Jpn. J. Cancer Res. **93**, 223–228, February 2002

Antitumor Effect of an Adeno-associated Virus Vector Containing the *Human Interferon-β* Gene on Experimental Intracranial Human Glioma

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We constructed an adeno-associated virus (AAV) vector containing the human interferon-β (*HuIFN-β*) gene (AAV-IFN-β) and investigated its antitumor effect against human glioma cells (U251-SP) inoculated into the brain of nude mice. Prior to this, we examined human glioma cells transduced with AAV-IFN-β using video-enhanced contrast differential interference contrast (VEC-DIC) microscopy. Infection of AAV-IFN-β induced apoptosis and secondary necrosis in human glioma cells. In *in vivo* experiments, we confirmed production of HuIFN-β and induction of heat-shock protein (HSP) in glioma cells transduced with AAV-IFN-β. Growth of the experimental gliomas was completely inhibited by six injections of AAV-IFN-β, starting 7 days after transplantation of glioma cells. In addition, the survival of mice treated with AAV-IFN-β was remarkably prolonged. These results indicate that AAV-IFN-β induces apoptosis of glioma cells and has a strong antitumor effect in this experimental glioma model.

Key words: AAV — IFN-β — Glioma — Gene therapy

Human interferon-β (HuIFN-β) is thought to be an important factor in the growth of human glioma because homozygous deletions of the class I *IFN* gene cluster, comprising multiple *IFN-α* genes and a single *IFN-β* gene, have been demonstrated in human glioma.[1, 2] Since 1980, HuIFN-β protein has been used in therapy for patients with glioma in Japan and has demonstrated potential antitumor effects.[3, 4] However, clinical responses have not been satisfactory. Recently, we found that *HuIFN-β* gene transfer to human glioma cells has additional antitumor effects which can not be induced by HuIFN-β protein alone; for example, stronger promotion of cytokine networks inducing interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α; and stronger induction of cytotoxic T lymphocytes (CTLs) into brain tumors. In order to develop more effective treatment for glioma using *HuIFN-β* gene, we have been examining *HuIFN-β* gene therapy using recombinant adeno-associated virus (AAV) vectors.

AAV vectors are one of the most promising alternatives to current viral delivery systems.[5] AAV belongs to the parvovirus family, a group of small single-stranded DNA viruses, and has many advantageous features for human gene therapy, including nonpathogenicity, targeted integrating capacity, and a broad host range. However, some problems occur with recombinant AAV vectors, including instability in packaging large genes and difficulty in mass production. In general, production of recombinant AAV vectors requires helper functions supplied by a second coinfecting virus, such as adenovirus or herpes virus; we currently use an adenovirus-free system in which adenovirus helper functions are supplied by nonreplicating adenovirus genomic plasmids.[6] These vectors are thought to be much safer than those prepared by the classic method using adenovirus itself for the helper function, which contain adenovirus proteins with high immunogenicity. In this experiment, we constructed AAV vectors containing the *HuIFN-β* gene (AAV-IFN-β) prepared by means of the adenovirus-free system, and observed killing of human glioma cells transduced with AAV-IFN-β *in vitro* using video-enhanced contrast differential interference contrast (VEC-DIC) microscopy. Furthermore, we evaluated the antitumor effects of this system against a human glioma cell line inoculated into the brain of nude mice.

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MATERIALS AND METHODS

Vectors AAV vector expressing the *HuIFN-β* gene (AAV-IFN-β) and AAV vector expressing the *β-galactosidase* gene (AAV-LacZ) when activated by cytomegalovirus-immediate early (CMV-IE) promoter were constructed in our laboratory (Fig. 1). Both vectors were prepared in an adenovirus-free system developed by Matsushita *et al*.[6] This method differs from the classic method in that the adenovirus helper functions are supplied by a non-replicating adenovirus genomic plasmid, in which the VA, E2A and E4 regions are included. These had titers of 9.1×10^{13} and 1.9×10^{13} particles/ml, respectively.

