Jpn. J. Cancer Res. **91**, 1028–1034, October 2000

Transduction of a Fiber-mutant Adenovirus for the *HSVtk* Gene Highly Augments the Cytopathic Effect towards Gliomas

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Suicide gene therapy utilizing the herpes simplex thymidine kinase (HSVtk)/ganciclovir (GCV) system has been performed to kill cancer cells. However, the low transduction efficiency of *HSVtk* gene into cancer cells critically limits its efficacy in cancer treatment in clinical situations. To improve delivery of the *HSVtk* gene into cancer cells, we transduced U-87MG and U-373MG glioma cells with adenovirus (Adv) vectors with a fiber mutant, F/K20, which has a stretch of 20 lysine residues added at the C-terminus of the fiber, for the *HSVtk* gene (Adv-TK-F/K20), and compared the cytopathic effect of Adv-TK-F/K20 with that of the Adv for HSVtk with wild-type fiber (Adv-TK). The cytopathic effect of Adv-TK-F/K20 in U-87MG and U-373MG cells was approximately 140 and 40 times, respectively, stronger than that of Adv-TK. At the same multiplicity of infection (MOI) in each cell line, Adv-TK-F/K20 induced a higher degree of apoptosis (U-87MG, 35%; U-373MG, 77%) than Adv-TK (U-87MG, 0.11%; U-373MG, 27%) in U-87MG (MOI 0.03) and U-373MG cells (MOI 0.1). Cleavage of poly(ADP-ribose)polymerase (PARP) was more marked in the cells that were infected with Adv-TK-F/K20 than in cells that were infected with Adv-TK. These results indicate that gene therapy utilizing Adv-TK-F/K20 may be a promising therapeutic modality for the treatment of gliomas.

Key words: Apoptosis — HSVtk — Fiber-mutant — Adenovirus — Glioma

Gene therapy utilizing the herpes simplex thymidine kinase (HSVtk)/ganciclovir (GCV) system has begun to be used clinically for the treatment of malignant brain tumors, and an antitumor effect has been detected in some small tumors.[1–3] However, Ram *et al.*[3] concluded that the low delivery of the *HSVtk* gene through retrovirus vectors *in vivo*, and the consequent low killing effect towards brain tumors are the most critical problems of this gene therapy approach. Thus, intense research efforts have been focused on this subject. Although transduction of adenovirus (Adv) vector is one of the most efficient gene delivery systems, the transduction efficiency of Adv vector was not sufficient to induce efficient expression of the transduced gene in gene therapy in clinical situations.[4, 5] To improve the efficacy of *HSVtk/GCV* gene therapy of cancers through Adv vectors, several approaches have been reported such as using Adv vectors capable of replication,[6] or protamine with Adv vectors.[7] We previously reported that utilizing an Adv vector with a fiber mutant, F/K20, which has a stretch of 20 lysine residues added at the C-terminus of the fiber, the transduction efficiency to gliomas was over 10 times higher than that utilizing the adenoviral vector with the wild-type fiber.[8] The combination of a fiber-mutant with a replication-competent Adv vector shows a highly augmented cytopathic effect in glioma cells.[9] In this study, we transduced U-87MG and U-373MG glioma cells with the Adv for the *HSVtk* gene with F/K20 (Adv-TK-F/K20), and compared the cytopathic effect induced by Adv-TK-F/K20 with that induced by the Adv for HSVtk with wild-type fiber (Adv-TK). We found that transduction of Adv-TK-F/K20 into the glioma cells enhanced the cytopathic effect in comparison with that induced by transduction of Adv-TK.

MATERIALS AND METHODS

Cell line The U-87MG and U-373MG glioma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Nissui Phar-

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maceutical, 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.

Generation of the adenoviral vectors The *Bst*BI fragment of pSKb2-CMTK, which consists of the *HSVtk* gene coding region driven by the cytomegalovirus (CMV) promoter and poly(A) sequence, was inserted into the *Cla*I site of the cosmid pAxcw,[10] generating pAx-CMTK. Recombinant adenovirus was generated by cotransfection of the cosmids using the method described by Miyake *et al*.[10]

