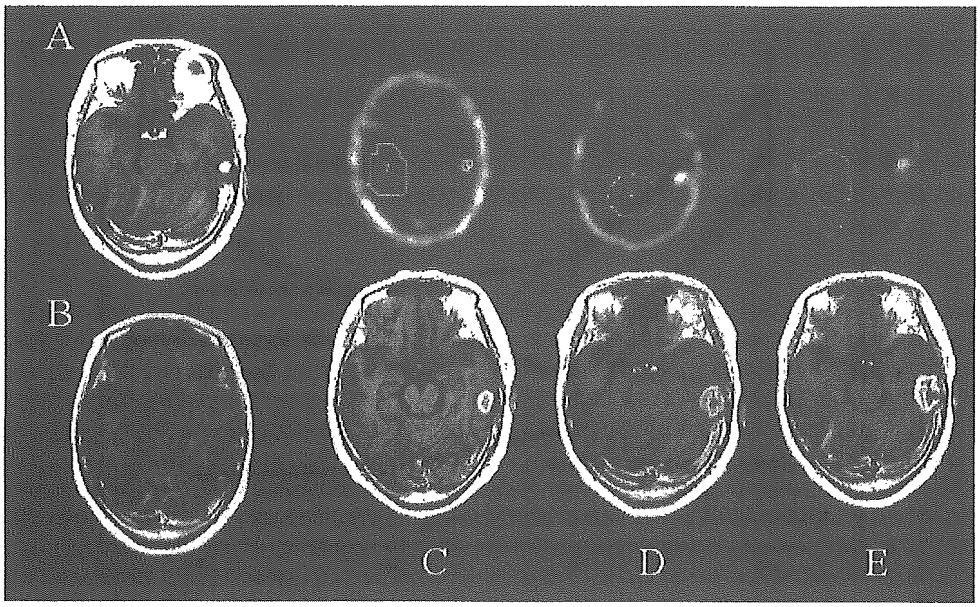

FIG. 6. Serial enhanced MR and ^{201}Tl SPECT studies obtained in the patient in Case 3. A: A small left temporal metastatic tumor from breast cancer was treated by GKS. B: Two months later, the tumor has completely disappeared. C: Six months later, tumor regrowth is apparent. Thallium-201 accumulation is low (3.10). The radioisotope diagnosis was radiation injury at this initial study. D: Eight months later the tumor continued to grow. Abnormally high Tl accumulation in the posterior part of the enhanced area (Tl index 5.18) is consistent with a radioisotope diagnosis of tumor recurrence. The tumor was treated again with GKS, using a peripheral dose of 20 Gy. E: Ten months later, the tumor continued to grow. Thallium-201 accumulated in the anterior portion of the area showing enhancement (Tl index 5.12). The patient exhibited sensory aphasia. The tumor was surgically removed 3 months after repeated GKS.

low enough to be definitive, which necessitated serial measurements. Eight months later MR imaging demonstrated a further increase in size, and the index was 3.43. The radioisotope diagnosis was radiation injury. A specimen obtained by MR imaging–guided stereotactic biopsy revealed no viable tumor cells (Fig. 5).

Case 3. Radiation Injury. This 58-year-old woman had multiple brain metastases from breast cancer. A small peripheral lesion in the temporal lobe was irradiated with 20 Gy to the 80% isodose line (Fig. 6A). Two months later MR imaging revealed complete remission (Fig. 6B). Eight months later MR imaging revealed tumor growth (Fig. 6C). There was an abnormally high Tl accumulation in the posterior part of a mass, which showed enhancement, and the Tl index was 5.18, indicating tumor recurrence. The tumor was irradiated again with a peripheral dose of 20 Gy to the 55% isodose line. Further tumor growth was seen on MR imaging 10 months later, that is 2 months after the second GKS. There was Tl accumulation in the anterior part of the area showing enhancement. The Tl index was still high at 5.12 (Fig. 6D). The patient exhibited sensory aphasia despite steroid administration. The tumor was surgically removed 3 months after the second GKS. The pathological examination confirmed pure radiation injury as shown in Fig. 7.

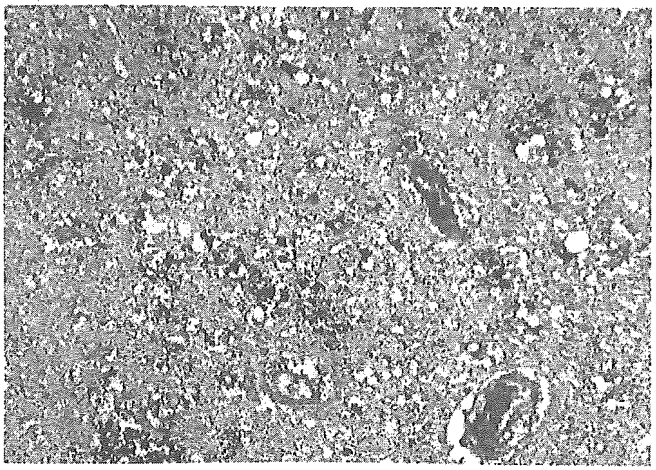

FIG. 7. Photomicrograph obtained in Case 3. The pathological examination confirmed radiation injury. H & E.

