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Apoptosis induced by adenovirus-mediated p14^{ARF} expression in U2OS osteosarcoma cells is associated with increased Fas expression ☆

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Abstract

The INK4A/ARF locus on chromosome 9 is a tumor suppressor gene frequently mutated in human cancers. In order to study the effects of p14ARF expression in tumor cells, we constructed a recombinant adenovirus containing p14ARF cDNA (Adp14^{ARF}). Adp14^{ARF} infection of U2OS osteosarcoma cells which has wild type p53 and mutant p14ARF revealed high levels of p14 (ARF) expression within 24 h. In addition, Adp14^{ARF}-mediated expressing of p14 (ARF) was associated with increased levels of p53, p21, and mdm2 protein. Growth inhibition assays following Adp14^{ARF} infection demonstrated that the growth of U2OS cells was inhibited relative to infection with control virus. Furthermore, TUNEL analysis as well as PARP cleavage assays demonstrated that Adp14^{ARF} infection was associated with increased apoptosis in U2OS cell line and that it was associated with Adp14^{ARF} induced overexpression of Fas and Fas-L. Addition of Fas-L neutralizing antibody NOK-1 decreased Adp14-mediated cell death, indicating that p14 (ARF) induction of the Fas pathway is associated with increased apoptosis. The finding that Adp14^{ARF} infection did not induce Fas expression in U2OS/E6 and MCF/E6 cells suggests that wild type p53 expression may be necessary for Adp14^{ARF}-mediated induction of Fas. The observation that overexpression of p53 by Adp53 infection in MCF-7 does not induce increased Fas protein levels nor apoptotic cell death suggests that p53 overexpression is required but not sufficient enough for apoptosis. These studies suggest there are other mechanisms other than induction of p53 in ARF-mediated apoptosis and gene therapy using Adp14^{ARF} may be a promising treatment option for human cancers containing wild type p53 and mutant or deleted p14 expression.

Keywords: p14ARF; Apoptosis; Fas; p53; Adenovirus

The *INK4a/ARF* gene locus produces two transcripts which use alternative first exons, E1α and E1β, each of which is joined through the identical splice acceptor site to sequences in exon 2 [1,2]. The exon 1α–exon 2–exon 3 transcript encodes the CDK4/6-specific inhibitor p16^{INK4a}, while the exon 1β–exon 2–exon 3 transcript uses an alternative reading frame and encodes ARF. p16^{INK4a} and ARF have no homology at the amino acid level [3]. Translation from the mouse β cDNA yields a polypeptide of 169 amino acids with a molecular mass of 19 kDa (p19^{ARF}) while human ARF protein is predicted to be 132 amino acids long with a molecular mass of 14 kDa. Mouse and human ARF polypeptides are 45% identical in exon 1β segments and 50% identical overall.

Homozygous deletion of the INK4a/ARF locus, which causes a loss of both the *INK4a* gene and the *ARF* gene, occurs frequently in a variety of human cancers. Thus, determining the individual contributions of each of the two genes to tumor suppression has been difficult [4,5]. However, mice lacking p19^{ARF} but retaining an

☆ *Abbreviations:* ARF, alternative reading frame; MDM2, mouse double minute 2; CMV, cytomegalovirus; pfu, plaque-forming unit; FACS, fluorescence-activated cell sorting; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; FITC, fluorescein isothiocyanate.

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intact $p16^{INK4a}$ gene develop multiple tumors [5], indicating that ARF, like INK4a, is also a bona fide tumor suppressor. ARF overexpression results in cell cycle arrest, however, this is not associated with ARF binding to CDKs or direct inhibition of cyclin-CDK activity [3]. Furthermore, in contrast to INK4a, there is no evidence that ARF exerts its growth-inhibitory effect through the pRB pathway [6]. ARF does require the presence of wild type p53 for induction of cell cycle arrest and cell lines that lack p53 or have p53 mutations are resistant to growth arrest by ectopic expression of ARF [5].

Cell cycle arrest and apoptosis are two cellular responses induced by a wide variety of DNA damaging agents. Cell cycle arrest provides a critical opportunity for cells to repair damaged DNA, while cells with extensive, and presumably irreparable, DNA damage can be eliminated by apoptosis. It is not surprising that one or both of these processes are frequently altered in cancer cells [7–11].

Fas (CD95/APO1), a death-domain bearing member of the tumor necrosis factor receptor superfamily, is a transmembrane protein that, on specific engagement with Fas-ligand initiates an apoptotic response in cells. Oligomerization of the Fas receptors following cross-linking with Fas ligand or agonistic antibodies 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 [12,13]. Several studies have examined the relationship between Fas and p53 expression [14]. Overexpression of p53 resulted in Fas induction in lung cancer cells [15]. 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 [16]. We also previously reported that overexpression of wild type p53 can induce Fas protein and that this effect was observed predominantly in cells which express mutant p53 or null for p53 [17].