The cosmid pL_{RI}CAZ2 was constructed as described previously.[8] The *Bst*BI fragment of pSKb2-CMTK was inserted into the *Cla*I site of pL_{RI}CAZ2, generating pL_{RI}-CMTK. Fiber-mutant Adv vectors were generated essentially as described previously.[8] Briefly, the fiber-mutant construct in the plasmid which consisted of the right two-thirds of the human adenovirus type 5 (Ad5) genome (pTR), was cotransfected with Ad5 DNA-TPC, efficiently yielding the recombinant Adv with the fiber-mutant which has a linker and a stretch of 20 lysine residues added at the C-terminus of the fiber (F/K20). The DNA-TPC from the mutant and Adv pL_{RI}-CMTK were then used to produce a second-step recombinant Adv with the HSVtk coding region to generate Adv-TK-F/K20. Adenovirus-mediated gene transduction was performed using the method described by Yoshida and Hamada.[11]

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 (Becton Dickinson, San Jose, CA). Two samples of cells for each experimental condition were analyzed and this was repeated twice. All of the assays were performed 7 days after infection with Adv vectors. Electron microscopic analysis for detection of apoptotic cell death was performed as described previously.[12]

MTT assay Three thousand U-87MG or 3000 U-373MG cells per well in 96-well microtiter plates were infected with Adv-TK-F/K20, Adv-TK, or Adv-lacZ at various multiplicities of infection (MOIs) (n=8 in each condition). On day 7, the percentage of surviving cells was determined using a modification of the MTT assay.[13] Briefly, 100 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, M-2128, St. Louis, MO) solution in double-distilled water was added to each well. The cells were incubated for 1 h at 37°C, and then 100 μl of 100% ethanol was added. Colorimetry was performed using a microplate reader (Model 3550, Bio-Rad, Hercules, CA). For each MOI, two independent experiments were performed, each representing the average of eight wells. Cell survival was expressed as the mean±standard error of the percentage of surviving cells relative to that in the respective control cells without Adv infection.

Immunoblot analysis Immunoblot analysis was performed using the enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, England), as previously described.[12] 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 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 (BioRad), 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). After washing with 5% dry milk in TBS, 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

Transduction of Adv-TK-F/K20 showed a more extensive cytopathic effect compared with transduction of Adv-TK in glioma cells U-87MG and U-373MG cells were infected with the Adv-TK-F/K20, Adv-TK, or control Adv for lacZ (Adv-lacZ)[10] at various MOIs in the presence or absence of GCV (20 μM), and the cytopathic effect was analyzed 7 days after infection with the MTT assay. In U-87MG cells, the MOI for cell death of 80% of the population (ED_{80}) of Adv-TK-F/K20 with GCV was 0.0018, whereas the ED_{80} of Adv-TK with GCV was 2.6

(Fig. 1A). In U-373MG cells, the ED_{80} of Adv-TK-F/K20 with GCV was 0.0068, whereas the ED_{80} of Adv-TK with GCV was 0.27 (Fig. 1B). Thus, the cytopathic effect of Adv-TK-F/K20 in U-87MG and U-373MG cells was approximately 140 and 40 times, respectively, stronger than that of Adv-TK.

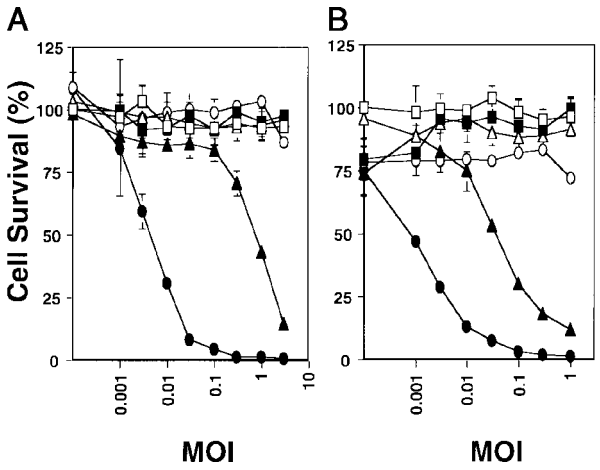

Fig. 1. Cytopathic effect of Adv-TK-F/K20, Adv-TK or Adv-lacZ towards U-87MG (A) and U-373MG (B) cells *in vitro* in the presence or absence of GCV (20 μM). MTT assays were performed as described in "Materials and Methods." Each point represents the mean±SD of 8 experiments. □ Adv-lacZ/GCV(−), ■ Adv-lacZ/GCV(+), △ Adv-TK/GCV(−), ▲ Adv-TK/GCV(+), ○ Adv-TK-F/K20/GCV(−), ● Adv-TK-F/K20/GCV(+).