Cells We used the human glioma cell line U251-SP, which has high transplantability into nude mice. The cells have no deletion of the *IFN-β* gene. Cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum, 0.1 m*M* nonessential amino acids, 5

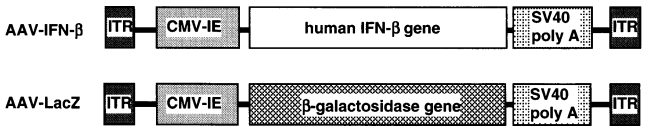

Fig. 1. Maps of the AAV-IFN-β and AAV-LacZ vector genomes.

m*M* L-glutamine, and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml).

Morphologic changes of cultured human glioma cells treated with AAV-IFN-β We used VEC-DIC microscopy to observe the morphologic changes of cultured human glioma cells treated with AAV-IFN-β. Cells were examined with an inverted Nomarski microscope equipped with a 100× DIC objective lens and a 2.5× insertion lens (Axiovert 35, Zeiss, Oberkochem, Germany). The coverslip, plated with cultured cells, was fixed with petroleum jelly (Vaseline) to a square hole made in the center of a plastic slide. The image was acquired with a 0.5-inch CCD camera (ZVS3C75DEC, Sony, Tokyo) and image contrast was enhanced with a high-speed digital image processor. The processed image was observed on a slightly overscanned video monitor and simultaneously saved on laser disc or by an S-VHS format recorder.

In order to confirm cell death, we used annexin-V as an apoptosis marker and propiodium iodide (PI) as a necrosis marker according to the Immunotech protocol.

Animals Female BALB/c nude mice (8–10 weeks old) were maintained and bred under pathogen-free conditions in our animal facility. Animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" prepared by the Office of the Prime Minister of Japan.

Production of experimental glioma in nude mouse brain We injected human glioma cells (U251-SP) suspended in phosphate-buffered saline (PBS) into the brain. Briefly, animals were anesthetized by intraperitoneal injection of pentobarbital (60–70 mg/kg), then held in a stereotaxic apparatus with an ear bar. A 2-μl aliquot of the cell suspension (2×10^5 cells) was injected with a Hamilton syringe using a microsyringe pump (Model 2000, Instech, Plymouth Meeting, PA). The site of the injection was 3 mm lateral from the midline, 4 mm behind the bregma, and 3 mm below the dura mater. Transplanted glioma cells grew to afford a brain tumor mass reaching about 2 mm in diameter 7 days after transplantation.

Injection of AAV vectors into an experimental glioma We injected 2 μl of AAV-IFN-β (3.8×10^{10} particles in 50 m*M* HEPES, 150 m*M* NaCl, pH 7.5) or AAV-LacZ (3.8×10^{10} particles in 50 m*M* HEPES, 150 m*M* NaCl, pH 7.5) over 4 min into the brain into which glioma cells had been transplanted. The needle was left in the tissue for an additional 3 min and then slowly withdrawn. Injection of vectors in some studies was performed every other day for a total of six injections, starting 7 days after cell transplantation.

Detection of HuIFN-β and heat-shock protein (HSP) in experimental glioma infected with AAV-IFN-β Nude mice injected with AAV-IFN-β were sacrificed 4 days after the last injection, and the brain was removed. The brain was homogenized and lysed by three cycles of freezing and thawing. The extract was examined by an enzyme immunoassay for HuIFN-β.[7] In this assay, HuIFN-β levels >2.5 IU/ml can be determined. Total protein was measured by a modified Lowry method.[8] Induction of HSP after injection of AAV-IFN-β was examined by western blot analysis.

Growth inhibition and survival time Growth-inhibitory effects on experimental gliomas were evaluated visually and by light microscopy 31 days after transplantation. In addition, the time to appearance of neurologic symptoms and the survival time were recorded.

RESULTS

Morphologic changes of cultured human glioma cells under VEC-DIC microscopy U251-SP cultured human glioma cells treated with AAV-IFN-β (30 000 vector genome titers/cell) were observed at magnifications of 2000× to 7500×. As shown in Fig. 2, after treatment the nucleoli and cytoplasm became unclear (stage 2), and blebbing of the cell membrane occurred; thereafter these changes increased (stage 2). After 1 h, some cells began to shrink (stages 2 and 3), form apoptotic bodies and undergo ballooning (stage 4). Between stage 2 and stage 4, the cells became annexin V-positive. After stage 3, almost all cells were PI-positive. The same morphologic changes were also observed in other human glioma cells, U251-MG and SK-MG-1 (data not shown). These morphologic changes (apoptotic cell death) were not observed in human glioma cells treated with human IFN-β protein (1000 IU), although cell growth was inhibited.