To study the function of p14ARF, we have constructed a recombinant replication-deficient adenovirus expressing human $p14^{ARF}$ ($Adp14^{ARF}$). Previously we reported that infection of cells with $Adp14^{ARF}$ resulted in high levels of $p14^{ARF}$ expression and induction of apoptosis in human breast cancer cell lines MCF-7 and MDA-MB-231 [18]. In this report, we provide evidences that $Adp14^{ARF}$ induces apoptosis in human breast and osteosarcoma cancer cells are associated with upregulation of Fas.

Results

Adenovirus-mediated $p14^{ARF}$ expression

$Adp14^{ARF}$-mediated $p14^{ARF}$ expression was examined by Western blot analysis following infection of human osteosarcoma cancer cells U2OS which have wild type p53 and mutant type p14 with $Adp14^{ARF}$. As shown in Fig. 1A, 24 h after infection with $Adp14^{ARF}$, there was a marked increase in $p14^{ARF}$ protein levels in U2OS cells. In U2OS cell lines, the increase in $p14^{ARF}$ protein following infection with $Adp14^{ARF}$ was dependent on the

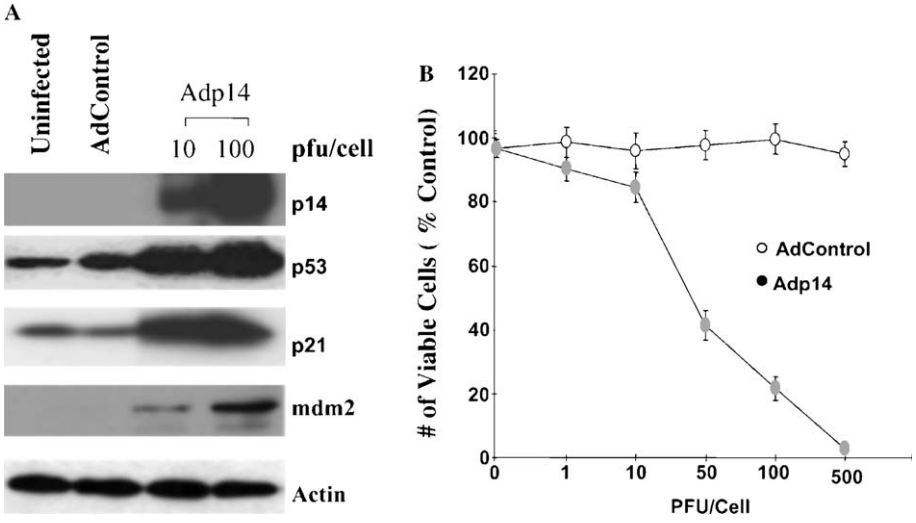

Fig. 1. Effects of $Adp14^{ARF}$ on expression of p14 and associated proteins and on cell viability following Adp14 infection. (A) Western blot analysis of p14, p53, p21, and mdm2 in U2OS cells following Adp14 infection. Cells were infected with 100 pfu/cell of AdControl and varying doses of Adp14. After 24 h incubation at 37 °C, whole cell lysates were collected and subjected to Western blot analysis as described in Materials and methods. Cell lines and adenoviral vectors used are shown on top of the panel; mock infected cells are labeled uninfected (Ui). (B) Dose dependent effects of Adp14 on the survival of U2OS cells. Cells were infected with Adp14 and incubated for 3 days. Viable cells were counted by trypan-blue exclusion assay. Results are expressed as percentage of change in the number of viable cells from cultures without any treatments. Data points represent the mean values of four replicates, with bars indicating SEM.

dose of the virus. Previous studies had indicated that $p14^{ARF}$ expression can alter the intracellular levels of p53 protein as well as the level of p21, a p53 inducible protein, in p53 WT cell lines. As shown in Fig. 1B, the levels of p53 in U2OS cells increased following infection with $Adp14^{ARF}$. Moreover, following 24 h infection of U2OS cells with $Adp14^{ARF}$, there was marked increase in p21 expression as well (Fig. 1A). Similar results were obtained in MCF-7 cells (data not shown). Thus, $p14^{ARF}$-mediated increase in p53 protein was also associated with increased levels of p21 protein in U2OS and MCF-7 cells.

Since previous studies have indicated that the induction of p53 protein by $p14^{ARF}$ resulted from the interaction of $p14^{ARF}$ with mdm2, a p53 binding protein, we also examined mdm2 levels following $Adp14^{ARF}$ infection. As shown in Fig. 1A, $Adp14^{ARF}$ infection of U2OS cells also results in a marked increase in mdm2 protein levels.