The HSVtk/GCV system has been reported to induce apoptosis.[14, 15] Therefore, we evaluated the effect of infecting Adv-TK-F/K20, Adv-TK or Adv-lacZ into U-87MG and U-373MG cells in the presence or absence of GCV on the degree of cell death and DNA fragmentation 7 days after Adv infection. In GCV-treated U-87MG cells, infection with Adv-TK-F/K20 induced remarkably extensive cell death (Fig. 2, upper right panel), whereas infection with Adv-TK (Fig. 2, upper middle panel) or Adv-lacZ (Fig. 2, upper left panel) did not induce a cytopathic effect. Similarly, in GCV-treated U-373MG cells, infection with Adv-TK-F/K20 (Fig. 2, lower right panel) induced more extensive cell death than that with Adv-TK (Fig. 2, lower middle panel), while infection with Adv-lacZ did not induce a cytopathic effect (Fig. 2, lower left panel). The percentage of cells that had died among U-87MG cells and among U-373MG cells infected with Adv-TK-F/K20 (85±3.0% and 89±1.8%, respectively) was much higher than the percentage of cells that had died among U-87MG cells and among U-373MG cells infected with Adv-TK (25±2.7% or 40±7.2%, respectively) in the presence of GCV (Fig. 3). The Br-dUTP uptake assay for the detection of DNA fragments revealed that in the GCV-treated U-87MG cells, 35% of the cells infected with Adv-TK-F/K20 contained fragmented DNA, while 0.11% of the cells infected with Adv-TK contained fragmented DNA (Fig. 4A). Although the degree of apoptosis in GCV-treated U-373MG cells infected with Adv-TK-F/

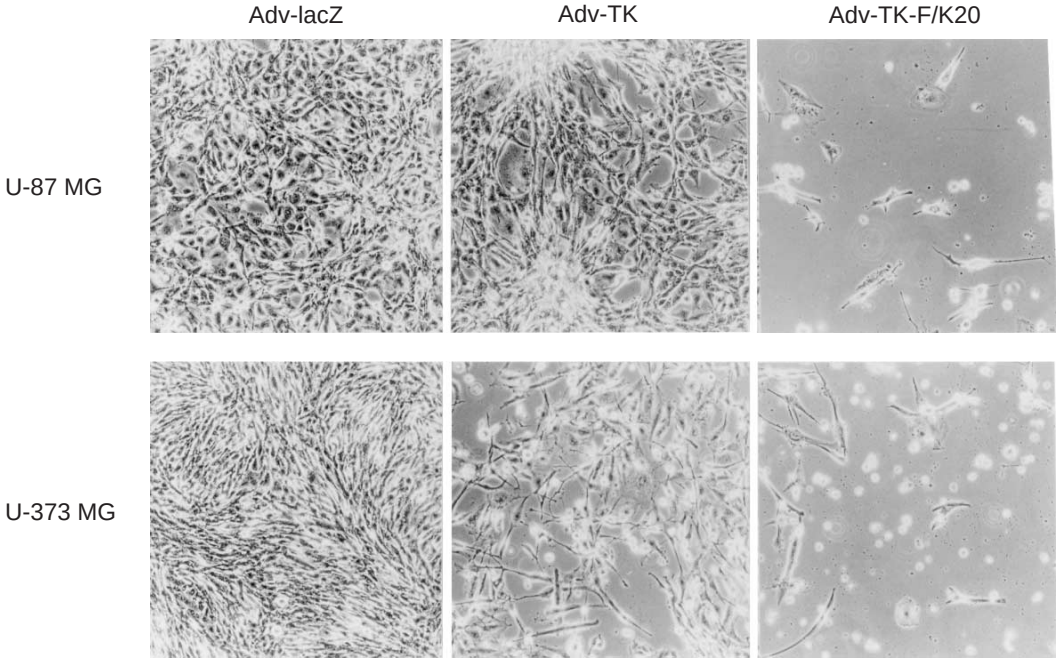

Fig. 2. Microscopic photographs of U-87MG (MOI 0.03) and U-373 MG cells (MOI 0.1) that had been infected with Adv-TK-F/K20, Adv-TK, or Adv-lacZ in the presence of GCV (20 μM). The cells were examined 7 days after infection (original magnification ×100).