Detection of HuIFN-β and HSP in experimental glioma injected with AAV-IFN-β As shown in Table I, we detected HuIFN-β in the extract obtained from brains of glioma-transplanted nude mice after AAV-IFN-β injection, but not in nude mice injected with PBS, HN (virus stock) solution, or AAV-LacZ. The levels of HuIFN-β were 0.12±0.02 and 0.32±0.04 IU/mg protein after one and six injections, respectively. An increase in HSP production was detected in experimental gliomas at 4 days after injection of AAV-IFN-β (Fig. 3).

Appearance of symptoms in glioma-transplanted mice treated with AAV-IFN-β The appearance of symptoms is shown in Table II. The average latent period until the appearance of hemiparesis or abnormal behavior was

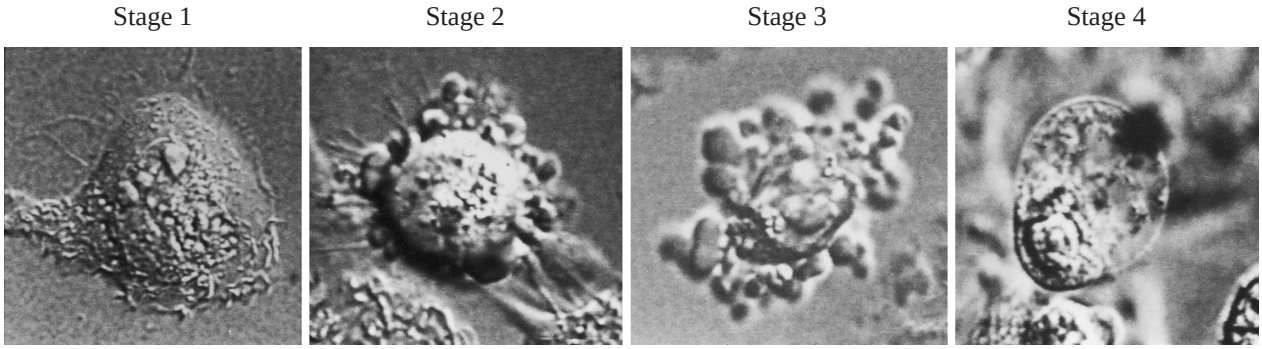

Fig. 2. Apoptosis and secondary necrosis of human glioma (U251-SP) cells transduced with AAV-IFN-β. Stage 1, preapoptotic stage (control); stage 2, nuclear condensation and membrane blebbing; stage 3, membrane blebbing and cell shrinkage; stage 4, formation of apoptotic bodies and ballooning.

Table I. Production of HuIFN-β in an Experimental Glioma Transduced with AAV-IFN-β

Group	HuIFN-β (IU/mg protein of brain)
(1) PBS-treated	ND
(2) HN-injected	ND
(3) AAV-L (once)	ND
(4) AAV-L (6 times)	ND
(5) AAV-I (once)	0.12±0.02
(6) AAV-I (6 times)	0.32±0.04

The values are mean±SD (n=6).
PBS, phosphate-buffered saline; HN, virus stock solution (50 m*M* HEPES, 150 m*M* NaCl, pH 7.5); AAV-L (once), single injection of AAV-LacZ; AAV-L (6 times), six injections of AAV-LacZ; AAV-I (once), single injection of AAV-IFN-β; AAV-I (6 times), six injections of AAV-IFN-β; ND, not detectable.

Table II. Appearance of Neurologic Symptoms in Glioma-transplanted Mice Transduced with AAV-IFN-β

Group	Days
(1) PBS-treated	21.4±3.5
(2) HN-injected	20.9±4.4
(3) AAV-L (once)	22.0±4.8
(4) AAV-L (6 times)	21.4±7.4
(5) AAV-I (once)	58.5±10.8
(6) AAV-I (6 times)	>120 in all mice

The values are mean±SD (n=6).
PBS, phosphate-buffered saline; HN, virus stock solution (50 m*M* HEPES, 150 m*M* NaCl, pH 7.5); AAV-L (once), single injection of AAV-LacZ; AAV-L (6 times), six injections of AAV-LacZ; AAV-I (once), single injection of AAV-IFN-β; AAV-I (6 times), six injections of AAV-IFN-β.

