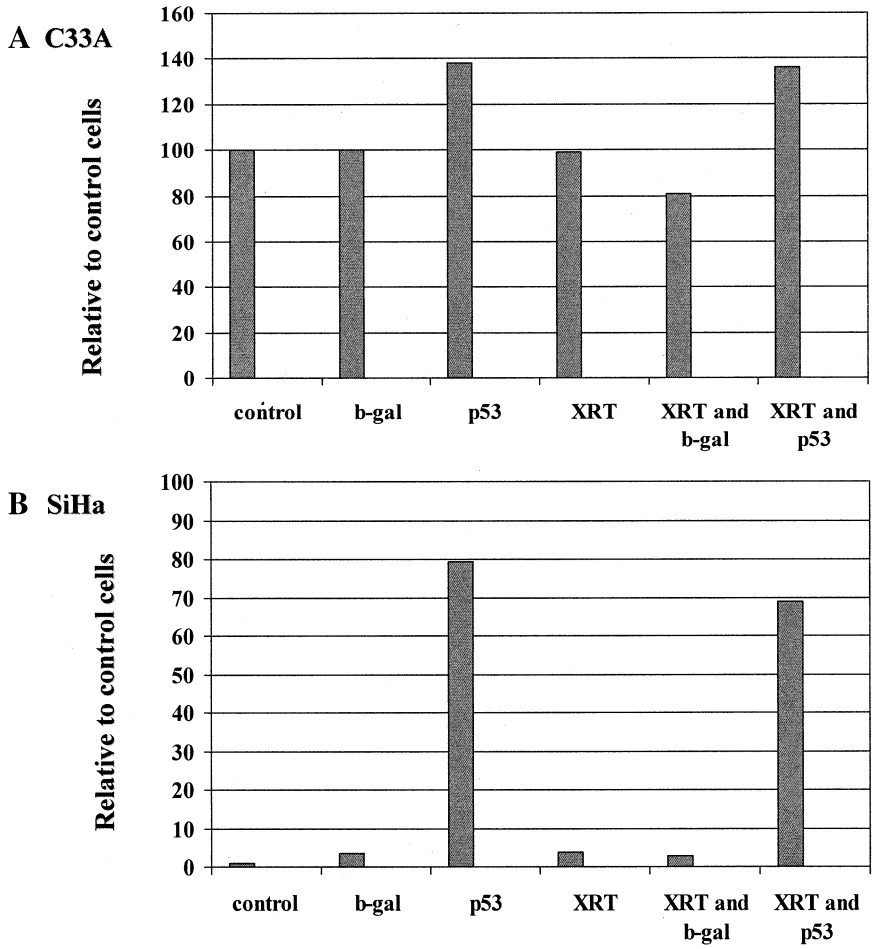

Fig. 2. Relative p53 expression. Western blot analysis for p53 expression 48 h after infection with Ad*p53*, Ad*CMVβ-gal* (β-gal), or mock-infection, with or without radiation (XRT) with 4.0 Gy prior to infection. (A) C33A (mutant *p53*, HPV negative); all cells show significant p53 expression, but cells infected with Ad*p53* had increased p53 protein levels. (B) SiHa (wild-type *p53*, HPV 16 positive); only cells infected with Ad*p53* show significant p53 expression. In both cell lines, radiation had no effect on p53 expression. Relative quantities of p53 were determined by densitometry. HPV = human papilloma virus.

overall incidence), they can occur and cause complete downregulation of p53 function [8]. And as inactivation of *p53* is an important step in the pathogenesis of cervical cancer, we explored the effect of wild-type *p53* transfection on a cell line containing a mutated *p53* gene, as well as on a cell line infected with HPV, which is more common in cervical cancers [9]. We introduced the wild-type *p53* gene into both irradiated and nonirradiated cervical cancer cells to assess the effects on their growth in vitro and to determine whether the mechanism of growth suppression is due to induction of apoptosis. Radiotherapy, followed by infection with recombinant adenovirus vector Ad*p53*, caused greater suppression of cervical cancer cells compared with either of these treatments alone. At least some of this growth inhibition was attributed to apoptosis, which was also increased when both treatments were used. Ad*p53* replaced wild-type p53 function in C33A cells, which contains a mutation in *p53*, and in SiHa cells, which harbors HPV 16 [15]. We suspect radiation-induced DNA damage triggered signaling events that were enhanced and sustained, prompting a strong response from p53-dependent apoptosis pathways. No difference was found in the ability of the virus to infect the host cell after radiation.

Combining radiotherapy and *p53*-gene therapy as treatment for colorectal, head and neck, ovarian, and lung cancers has shown promising results, with enhanced radiosensitivity when cells were infected with Ad*p53* approximately 2 days prior to radiation [26,27]. These results suggest that combining radiation with Ad*p53* may be an effective mode of therapy in cervical cancer as well. In the present study, we reversed the order of treatment by irradiating cells first and then infecting them with Ad*p53* 4–12 h after irradiation. Exposure to ionizing radiation results in a variety of DNA lesions to which cells respond differently. This could mean growth arrest, DNA repair, or lethality, depending on the cell type, radiation dose, and amount of DNA damage [28–31]. Cell death after irradiation may take the form of necrosis, apoptosis/interphase death, or reproductive/mitotic death [32,33]. A wide variety of genes, most notably *p53*, influence the complex signaling pathways, modulating cellular response to DNA damage [30].

