Co-transduction of Apaf-1 and Caspase-9 Augments Etoposide-induced Apoptosis in U-373MG Glioma Cells

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Several apoptosis-related genes have been reported to be involved in chemotherapy-induced apoptosis in cancers. An assessment of the relationship between expression of those genes and the degree of chemotherapy-induced apoptosis may be useful in improving the efficacy of cancer therapy. We transduced Apaf-1 (apoptotic protease-activating factor-1) and caspase-9 into U-373MG glioma cells using adenovirus (Adv) vectors in the presence of etoposide and evaluated the degree of apoptosis. The degree of apoptosis in etoposide-treated U-373MG cells infected with Adv for Apaf-1 (Adv-APAF1) was higher (27%) than that in cells infected with control Adv (14%), that in cells infected with Adv for caspase-9 (Adv-Casp9) was higher (34%) than that in cells infected with Adv-APAF1, and that in cells infected with both Adv-APAF1 and Adv-Casp9 was the highest (41%). Treatment with etoposide increased expression of p53 and decreased expression of Bcl-XL in U-373MG cells which harbored mutant p53. These results indicate that the expression of Apaf-1 and caspase-9 may be important determinants in predicting the sensitivity of cancers to chemotherapy. Adv-mediated co-transduction of Apaf-1 and caspase-9 should render cancer cells highly sensitive to chemotherapy.

Key words: Apoptosis — Etoposide — Apaf-1 — Caspase-9 — Glioma

Cancers are often refractory to conventional therapies such as chemotherapy; to improve therapy, it would be useful to be able to predict the efficacy of chemotherapy by analysis of the status or expression level of apoptosis-related genes and to be able to induce genes that augment cytotoxicity. The p53 gene has been reported to play a critical role in the success of chemotherapy.1) Treatment with chemotherapeutic agents induces nuclear accumulation of p53,2) and DNA binding by activated p53.3) Cancers harboring functional p53 are sensitive to chemotherapy,4) whereas those with mutated p53 are resistant to chemotherapeutic agents.1) Induced expression of wild-type p53 augments the efficacy of chemotherapy.6) Recently, Apaf-1 (apoptotic protease-activating factor-1) and caspase-9 have been reported to be essential downstream components of p53 in Myc-induced apoptosis of early passage mouse embryo fibroblasts.7) Apaf-1 and caspase-9 profoundly suppress the tumorigenicity of Myc-Ras-transformed mouse embryo fibroblasts.7) As Apaf-1 and caspase-9 are downstream components of wild-type p53 in cancers, they may facilitate chemotherapeutic agent-induced cytotoxicity. Indeed, over-expression of Apaf-1 alone promotes apoptosis in chemotherapeutic agent-treated HL-60 cells and prevention of caspase-9 activation blocked drug-induced apoptosis in cancer cells sensitized by E1A, an adenoviral oncoogene.8, 9)

In this study, we transduced U-373MG cells harboring mutated p53 with Apaf-1 and/or caspase-9 using adenovirus (Adv) vectors, and evaluated the degree of apoptosis in the presence of etoposide, a chemotherapeutic drug.