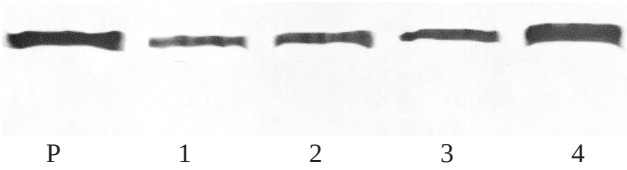

Fig. 3. Production of heat-shock protein in an experimental glioma treated with AAV-IFN-β. P, positive control; 1, PBS-treated; 2, HN (virus stock) solution; 3, AAV-LacZ; 4, AAV-IFN-β. A more intense band was confirmed in line 4.

21.4±3.5 days in mice injected with PBS (sham operation), 20.9±4.4 days in mice treated with HN solution (50 m*M* HEPES, 150 m*M* NaCl, pH 7.5), 22.0±4.8 days in mice injected with AAV-LacZ once, 21.4±7.4 days in mice injected with AAV-LacZ six times, and 58.5±10.8 days in mice injected with AAV-IFN-β once. In all mice receiving AAV-IFN-β six times, no neurologic symptoms were present at the end of the experimental period (120 days). The latent period was prolonged significantly in mice injected with AAV-IFN-β.

Antitumor effects of AAV-IFN-β on cerebrally transplanted human glioma Using light microscopy in our previous experiments, we had found no tumor suppression even when $>5\times10^4$ IU HuIFN-β was intratumorally injected.[9] The results of our present experiments are shown in Table III and Fig. 4. One injection of AAV-IFN-β remarkably inhibited tumor growth: the maximum diameter of tumors was 1.9±0.8 mm in mice treated with AAV-IFN-β versus 5.3±1.5 mm, 5.1±2.0 mm, 4.8±1.9 mm, and 6.2±2.6 mm in mice treated with PBS, HN solution, AAV-LacZ ×1 and AAV-LacZ ×6, respectively. Six injections

Table III. Antitumor Effects of AAV-IFN-β on Transplanted Human Gliomas in Nude Mice

Group	Tumor-bearing	Tumor size (mm)
(1) PBS-treated	5/5	5.3±1.5
(2) HN-injected	5/5	5.1±2.0
(3) AAV-L (once)	5/5	4.8±1.9
(4) AAV-L (6 times)	5/5	6.2±2.6
(5) AAV-I (once)	5/5	1.9±0.8
(6) AAV-I (6 times)	0/5	ND

Tumor size is presented as the maximum diameter 31 days after transplantation (24 days after first AAV vector injection). The values are mean±SD (n=5).
PBS, phosphate-buffered saline; HN, virus stock solution (50 m*M* HEPES, 150 m*M* NaCl, pH 7.5); AAV-L (once), single injection of AAV-LacZ; AAV-L (6 times), six injections of AAV-LacZ; AAV-I (once), single injection of AAV-IFN-β; AAV-I (6 times), six injections of AAV-IFN-β; ND, not detectable.

Table IV. Median Survival Time (MST) of BALB/c Nude Mice after Human Glioma Transplantation

Mice	MST (Days)
PBS-treated	44.3±6.4
HN-injected	48.0±10.2
AAV-L (once)	45.2±8.2
AAV-L (6 times)	42.0±11.4
AAV-I (once)	63.7±15.3
AAV-I (6 times)	>120 days in all mice

The values are mean±SD (n=6).
PBS, phosphate-buffered saline; HN, virus stock solution (50 m*M* HEPES, 150 m*M* NaCl, pH 7.5); AAV-L (once), single injection of AAV-LacZ; AAV-L (6 times), six injections of AAV-LacZ; AAV-I (once), single injection of AAV-IFN-β; AAV-I (6 times), six injections of AAV-IFN-β.

of AAV-IFN-β induced complete remission of tumors in all mice.

