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

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Adeno-associated virus (AAV) has attracted considerable interest as a potential vector for gene therapy because of its wide host range, high transduction efficiency, and lack of cytopathogenicity. In this experiment, we evaluated the efficacy of AAV vector containing the herpes simplex virus thymidine kinase (HSV-tk) gene on human gliomas transplanted into the brain of nude mice. Complete regression of the tumors was observed after multiple AAV-tk injections followed by intraperitoneal ganciclovir (GCV) administration, and the survival of mice treated with AAV-tk vector and GCV administration was markedly prolonged. These results suggest that AAV-tk vectors may be useful for gene therapy against malignant gliomas in humans.

Key words: AAV — HSV-tk — GCV — Glioma — Gene therapy

Advances in gene therapy will depend in large part on the development of delivery systems capable of efficiently introducing DNA into target cells. Retrovirus and adenovirus vectors are the main systems which have thus far been applied for human gene therapy. Retrovirus vectors have been studied extensively and although their preparation is not difficult, their use is limited because gene delivery requires that the target cells are dividing, and the viruses are rapidly inactivated in the blood. Furthermore, there is a risk that pathogenic replication-competent retroviruses (RCRs) will be produced. In contrast, adenovirus vectors are capable of efficiently delivering a gene to both dividing and non-dividing cells. However, adenoviruses express proteins that trigger an immune response and induce inflammation and an allergic response. This immune response may limit the length of time during which gene expression can be maintained in the target cell.

Adeno-associated virus (AAV), which is a human paravovirus with a single-stranded DNA genome of approximately 4.6 kb encapsulated in an icosahedral virion 20 to 24 nm in diameter,1) has no cytopathic effect in humans. AAV depends on coinfection with a helper virus (either adenovirus or herpesvirus) for efficient DNA replication.2, 3) Therefore, AAV vectors have considerable potential for human gene therapy.

The herpes simplex virus thymidine kinase (HSV-tk) gene under transcriptional control of the cytomegalovirus immediately-early (CMV-IE) promoter. It was prepared in an adenovirus-free system5) and had a titer of 4.8x1013 particles/ml. The “bystander effect” would allow effective killing of invasive tumor cells in normal tissue. Therefore, the transfer of the gene coding for HSV-tk followed by GCV administration has been studied in several types of cancers. Specifically, the therapeutic effects of HSV-tk gene transfer using retroviruses followed by GCV administration have been studied in patients with malignant gliomas.9) However, the results were not optimal because in vivo gene transduction efficiency was very low. Therefore, there is a need to develop new approaches using safe vectors that have high transduction efficiency. AAV vectors containing the HSV-tk gene are considered promising from this point of view.

Gliomas in Mice, in Conjunction with Ganciclovir Administration

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Malignant gliomas, including glioblastoma, are formidable neoplasms that are resistant to the multimodality treatment of surgery, radiation, chemotherapy, and immunotherapy.7) The average survival time of patients with malignant gliomas is less than 2 years. Therefore, the development of new therapeutic approaches such as gene therapy is very important. Here, we describe an in vivo basic study on the effect of an AAV vector containing HSV-tk gene (AAV-tk), in combination with ganciclovir administration, on the growth of malignant gliomas in mice.

MATERIALS AND METHODS

Cell line The human glioma cell line U251-SP was grown in Eagle’s minimum essential medium with 10% fetal calf serum, 1% nonessential amino acids, 5 mM L-glutamine, and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin).

Vector The AAV-tk vector contains HSV-tk gene under transcriptional control of the cytomegalovirus immediately-early (CMV-IE) promoter. It was prepared in an adenovirus-free system5) and had a titer of 4.8x1013 particles/ml.

Animals Female BALB/c nude (8–10 weeks old) were maintained and bred under a pathogen-free condition in the
animal facility of the Nagoya University School of Medicine. Animal experiments were performed according to the principles enunciated in the “Guide for the Care and Use of Laboratory Animals” prepared by the Office of the Prime Minister of Japan.