MATERIALS AND METHODS

Cell line and reagents The U-373MG glioma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). U-373MG cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. 4’-Desmethyllepipodophyllotoxin 9-[4,6-O-ethylidene-β-D-glucopyranoside] (etoposide) was purchased from Sigma (E-1383, St. Louis, MO), and dissolved in dimethylsulfoxide (DMSO, #043-07216, Wako, Osaka).
Generation of the adenoviral vectors We utilized the Cre/loxP system for constructing Adv for Apaf-1 (Adv-Apaf1) and caspase-9 (Adv-Casp9), to avoid the damage to the 293 producer cells. The 657 bp fragment from the AvaI (nt 884) to the AvaI (nt 1541) site in the CA promoter region was deleted from pCALNL, which the product was designated pCdNLNL. A Nol linker was inserted into the EagI/Clal (blunt end) of pCdNLNL, affording pCdL2. The SnaBI/BglII site of pCdL2 was inserted into the SnaBI/BglII site of pCACC, which generated pCdclL2. The AavrI/NsiI (blunt end) fragment from pDNA3-hApaf1 containing the full-length human Apaf-1 cDNA was inserted into the EcoRI site (blunt end) of pCdclL2, which generated pCdclL2-Apaf-1. The cosmid pAxCdclL2-Apaf-1 was generated by inserting the Clal expression cassette from pCdclL2-Apaf-1 into the Clal site of the cosmid pAxCyw1.13) The EcoRI/BamHI fragment from pT773-U18-caspase9,14) containing the full-length human caspase-9 cDNA, was inserted into the EcoRI/BglIII site of pCdclL2, which generated pCdclL2-caspase-9. The cosmid pAxCdclL2-caspase-9 was generated by inserting by the Clal expression cassette from pCdclL2-caspase-9 into the Clal site of the cosmid pAxCyw1. The on/off switching unit, pCdclL2-Apaf-1 or pCdclL2-caspase-9, consisted of the Cd promoter and poly(A) sequence, flanked by a pair of loxP sites, the Apaf-1 or caspase-9 gene, and another poly(A) signal, respectively. Neither the CdclL2-Apaf-1 nor the CdclL2-caspase-9 construct expresses the respective gene without NCre. In the presence of NCre, which contains the Cre recombining coding region, the Cd promoter and respective gene are joined together, resulting in the Adv expressing the respective gene under the control of the Cd promoter.

pAxCdclL2-Apaf-1 and pAxCdclL2-caspase-9 were co-transfected with the genomic DNA-terminal peptide complex of Adv type 5 (Ad5dix); the recombinant Advs were generated using the method described by Miyake et al.13) Adv-mediated gene transduction was performed as described by Yoshida and Hamada.11) The AxCdLNL-Apaf-1 and AxCdLNL-caspase-9 were always co-infected with AxCANCRe,10) at a ratio of MOIs (multiplicities of infection) of 2:1. The total MOI of Adv used to infect each cell was kept the same in all experiments, by supplementing with the Adv from which E1 and E3 had been deleted (Adv-dE).10)

Assessment of cell death The degree of cell death was assessed by determining the percentage of cells that had died, the percentage of hypodiploid cells, and the degree of DNA fragmentation. To determine the percentage of cells that had died, the cells that adhered to the plate and those that were detached were stained with 0.2% trypan blue. The cells were then counted using a hemocytometer. The percentage of hypodiploid cells was determined by the method described previously.13) Briefly, ethanol-permeabilized cells were stained with propidium iodide and then analyzed using the CELLQuest software on a FACSscan (Becton Dickinson, San Jose, CA). The DNA fluorescence gate was set up to exclude cell aggregates and debris. The percentage of cells that had undergone apoptosis was assessed in terms of the ratio of the fluorescent area smaller than the G0/G1 peak to the total area of fluorescence. DNA fragments in apoptotic cells were detected using the “APO-BRDU” kit (Pharmingen, San Diego, CA), according to the manufacturer’s instructions. Briefly, the 3′-hydroxyl ends of the DNA in apoptotic cells were labeled with bromodeoxyuridine triphosphate nucleotides (Br-dUTP) by terminal deoxynucleotidyl transferase and Br-dUTP was stained by applying a fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody. The samples were analyzed by FACSscan. Two samples of cells for each experimental condition were analyzed and each experiment was repeated twice. All of the assays were performed 48 h after infection with Adv vectors. Electron microscopical analysis for apoptotic cell death was performed as described previously.15)

Detection of Fas FACS analysis of Fas expression on the cell surface was performed as described previously.15) Briefly, one million cells were incubated with 0.25 μg of FITC-conjugated mouse anti-human CD95 antibody (Pharmingen, #33454X) for 20 min at 4°C, washed twice, resuspended in phosphate-buffered saline (PBS) containing 5% FBS, and analyzed by FACSscan using CELLQuest software according to the manufacturer’s instructions (Becton Dickinson). An isotype-matched control antibody was used for negative control staining.