Survival of glioma-transplanted mice treated with AAV-IFN-β As shown in Table IV, the average survival time was 44.3±6.4 days in mice treated with PBS (sham), 48.0±10.2 days in mice treated with HN, 45.2±8.2 days in mice injected with AAV-LacZ once, 42.0±11.4 days in mice injected with AAV-LacZ six times, and 63.7±15.3 days in mice injected with AAV-IFN-β once. All mice injected with AAV-IFN-β six times were alive at the end of the experimental period (120 days).

DISCUSSION

Gene therapy for malignant brain tumors has focused on retroviral or adenoviral vectors to deliver suicide genes such as herpes simplex virus-thymidine kinase (*HSV-tk*) gene, which renders them sensitive to ganciclovir (GCV). In 1997, Ram *et al.* reported that intratumoral implantation of murine cells modified to produce retroviral vectors containing the *HSV-tk* gene, followed by GCV treatment, induced antitumor activity only in smaller brain tumors (1.4±0.5 ml). Furthermore, they suggested that techniques

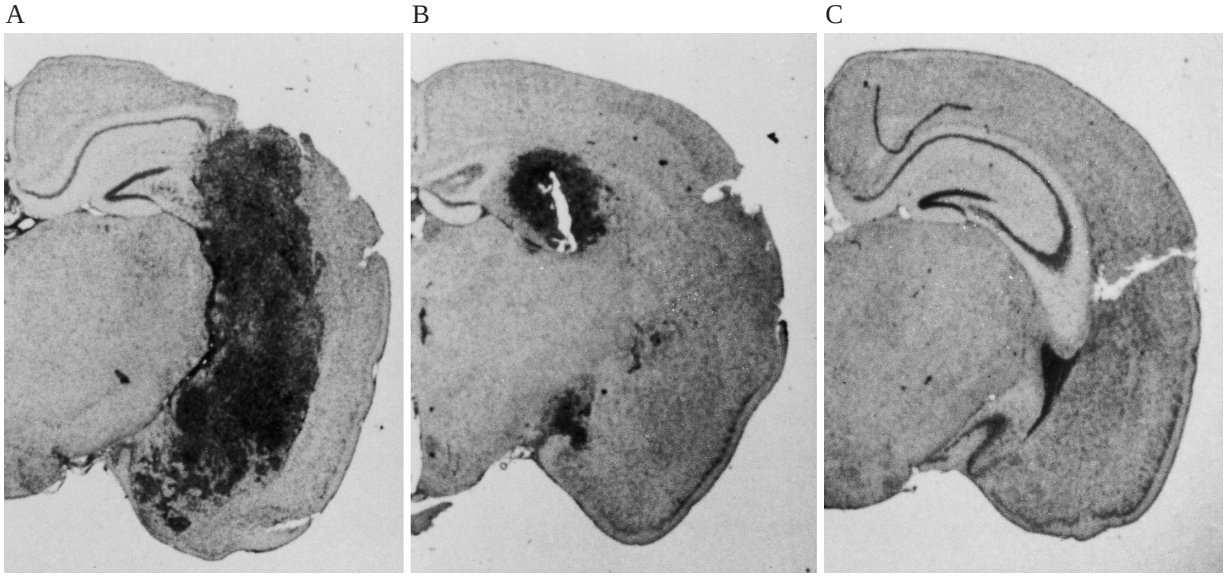

Fig. 4. Antitumor effect of AAV-IFN-β against human glioma (U251-SP) cells transplanted into the brain of nude mice. Histologic findings 31 days after glioma transplantation. A, PBS treatment; B, AAV-IFN-β was injected once on day 7; C, AAV-IFN-β was injected six times.

to improve delivery and distribution of the therapeutic gene would be needed if clinical utility was to be achieved with this approach.[10] Adenoviral vectors have higher transduction efficacy than retroviral vectors. Trask *et al.* presented two patients treated with 10^{11} IU who exhibited central nervous system (CNS) toxicity with persistent mental status changes.[11] This may have been a consequence of inflammatory responses to adenoviral vectors. Successful gene therapies for malignant brain tumors need a safer gene delivery system. AAV vectors are promising candidates because they are not pathogenic in human beings.