$Adp14^{ARF}$-mediated cytotoxicity in U2OS osteosarcoma cells

The effect of $Adp14^{ARF}$ infection on cytotoxicity of human cancer cells was assessed using a trypan blue exclusion assay. As shown in Fig. 1B, 3 days following infection with $Adp14^{ARF}$, there was marked cytotoxicity in both U2OS osteosarcoma cells. Following infection with $Adp14^{ARF}$ at 500 pfu/cell, >80% of cells were not viable. In contrast, following infection with 500 pfu/cell of AdControl, >90% of cells were viable. The IC_{50} for $Adp14^{ARF}$ was between 10 and 50 pfu/cell for U2OS cells (Fig. 1B), while that for the control virus was over 5000 pfu/cell for U2OS cells (data not shown). Thus, $Adp14^{ARF}$ is approximately 100 times more cytotoxic to U2OS osteosarcoma cells compared to control virus.

$p14^{ARF}$ expression induces apoptosis in U2OS osteosarcoma cells

We next investigated the mechanism of $Adp14^{ARF}$ induced cytotoxicity in U2OS cells. As shown in Fig. 2A, PI staining indicated that uninfected or AdControl-infected U2OS cells had a small sub-G1 population. In contrast, cells infected with $Adp14^{ARF}$ exhibited a marked increase in the proportion of sub-G1 population of cells (23.6% versus 10.4% for control virus infected U2OS). Similar results were obtained using TUNEL assay for evaluation of $Adp14^{ARF}$ induced apoptosis. As shown in Fig. 2B, $Adp14^{ARF}$ infection of U2OS cells resulted in a marked shift of the fluorescent peak. These results are consistent with induction of apoptosis in U2OS cell lines following infection with $Adp14^{ARF}$.

The process of apoptosis involves the activation of a cascade of proteolytic enzymes belonging to the caspase family resulting in cleavage of several key cellular components including poly (ADP-ribose) polymerase (PARP) and CPP32. We therefore examined the cleavage of these proteins following $Adp14^{ARF}$ infection. As shown in Fig. 3A, infection of U2OS cells with $Adp14^{ARF}$ resulted in the appearance of a specific M_r 85000 cleavage product of PARP, which was not observed in uninfected cells or cells infected with a control virus (AdControl).

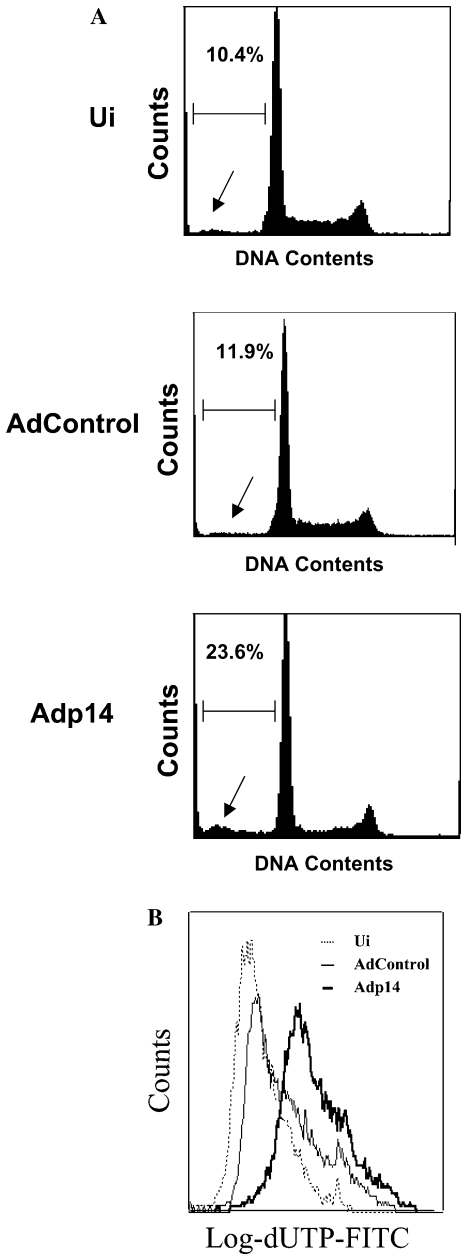

Fig. 2. Cell cycle and apoptosis analysis of Adp14 infected cells. (A) U2OS Cells (1×10^6) were infected with Adp14 (100 pfu), AdControl (100 pfu) or uninfected for 3 days and subjected to FACS analysis using propium iodide. Cells were analyzed for apoptosis as represented by the appearance of a sub-G1 population. (B) Flow cytometric TUNEL analyses for apoptosis on U2OS cells. Cells (1×10^6) were infected with Adp14 (100 pfu), AdControl (100 pfu) or uninfected for 3 days and subjected to TUNEL analysis.

