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Adenovirus-mediated Transfer of *Fas Ligand* Gene Augments Radiation-induced Apoptosis in U-373MG Glioma Cells

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Most malignant astrocytomas (gliomas) express a high level of Fas, whereas the surrounding normal tissues such as neurons and astrocytes express a very low level of Fas. Thus, transduction of Fas ligand would selectively kill malignant astrocytoma cells. On the other hand, glioma cells harboring p53 mutation have been reported to be resistant to conventional therapies including radiation. To override the resistance mechanism of glioma cells with p53 mutation to radiation, we transduced U-373MG malignant astrocytoma (glioma) cells harboring mutant p53 with Fas ligand via an adenovirus (Adv) vector in combination with X-ray irradiation, and evaluated the degree of apoptosis. The degree of apoptosis in U-373MG cells infected with the Adv for Fas ligand (Adv-FL) and treated with irradiation (81%) was much higher than that in U-373MG cells infected with Adv-FL and not treated with irradiation (0.8%) or that in U-373MG cells infected with the control Adv for lacZ and treated with irradiation (5.0%). In U-373MG cells infected with Adv-FL, irradiation increased the expression of Fas ligand. Coincident with the increase in Fas ligand, there was a marked reduction in the caspase-3 level and a marked increase in the cleaved form of poly(ADP-ribose) polymerase (PARP), which are downstream components of Fas ligand-mediated apoptosis. This suggests that the enhanced activation of caspase-3 by the transduction of Fas ligand combined with irradiation, induced extensive apoptosis in U-373MG cells. In summary, transduction of Fas ligand may override the resistance mechanism to radiotherapy in glioma cells harboring p53 mutation.

Key words: Apoptosis — Radiation — Fas ligand — Glioma — Adenovirus

Various genes are involved in the mechanism of the resistance of cancer cells to treatment, including radiation. Since the *p53* tumor suppressor gene plays a critical role in radiation-induced apoptosis, mutation of p53 increases the radioresistance of cancer cells, including gliomas.[1–4] Apoptosis-related genes such as wild-type *p53* and *Bax* have been transduced into cancer cells to override the radioresistance mechanism of cancer cells harboring mutant p53.[5, 6]

It has been reported that nearly all malignant astrocytomas express a high level of Fas,[7, 8] while neurons and astrocytes express a low level of Fas, if any, and consequently are resistant to Fas ligand-mediated killing.[9, 10] Hence, induction of Fas ligand may selectively kill malignant astrocytomas.[11] Caspase-3, a mediator of apoptosis, is a common downstream component of Fas ligand- and radiation-induced apoptotic pathways.[12–14] Therefore, transduction of Fas ligand may augment radiation-induced apoptosis in malignant astrocytomas through enhanced activation of caspase-3, and spare normal neurons and astrocytes. In this study, we transduced the U-373MG malignant astrocytoma (glioma) cell line harboring mutant p53 with Fas ligand via an adenovirus vector driven by myelin basic protein (MBP) promoter, a glioma-specific promoter, concomitant with X-ray irradiation. We found that transduction of Fas ligand with irradiation induced extensive apoptosis in U-373MG cells.

MATERIALS AND METHODS

Cell culture and treatment of cells 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 m*M* glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Irradiation of cell cultures was performed as indicated at room temperature by placing the cell cultures in an M-150WE X-ray machine (SOFTEX, Ebina) with a 0.5 filter

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at a dose rate of 1.0 Gy/min. Irradiation (40 Gy) was applied immediately after the adenovirus (Adv) was added to the cell culture.