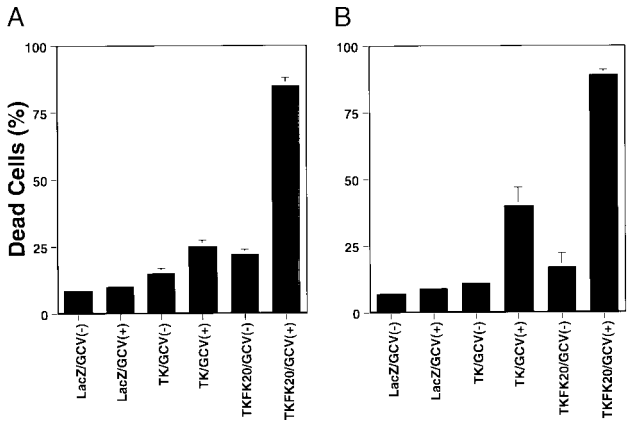

Fig. 3. Percentage of cells that had died among U-87MG (A) (MOI 0.03) and U-373MG (B) cells (MOI 0.1), as measured by trypan blue exclusion, 7 days after infection with Adv-TK-F/K20, Adv-TK, or Adv-lacZ in the presence or absence of GCV (20 μM). The mean±standard deviation of the percentage of dead cells in three preparations of two independent experiments is shown.

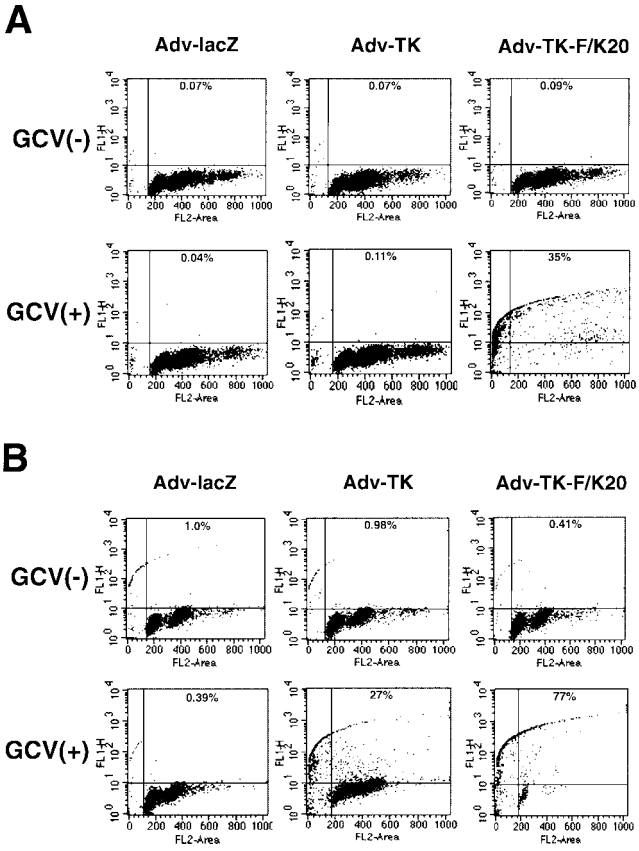

Fig. 4. DNA fragmentation of U-87MG (A, MOI 0.03) or U-373MG cells (B, MOI 0.1) infected with Adv-TK-F/K20, Adv-TK, or Adv-lacZ in the presence or absence of GCV (20 μM). The assay was performed as described in "Materials and Methods" 7 days after infection. The *X*-axis represents the propidium iodide-related 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.

K20 was greater than that in GCV-treated U-87MG cells infected with Adv-TK-F/K20, the U-373MG cells showed a similar pattern (Fig. 4B). The percentage of cells with fragmented DNA among U-373MG cells infected with Adv-TK-F/K20 was 77%, whereas that among U-373MG cells infected with Adv-TK was 27% in the presence of GCV (Fig. 4B). These results indicate that infection with Adv-TK-F/K20 induced remarkably extensive apoptotic cell death in comparison with that induced by infection with Adv-TK in two GCV-treated glioma cell lines. Electron microscopic analysis of U-87MG and U-373MG cells infected with Adv-TK-F/K20 in the presence of GCV, revealed condensed chromatin in the nuclei (Fig. 5, upper right panel) and apoptotic bodies (Fig. 5, lower right panel) 7 days after infection, which are features of apoptotic cell death.