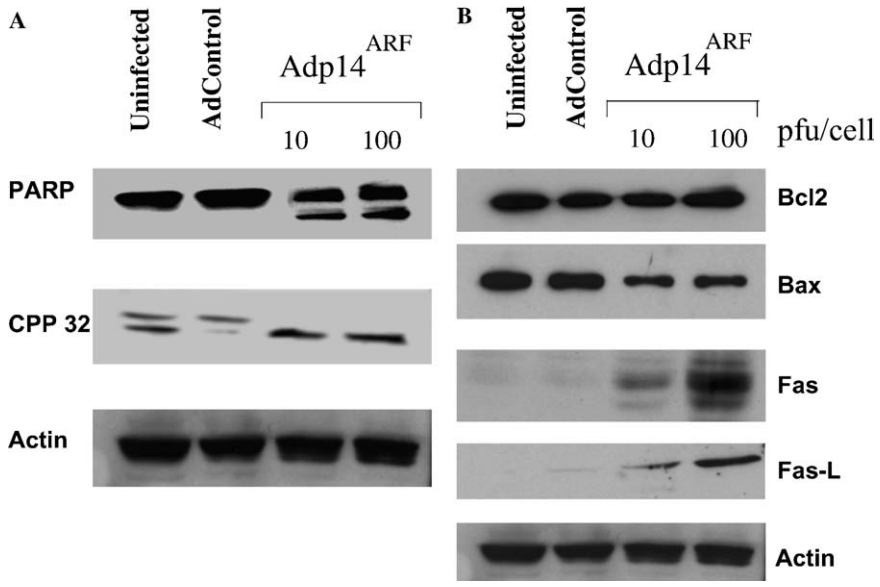

Fig. 3. Western blot analyses of apoptosis related proteins. Cells (5×10^6) were infected with 100 pfu/cell of AdControl and 10, 100 pfu/cell of Adp14. After 3 days of incubation, whole cells were collected and subjected to Western blot analysis. (A) PARP, CPP32, and actin in U2OS cells. (B) Bcl2, Bax, Fas, Fas-L, and actin in U2OS cells.

Since degradation of CPP32 has also been reported to be associated with apoptosis, we also examined the levels of CPP32 protein in uninfected U2OS cells or cells infected with AdControl virus or Adp14^{ARF} virus. As shown in Fig. 3A, the CPP32 cleavage protein level increased in U2OS cells following infection with increasing doses of Adp14^{ARF}, but remained unchanged in cells infected with AdControl. These results provide additional evidence that overexpression of p14 following Adp14 infection results in apoptosis in U2OS osteosarcoma cells.

Role of Fas induction in p14^{ARF}-mediated apoptosis

To elucidate the mechanism of p14 induced apoptosis in U2OS cells, we examined the expression of several proteins involved in apoptosis including Bcl2, Bax, Fas, and Fas-L by Western blot analysis. As shown in Fig. 3B, although Bcl2 expression was unchanged and Bax expression was little decreased, both Fas and Fas-L protein levels were increased in Adp14^{ARF} infected U2OS cells compared to uninfected or AdControl infected U2OS cells. These studies suggest a role for Fas/FasL in ARF-mediated cytotoxicity in U2OS cells.

Apoptosis and Fas expression by p14 is p53 dependent

To test the effect of p53 on Adp14 induced Fas expression, we made U2OS/E6 cells which were transfected with human papillomavirus E6 gene to inactivate p53 protein. Fig. 4A showed p53 protein was successfully deleted from U2OS cells. U2OS/E6 cells showed decreased FAS and Fas-L expression following infection with Adp14^{ARF} compare with U2OS cells (Fig. 4B). U2OS/E6 cells were also more resistant to Adp14 compare with U2OS cells (data not shown). These results