Generation of the adenoviral vectors pAxCALNL-hFL was constructed as described previously.[11] The on/off switching unit CALNL-hFL consisted of the chicken β-actin (CA) promoter, the *neo* gene and a poly(A) sequence flanked by a pair of loxP sites, *Fas ligand* gene, and another poly(A) signal.[15] The CALNL-hFL without Cre recombinase expresses only the *neo* gene and does not express the *Fas ligand* gene. In the presence of Cre recombinase, the *neo* gene between the loxP sites is excised, and the CA promoter and *Fas ligand* gene are joined together, resulting in the Adv expressing *Fas ligand* gene under the control of the CA promoter.[15] The recombinant adenovirus AdexMBP-NL-Cre that carries the *Cre recombinase* gene with a nuclear localization signal under the control of the mouse MBP promoter, was constructed as described previously.[16] The cosmid pAxCALNL-hFL was co-transfected with the genomic DNA-terminal protein complex of adenovirus type 5 (Ad5dlX), and the recombinant adenoviruses were generated according to the method described by Miyake *et al*.[17] Adv-mediated gene transduction was performed as described previously.[18] AxCALNL-hFL was always co-infected with AdexMBP-NL-Cre at a ratio of multiplicities of infection (MOIs) of 1:5. The total MOI of adenovirus used to infect each cell preparation, was kept the same in all experiments by supplementing with the Adv for lacZ (Adv-lacZ).[15, 17]

Assessment of cell death The degree of cell death was assessed by determining the percentage of cells that had died, 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. 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 the Br-dUTP were stained by a fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody. The samples were analyzed by FACScan. Two samples of cells for each experimental condition were analyzed and repeated more than twice. All of the assays were performed 3 days after infection with Adv vectors. Electron microscopic analysis for apoptotic cell death was performed as described previously.[11]

Detection of Fas ligand and Fas Fluorescence-activated cell sorter (FACS) analysis of Fas ligand and Fas expression on the surface of U-373MG cells was performed as described previously.[11] Briefly, for FACS analysis of Fas ligand, the cells were detached, washed, and incubated with 5 ml (per 10^6 cells) of biotinylated mouse anti-human Fas ligand antibody (Sumitomo Electric, #SE-50082X, Yokohama) at 4°C for 20 min. After having been washed twice with phosphate-buffered saline (PBS) containing 5% FBS, 1×10^6 cells were incubated with 0.5 mg of streptavidin-phycoerythrin conjugate (Pharmingen, #13025D, San Diego, CA) at 4°C for 20 min, washed twice with PBS, and analyzed by FACScan using CELLQuest software according to the manufacturer's instructions (Becton Dickinson, San Jose, CA). An isotype-matched control antibody was used as a negative control. For FACS analysis of Fas, 1×10^6 cells were incubated with 0.25 mg of FITC-conjugated mouse anti-human CD95 antibody (Pharmingen, #33454X) at 4°C for 20 min, washed twice, resuspended in PBS with 5% FBS, and analyzed by FACScan. An isotype-matched control antibody was used as a negative control.

Immunoblot analysis Immunoblot analysis was performed using the ECL kit (Amersham, Buckinghamshire, England), as previously described.[11] Briefly, cells were lysed in 2× lysis buffer (10 m*M* Tris/HCl pH 8.0, 0.2% NP40, 1 m*M* EDTA) for 15 min on ice, and this was centrifuged at 18 500*g* at 4°C for 2 min. The protein content of the supernatant was quantified using the DC Protein Assay Kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. An equal volume of 2× Laemmli buffer was added to the supernatant, and the mixture 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 m*M* Tris-HCl pH 7.5, 150 m*M* 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), and mouse anti-β-actin monoclonal antibody (Sigma, #A-5441, St. Louis, MO). After having been washed the membranes were incubated with 30 μl (per 15 ml) of horseradish peroxidase-conjugated rabbit anti-mouse IgG+A+M (H+L) (ZYMED Laboratories, #61-6420, San Francisco, CA) using the ECL kit, according to the manufacturer's instructions (Amersham).