Discussion

Over the last two decades extensive research focusing on various methods, such as positron emission tomography scanning using ^{18}F-fluorodeoxyglucose or L-methyl-^{11}C-methionine, SPECT using ^{201}Tl and ^{99m}-Tc-hexakis-2-methoxy-isobutylisonitrile, and MR spectroscopy, has provided invaluable information facilitating the differential diagnosis of tumor recurrence from radiation injury.[1–3,6,7,11] Thallium-201, already used as a cardiomuscle tracer, was discovered to accumulate in various brain tumors, and reports on differential diagnosis of brain tumors and assessment after radiotherapy have been published.[4,9] We have endeavored to differentiate regrowth of lesions after GKS by using this classic tracer. We introduced scatter correction with the triple-energy window method, a more sensitive detection technique, which has a higher Tl index than conventional methods. This allowed us to differentiate tumor

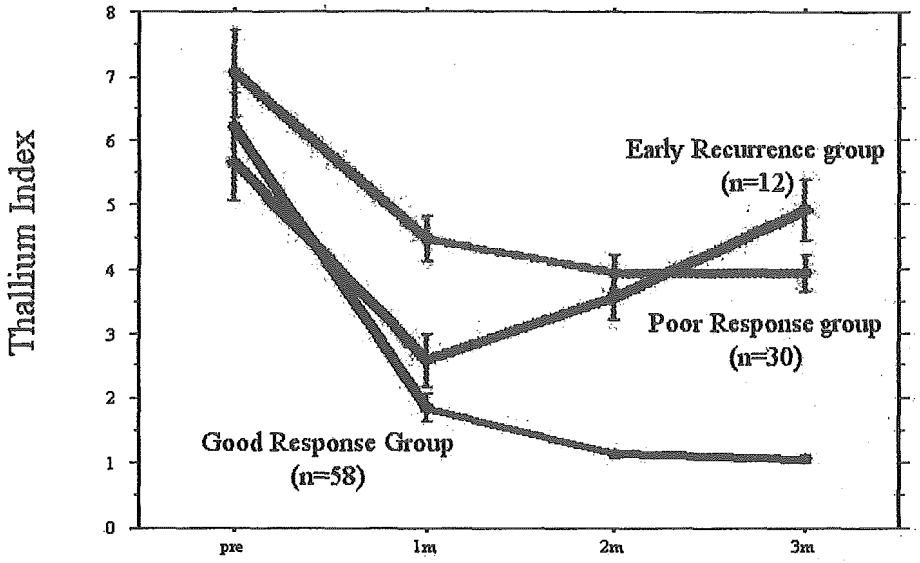

FIG. 8. Monthly serial Tl indices after GKS for 100 metastatic lesions were obtained in a pilot study. There were three patterns of Tl index change. 1: Good response type showing a continuous decrease to 1, within 2 or 3 months after GKS (blue, 58 lesions). 2) Early recurrence type demonstrating a transient decrease at 1 month but an increase to 5, within 3 months after GKS (red, 12 lesions). 3) Poor response type showing a transient decrease at 1 month and a fluctuation between 3 and 5, at 2 to 3 months after GKS (green, 30 lesions).

recurrence from radiation injury with higher sensitivity than would have been possible with conventional measurement methods. Furthermore, MR imaging following SPECT studies is performed within 1 hour after simultaneous intravenous injection of ^{201}Tl and Gd during serial measurements. These innovations make it possible to examine patients with tumor regrowth easily and quickly.

In a lesion that starts to grow again after GKS, tumor recurrence and radiation injury are not mutually exclusive because both components may be present. In this situation, it is crucial to ascertain when the recurrent components begin to grow and require retreatment. We identify a gray zone in which the Tl index is between 3 and 5 to determine whether a mixed lesion represents true recurrence. In cases with a Tl index in this gray zone, ^{201}Tl SPECT studies are repeated once per month until the Tl index exceeds 5 or is less than 3. A frequency of observation of every other month is considered adequate for serial measurements. With radiation injury, the Tl index usually fluctuates, whereas in a recurrent tumor it usually increases monthly. This pattern provides important information facilitating differentiation.

The cut-off value we used was derived from a pilot study of monthly serial Tl measurements after GKS in 100 metastatic lesions (Fig. 7). That study demonstrated three patterns of Tl index change, which were classified as follows. 1) Good response: showing a continuous decrease to 1, within 2 to 3 months after GKS (58 lesions). 2) Early recurrence: demonstrating a transient decrease at 1 month and an increase to 5 at 3 months (12 lesions). 3) Poor response: showing a transient decrease at 1 month and fluctuation between 3 and 5 at 2 to 3 months after GKS (30 lesions).

Setting the cut-off value at 5 was based on these results, as we previously reported.[9] The "poor response" type represents tumor recurrence followed later by radiation injury. This is why we consider values between 3 and 5 to represent a gray zone.

Thallium-201 chloride SPECT scanning does have limitations. The first is that a simple interinstitutional comparison of Tl indices is not possible because measurement methods are institute specific. Thus, serial measurements must be performed under the same conditions at the same institute. Second, steroid use decreases the Tl index to a variable degree, as reported by Namba, et al.[5] This reduction may be attributed to steroid administration, which decreases the uptake of ^{201}Tl into viable tumor cells and the inflammatory portion of a radiation injury lesion. This phenomenon must be kept in mind, especially with serial Tl measurements. Finally, highly inflammatory lesions associated with radiation injury, such as occurs after repeated GKS or with high-dose GKS, may produce a Tl index greater than 5, as in our Case 3.

Conclusions

Thallium-201 chloride SPECT scanning can be effective for differentiating tumor recurrence from radiation injury in metastatic brain tumors treated with GKS. The technique is appropriate as long as the technical limitations of the method are kept in mind.