Immunoblot analysis Immunoblot analysis was performed using the ECL kit (Amersham, Buckinghamshire, England), as previously described.13) Briefly, cells were lysed in 2× lysis buffer (10 mM Tris/HC1 pH 8.0, 0.2% NP40, 1 mM EDTA) for 15 min on ice, centrifuged at 18 500g for 2 min, and the protein content of the supernatant was quantified using the DC Protein Assay Kit (Pharmingen, San Diego, CA). The DNA fluorescence kit (Pharmingen, San Diego, CA), according to the manufacturer’s instructions. An equal volume of 2× Laemmli buffer was added to the supernatant, and the mix was boiled for 5 min. Equal amounts of protein from each extract (5 μg per lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking with 5% dry milk in TBS (10 mM Tris/HC1 pH 7.5, 150 mM sodium chloride), the membranes were incubated with the primary antibody for 1 h. We used mouse anti-human caspase-3 antibody (Transduction Laboratories, #C31720, Lexington, KY), mouse anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibody (BIOMOL Research Laboratories, USA-250, Plymouth Meeting, PA), mouse anti-human p53 mon-
oclonal antibody (Oncogene Science, #OP09, Cambridge, MA), mouse anti-human Bax monoclonal antibody (Medical and Biological Laboratories, #M010-3, Nagoya), rabbit anti-human Bcl-x polyclonal antibody (Transduction Laboratories, #B22630), rabbit anti-human caspase-9 polyclonal antibody (IMGENEX, #IMG-123, San Diego, CA), rabbit anti-Apaf-1 polyclonal antibody (ProSci, #2013, Poway, CA), and mouse anti-β-actin monoclonal antibody (Sigma, #A-5441). After washing, the membranes were incubated with 30 µl (per 15 ml) of horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham, #NA9340) for Apaf-1, caspase-9, and Bcl-X<sub>l</sub> using the ECL (enhanced chemiluminescence) kit, according to the manufacturer’s instructions (Amer-

RESULTS

Co-transduction of Apaf-1 and caspase-9 strongly enhanced etoposide-induced apoptosis in U-373MG cells For the transduction of Apaf-1 and caspase-9 genes, we infected U-373MG cells with Adv-APAF1 and/or Adv-Casp9, with or without etoposide (50 µM), and evaluated the expression of Apaf-1 and caspase-9 (Fig. 1). Transfection of Adv-APAF1 or Adv-Casp9 into U-373MG cells induced the expression of exogenous Apaf-1 (Fig. 1, lanes 3, 4, 7), or caspase-9 (Fig. 1, lanes 5, 6, 7, 8), respectively. Co-infection of Adv-APAF1 and Adv-Casp9 in the presence of etoposide did not increase the expression of Apaf-1 (Fig. 1, lane 8), possibly because Apaf-1 was cleaved as a result of an apoptotic signal and the anti-

body against Apaf-1 used in this study did not recognize the cleaved product. Similarly, the expression level of caspase-9 after co-infection of Adv-APAF1 and Adv-

Casp9 in the presence of etoposide (Fig. 1, lane 8) was lower than that in the absence of etoposide (Fig. 1, lane 7), possibly due to the cleavage of caspase-9, through the activation of an apoptotic cascade. To evaluate whether the degree of apoptosis induced by etoposide was increased by transduction of either Apaf-1 or caspase-9, or co-transduction of Apaf-1 and caspase-9, U-373MG cells were infected with either Adv-APAF1 (MOI 60) or Adv-Casp9 (MOI 60) or co-infected with both Ads, in the presence or absence of etoposide. The resulting percentage of cell death and degree of apoptosis, assessed as the percentage of hypodiploid cells or degree of DNA fragmentation in U-373MG cells, were evaluated. In the presence of etoposide, U-373MG cells after infection with Adv-APAF1 (Fig. 2A, upper right panel) were more effectively killed than those infected with control Adv-dE (Fig. 2A, upper middle panel). In addition, U-