RESULTS AND DISCUSSION

Fas ligand expression induced by infection with the Adv for Fas ligand was upregulated by X-ray irradiation The Fas-mediated pathway has been reported to be involved in the irradiation-induced apoptosis of glioma cells.[19] To evaluate whether X-ray irradiation (40 Gy) alters the level of Fas ligand expression on the surface of

U-373MG cells harboring mutant p53, we examined the expression of Fas ligand 2 days after infection with the Adv for Fas ligand (Adv-FL). For glioma-specific expression of Fas ligand, we used an Adv carrying the *Cre recombinase* gene controlled by the MBP promoter, together with the Adv for CALNL-FL, in which a spacer DNA with a poly(A) addition signal flanked by a pair of loxP sequences would be excised by the Cre recombinase. Infection of Adv-FL at MOI 100 indicates the co-infection of AxCALNL-hFL at MOI 100 with AdexMBP-NL-Cre at MOI 500. FACS analysis revealed that infection of Adv-FL at MOI 100 induced the expression of Fas ligand (Fig. 1, No. 3), which was not expressed constitutively on the surface of U-373MG cells (Fig. 1, No. 2). The expression of Fas ligand was markedly upregulated in the radiation-treated U-373MG cells infected with Adv-FL (Fig. 1, No. 5), whereas it was not upregulated in the radiation-treated U-373MG cells that were not infected with Adv-FL (Fig. 1, No. 4). It should be noted that expression of other apoptosis-related genes (*Apaf-1* or *caspase-9*) transferred by Adv was not upregulated by irradiation in U-373MG cells, indicating that the Adv-mediated transgene expression was not enhanced by irradiation in general (data not shown). These results indicate that X-ray irradiation remarkably upregulated Fas ligand expression in Fas ligand-transduced U-373MG cells.

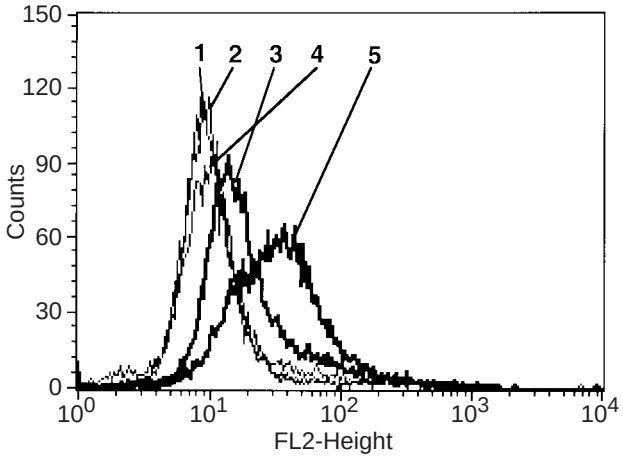

Fig. 1. Expression of Fas ligand on the surface of U-373MG cells as measured by FACS after infection of the cells with Adv-FL (MOI 100) (which indicates AxCALNL-hFL at MOI 100 with AdexMBP-NL-Cre at MOI 500), or control Adv-lacZ (MOI 600) with or without irradiation (40 Gy). The U-373MG cells were stained with anti-Fas ligand antibody as described in "Materials and Methods." The total MOI was kept constant with control Adv-lacZ. The data are presented as the log peak fluorescence intensity of: (1) U-373MG cells stained with isotype-matched control 2 days after infection with Adv-lacZ; (2) U-373MG stained with anti-Fas ligand antibody 2 days after infection with Adv-lacZ; (3) U-373MG stained with anti-Fas ligand antibody 2 days after infection with Adv-FL; (4) U-373MG stained with anti-Fas ligand antibody 2 days after infection with Adv-lacZ and irradiation; and (5) U-373MG stained with anti-Fas ligand antibody 2 days after infection with Adv-FL and irradiation.