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Recombinant Sendai Virus Vector Induces Complete Remission of Established Brain Tumors through Efficient *Interleukin-2* Gene Transfer in Vaccinated Rats

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Abstract **Purpose:** Sendai virus (SeV), a murine parainfluenza virus type I, replicates independent of cellular genome and directs high-level gene expressions when used as a viral vector. We constructed a nontransmissible recombinant SeV vector by deleting the *matrix (M)* and *fusion (F)* genes from its genome (SeV/ΔMΔF) to enhance its safety. We also estimated the therapeutic efficacy of the novel vector system against a rat glioblastoma model.

Experimental Design: We administered the recombinant SeV vector carrying the *lacZ* gene or the *human interleukin-2* (*hIL-2*) gene into established 9L brain tumors *in vivo* simultaneous with peripheral vaccination using irradiated 9L cells. Sequential monitoring with magnetic resonance imaging was used to evaluate the therapeutic efficacy.

Results: We found extensive transduction of the *lacZ* gene into the brain tumors and confirmed sufficient amounts of interleukin 2 (IL-2) production by hIL2-SeV/ΔMΔF both *in vitro* and *in vivo*. The magnetic resonance imaging study showed that the intracerebral injection of hIL2-SeV/ΔMΔF brought about significant reduction of the tumor growth, including complete elimination of the established brain tumors. The ^{51}Cr release assay showed that significant amounts of 9L-specific cytotoxic T cells were induced by the peripheral vaccination. Immunohistochemical analysis revealed that $CD4^+$ T cells and $CD8^+$ T cells were abundantly infiltrated in the target tumors.

Conclusion: The present results show that the recombinant nontransmissible SeV vector provides efficient *in vivo* gene transfer that induces significant regression of the established brain tumors and suggest that it will be a safe and useful viral vector for the clinical practice of glioma gene therapy.

Glioblastoma is the most common malignant brain tumor. It is considered incurable despite multimodal approaches of therapy, including surgery, radiotherapy, and chemotherapy (1). The therapeutic application of the gene transfer technique has been expected as a new therapeutic option for glioblastoma (2). The efficacy, however, has been limited by low levels of transgene transduction and poor distribution of the target molecules throughout the tumor tissues (3). The successful gene therapy in clinical practice will depend on the development of novel vector systems capable of wide distribution and efficient transduction of the target genes into the tumor cells.

Recent advances to manipulate the genome of negative-strand RNA viruses have led to the development of a new class of viral vectors for gene transfer approaches (4). Sendai virus (SeV) is a murine parainfluenza virus type I belonging to the family Paramyxoviridae and is a single-stranded RNA virus. The genome of SeV is a linear and nonsegmented negative-strand RNA of ~15.4 kb. It contains six major genes that are arranged in tandem on its genome; it is tightly encapsidated with the nucleoprotein (NP) and is further complexed to phosphoprotein (P) and large protein (L; the catalytic subunit of the polymerase). This viral ribonucleoprotein complex constitutes the internal core structure of the virion. The viral envelope contains two spike proteins, hemagglutinin-neuraminidase (HN) and fusion (F), which mediate the attachment of virions and the penetration of ribonucleoproteins into infected cells, respectively. Matrix (M) protein functions in virus assembly and budding.

For the use of gene therapy vectors, the promising characteristics of SeV are as follows: (*a*) an exclusively cytoplasmic replication cycle without any risk of integration into the genomic DNA, (*b*) transduction efficacy that is not dependent on the cell cycle of target cells, (*c*) no homologous recombination between

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different SeV genomes or to wild-type virus, (*d*) the remarkably brief contact time that is necessary for cellular uptake, (*e*) a high and adjustable expression of virally encoded genes in a broad range of host cells, and (*f*) the lack of association with any disease process in humans (4). Indeed, the SeV vector has been shown to produce 2 to 3 logs higher transfection efficiency than the adenoviral vectors or lipofection (5, 6), and high gene expressions have been noticed in a broad range of tissues, including the airway epithelial cells, vasculature tissues, skeletal muscle, activated T cells, stem cells, and neural tissues (5–12). In addition to these biological features of the SeV vector, we have constructed a nontransmissible recombinant SeV vector by deleting the M and F genes from its genome to enhance its safety (13–15).

We previously verified the efficacy of the intracerebral (i.c.) transplantation of interleukin 2 (IL-2)–producing cells in the rat brain tumor model (16, 17). For clinical application of the cytokine gene therapy strategy, however, the cell-mediated therapy needs a large amount of cytokine-producing cells to cover the whole tumor areas in the human brain. The SeV vector–mediated strategy would be superior in the wide distribution of the transgene products achieved by a small amount of viral solution, and it is expected to be especially suitable for delivery of the secreted proteins, such as cytokines. In the present study, we examined the therapeutic potentials of the nontransmissible recombinant SeV vector carrying *human IL-2* gene in a cytokine gene therapy against brain tumors.

Materials and Methods

Cells and animals. Rat 9L gliosarcoma, C6 glioma, and rhesus monkey LLC-MK_2 kidney cell lines were maintained in DMEM supplemented with 10% FCS in a humidified atmosphere of 5% CO_2. Male Fisher 344 rats, weighing between 200 and 240 g (7-8 weeks old), were used as indicated in the experiments. These animals were maintained in a specific pathogen-free environment in accordance with the Laboratory Animal Resources Commission Standards.