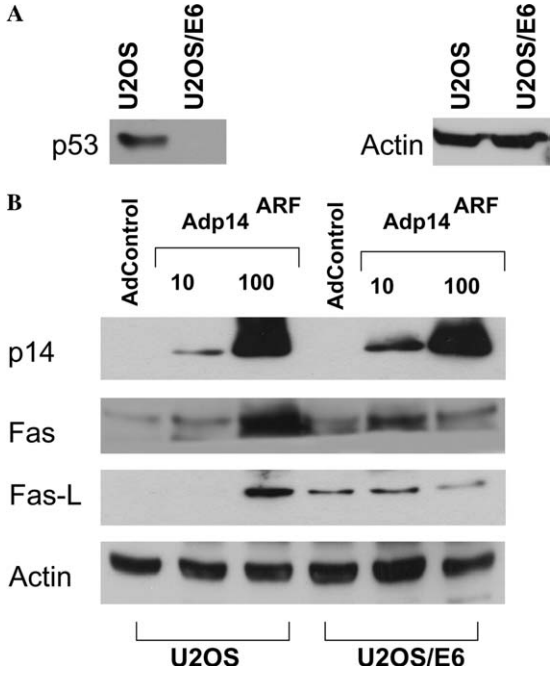

Fig. 4. Effects of Adp14 infection on Fas and Fas-L expression are associated with p53 status on cells. (A) U2OS Cells were infected with E6 virus to delete p53 protein. Western blot analysis showed p53 was successfully deleted from U2OS cells. (B) Western blot analyses of p14, Fas, Fas-L, and actin in U2OS and U2OS/E6 cells. Cells (5×10^6) were infected with 10 and 100 pfu/cell of Adp14 and 100 pfu/cell of AdControl. After 3 days of incubation, whole cells were collected and subjected to Western blot analysis.

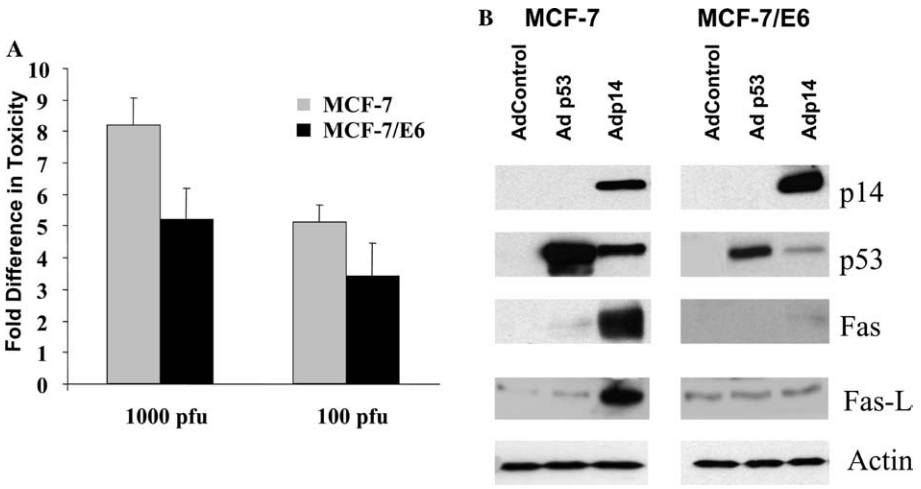

Fig. 5. Effect of Adp14 infection on cell cytotoxicity associated p53 status on cells. (A) Cells (1×10^3) were infected with 100 and 1000 pfu/cell of Adp14 to MCF-7 and MCF-7/E6 cells. After 5 days of incubation cell viability was accessed by MTT assay. The IC_{50s} for Adp14 and AdControl were calculated for each cell line assuming the survival rate of uninfected cells was 100%. Results shown are the ratios of the IC_{50s} following Adp14 infection relative to the IC_{50s} of AdControl in each cell line. Data points represent the mean values of four replicates, with bars indicating SEM. (B) Western blot analyses of p14, p53, Fas, Fas-L, and actin in MCF-7 and MCF-7/E6 cells. Cells (5×10^6) were infected with 100 pfu/cell AdControl, Adp53, and Adp14. After 3 days of incubation, whole cells were collected and subjected to Western blot analysis.

suggest p53 role on Fas and Fas-L expression after p14 overexpression. Shown in Fig. 5A are the cytotoxicity results of $Adp14^{ARF}$ infection in MCF-7 cells which containing wild type p53 and MCF-7/E6 cells which were transfected with human papillomavirus E6 gene which binds to and inactivate p53. We have previously shown that $Adp14^{ARF}$ infection results in increased cell death in MCF-7 cells relative to control virus [18]. As shown in Fig. 5A, the cytotoxicity of $Adp14^{ARF}$ is diminished in cells containing reduced levels of wild type p53 (MCF-7/E6). Furthermore, the decreased toxicity of $Adp14^{ARF}$ in MCF-7/E6 cells is also associated with decreased FAS expression in these cells following infection with $Adp14^{ARF}$.

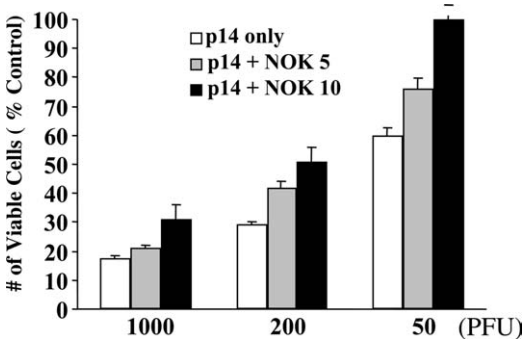

Fig. 6. Inhibition of Adp14-mediated cytotoxicity by anti Fas-L antibody in MCF-7 cells. Cells (1×10^3) were infected with 50, 200, and 1000 pfu/cell of Adp14. 5 and 10 μg/ml of NOK-1 was added 24 h after Adp14 infection. After 5 days of incubation cell viability was accessed by MTT assay. Results are expressed as percentage of change in the number of viable cells from cultures without any treatments. Data points represent the mean values of four replicates, with bars indicating SEM.