Infection with Adv-FL greatly enhanced radiation-induced apoptosis in U-373MG cells To assess whether the transduction of Fas ligand augmented radiation-induced apoptosis, U-373MG cells were infected with Adv-FL (MOI 100) or control Adv-lacZ with or without X-ray irradiation (40 Gy). To exclude the cytotoxicity of Adv itself, we used the same MOIs of Adv-FL as those of control Adv-lacZ for the infection of U-373MG cells. Three days after infection and irradiation, the percentage of cell death and degree of DNA fragmentation in the U-373MG cells were analyzed. The U-373MG cells infected with Adv-FL concomitantly with irradiation (Fig. 2A, lower right panel) were more effectively killed than those infected with Adv-FL without irradiation (Fig. 2A, upper right panel), or those infected with control Adv-lacZ with irradiation (Fig. 2A, lower left panel). The percentage of dead cells among U-373MG cells infected with Adv-FL and treated with irradiation (64±4.8%) was much higher than that among U-373MG cells infected with Adv-FL without irradiation (7.5±2.1%), or that among cells infected with control Adv-lacZ with irradiation (11±2.6%, Fig. 2B). The Br-dUTP uptake assay for the detection of DNA fragments revealed that the percentage of apoptotic cells among U-373MG cells infected with Adv-FL and treated with irradiation (81%) was much higher than that among cells infected with Adv-FL without irradiation (0.8%), or that among cells infected with control Adv-lacZ with irradiation (5.0%, Fig. 2C). These results indicate that the degree of apoptosis in the radiation-treated U-373MG cells infected with Adv-FL was markedly higher than that in the cells infected with Adv-FL without irradiation, or that in the cells infected with Adv-lacZ with irradiation. Similar results were obtained in U-373MG cells treated with X-ray irradiation at a single dose of 20 Gy (data not shown). It should be noted that the dose of 40 Gy seems to be too high, since usual radiotherapy utilizes a fractionated irradiation protocol of around 3 Gy per shot. Recently, radiosurgery has been reported to prolong survival in patients with malignant gliomas.[20, 21] The minimum peripheral doses of radiosurgery were 6 to 24 Gy, indicating that parts of the central doses would be more than 24 Gy. Therefore, the results of this study may be useful to improve the therapeutic effect of radiosurgery for malignant gliomas.

Electron microscopic analysis of U-373MG cells infected with Adv-FL and treated with irradiation revealed condensed chromatin in the nuclei (Fig. 2D: middle panel) and apoptotic bodies 3 days after infection and irradiation