Recombinant Sendai virus vector. Genome order of the SeV full length genome used was as follows: the leader (ld) at the 3′-end followed by viral genes, *nucleocapsid (NP), phospho (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN),* and *large proteins (L)*. Finally, a small trailer (tr) sequence was placed at the 5′-end (Fig. 1). We utilized both the *M* and *F* genes–deleted SeV vector (SeV/ΔMΔF) in the experiment. F protein is essential for viral infection and M protein functions in virus assembly and budding (13, 14). Therefore, SeV/ΔMΔF is nontransmissible with loss of particle formation from infected cells (15). SeV/ΔMΔF carrying *human IL-2* gene (hIL2-SeV/ΔMΔF) and *lacZ* gene (lacZ-SeV/ΔMΔF) were constructed as previously described (13). In brief, human IL-2 (accession no. U25676) cDNA was amplified with a pair of *Not*I-tagged primers that contained SeV-specific transcriptional regulatory signal sequences, 5′-ACTTGCGGCCGCGTTTAAACGGCGCGCCATGTACAGGATG-CAACTCCTGTC-3′ and 5′-ATCCGCGGCCGCGATGAACTTTCACCC-TAAGTTTTTCTTACTACGGATTTAAATGGCGCGCCA-3′. The amplified fragment was introduced into the *Not*I site of the parental pSeV18+/ΔMΔF. Thus, the cDNA of hIL2-SeV/ΔMΔF (phIL2-SeV/ΔMΔF) was constructed. The cDNA of lacZ-SeV/ΔMΔF (placZ-SeV/ΔMΔF) was constructed in similar manner using the amplified fragment of lacZ (18). phIL2-SeV/ΔMΔF and placZ-SeV/ΔMΔF were transfected into LLC-MK_2 cells after infection of the cells with vaccinia virus vTF7-3 (19), which expresses T7 polymerase. The T7-driven recombinant hIL2-SeV/ΔMΔF and lacZ-SeV/ΔMΔF RNA genomes were encapsulated by N, P, and L proteins, which were derived from their respective cotransfected plasmids. The recovered SeV vectors were propagated using both M and F protein–expressing packaging cell lines (15). The virus titers were determined using infectivity and were expressed in cell infectious units. The SeV vectors were stored at −80°C until use.

Kinetic analysis of interleukin-2 production. LLC-MK_2 cells (10^6) grown in six-well plates were infected at a multiplicity of infection of 10 for 1 hour with hIL2-SeV/ΔMΔF and incubated in serum-free MEM at 37°C. The culture supernatants were collected every 24 hours, with immediate addition of MEM to the remaining cells. IL-2 protein in the supernatant was quantified by ELISA using the human IL-2 ELISA kit (Biosource International, Inc., Camarillo, CA).

Brain tumor model and treatment. The animals were anesthetized and placed in a stereotaxic apparatus. A burr hole was made at 4 mm posterior to bregma and 3 mm right to midline. A 25-gauge needle was inserted to the point of 3 mm ventral from dura where 1×10^5 syngeneic 9L tumor cells in 10 μL medium were slowly injected. Treatment was started 3 days (day 3) after i.c. inoculation of 9L tumor cells (day 0). The animals received i.c. administration of hIL2-SeV/ΔMΔF or lacZ-SeV/ΔMΔF and/or s.c. vaccination with irradiated wild-type 9L tumor cells. For i.c. administration, 1×10^7 cell infectious units of SeV vector in 10 μL PBS were used in the same stereotactic coordinates. For s.c. vaccination, wild-type 9L cells were irradiated at 30 Gy and 1×10^6 cells in 100 μL medium were injected into the lower abdominal quadrant (16, 17). The animal experimentation was reviewed and approved by the Institutional Animal Care and Use Committee of Chiba Cancer Center Research Institute.

Magnetic resonance imaging study. To estimate i.c. tumor volume sequentially, all the animals were examined with magnetic resonance imaging (MRI) every 7 days started on day 7 after the tumor inoculation. Rats were anesthetized with 50 mg/kg pentobarbital and injected with 0.2 mL gandolinium-diethylenetriaminepentaacetic acid (Gd-DTPA, 0.8-1.0 mL/kg). Coronal T_1-weighted images (TR 500 milliseconds, TE 11 milliseconds, 3 mm thickness, gapless) were obtained with a 1.5-T MR device (Signa Advantage, General Electric, Milwaukee, WI). Tumor volume (mm^3) was calculated as the sum of the Gd-DTPA–enhanced portion of each MR-imaged area (mm^2) times the imaged thickness. The estimated tumor volumes on MRI have a linear correlation with actual tumor weights obtained immediately after the imaging study (20).

Immunohistochemistry. Tumor-bearing rats were perfused through the ascending aorta with 4% paraformaldehyde and brains were

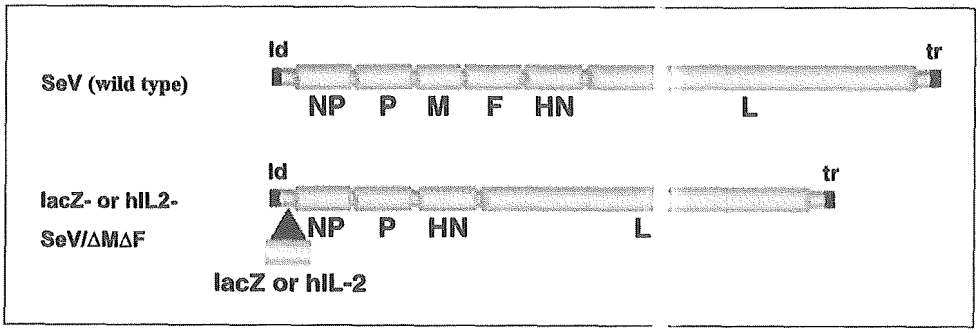

Fig. 1. Schematic genome structures of wild-type SeV and the both *M* and *F* gene–deleted SeV vector carrying *lacZ* or *human IL-2* gene. The open reading frame of the *lacZ* or *human IL-2* gene was inserted with the SeV-specific transcriptional regulatory signal sequences, end and start signals between the leader (*ld*) and the *NP* gene.