We previously evaluated gene delivery with recombinant AAV vectors for treatment of experimental glioma.[12] At that time, 30 to 40% of cells along the needle track expressed β-galactosidase when 1.6×10^{10} AAV-LacZ particles were directly injected into brain tumors *in vivo*. Biodistribution of AAV vectors was at most 5 mm from the needle track. Furthermore, we demonstrated that a single injection of high-titer AAV-tk-IRES-IL-2 induced remarkable growth inhibition of human glioma transplanted into the brains of nude mice, although we did not achieve complete regression. We also evaluated the antitumor effects of AAV-tk without IL-2 on the same experimental glioma. Complete regression of tumors was accomplished after three injections of AAV-tk, followed by GCV treatment; all mice were cured.[13]

In the present study, we evaluated the antitumor effects of AAV-IFN-β on cultured human glioma cells. We observed cell death induced by AAV-IFN-β using VEC-DIC microscopy, a new technology which has rapidly advanced since 1980. VEC-DIC achieves an optical resolution enabling observation of microstructures in viable cells over time at the nanometric level. In the present study, morphologic changes in U251-SP cultured human glioma cells transduced with AAV-IFN-β were continuously observed in this system. We confirmed that AAV-IFN-β induced both apoptosis and secondary necrosis in cultured human glioma cells. When we infected U251-SP cells with AAV-IFN-β at vector genome titers of 30 000, we could observe the apoptotic process in almost all cells. The typical apoptosis process may be divided into four stages: (i) pre-apoptotic stage, (ii) cytoplasmic shrinkage, (iii) membrane blebbing, and (iv) ballooning.[14] In stage 1, no visible changes were seen in VEC-DIC microscopy. However, it is reported that electron microscopic observation has revealed the disappearance and reappearance of microvilli during stage 1.[15, 16] In stage 2, the cells began to show shrinkage and membrane blebbing. It is reported that the microvilli disappeared irreversibly at this stage.[15, 16] Stage 3 is the most dynamic stage in the apoptotic process, resulting in the formation of apoptotic bodies. Probably DNA fragmentation occurs in the late period of this stage. In stage 4, the cells take a large balloon-like appearance and then die. We observed the entire apoptotic process when AAV-IFN-β infected human glioma cells. On the other hand, apoptotic cell death was not observed when the cells were treated with human IFN-β protein (1000 IU)

The reason for this difference is not clear yet, but there are various possible differences between the effects of *IFN-β* gene transfer and its protein. The intracellular concentration of IFN-β mRNA, for example, may be related to the susceptibility to IFN-β. Second, *IFN-β* gene transfer can induce a longer activation of the JAK-STAT pathway than its protein. Further study is needed to elucidate the mechanisms involved. Increased HSP production was detected *in vitro* (data not shown) and *in vivo* (Fig. 3). Melcher *et al.* demonstrated that induction of necrosis is one of the most important factors in activating tumor immunogenicity, and that the activation was induced by HSP.[17] If AAV-IFN-β is administered to a malignant glioma in immune-competent living things, including humans, the evidence that AAV-IFN-β induced apoptosis and necrosis of glioma cells suggests the possibility that the host immune system is activated via HSP production. The nature of the relationship between IFN-β and HSP is still unclear, but we consider that some linkage may exist. These results suggest that gene therapy using AAV-IFN-β has the potential to treat malignant glioma successfully.

(Received August 2, 2001/Revised November 5, 2001/2nd Revised November 29, 2001/Accepted December 4, 2001)

REFERENCES

1) Miyakoshi, J., Dobler, K. D., Allalunis-Turner, J., McKean, J. D. S., Petruk, K., Allen, P. B. R., Aronyk, K. N., Weir, B., Huyser-Wierenga, D., Fulton, D., Urtasun, R. C. and Day, R. S., III. Absence of IFNA and IFNB genes from human malignant glioma cell lines and lack of correlation with cellular sensitivity to interferons. *Cancer Res.*, **50**, 278–283 (1990).
2) James, C. D., Carlbom, J. H. E., Nordenskjold, M., Cavenee, W. K. and Collins, V. P. Chromosome 9 deletion mapping reveals interferon α and interferon β-1 gene deletion in human glial tumors. *Cancer Res.*, **51**, 1684–1688 (1991).
3) Nagai, M. and Asai, T. Clinical effect of interferon in malignant brain tumors. *Neurosurg. Rev.*, **7**, 55–64 (1984).
4) Yoshida, J., Kajita, Y., Wakabayashi, T. and Sugita, K. Long-term follow-up results of 175 patients with malignant glioma: importance of radical tumour resection and postoperative adjuvant therapy with interferon, ACNU and radiation. *Acta Neurochir.* (Wien), **127**, 55–59 (1994).
5) Samulski, R. J. Adeno-associated virus-based vectors for

