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 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. Since 1980, HuIFN-β protein has been used in therapy for patients with glioma in Japan and has demonstrated potential antitumor effects. 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)-β, 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. 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. 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.

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. 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 9.1×1013 and 1.9×1013 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 mM nonessential amino acids, 5 E-mail: jyoshida@med.nagoya-u.ac.jp
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. Induction of HSP after injection of AAV-IFN-β was examined by western blot analysis.

**RESULTS**

**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 over-scanned 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 propidium 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 mM HEPES, 150 mM NaCl, pH 7.5) or AAV-LacZ (3.8×10^10 particles in 50 mM HEPES, 150 mM 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
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21.4 ± 3.5 days in mice injected with PBS (sham operation), 20.9 ± 4.4 days in mice treated with HN solution (50 mM HEPES, 150 mM 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 × 10⁴ IU HuIFN-β was intratumorally injected. 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

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**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 mM HEPES, 150 mM 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 mM HEPES, 150 mM 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 AA V-IFN-β induced complete remission of tumors in all mice.

Survival of glioma-transplanted mice treated with AA V-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 AA V-LacZ once, 42.0±11.4 days in mice injected with AA V-LacZ (6 times), 63.7±15.3 days in mice injected with AA V-IFN-β once. All mice injected with AA V-IFN-β six times were alive at the end of the experimental period (120 days).

Table III. Antitumor Effects of AA V-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) AA V-L (once) | 5/5       | 4.8±1.9         |
| (4) AA V-L (6 times) | 5/5      | 6.2±2.6         |
| (5) AA V-I (once)   | 5/5           | 1.9±0.8         |
| (6) AA V-I (6 times)| 0/5         | ND              |

Tumor size is presented as the maximum diameter 31 days after transplantation (24 days after first AA V vector injection). The values are mean±SD (n=5).

PBS, phosphate-buffered saline; HN, virus stock solution (50 mM HEPES, 150 mM NaCl, pH 7.5); AA V-L (once), single injection of AA V-LacZ; AA V-L (6 times), six injections of AA V-LacZ; AA V-I (once), single injection of AA V-IFN-β; AA V-I (6 times), six injections of AA V-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  |
| AA V-L (once) | 45.2±8.2   |
| AA V-L (6 times) | 42.0±11.4 |
| AA V-I (once) | 63.7±15.3  |
| AA V-I (6 times) | >120 days | in all mice

The values are mean±SD (n=6).

PBS, phosphate-buffered saline; HN, virus stock solution (50 mM HEPES, 150 mM NaCl, pH 7.5); AA V-L (once), single injection of AA V-LacZ; AA V-L (6 times), six injections of AA V-LacZ; AA V-I (once), single injection of AA V-IFN-β; AA V-I (6 times), six injections of AA V-IFN-β.

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
to improve delivery and distribution of the therapeutic gene would be needed if clinical utility was to be achieved with this approach. 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. 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. 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. Bio-distribution 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.

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. 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. In stage 2, the cells began to show shrinkage and membrane blebbing. It is reported that the microvilli disappeared irreversibly at this stage. 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. 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.

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