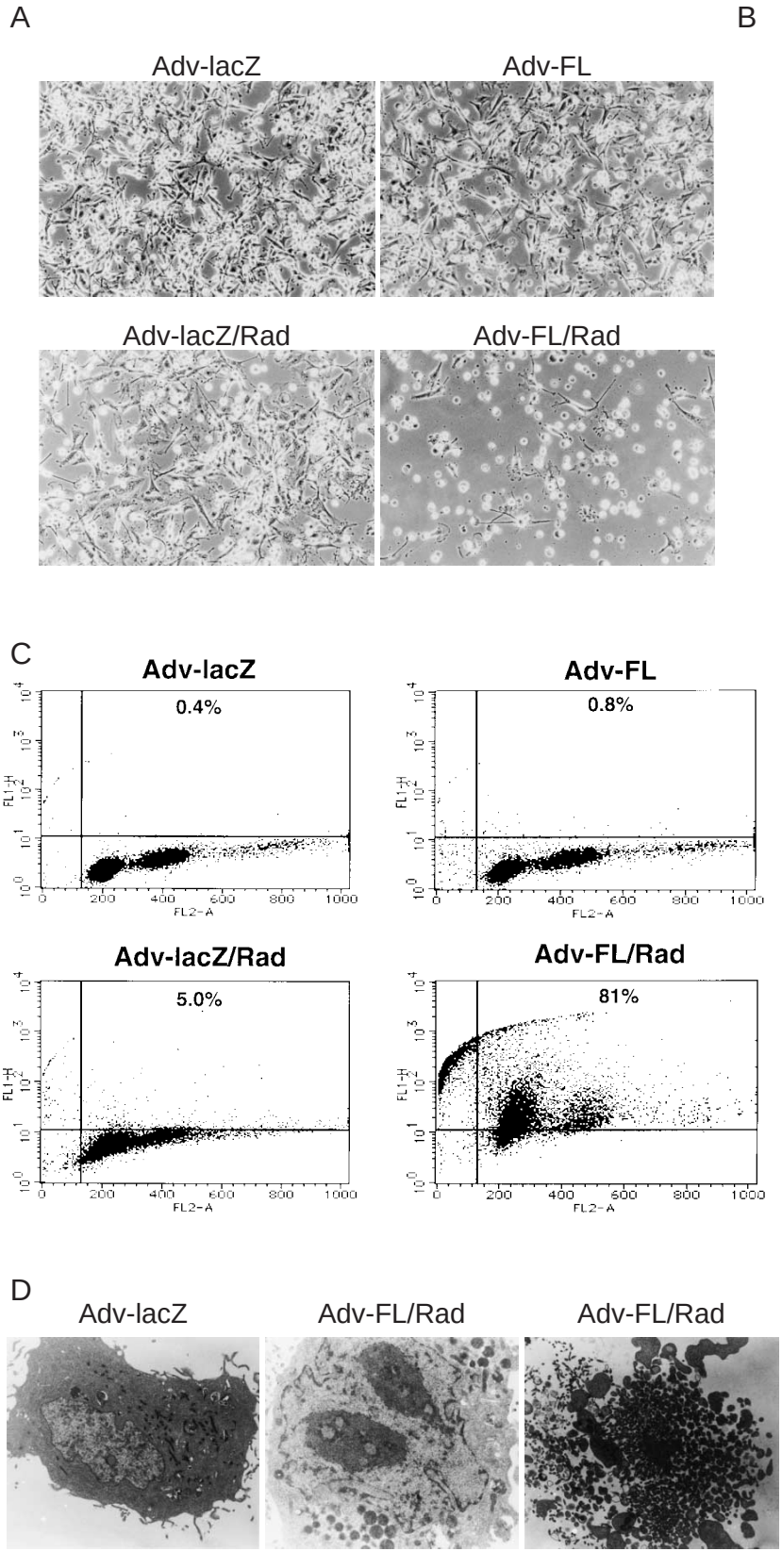

Fig. 2. A. Photomicrographs of U-373MG cells infected with Adv-FL or Adv-lacZ, with or without irradiation treatment (Rad). Cells were examined 3 days after infection (original magnification ×100). The MOIs used in these experiments were the same as those in Fig. 1. B. Percentage of cells that had died among the U-373MG cells, as measured by trypan blue exclusion, 3 days after infection with Adv-FL or Adv-lacZ, with or without irradiation treatment. The MOIs used in these experiments were the same as those in Fig. 1. The mean±standard deviation of the percentage of dead cells in three preparations under two separate experimental conditions, is shown. C. DNA fragmentation of U-373MG cells infected with Adv-FL or Adv-lacZ, with or without irradiation treatment. The MOIs used in these experiments were the same as those in Fig. 1. The assay was performed as described in "Materials and Methods" 3 days after infection. The *X*-axis represents the propidium iodide-related fluorescence and the *Y*-axis represents the Br-dUTP-related fluorescence. The points in the upper left and upper right areas of each panel represent apoptotic cells with fragmented DNA. The percentage of apoptotic cells with fragmented DNA is shown in each panel. D. Ultrastructural analysis of U-373MG cells after infection with Adv-FL with irradiation or Adv-lacZ without irradiation. The MOIs used in these experiments were the same as those in Fig. 1. Left panel: U-373MG cells 3 days after infection with Adv-lacZ (×5000); middle and right panels: U-373MG cells 3 days after infection with Adv-FL and treatment with irradiation (middle, ×7000; right, ×5000). Condensation of chromatin (middle panel) and apoptotic bodies (right panel), the hallmarks of apoptosis, appeared in the U-373MG cells 3 days after infection with Adv-FL and irradiation.