Adv-TK-F/K20 infection induced more marked cleavage of PARP than Adv-TK infection in glioma cells

The apoptotic pathway induced by the transduction of HSVtk with GCV treatment is mediated through the activation of caspases.[14] Thus, we examined the expression of caspase-3 and PARP 5 days after infection with Adv-TK-F/K20, Adv-TK or Adv-lacZ in the presence or absence of GCV. In accordance with the report by Wei *et al.*,[17] the level of caspase-3 in GCV-treated HSVtk-transduced U-87MG and U-373MG cells (Fig. 6, A and B; lanes 4, 6) was higher than that in the respective GCV-treated Adv-lacZ-transduced cells (Fig. 6, A and B; lane 2). In the GCV-treated U-87MG cells infected with Adv-TK-F/K20, the level of the uncleaved form of the PARP molecules (p116) was reduced, and the cleaved form (p85), a substrate on which caspase-3 acts, appeared (Fig. 6A, lane 6). In contrast, p85 did not appear in the GCV-treated U-87MG cells infected with Adv-TK (Fig. 6A, lane 4). Similarly, the level of p85 in the GCV-treated U-373MG cells infected with Adv-TK-F/K20 (Fig. 6B, lane 6) was higher than that in the GCV-treated U-373MG cells infected with Adv-TK (Fig. 6B, lane 4). These results suggest that a greater percentage of the caspases in the GCV-treated cells infected with Adv-TK-F/K20, was activated than that in the GCV-treated cells infected with Adv-TK, although the antibody for caspase-3 used in this study did not recognize the cleaved product of caspase-3. The HSVtk/GCV system kills cancer cells by apoptosis through p53 accumula-

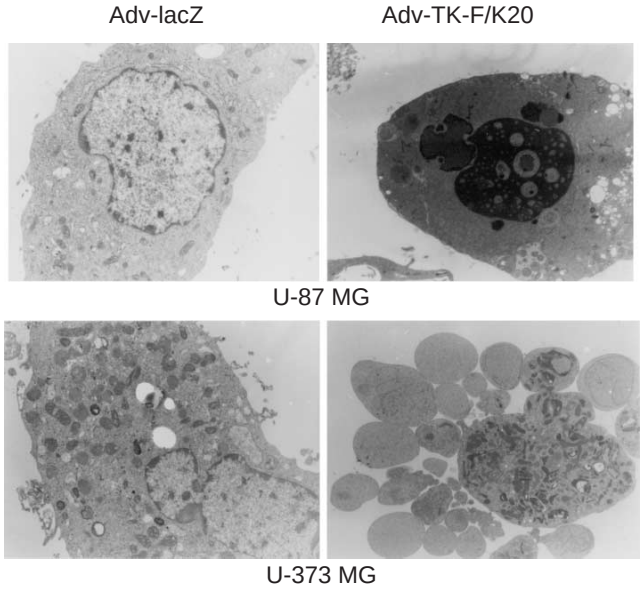

Fig. 5. Ultrastructural analysis of U-87MG (MOI 0.03) and U-373MG cells (MOI 0.1) 7 days after being infected with Adv-lacZ or Adv-TK-F/K20 in the presence of GCV (20 μM). Upper left panel, U-87MG cells 7 days after being infected with Adv-lacZ (×8000); upper right panel, U-87MG cells 7 days after being infected with Adv-TK-F/K20 in the presence of GCV (×5000); lower left panel, U-373MG cells 7 days after being infected with Adv-lacZ (×7000); lower right panel, U-373MG cells 7 days after being infected with Adv-TK-F/K20 in the presence of GCV (×4000).