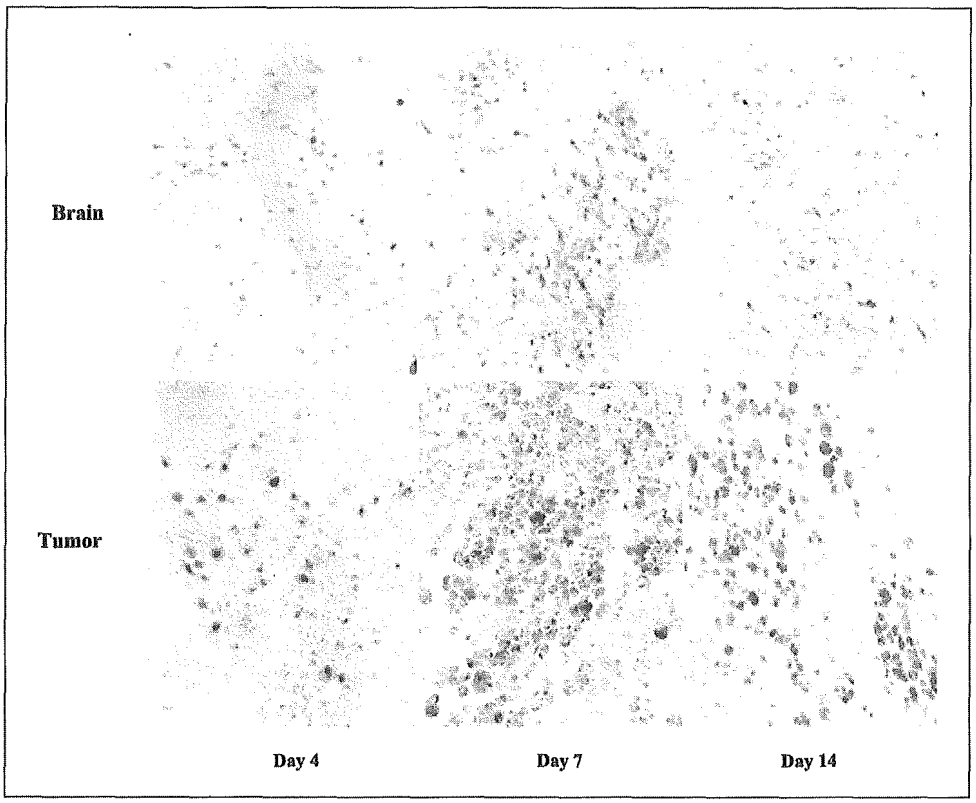

Fig. 2. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of rat brain tissues (*top*) and 9L brain tumors after 7 days of growth in the brain (*bottom*) administered *in situ* with lacZ-SeV/ΔMΔF. Four, seven, and fourteen days after administration of the vector, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining was done (×200 magnification). Maximal expression or accumulation of β-galactosidase was obtained on day 7 after injection of the vector, and the expression was maintained on day 14 both in the brain tissues and in the brain tumors.

removed. Frozen tissue sections of the brain specimens at 10 μm thickness slices were reacted with anti-CD4 antibody (W3/25, Serotec, Oxford, United Kingdom), anti-CD8 antibody (OX-8, Serotec), and anti-human IL-2 antibody (R&D Systems, Minneapolis, MN). Tissues were then reacted with horseradish peroxidase–conjugated goat anti-mouse IgG and stained with 3,3′-diaminobenzidine tetrahydrochloride (Nichirei, Tokyo, Japan). β-galactosidase expression was detected using β-Galactosidase Staining Kit (Mirus, Madison, WI).

CTL assay. To test lymphocytes for their antitumor cytotoxicity, a standard 4-hour ^{51}Cr release assay was done. Spleen cells (1×10^6 cells) were harvested from rats on day 14 after treatment and were cultured in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} mol/L 2-mercaptoethanol. They were stimulated *in vitro* with irradiated 9L cells for 5 days. Syngeneic 9L or allogeneic C6 cells were used as ^{51}Cr-labeled targets and were cultured with the spleen cells at various effector-to-target cell ratios. After 4-hour incubation, radioactivities in the culture supernatants were counted with an automatic γ-counter. Specific cytotoxic activity was calculated as follows: 100 × ([experimental counts per minute − spontaneous counts per minute] / [maximal counts per minute − spontaneous counts per minute]). The maximal counts per minute were released by adding 1% NP40 to wells in experiments.

Statistics. Comparison of tumor volumes in each treatment group was done with the unpaired *t* test. The Kaplan-Meier method was used to estimate the survival rates and the Cox-mantel log-rank test was used to compare the survival differences in each treatment group. All of the statistical analyses were done with the StatView software (SAS Institute, Inc., Cary, NC).