Production of glioma in the brain of nude mice To produce gliomas in the brain of nude mice, U251-SP human glioma cells suspended in PBS or culture medium without serum were injected into the brain. Briefly, animals were anesthetized by intraperitoneal (i.p.) injection of Nembutal (60–70 mg/kg body weight) and held in a stereotaxic apparatus (SAS-1405, ASI Instruments, Warren, MI). A 2 µl aliquot of the cell suspension (approximately 2×10^6 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.

Protocol for in vivo transduction AAV-tk (9.6×10^9 particles in 2 µl) was injected into the brain where glioma cells had been transplanted on day 7 (single injection) or on days 7, 10, and 13 (multiple injections). In the case of a single injection, 100 mg/kg GCV was administered i.p., twice daily, in 0.5 ml of saline, for 6 days after vector injection. Following multiple injections, the same volume of GCV was administered i.p., twice daily, in 0.5 ml saline, for 2 days after vector injection and the procedure was repeated three times. On the 31st day after transplantation, animals were killed and the tumor was checked both visually and by light microscopy.

RESULTS

Antitumor effect of AAV-tk According to the experimental protocol, AAV-tk vector was injected into tumors, followed by GCV administration. The size of transplanted tumors was about 2 mm in diameter at the start of the treatment. The results are shown in Table I. A single injection of AAV-tk vector followed by GCV administration for 6 days remarkably inhibited tumor growth: the maximum diameter of the tumors was 2.8±0.5 mm for mice treated with AAV-tk and GCV, versus 6.1±1.3 mm, 5.9±0.9 mm, 5.7±1.2 mm for mice treated with phosphate-buffered saline (PBS), GCV alone or AAV-tk vector alone, respectively. Three injections of AAV-tk vector followed by GCV administration induced the complete remission of the tumors in five of six mice. In the one mouse with a residual tumor, the tumor was very small with a diameter of 1.3 mm. Microscopic findings are shown in Fig. 1 (low magnification) and Fig. 2 (high magnification). In normal brain, no necrosis or inflammatory response was observed, except for the scars of the needle track.

Survival We investigated whether the AAV-tk vector and GCV treatment were able to cure mice with intracerebrally transplanted human glioma cells. When AAV-tk vector was injected once into mice followed by GCV treatment, survival was significantly prolonged: 63.5±4.7 days versus 40.0±2.2 days, 39.7±2.9 days, and 38.3±5.2 days in mice treated with PBS, GCV alone, and AAV-tk vector alone, respectively. When injected three times with AAV-tk vector followed by GCV treatment, six of seven mice were alive at the end of the experimental period (120 days), while all mice treated with GCV or AAV-tk vector alone were dead by 45 days after transplantation (Table II).

DISCUSSION

Gene therapy has been proposed for several inherited and acquired diseases. However, there is little evidence that current delivery systems can achieve complete regression or cure. Retrovirus vectors have low transduction efficiencies and adenovirus vectors cannot be injected repeatedly because of their high immunogenicity. Therefore, a new delivery system with high transduction efficiency is needed.

Recombinant AAV vectors are physically stable, elicit less of a host immune response, and can efficiently transduce genes into a wide variety of dividing and non-dividing cells. Recombinant AAV vectors are physically stable, elicit less of a host immune response, and can efficiently transduce genes into a wide variety of dividing and non-dividing cells. Our previous study demonstrated that a single injection of high-titer AAV-tk-IRE-IL-2 remarkably inhibited the growth of human gliomas transplanted into the brain of nude mice. However, complete regression of the
tumors was not achieved. Here, we evaluated the safety and antitumor effect of an AAV-tk vector in transplanted gliomas injected multiple times with the vector. The AAV vector was prepared by using an adenovirus-free system so there would be no induced immunogenicity due to contaminating adenovirus. Repeated injections of AAV vector alone did not result in a cytopathic effect, but a remarkable antitumor effect was induced even after a single injection.