We have previously shown that infection of MCF-7 cells with an adenoviral vector expressing wild type p53 (Adp53) resulted in cell cycle arrest but not apoptosis [17]. As shown in Fig. 5B, while $Adp14^{ARF}$ infection in MCF-7 cells results in increased p53 expression and a concomitant increase in FAS expression, infection with Adp53 results in p53 overexpression without induction of FAS. Taken together these results indicate that wild type p53 overexpression may be necessary, but not necessarily sufficient for $p14^{ARF}$-mediated cytotoxicity (Fig. 5B). Furthermore, the pretreatment of MCF-7 cells with neutralizing antibody against Fas-L (NOK-1) reduced toxicity of $Adp14^{ARF}$ (Fig. 6). These results suggest that the Fas pathway is involved in the induction of apoptosis in MCF-7 cells by $Adp14^{ARF}$. These studies also suggest that $p14^{ARF}$-mediated apoptosis involves other cellular change mechanisms in addition to p53 overexpression.

Discussion

Recent studies have shown that the INK4A/ARF gene can function as a tumor suppressor gene and that $p14^{ARF}$ can inhibit cell growth. In previous report we constructed an adenoviral vector expressing $p14^{ARF}$ to study the function of $p14^{ARF}$ overexpression in human breast cancer cells [18]. In this report we also found that overexpression of $p14^{ARF}$ results in apoptosis in U2OS cells. This was demonstrated by TUNEL assays as well as cleavage of PARP and CPP32. The mechanism of $p14^{ARF}$ inducing apoptosis in the cells involves overexpression of both FAS and FAS ligand and is inhibited at least in part, by incubation of cells with antibody directed against FAS ligand.

Previous studies by our laboratory have shown that overexpression of wild type p53 in MCF-7 cells using a recombinant adenoviral vector resulted in overexpression of p21 and increased G1 cell cycle arrest without induction of apoptosis [19]. Furthermore, we found that overexpression of wild type p53 in MCF-7 cells did not result in increased expression of FAS, while other studies showed that enhanced expression of FAS and wild type p53 expression in MCF-7 cells did result in increased apoptosis [17]. In this study, we report that overexpression of $p14^{ARF}$ in U2OS and MCF-7 cells resulted in increased expression of both p53 and Fas and induction of apoptosis. These effects of $p14^{ARF}$ expression involve other changes in cells in addition to induction of wild type p53 while previous studies had suggested the effect of $p14^{ARF}$ is mediated through p53 [20]. The results proposed in this report as well as previous studies from our laboratory [18] suggest that the induction of wild type p53 by p53 adenoviral vector is by itself not sufficient to induce p53-mediated apoptosis in cells, indicating that other effects of $p14^{ARF}$ are needed to induce apoptosis in addition to p53 regulation.

CDKIs, including p21 and p16, have been proposed as candidates for gene therapy of cancer [19,21]. $p14^{ARF}$ may also represent a good choice for gene therapy of cancer as it can produce not only cell cycle arrest but also it can induce apoptosis in some cancer cells. Additional studies on the usefulness of $Adp14^{ARF}$ as a vector for gene therapy in combination with chemotherapy should be explored.

Materials and methods

Cell culture. Human breast cancer cell line MCF-7 (ATCC HTB-22, wild type p53, mutant type p14), MCF-7/E6 (kindly provided by Dr. Albert J Fornace Jr, NCI, NIH, Bethesda, MD, USA), and osteosarcoma cell line U2OS (ATCC HTB96, wild type p53, mutant type p14) were cultured in Improved minimal essential medium (IMEM) (Life Technologies, Gaithersburg, MD, USA) containing 10% fetal bovine serum as described previously [22]. U2OS/E6 cells were obtained after transduction of human papillomavirus E6 and selection with neomycin for 10 days. p53 deletion was confirmed with Western blot analysis.