Results

Sendai virus vector–mediated transduction of β-galactosidase gene into glioma tissue. The efficiency of i.c. transduction of the *β-galactosidase* gene by SeV vector was examined in brain tumors and normal brain tissues removed 4, 7, and 14 days after administration of lacZ-SeV/ΔMΔF. When injected into the brain tumor, the typical appearance of the vector-injected tissues was scattered colonies of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–positive cells, which were composed of transduced tumor cells from the injected lacZ-SeV/ΔMΔF (Fig. 2). Nontransduced tumor cells were seen between the scattered 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside–stained colonies. Maximal expression or accumulation of

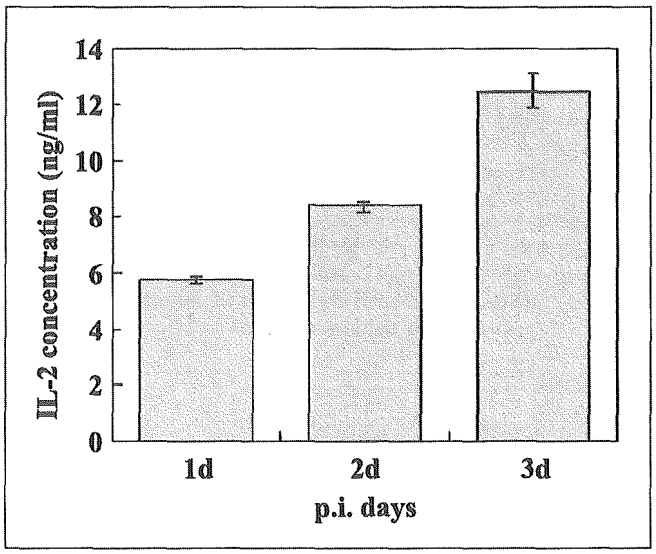

Fig. 3. Kinetics of the expression of IL-2 protein in the cells infected with hIL2-SeV/ΔMΔF *in vitro*. LLC-MK_2 cells (10^6) were infected at a multiplicity of infection of 10 with hIL2-SeV/ΔMΔF. The culture supernatants were collected every 24 hours and the amounts of IL-2 protein were quantified by ELISA. Substantial amount of IL-2 was secreted even at 1 day after the infection. The maximum production of 12.5 ng/mL was observed at day 3.

β-galactosidase was obtained on day 7 and the expression level was maintained on day 14 after injection of the vector. Transduction of normal brain surrounding the tumor was scarcely detected with the exception of the choroid plexus. When injected into the normal brain tissues, transductions of neurons and glial cells were also observed at lower efficiency compared with the case of intratumoral injection. Ependymal cells were not transduced by the intraparenchymal injection of the vector.

In vitro *kinetics of interleukin-2 production by cells infected with hIL2-SeV/ΔMΔF.* To clarify the transgene expression induced by hIL2-SeV/ΔMΔF, the amount of IL-2 production by the cells infected with hIL2-SeV/ΔMΔF was investigated by ELISA. Sufficient amount of IL-2 protein (5.8 ng/mL) was detected at day 1 after the vector infection and increased to 12.5 ng/mL at 3 days after the infection (Fig. 3).

Antitumor effects of i.c. administration of hIL2-SeV/ΔMΔF. All the naive rats inoculated with 9L cells in the brain developed progressive tumors. We examined the therapeutic effect of i.c. administration of hIL2-SeV/ΔMΔF combined with s.c. vaccination by measuring the tumor volumes with serial Gd-enhanced MRI (Fig. 4A). The tumor volumes on day 21 in the rats treated with the i.c. administration of hIL2-SeV/ΔMΔF and the vaccination (86.5 ± 63.8 mm^3, $n = 10$) were significantly smaller than the following groups: untreated (286 ± 51.2 mm^3, $n = 10$, $P < 0.0001$); vaccination alone (197 ± 48.9 mm^3, $n = 10$, $P = 0.0005$); i.c. administration of lacZ-SeV/ΔMΔF combined with the vaccination (233 ± 73.2 mm^3, $n = 6$, $P = 0.0012$); and i.c. administration of hIL2-SeV/ΔMΔF alone (256 ± 53.2 mm^3, $n = 6$, $P = 0.0001$; Fig. 4B). When treated with the combination strategy, all the inoculated tumors became visible by MRI on day 21 and the established brain tumors were completely eliminated in 3 of 10 rats (Fig. 4A). There was no difference in the tumor volumes on day 21 between the three cured tumors (81.6 ± 74.9 mm^3) and the other seven tumors (88.6 ± 67.1 mm^3). Accordingly, the lifetime of the rats treated with i.c. administration of hIL2-SeV/ΔMΔF vector combined with the vaccination was significantly prolonged compared with the untreated control rats or the rats treated otherwise ($P < 0.05$, log-rank test; Fig. 4C). All the cured animals completely rejected