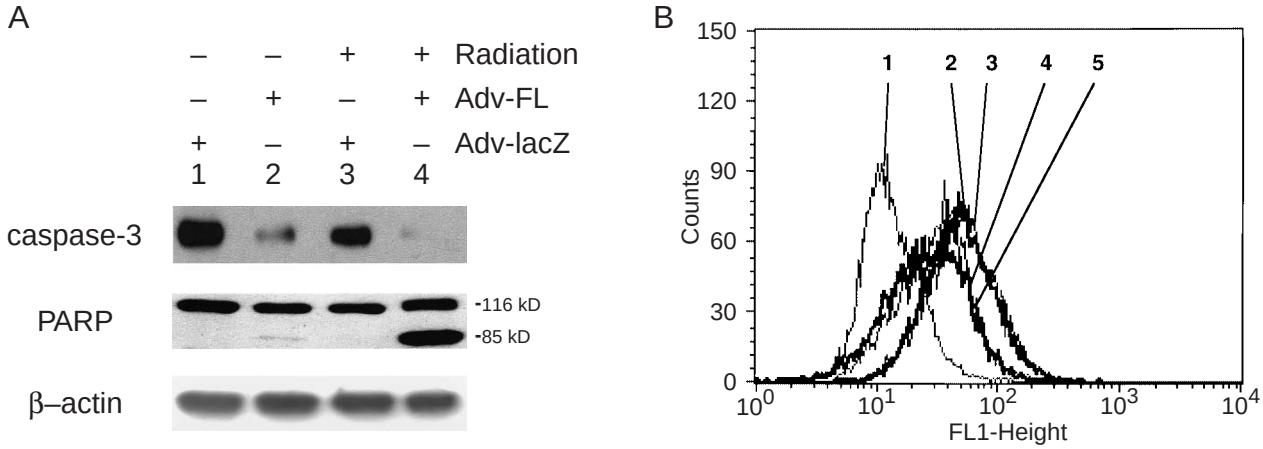

Fig. 3. A. Immunoblot analysis of the caspase-3, PARP and β-actin proteins extracted from U-373MG cells 2 days after infection with Adv-FL or Adv-lacZ, with or without irradiation. The MOIs used in these experiments were the same as those in Fig. 1. B. Expression of Fas on the surface of U-373MG cells, as measured by FACS, after infection of the cells with Adv-FL or Adv-lacZ, with or without irradiation. The MOIs used in these experiments were the same as those in Fig. 1. The U-373MG cells were stained with anti-Fas antibody as described in "Materials and Methods." The data are presented as the log peak fluorescence intensity of: (1) U-373MG cells stained with isotype-matched control 2 days after infection with Adv-lacZ; (2) U-373MG stained with anti-Fas antibody 2 days after infection with Adv-lacZ; (3) U-373MG stained with anti-Fas antibody 2 days after infection with Adv-FL; (4) U-373MG stained with anti-Fas antibody 2 days after infection with Adv-lacZ and irradiation; and (5) U-373MG stained with anti-Fas antibody 2 days after infection with Adv-FL and irradiation.

(Fig. 2D: right panel); these are features of apoptotic cell death.

Adv-FL infection in radiation-treated U-373MG cells reduced the caspase-3 level and cleaved PARP, despite the downregulation of Fas expression by irradiation To evaluate the mechanism of apoptosis in U-373MG cells after infection with Adv-FL and irradiation, we examined the expression of Fas, caspase-3 and PARP. Immunoblot analysis for caspase-3 revealed that Adv-FL infection reduced the level of caspase-3, and with irradiation, Adv-FL infection markedly reduced the level of caspase-3 (Fig. 3A, lanes 2, 4). In accordance with the downregulation of caspase-3, Adv-FL infection increased the cleaved form of PARP (p85) in the U-373MG cells (Fig. 3A, lanes 2, 4). The level of p85 in the radiation-treated U-373MG cells infected with Adv-FL (Fig. 3A, lane 4) was much higher than that in the U-373MG cells infected with Adv-FL without irradiation (Fig. 3A, lane 2). On the other hand, the expression of Fas on the surface of radiation-treated U-373MG cells (Fig. 3B, Nos. 4, 5) was lower than that on the surface of U-373MG cells that were not irradiated, irrespective of whether the cells were infected with Adv-FL or not (Fig. 3B, Nos. 2, 3).