Fig. 1. Antitumor effect of adeno-associated virus thymidine kinase vector (AAV-tk) and ganciclovir (GCV) against human glioma (U251-SP) cells transplanted into the brain of nude mice. Histological findings 31 days after glioma transplantation. A, no treatment; B, AAV-tk vector was injected once on day 7 and GCV was administered intraperitoneally twice daily for 6 days after vector injection; C, AAV-tk vector was injected on day 7 and GCV was administered intraperitoneally twice daily for 2 days after vector injection, after which this sequence was repeated two additional times.

Fig. 2. Histological findings of the residual tumors. A, High magnification of the residual tumor in a mouse without treatment, showing highly cellular tissue and aggressively invasive tumor cells (magnification ×400); B, High magnification of the residual tumor in a mouse injected with AAV-tk once, showing mixed appearance of highly cellular tissue and necrosis (magnification ×400).
followed by GCV. Three successive injections followed by GCV administration caused complete regression of tumors in five of six mice and all the mice survived. This is the first demonstration of complete regression of malignant gliomas using an AAV vector with the HSV-tk gene. The regression was probably due to two important features of the AAV delivery system: (i) high-titer AAV vector was used (more than 10^13 particles/ml), and (ii) multiple injections of AAV vector were possible because of the absence of contaminating adenovirus. In this protocol, GCV was administered 12 h after vector injection, at a high dose (100 mg/kg). However, similar results were obtained even when GCV was injected 2 or 4 days after vector injection, or when the GCV concentration was decreased to 25 mg/kg (data not shown). Cool et al. demonstrated that two doses of 15 mg GCV/kg per day were optimal to treat 9L glioma transplanted into the brain of Fisher 344 rats. However, we needed to administer more than 25 mg/kg GCV to obtain complete regression of U251-SP gliomas transplanted into the brain of nude mice.

In our previous study using AAV-LacZ, an AAV-based vector encoding the LacZ gene, we obtained a 10–20% transduction efficiency following a single injection. As we obtained complete regression after multiple injections, there is probably a "bystander effect." Cell death of the HSV-tk-negative population is considered to be mediated via apoptosis, and cell-to-cell contact is necessary for the transfer of cytotoxic molecules (probably GCV monophosphate) from HSV-tk-positive cells to cells without the HSV-tk gene through a gap-junction. Some investigators could not demonstrate this effect in nude mice and suggested that it might be related to the host immune response. However, in our experiments in nude mice, we believe that the complete tumor regression involves a bystander effect via cell-to-cell contact.

Finally, our results demonstrate that in vivo gene transfer of an the HSV-tk gene by means of an AAV vector, followed by GCV treatment, is able to cure mice transplanted with human glioma cells. These findings suggest that AAV-tk vectors may be useful for human gene therapy against malignant gliomas.

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| Group       | Survival day (mean±SD) |
|-------------|------------------------|
| (1) control | 40.0±2.2               |
| (2) GCV     | 39.7±2.9               |
| (3) AAV-tk  | 38.3±5.2               |
| (4) AAV-tk + GCV | 63.5±4.7          |
| (5) AAV-tk(3) | 38.5±2.4             |
| (6) AAV-tk(3) + GCV |            |

Groups: (1) control. no treatment; (2) GCV, ganciclovir (100 mg/kg) was administered intraperitoneally twice daily for 6 days beginning on day 7; (3) AAV-tk, adeno-associated virus thymidine kinase vector (9.0×10^10 particles), was injected into the brain once on day 7; (4) AAV-tk + GCV, AAV-tk vector was injected once on day 7 and GCV was administered intraperitoneally twice daily for 6 days after vector injection; (5) AAV-tk(3), AAV-tk vector was injected on days 7, 10 and 13 (total three times); (6) AAV-tk(3) + GCV, AAV-tk vector was injected on days 7 and GCV was administered intraperitoneally twice daily for 2 days after vector injection, after which this sequence was repeated two additional times. n=6.

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