human gene therapy. *In* "Gene Therapy from Laboratory to the Clinic," ed. K. M. Hui, pp. 232–271 (1994). World Scientific, Singapore.
6) Matsushita, T., Elliger, S., Elliger, C., Podsakoff, G., Villarreal, L., Kurtzman, G. J., Iwaki, Y. and Colosi, P. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.*, **5**, 938–945 (1998).
7) Yamazaki, S., Kuramoto, K., Matsuo, E., Hosoi, K., Hara, M. and Shimizu, H. Highly sensitive enzyme immunoassay of human interferon-β1. *J. Immunoassay*, **10**, 57–73 (1989).
8) Hess, H. H., Lees, M. B. and Derr, J. E. A linear Lowry-Folin assay for both water-soluble and sodium dodecyl sulfate-solubilized proteins. *Anal. Biochem.*, **85**, 295–300 (1978).
9) Enomoto, H., Yoshida, J., Kageyama, N., Ueda, R., Kato, T. and Ota, K. Anti-tumor activity of human recombinant TNF against human malignant glioma cell lines and combination effect with HuIFN-β. *Jpn. J. Cancer Chemother.*, **13**, 1953–1961 (1986).
10) Ram, Z., Culver, K. W., Oshiro, E. M., Viola, J. J., DeVroom, H. L., Otto, E., Long, Z., Chiang, Y., McGarrity, G. J., Muul, L. M., Katz, D., Blaese, R. M. and Oldfield, E. H. Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat. Med.*, **3**, 1354–1361 (1997).
11) Trask, T. W., Aguilar-Cordova, E., Goodman, J. C., Guevara, R., Wyde, P., Shine, H. D. and Grossman, R. G. A phase I study of adenoviral vector delivery of the HSV-tk gene and the intravenous administration of ganciclovir in adults with malignant tumors of the central nervous system. *ASGT (Suppl.)*, 112 (1998).
12) Okada, H., Miyamura, K., Itoh, T., Hagiwara, M., Wakabayashi, T., Mizuno, M., Colosi, P., Kurtzman, G. and Yoshida, J. Gene therapy against an experimental glioma using adeno-associated virus vectors. *Gene Ther.*, **3**, 957–964 (1996).
13) Mizuno, M., Yoshida, J., Colosi, P. and Kurtzman, G. J. Adeno-associated virus vector containing the herpes simplex virus thymidine kinase gene causes complete regression of intracerebrally implanted human gliomas in mice, in conjunction with ganciclovir administration. *Jpn. J. Cancer Res.*, **89**, 76–80 (1998).
14) Okamoto, K., Mizuno, M., Nakahara, N., Natsume, A., Yoshida, J., Mori, T., Hori, S. and Kobayashi, H. Process of apoptosis induced by TNF-α in murine fibroblast Ltk– cells: continuous observation with video enhanced contrast microscopy. *Apoptosis* (2002), in press.
15) Laster, S. M. and Mackenzie, J. M., Jr. Bleb formation and F-actin distribution during mitosis and tumor necrosis factor-induced apoptosis. *Microsc. Res. Tech.*, **34**, 272–280 (1996).
16) Katsen, A. D., Vollmar, B., Mestres-Ventura, P. and Menger, M. D. Cell surface and nuclear changes during TNF-α-induced apoptosis in WEHI 164 murine fibrosarcoma cells. *Virchows Arch.*, **433**, 75–83 (1998).
17) Melcher, A., Todryk, S., Handwick, N., Ford, M., Jacobson, M. and Vile, R. G. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. *Nat. Med.*, **4**, 581–587 (1998).