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![Fig. 1. Immunoblot analysis of Apaf-1, caspase-9 and β-actin protein extracted from U-373MG cells 48 h after infection with Adv-APAF1, Adv-Casp9, Adv for NCre (Adv-Cre), or Adv-dE in various combinations in the presence and absence of etoposide (50 µM). The MOI of Adv used to infect the particular glioma cells is noted in parentheses. The total MOI was kept constant by supplementing with Adv-dE. Lane 1, U-373MG infected with Adv-dE (MOI 180) and treated with DMSO; lane 2, U-373MG infected with Adv-dE (MOI 180) and treated with etoposide; lane 3, U-373MG co-infected with Adv-APAF1 (MOI 60), Adv-Cre (MOI 30) and Adv-dE (MOI 90) and treated with DMSO; lane 4, U-373MG co-infected with Adv-APAF1 (MOI 60), Adv-Cre (MOI 30) and Adv-dE (MOI 90) and treated with etoposide; lane 5, U-373MG co-infected with Adv-Casp9 (MOI 60), Adv-Cre (MOI 30) and Adv-dE (MOI 90) and treated with DMSO; lane 6, U-373MG co-infected with Adv-Casp9 (MOI 60), Adv-Cre (MOI 30) and Adv-dE (MOI 90) and treated with etoposide; lane 7, U-373MG co-infected with Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), and Adv-Cre (MOI 60) and treated with etoposide.](image-url)
Fig. 2. A. Photomicrographs of U-373MG cells that had been infected with Adv-dE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), or Adv-APAF1 (MOI 60) and Adv-Casp9 (MOI 60), in the presence or absence of etoposide (ET). Cells were examined 48 h after infection (original magnification ×100). The total MOI in all of the experiments was kept the same by supplementing with Adv-dE. B. Percentage of dead cells in the U-373MG cells, measured by trypan blue exclusion 48 h after infection with Adv-dE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), Adv-Cre, or co-infection with those Adv's, in the presence or absence of etoposide. The total MOI was kept constant by supplementing with Adv-dE. The mean ± standard deviation of the percentage of dead cells in three preparations under two separate experimental conditions is shown. Experiments were repeated twice and representative results are shown. Data are normalized to the rate of spontaneous cell death occurring in U-373MG cells infected with control Adv-dE treated with DMSO (<10%). * P<0.01. C. FACS analysis of the percentage of hypodiploid cells, 48 h after infection of U-373MG cells with Adv-dE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), Adv-Cre, or co-infection with those Adv's, in the presence or absence of etoposide. The total MOI was kept constant by supplementing with Adv-dE. D. DNA fragmentation of U-373MG cells infected with Adv-dE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), Adv-Cre, or co-infected with those Adv's in the presence or absence of etoposide. The total MOI was kept constant by supplementing with Adv-dE. The assay was performed as described in the “Materials and Methods” 48 h after infection. Experiments were repeated twice and representative results are shown. E. Ultrastructural analysis of U-373MG cells infected with Adv-dE (MOI 180) and treated with DMSO, or co-infected with Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), and Adv-Cre (MOI 60) in the presence of etoposide. Upper panels: U-373MG cells 48 h after infection with Adv-dE and treated with DMSO (left, ×5000; right, ×40 000); lower panels: U-373MG cells 48 h after co-infection with Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), and Adv-Cre (MOI 60) in the presence of etoposide (left, ×8000; right, ×40 000). Forty-eight hours after co-infection with Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), and Adv-Cre (MOI 60) in the presence of etoposide, most of the U-373MG cells showed mitochondrial damage (arrowhead in lower right panel), whereas 48 h after infection with Adv-dE in the absence of etoposide, U-373MG cells showed normal mitochondria (arrowhead in upper right panel) and nuclei (arrow in upper left panel). Condensation of chromatin (arrow in lower left panel), a hallmark of apoptosis, appeared in U-373MG cells 48 h after co-infection with Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), and Adv-Cre (MOI 60), in the presence of etoposide.
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among those infected with Adv-APAF1 and treated with etoposide (Fig. 2B; \( P < 0.01 \), Student’s \( t \) test). The percentage of cells that died among U-373MG cells infected with Adv-APAF1 and Adv-Casp9, and treated with etoposide (28±1.5%) was higher than among those infected with Adv-APAF1 and Adv-Casp9 in the absence of etoposide (17±3.0%), or with Adv-Casp9 in the presence of etoposide (Fig. 2B; \( P < 0.01 \), Student’s \( t \) test).

The percentage of hypodiploid cells reflects the degree of apoptosis. In the presence of etoposide, the percentage of hypodiploid cells in U-373MG cells infected with Adv-APAF1 was higher (27%) than that in cells infected with control Adv-deE (12%), that in cells infected with Adv-Casp9 was higher (34%) than that in cells infected with Adv-APAF1, and that in cells infected with Adv-APAF1 and Adv-Casp9 was the highest (42%) (Fig. 2C).