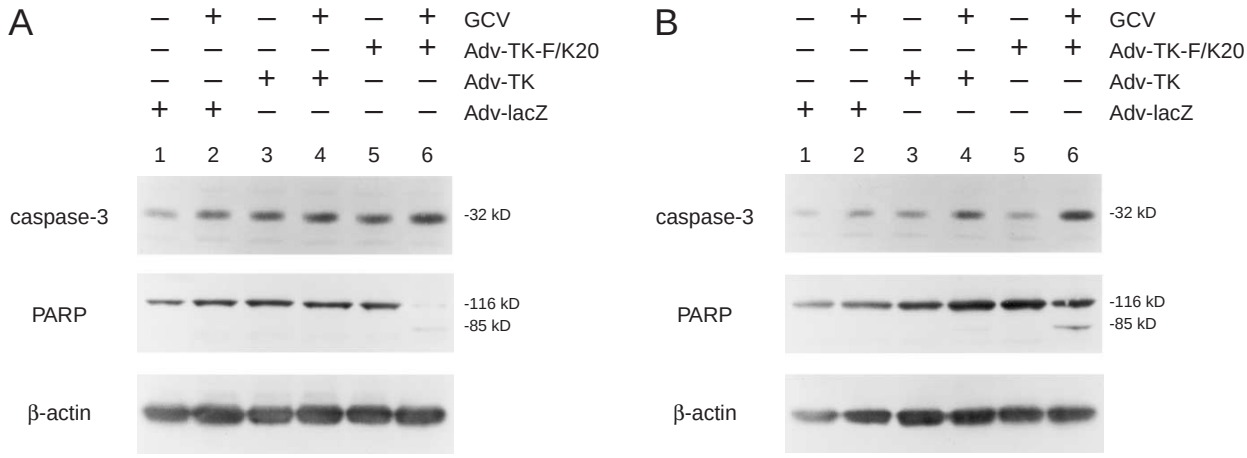

Fig. 6. Immunoblot analysis of caspase-3, PARP and β-actin protein extracted from U-87MG cells (A, MOI 0.03) and U-373MG cells (B, MOI 0.1) 5 days after being infected with Adv-TK-F/K20, Adv-TK, or Adv-lacZ in the presence or absence of GCV (20 μM).

tion and increased expression of Fas or Bax, leading to the activation of caspases.[14, 16, 17] In accordance with the higher degree of apoptosis induced by Adv-TK-F/K20 than by Adv-TK (Fig. 4), the level of the cleaved product of PARP, p85, in glioma cells infected with Adv-TK-F/K20 was higher than that in the respective cells infected with Adv-TK (Fig. 6). This suggests that the apoptosis induced by Adv-TK-F/K20 or Adv-TK was mediated at least through caspases.

The high transduction efficiency obtained by the F/K20 mutant Adv is beneficial for the gene therapy of gliomas. First, an increase in the transduction efficiency would augment expression of the transduced genes, leading to an increased cytopathic effect toward glioma cells. In U-87MG cells, the transduction efficiency of Adv-lacZ with F/K20 (Adv-lacZ-F/K20) was 42 times higher than that of Adv-lacZ,[8] whereas the cytopathic effect of Adv-TK-F/K20 was 140 times stronger than that of Adv-TK (Fig. 1). Similarly, in U-373MG cells, the transduction efficiency of Adv-lacZ-F/K20 was 9 times higher than that of Adv-lacZ,[8] while the cytopathic effect of Adv-TK-F/K20 was 40 times stronger than that of Adv-TK (Fig. 1). The high transduction efficiency of Adv-TK-F/K20 led to high expression of the *HSVtk* gene, which resulted in a remark-

ably augmented cytopathic effect of Adv-TK-F/K20 in the glioma cells. It should be noted that the ratio of the cytopathic effect induced by Adv-TK-F/K20 versus Adv-TK was higher than the ratio of the transduction efficiency of Adv-lacZ-F/K20 versus Adv-lacZ. This may occur because the higher transduction of the *HSVtk* gene by Adv-TK-F/K20 compared with that by Adv-TK may markedly enhance the killing effect towards glioma cells through the bystander effect.[1, 15] However, careful side-by-side, quantitative comparison of the lacZ expression and GCV-killing effect is required to test this hypothesis. For clinical application of this vector, it would be important to conduct *in vivo* experiments utilizing this vector. Unfortunately, the concentration of this fiber-mutant adenovirus achieved was not high enough for conducting animal experiments. Further investigations such as utilization of a special column for the concentration of this vector are needed to override this technical difficulty.

Another advantage of using Adv-TK-F/K20 is that the enhancement of transduction efficiency reduces the total dose of Adv required to obtain the same level of cytopathic effect. Injection of Adv doses above 10^{10} pfu into the human brain may be toxic.[18] Thus, it is critical to minimize the total dose of Adv as much as possible. It is highly advantageous to use the Adv with F/K20 to reduce the toxicity of Adv infection, because it shows high transduction efficiency to gliomas alone and not to normal cells.[9] Production of Adv-TK-F/K20 in which the *HSVtk* gene is driven by a glioma-specific promoter such as E2F[19] or myelin basic protein,[20] would be a promising approach to further restrict undesired viral transduction. Careful basic and clinical studies are required to evaluate the undesirable effects of Adv-TK-F/K20 in the normal human brain.

ACKNOWLEDGMENTS

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 April 28, 2000/Revised June 27, 2000/2nd Revised July 11, 2000/Accepted July 14, 2000)

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