Construction of adenoviral vectors and infection procedure. Human $p14^{ARF}$ cDNA that expresses full-length human $p14^{ARF}$ protein was a kind gift from Dr. K.H. Vousden of the National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD. A recombinant adenovirus containing human $p14^{ARF}$ cDNA, designated $Adp14^{ARF}$, was constructed by homologous recombination using methods described previously [19,21,23]. Briefly, human $p14^{ARF}$ cDNA was cloned into an adenovirus shuttle vector containing Ad5 backbone. Following transfection with Ad5 genomic DNA into 293 cells, recombinant plaques were screened for the presence of $p14^{ARF}$ sequences by polymerase chain reaction (PCR) using the following primers: 5′-AGTGAGGGTTTTCGTGGTTCAC-3′ (upstream) and 5′-ATGACCTGGTCTTCTAGGAAGCGG-3′ (downstream). The control adenovirus (AdControl) used in this study is identical to $Adp14^{ARF}$ except that it does not contain any transgene.

Infection with $Adp14^{ARF}$ and the control virus (AdControl) was accomplished by incubating cells with various doses of each virus (expressed as pfu/cell) in serum-free medium for 2 h, followed by the addition of medium containing 10% serum and further incubation at 37 °C for the required time.

Cytotoxicity assays. Cell viability was assessed following appropriate treatment by microscopic examination of trypan-blue stained cells or was measured using the standard MTT assay, as described [24]. Cells (1500/well) were plated in each 96-well plate and incubated at 37 °C overnight. Following exposure to different concentrations of Adp14 or AdControl for 7 days at 37 °C, MTT (50 μl of 2 mg/ml) in 1× PBS was added to each well and the cells incubated 4 h at 37 °C. Following centrifugation of plates at 500*g* for 10 min, DMSO (120 μl) was added to each well and incubated for 1 h at room temperature on an orbital shaker. The OD_{570} was determined using Ultra-microplate reader (ELx 808, Bio-tek instrument, Winooski, VT, USA). Cytotoxicity was expressed as the percentage of OD_{570} relative to that of untreated cells.

Cell cycle analysis. Cells were plated in 10-cm dishes ($1–2 \times 10^6$ cells/dish) 1 day prior to infection. Cells were exposed to various concentrations of $Adp14^{ARF}$ (0, 1, 10, and 100 pfu/cell) for increasing times at 37 °C. Cells were harvested, fixed with 2% paraformaldehyde, and stained for cell cycle analysis using propidium iodide (PI) [25]. DNA content was measured using a flow cytometer (FACSCalibur, Becton–Dickinson, Mountain View, CA, USA) and cell cycle analysis was performed with the ModFit software (Becton–Dickinson). Analysis of the sub-G1 (sub-diploid) population was done by measuring the fluorescence activity of propidium iodide (PI)-stained DNA of permeabilized and fixed cells on the FACSCalibur as detailed above [22,25].

$$\%\text{sub-G1 population} = \frac{\text{Number of cells below G1}}{\text{Total number analyzed}} \times 100\%.$$

TUNEL assay. For detection of apoptosis by the TUNEL method, cells were plated in 15-cm dishes. When cells were 70% confluent, $Adp14^{ARF}$ was added for varying times. The cells were harvested by gentle pipetting and collected by centrifugation at 1000*g* for 5 min. Cells were washed with ice cold PBS and then fixed with 4% paraformaldehyde. TUNEL assay was performed using MEBSTAIN Apoptosis kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions.

Western blot analysis. Cells were plated in 15-cm dishes. When cells were 70% confluent, adenoviral vectors were added. Following incubation at 37 °C, the cells were harvested by gentle scraping, and whole cell lysates were prepared and subjected to Western blot analysis as described previously [22,25]. Blots were probed with 2 μg/ml of antibody reactive with $p14^{ARF}$ (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (Ab-1; Oncogene, Cambridge, MA, USA), CPP32 (K-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PARP (Ab-2; Calbiochem, San Diego, CA, USA), FAS (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and FAS-L (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

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References

[1] L. Mao, A. Merlo, G. Bedi, G.I. Shapiro, C.D. Edwards, B.J. Rollins, D. Sidransky, A novel p16INK4A transcript, Cancer Res. 55 (1995) 2995–2997.