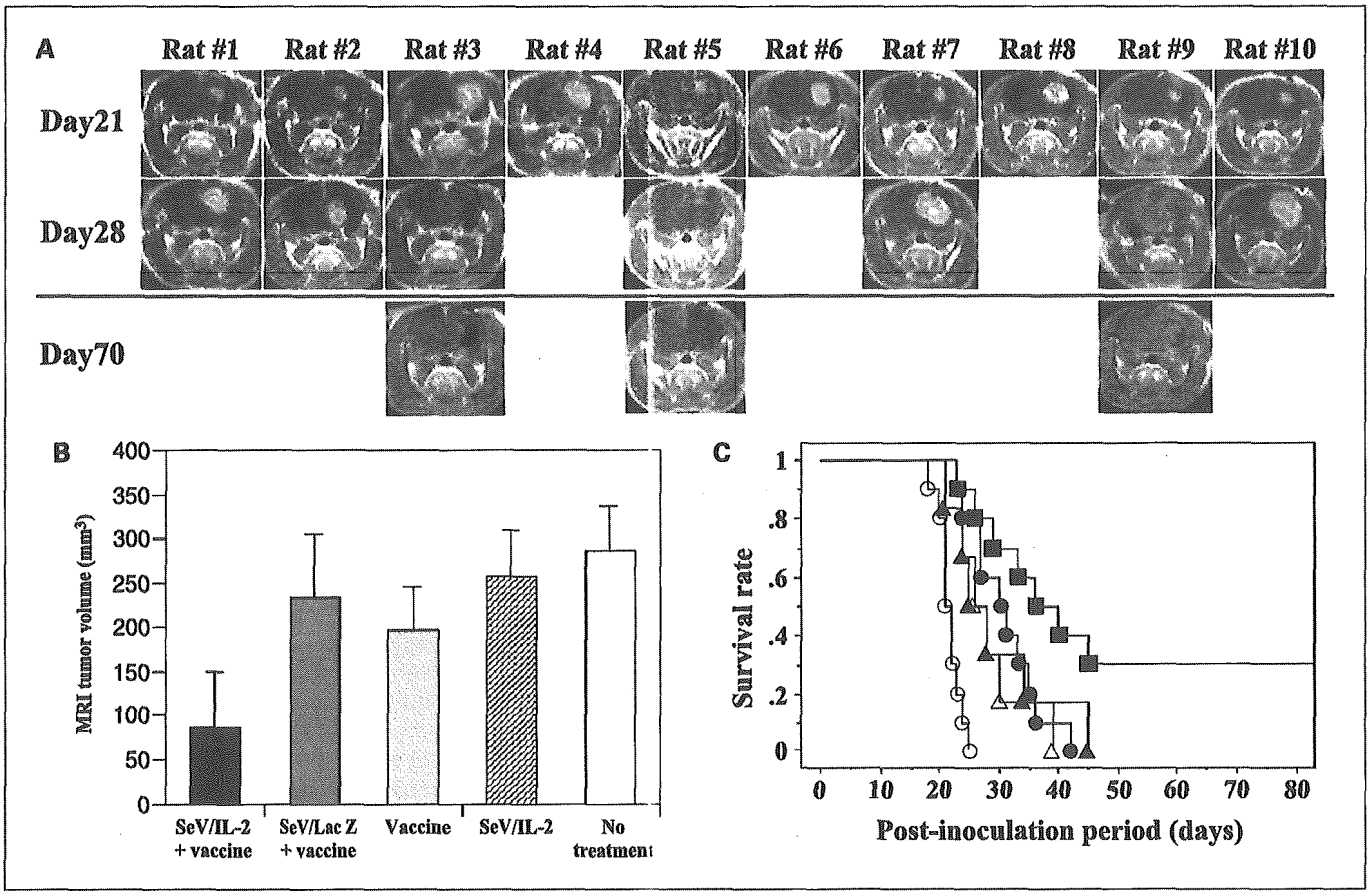

Fig. 4. Antitumor effects of i.c. administration of hIL2-SeV/ΔMΔF. *A*, Gd-DTPA – enhanced MRI images of all the 9L brain tumors treated with i.c. administration of hIL2-SeV/ΔMΔF and s.c. vaccination of irradiated wild-type 9L cells. All the animals were examined with MRI every 7 days, starting on day 7 after tumor inoculation. When treated with the combination strategy, all the i.c. tumors became visible at latest on day 21. The established brain tumors completely disappeared on day 28 in 3 of 10 rats examined (rats 3, 5, and 10). There was no difference in the tumor volumes on day 21 between the three eliminated tumors (81.6 ± 74.9 mm^3) and those of the seven other tumors (88.6 ± 67.1 mm^3). *B*, mean volumes of the 9L brain tumors measured by Gd-enhanced MRI on day 21. I.c. administration of hIL2-SeV/ΔMΔF combined with the vaccination (86.5 ± 63.8 mm^3, $n = 10$) resulted in significantly smaller volumes than the following groups: untreated (286 ± 51.2 mm^3, $n = 10$, $P < 0.0001$), vaccination alone (197 ± 48.9 mm3, $n = 10$, $P = 0.0005$), i.c. administration of lacZ-SeV/ΔMΔF combined with the vaccination (233 ± 73.2 mm^3, $n = 6$, $P = 0.0012$), and i.c. administration of hIL2-SeV/ΔMΔF alone (256 ± 53.2 mm^3, $n = 6$, $P = 0.0001$). Columns, mean; bars, SD. *C*, Kaplan-Meier survival analysis of the tumor-bearing rats. ○, untreated; ●, treated with the vaccination alone; ▲, treated with i.c. administration of lacZ-SeV/ΔMΔF and the vaccination; △, treated with i.c. administration of hIL2-SeV/ΔMΔF alone; ■, treated with i.c. administration of hIL2-SeV/ΔMΔF and the vaccination. Statistical analysis with log-rank test showed that the rats treated with i.c. administration of hIL2-SeV/ΔMΔF and the vaccination survived significantly longer than the other treatment groups ($P < 0.05$).

a second challenge of wild-type 9L inoculation in the brain and manifested no adverse effect during the 3-month follow-up period.

Induction of tumor-specific cytotoxic T cells. We used the standard ^{51}Cr release assay to evaluate the cytotoxic activity of spleen cells from the treated rats or those from naive rats against syngeneic 9L cells or allogeneic C6 cells. The cytotoxicity to 9L targets was strongly induced by the spleen cells from the rats treated with s.c. vaccination of the irradiated wild-type 9L cells (Fig. 5). In contrast, the cytotoxicity of the same effector cells to the C6 targets was not observed. The spleen cells from the tumor-bearing rats that were treated with i.c. administration of hIL2-SeV/ΔMΔF alone did not induce significant cytotoxicity against the 9L cells. These data suggest that the irradiated wild-type 9L cells were sufficiently immunogenic in the peripheral tissue and that the s.c. vaccination with these cells could induce 9L-specific cytotoxic T cells.