Cells are most sensitive to the effects of radiation during

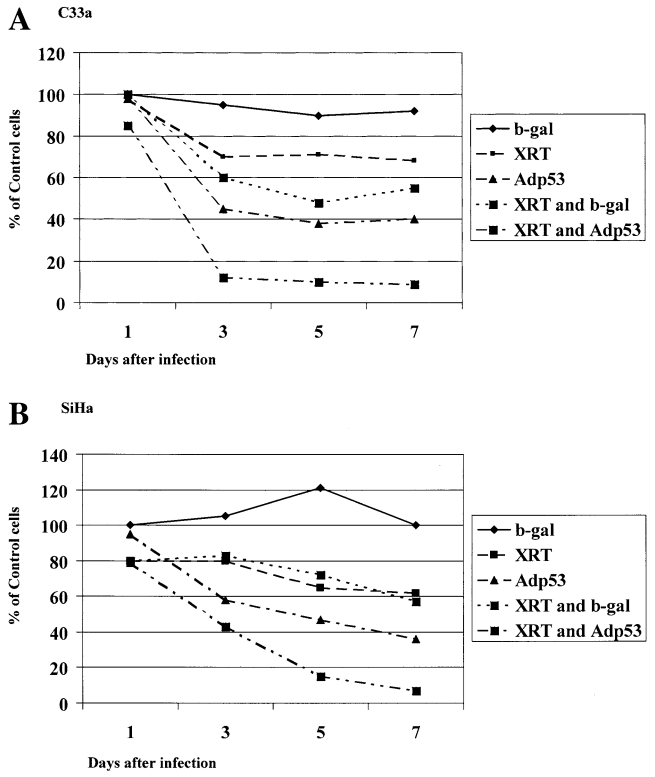

Fig. 3. Growth as a percentage of control. Growth curves using the sulforhodamine B assay. Cells were placed in one of six treatment groups: control untreated, infected with Ad*CMVβ-gal*, infected with Ad*p53*, treated with radiation (XRT) 4.0 Gy, radiation plus Ad*CMVβ-gal*, or radiation plus Ad*p53*. Cells were grown in 96-well plates in triplicate, fixed, and counted on days 1, 3, 5, and 7 after infection. Growth curves show relative cell growth of treated cells with the control untreated cells. Experiments were repeated at least three times, a representative result is shown. (A) C33A cells (p53 mutant), MOI of 5 used. (B) SiHa cells (HPV16 positive), MOI 25 used. HPV = human papilloma virus.

mitosis [34]. In our attempt to try to maximize the therapeutic potential of radiotherapy, we pretreated asynchronous cells with radiation, hoping to target the population of cells undergoing division before introducing Ad*p53*. This should lead to cell cycle arrest and interfere with immediate death from radiation. A normal function of p53 is to cause a G_1-S phase arrest following irradiation [35–37]. The p53-mediated delay in the cell cycle process after irradiation may allow time for repair of DNA damage and prevent cells from entering the S phase of the cell cycle [6,7,28]. One hypothesis is that a loss of normal p53 function would make cells more sensitive to ionizing radiation, because irradiated cells would enter into DNA synthesis without delay, before repairing potentially lethal DNA lesions. However, a study of head and neck cell lines found that p53 mutation status did not correlate with radiosensitivity [7]. Lee and Bernstein [6], in fact, found p53 mutations to correlate with resistance to ionizing radiation.

Regardless of p53 status, all cells undergo G_2 delay after irradiation [36–39]. This arrest is reported to be transient, lasting 4 to 6 h, and dose related [31,40]. With these fundamentals of cellular kinetics in mind, we hypothesize that radiation may enhance p53-induced apoptosis by introducing G_2 arrest, blocking survivors from entering the phase of the cell cycle against which the second agent, Ad*p53*, may be more effective. Over a period of time, the G_2 block began wearing off and as the p53 protein level was now high, a large cohort of DNA-damaged cells in synchrony increased cell-signaling events, enhancing p53-mediated apoptosis.