The Br-dUTP uptake assay—for the detection of DNA fragments—revealed that in the presence of etoposide, the percentage of apoptotic cells in U-373MG cells infected with Adv-APAF1 was higher (27%) than that in cells infected with control Adv-deE (14%), that in cells infected with Adv-Casp9 was higher (34%) than that in cells infected with Adv-APAF1, and that in cells infected with Adv-APAF1 and Adv-Casp9 was the highest (41%) (Fig. 2D). These results indicate that in the presence of etoposide, the degree of apoptosis in U-373MG cells infected with Adv-APAF1 was higher than that in cells infected with control Adv-deE, that in cells infected with Adv-Casp9 was higher than that in cells infected with Adv-APAF1, and that in cells infected with Adv-APAF1 and Adv-Casp9 was highest. In addition, it seems that the combination of Apaf-1, caspase-9 and etoposide gave a more-than-additive effect, since the degree of apoptosis induced by infection with Adv-APAF1 and Adv-Casp9 in the presence of etoposide was much higher than the sum of those induced by infection with Adv-APAF1 and Adv-

Fig. 3. A. Immunoblot analysis of the p53 (A-1), Bax (A-1), Bcl-X\(_L\) (A-1), caspase-3 (A-2), PARP (A-2) and \(\beta\)-actin (A-2) proteins extracted from U-373MG cells 48 h after infection with Adv-deE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), Adv-Cre, or co-infection with those Adv’s, in the presence or absence of etoposide. The MOI of Adv used to infect the U-373MG cells is the same as in Fig. 1. B. Expression of Fas on the surface of U-373MG cells measured by FACS after infection with Adv-deE, Adv-APAF1 (MOI 60), Adv-Casp9 (MOI 60), Adv-Cre, or co-infection with those Adv’s in the presence or absence of etoposide. The U-373MG cells were stained with the anti-Fas antibody as described in “Materials and Methods.” The total MOI was kept constant by supplementing with Adv-deE. The data are presented as the log peak fluorescence intensity of each cell line stained with (1) isotype-matched control, (2) anti-Fas antibody, (3) anti-Fas antibody 48 h after co-infection with Adv-APAF1, Adv-Casp9 and Adv-Cre in the absence of etoposide, and (4) anti-Fas antibody 48 h after co-infection with Adv-APAF1, Adv-Casp9 and Adv-Cre in the presence of etoposide.
Casp9 in the absence of etoposide and by infection with Adv-dE in the presence of etoposide (Fig. 2, C and D).

Electron microscopic analysis of U-373MG cells co-infected with Adv-APAF1 and Adv-Casp9 in the presence of etoposide, revealed condensed chromatin in the nuclei (Fig. 2E, lower left panel) 48 h after infection, a feature of apoptotic cell death. Moreover, in U-373MG cells co-infected with Adv-APAF1 and Adv-Casp9 in the presence of etoposide, most of the mitochondria were damaged (Fig. 2E, lower right panel), although infection with control Adv-dE in the absence of etoposide did not induce such damage (Fig. 2E, upper right panel).

**Treatment with etoposide increased expression of p53 and decreased expression of Bcl-XL**

To evaluate the mechanism of apoptosis in U-373MG cells induced by infection with Adv-APAF1 and/or Adv-Casp9 in the presence of etoposide, we examined the expression of apoptosis-related genes including p53, Bax, Bcl-XL, caspase-3, PARP, and Fas. Expression of another apoptosis-related gene, Bcl-2, in U-373MG cells was extremely low (data not shown). It was found that treatment with etoposide increased the expression level of p53 and decreased the expression level of Bcl-XL in U-373MG cells (Fig. 3A-1, lanes 2, 4, 6 and 8). No alterations were observed in the levels of Bax (Fig. 3A-1), caspase-3 (Fig. 3A-2), or Fas (Fig. 3B), although the cleaved form (p85) of PARP appeared in the U-373MG cells treated with etoposide (Fig. 3A-2, lanes 2, 4, 6 and 8). PARP is a substrate on which caspase-3 acts, so this result might suggest the activation of caspase-3, although the antibody used in this study did not recognize the cleaved product of caspase-3. Possibly because caspase-3 was abundantly expressed, the expression levels of caspase-3 were not reduced despite the cleavage of PARP.