[2] S. Stone, P. Jiang, P. Dayananth, S.V. Tavtigian, H. Katcher, D. Parry, G. Peters, A. Kamb, Complex structure and regulation of the P16 (MTS1) locus, Cancer Res. 55 (1995) 2988–2994.
[3] D.E. Quelle, F. Zindy, R.A. Ashmun, C.J. Sherr, Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest, Cell 83 (1995) 993–1000.
[4] M. Serrano, H. Lee, L. Chin, C. Cordon-Cardo, D. Beach, R.A. DePinho, Role of the INK4a locus in tumor suppression and cell mortality, Cell 85 (1996) 27–37.
[5] T. Kamijo, F. Zindy, M.F. Roussel, D.E. Quelle, J.R. Downing, R.A. Ashmun, G. Grosveld, C.J. Sherr, Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF, Cell 91 (1997) 649–659.
[6] C.J. Sherr, J.D. Weber, The ARF/p53 pathway, Curr. Opin. Genet. Dev. 10 (2000) 94–99.
[7] S.J. Martin, D.R. Green, Apoptosis and cancer: the failure of controls on cell death and cell survival, Crit. Rev. Oncol. Hematol. 18 (1995) 137–153.
[8] H. Nojima, Cell cycle checkpoints, chromosome stability and the progression of cancer, Hum. Cell 10 (1997) 221–230.
[9] H. Pan, C. Yin, T. Van Dyke, Apoptosis and cancer mechanisms, Cancer Surv. 29 (1997) 305–327.
[10] L. Philipson, Cell cycle exit: growth arrest, apoptosis, and tumor suppression revisited, Mol. Med. 4 (1998) 205–213.
[11] C.J. Sherr, Cancer cell cycles, Science 274 (1996) 1672–1677.
[12] J.P. Medema, R.E. Toes, C. Scaffidi, T.S. Zheng, R.A. Flavell, C.J. Melief, M.E. Peter, R. Offringa, P.H. Krammer, Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis, Eur. J. Immunol. 27 (1997) 3492–3498.
[13] J.P. Medema, C. Scaffidi, F.C. Kischkel, A. Shevchenko, M. Mann, P.H. Krammer, M.E. Peter, FLICE is activated by association with the CD95 death-inducing signaling complex (DISC), EMBO J. 16 (1997) 2794–2804.
[14] R. Takahashi, Role of p53 tumor suppressor gene and Fas/Apo-1 in induction of apoptosis and differentiation of cancer cells, Leukemia 11 (Suppl. 3) (1997) 331–333.
[15] L.B. Owen-Schaub, W. Zhang, J.C. Cusack, L.S. Angelo, S.M. Santee, T. Fujiwara, J.A. Roth, A.B. Deisseroth, W.W. Zhang, E. Kruzel, et al., Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression, Mol. Cell Biol. 15 (1995) 3032–3040.
[16] M. Muller, S. Strand, H. Hug, E.M. Heinemann, H. Walczak, W.J. Hofmann, W. Stremmel, P.H. Krammer, P.R. Galle, Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53, J. Clin. Invest. 99 (1997) 403–413.
[17] A.N. Rakkar, Y. Katayose, M. Kim, C. Craig, E. Ohri, Z. Li, K.H. Cowan, P. Seth, A novel adenoviral vector expressing human Fas/CD95/APO-1 enhances p53-mediated apoptosis, Cell Death Differ. 6 (1999) 326–333.
[18] X. Deng, M. Kim, D. Vandier, Y.J. Jung, T. Rikiyama, M.K. Sgagias, M. Goldsmith, K.H. Cowan, Recombinant adenovirus-mediated p14(ARF) overexpression sensitizes human breast cancer cells to cisplatin, Biochem. Biophys. Res. Commun. 296 (2002) 792–798.
[19] D. Katayose, R. Wersto, K.H. Cowan, P. Seth, Effects of a recombinant adenovirus expressing WAF1/Cip1 on cell growth, cell cycle, and apoptosis, Cell Growth Differ. 6 (1995) 1207–1212.
[20] F.J. Stott, S. Bates, M.C. James, B.B. McConnell, M. Starborg, S. Brookes, I. Palmero, K. Ryan, E. Hara, K.H. Vousden, G. Peters, The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2, EMBO J. 17 (1998) 5001–5014.
[21] C. Craig, M. Kim, E. Ohri, R. Wersto, D. Katayose, Z. Li, Y.H. Choi, B. Mudahar, S. Srivastava, P. Seth, K. Cowan, Effects of adenovirus-mediated p16INK4A expression on cell cycle arrest are determined by endogenous p16 and Rb status in human cancer cells, Oncogene 16 (1998) 265–272.
[22] M. Kim, Y. Katayose, Q. Li, A.N. Rakkar, Z. Li, S.G. Hwang, D. Katayose, J. Trepel, K.H. Cowan, P. Seth, Recombinant adenovirus expressing Von Hippel–Lindau-mediated cell cycle arrest is associated with the induction of cyclin-dependent kinase inhibitor p27Kip1, Biochem. Biophys. Res. Commun. 253 (1998) 672–677.
[23] C. Craig, R. Wersto, M. Kim, E. Ohri, Z. Li, D. Katayose, S.J. Lee, J. Trepel, K. Cowan, P. Seth, A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells, Oncogene 14 (1997) 2283–2289.
[24] D. Gerlier, N. Thomasset, Use of MTT colorimetric assay to measure cell activation, J. Immunol. Methods 94 (1986) 57–63.
[25] Y. Katayose, M. Kim, A.N. Rakkar, Z. Li, K.H. Cowan, P. Seth, Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27, Cancer Res. 57 (1997) 5441–5445.