The extent of radiation-induced apoptosis varies enormously among different tumor types [41]. Although wild-type *p53* is required for radiation-induced cell death by apoptosis, it currently remains unclear what determinants regulate whether a cell will arrest in G_1 or undergo apoptosis [6,38,42,43]. Some reports suggest that the extent of DNA damage is not important in a cell's decision to select apoptosis versus G_1 arrest [44]. We speculate that if sufficient DNA damage is incurred to the cells from radiation prior to the Ad*p53* infection, by the time functional protein p53 is overexpressed, DNA damage will be so severe and

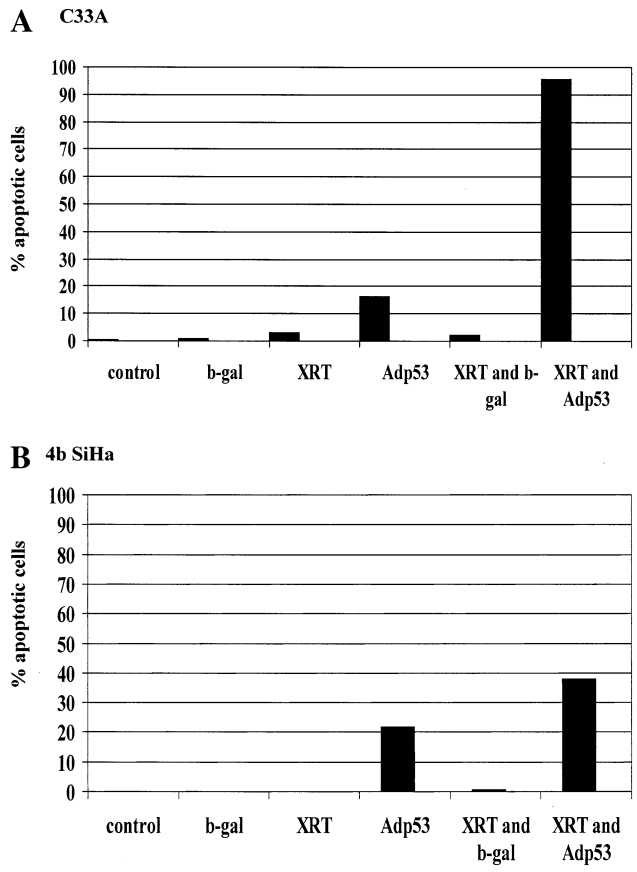

Fig. 4. Apoptosis after Adp53 and irradiation. The percentage of apoptotic cells was determined by using the TUNEL assay and flow cytometry. Cells were fixed and stained with the TUNEL reaction mixture 72 h after one of the six conditions shown, and percentage of apoptotic cells was determined. (A) The results of a typical experiment with C33A (mutant p53); (B) SIHA (HPV 16 positive). HPV = human papilloma virus; TUNEL =.

overwhelming, that the cell will deem the damages irreparable, and death by apoptosis will occur, rather than cell cycle arrest to allow for cell repair. Furthermore, we propose that the radiation-induced cellular synchrony at G_2, with subsequent progression of cells containing unrepaired DNA damage into mitosis and other cell cycles, leads to abundant cell signaling and facilitates apoptosis through a p53-dependent process. To support our hypothesis and determine the extent of apoptosis in our cells, we used the TUNEL assay. Morphologic changes suggestive of apoptosis, such as cellular shrinkage and formation of apoptotic bodies, supported the assay results. Only cells infected with Ad*p53* underwent apoptosis; and cells irradiated prior to Ad*p53* infection displayed greater apoptotic activity. Interestingly, despite the influence of radiation on apoptosis, we found no measurable increase in p53 protein expression after radiation.

Increased apoptosis occurs through several known mechanisms in the cell. One possibility is through the function of the retinoblastoma gene product (RB). RB targets E2F-1 to influence whether cells respond to p53 by undergoing growth arrest or apoptosis. However, both of the cell lines used in this study have nonfunctional RB, as SiHa is infected with HPV whose E7 protein blocks RB function, and C33A contains an inherent mutation in the *RB* gene [15]. Nonfunctioning or mutated RB is unable to bind E2F-1, therefore deregulating the function of this target protein, which induces both growth promoting and apoptotic signals. The combination of p53 induction and expression of E2F-1 appear to induce apoptotic cell death overcoming radiation-induced G_1 arrest [36,44]. Wu and Levine [45] demonstrated overexpression of E2F-1 abolished p53-mediated growth arrest and allowed progression into the S-phase and subsequently apoptosis. Since this pathway is disabled in both of our cell lines, another possibility is that it occurs through the effect of p53 on Bax and Bcl-2 levels. Bax accelerates apoptotic cell death, whereas Bcl-2 suppresses apoptosis. The relative levels of Bax and Bcl-2 expression are important in regulation of the cell. It is known that p53 can increase the level of Bax expression relative to Bcl-2 to favor apoptosis, which may enhance the susceptibility of the cells to apoptotic death after irradiation [44]. Therefore, evaluating the relative expression of these gene products after irradiation and Ad*p53* infection may help clarify this issue.

In this study, our results were similar to others, in that combination therapy was more effective than either radiation or Ad*p53* alone. Our findings suggest that p53 has an important role in inducing apoptosis in human cervical carcinoma cells after irradiation. However, the cell type, physiologic state of the cell, induction of other proteins that modulate cell death, and other factors may affect the timing and magnitude of the effect of p53 before or after irradiation.

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