**DISCUSSION**

We report here that in the presence of etoposide, apoptosis in U-373MG cells was enhanced most strongly by co-infection with Adv-APAF1 and Adv-Casp9 (Fig. 2). In the presence of dATP and cytochrome c, which are released from mitochondria in response to various apoptosis-inducing stimuli, Apaf-1 binds to procaspase-9, and through oligomerization causes the cleavage and activation of procaspase-9, resulting in activation of caspase-3.12, 17, 18

The proteolytic activity of caspase-9 in a complex with Apaf-1 is several orders of magnitude higher than that of the free enzyme.19 This is consistent with the finding that combined transduction of Apaf-1 and caspase-9 in the presence of etoposide induced the most extensive apoptosis in U-373MG cells.

Apaf-1 and caspase-9 play central roles in mitochondria-dependent apoptosis, by opening the permeability transition pore.9, 20, 21 The apoptosis induced by chemotherapeutic agents such as etoposide, which can be antagonized by Bcl-2 and Bcl-XL, is also entirely controlled by a mitochondrial pathway.22, 23 Etoposide induces release of mitochondrial cytochrome c followed by activation of caspase-9 and caspase-3.24 – 26 This suggests that the U-373 MG cells treated with etoposide and Apaf-1 and caspase-9 induction underwent large-scale apoptosis through damage to mitochondria and possibly release of cytochrome c from mitochondria.27 In this study, electron microscopic analysis demonstrated damage to the mitochondria 48 h after infection with Adv-APAF1 and Adv-Casp9 in the presence of etoposide (Fig. 2E). It is to be noted that there are some discrepancies between the morphology and degree of cell death (Fig. 2, A and B), or between the degree of cell death and apoptosis (Fig. 2, B and C or D). For instance, the U-373MG cells after infection with Adv-Casp9, although the degrees of cell death are roughly equal (Fig. 2, A and B). In addition, the U-373MG cells after infection with Adv-APAF1, Adv-Casp9, or Adv-APAF1 and Adv-Casp9 in the absence of etoposide showed significantly increased cell death in comparison with control cells. However, the percentages of hypodiploid cells (reflecting the degree of apoptosis) in these cells or cells showing DNA fragmentation were roughly equal (Fig. 2, B, C, and D). This suggests that the mode of cell death after infection with Adv-APAF1 and/or Adv-Casp9 might not only apoptosis, but also some other type of cell death, such as necrosis. Indeed, mitochondrial damage induced by various stimuli such as transduction of Bax leads to necrotic as well as apoptotic cell death.28, 29 Thus, transduction of Apaf-1 or caspase-9, which would lead to mitochondria-dependent apoptosis, might possibly induce not only apoptotic, but also necrotic cell death. The increase of cell death after infection with Adv-APAF1 and Adv-Casp9 in the presence of etoposide might be due to necrosis as well as apoptosis, although the main mode of cell death was apoptosis.

Interestingly, treatment with etoposide in U-373MG cells harboring mutated p53 induced increased expression of p53 and decreased expression of Bcl-XL (Fig. 3A-1). As p53 gene mutations are associated with decreased sensitivity of cancers to etoposide, the result suggests that increased expression of mutated p53 would induce an anti-apoptotic effect against etoposide-induced apoptosis in U-373MG cells.30 On the other hand, as Bcl-XL protects cells against etoposide-induced apoptosis, the decreased expression of Bcl-XL would augment apoptosis in U-373MG cells.22 Further investigations are required to determine which apoptosis-related genes are involved in etoposide-induced apoptosis.

It has been reported that various proapoptotic genes including p53,1, 3, 5 Bax,30, 31 Fas,32, 33 FADD,34
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We thank Dr. S. Fukuda for assistance with the electron microscopic studies, and Dr. H. Shinoura for technical assistance. This work was supported in part by a Special Grant for Advanced Research on Cancer from the Japanese Ministry of Education, Science, Sports and Culture and grants from the Japanese Ministry of Health and Welfare and the Takeda Science Foundation. (Received November 13, 2000/Revised January 18, 2001/ Accepted January 24, 2001)

ACKNOWLEDGMENTS

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