These results demonstrate that Adv-mediated transfer of Fas ligand induced extensive apoptosis in radiation-treated U-373MG cells. PARP, on which caspase-3 acts, was markedly cleaved in the Fas ligand-transduced, radiation-treated U-373MG cells (Fig. 3A, lane 4). The level of caspase-3 in these cells was markedly low, and this may be due to the activation and cleavage of caspase-3 by apoptosis, although the antibody used in this study could not recognize the cleaved product of caspase-3. This suggests that irradiation augmented the Fas ligand-induced activation of caspase-3 (Fig. 3A, lanes 2, 4), resulting in extensive apoptosis. Interestingly, the level of surface Fas was lower in the radiation-treated U-373MG cells (Fig. 3B, Nos. 4 and 5). Hence, the elevation of Fas ligand expression in the Fas ligand-transduced, radiation-treated U-373MG cells overrode the reduction in Fas expression induced by irradiation, leading to extensive Fas-mediated apoptosis. What is the mechanism of the radiation-induced increase of Fas ligand after Adv-FL infection in U-373MG cells? Since the expression of Fas ligand transcript is regulated under the CA promoter, its transcription is unlikely to be influenced by irradiation itself. Indeed, the Adv-mediated transgene expression was not enhanced by irradiation in general, because the expression of other apoptosis-related genes (*Apaf-1* or *caspase-9*) transferred by Adv was not affected by irradiation in U-373MG cells. Similarly, the cre expression of the co-infecting virus vector should not be enhanced, since the *cre* gene was also driven by the CA promoter. Irradiation did not alter the expression levels of other apoptosis-related proteins, such as Bax, Bcl-X_L, Bcl-2, caspase-3 or PARP, suggesting that it did not generally stabilize the transcript of apoptosis-related genes or apoptosis-related proteins (data not

shown). Accordingly, irradiation may upregulate Fas ligand expression through a specific mechanism involved in Fas ligand-mediated apoptotic pathways. In other cancer cells, irradiation as well as chemotherapeutic agents have been reported to upregulate Fas ligand expression.[22, 23] This mechanism might involve ceramide as a signaling intermediate.[24] Further investigations are required to clarify the mechanism of upregulation of Fas ligand in irradiated U-373MG cells.

Various mitochondria-related proapoptotic genes, including p53,[1–3] Bax,[6, 25] and caspase-9,[26] have been reported to be involved in radiation-induced apoptosis. In contrast, Fas-mediated signals, which are transduced by FADD or caspase-8, are not required in the radiation-induced apoptotic pathways.[27–31] This suggests that the mitochondria-dependent pathway plays a critical role in radiation-induced apoptosis, and that the radioresistance of cancer cells with p53 mutation may be due to blockage of this pathway by the p53 mutation.[32] On the other hand, the Fas signaling pathway in glioma cells with mutant p53 is intact.[11, 33, 34] Thus, the Adv-mediated transduction of Fas ligand overrode the radioresistance mechanism in the glioma cells, even though they harbor mutant p53, since it markedly activated the intact Fas-mediated pathway through the elevated Fas ligand expression induced by irradiation.

It has been reported that Fas ligand is expressed in many human glioma cell lines and glioma cells *ex vivo*.[35–37] Thus, the level of Fas ligand expression may determine or predict the therapeutic effect of irradiation in glioma cells. Finally, gene therapy utilizing Adv-FL would augment the sensitivity of glioma cells to irradiation, even in glioma cells that harbor p53 mutation and thereby are resistant to radiotherapy. Thus, Adv-mediated transduction of Fas ligand is a promising approach towards overriding the radioresistance mechanism in gliomas. The low level of Fas expression in the surrounding normal tissues and utilization of the MBP promoter in the Adv vector may ensure the selective killing of gliomas upon transduction of Fas ligand.

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