Immunohistochemical analysis. To ascertain the gene expression at protein level, we analyzed the immunoreactivity of IL-2 protein in the hIL2-SeV/ΔMΔF-injected tumors and confirmed that IL-2 protein was diffusely expressed in the treated tumors (Fig. 6A). We also immunohistochemically examined the presence of $CD4^+$ T cells and $CD8^+$ T cells in the tumors. Diffuse and dense infiltrations of $CD4^+$ T cells and $CD8^+$ T cells were observed in the tumors that were treated with i.c. administration of the hIL2-SeV/ΔMΔF and concurrent s.c. vaccination (Fig. 6B). In the tumors treated either with i.c. administration of the lacZ-SeV/ΔMΔF and the vaccination or i.c. administration of the hIL2-SeV/ΔMΔF alone, infiltrations of these cells were sparsely detected.

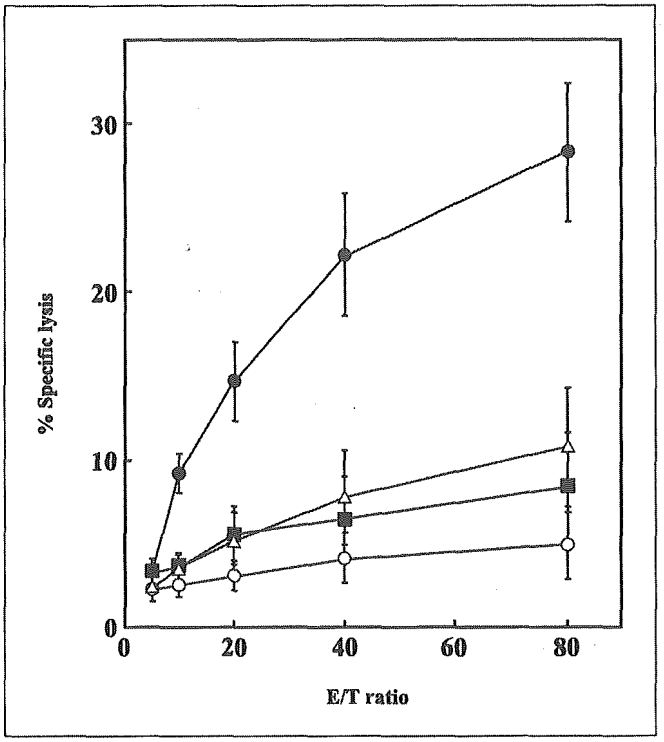

Fig. 5. Cytolytic activity by spleen cells from the treated rats was assessed with a standard ^{51}Cr-release assay against syngeneic 9L or allogeneic C6 targets. ○, spleen cells from naive rats against 9L targets; ●, spleen cells from the rats s.c. vaccinated with irradiated 9L against 9L targets; ■, spleen cells from the vaccinated rats against C6 targets. △, spleen cells from rats treated with i.c. administration of hIL2-SeV/ΔMΔF alone. The vaccination with irradiated 9L cells could induce 9L-specific cytotoxic T cells.

Discussion

We herein first showed that the nontransmissible recombinant SeV vector could transfer genes efficiently into the glioma cells *in vivo*, and this directly correlated with the therapeutic efficacy against the established brain tumors. Even complete elimination of the established brain tumors could be achieved in some cases by the gene therapy strategy using i.c. administration of SeV vector carrying *human IL-2* gene with s.c. vaccination. The SeV vector provided substantive expression of IL-2 protein in the glioma tissues, which would have reached a level necessary to induce significant proliferation and expansion of the peripherally activated tumor-specific T cells.

We have previously reported that transplantation of the IL-2-producing cells into glioma tissues could eliminate approximately half of the established brain tumors in animals immunized with an irradiated whole tumor cell vaccine (16). Although the amount of IL-2 produced by the cells transduced with hIL2-SeV/ΔMΔF was several times greater than that of the IL-2-producing cells utilized in the previous experiment, the cellular infiltration and the cure rate obtained with the hIL2-SeV/ΔMΔF treatment were comparable with those of the cell-mediated therapy. This result would partly be explained by the kinetics of the IL-2 expression in the animals treated with hIL2-SeV/ΔMΔF. The *in vivo* protein expression would have reached its peak 4 days or later after injection of the vector. However, because the tumor doubling time of human glioblastoma is presumed to be longer than the experimental 9L gliosarcoma model, the time lag to reach maximum IL-2 production is not considered to be a critical factor in clinical practice (21). In contrast, the main advantage of virus vector application would be the wide distribution of the transgene products achieved by a small amount of viral solution to be i.c. injected compared with the cell-mediated therapy that needs a large amount of therapeutic cells to cover the whole tumor areas in the human brain.

For the clinical application of this strategy, it would be important to verify the reasons for the difference in the therapeutic efficacy of hIL2-SeV/ΔMΔF among individual animals. This experimental brain tumor model using 9L cells has a quite stable property; the animals are 100% fatal between 18 and 25 days after inoculation and all the untreated tumors are visible by MRI on day 7 with a mean size of 8.0 ± 6.0 mm^3 (16, 17). When treated with hIL2-SeV/ΔMΔF and the vaccination, the observed tumor volumes on day 21 were not different between the eliminated tumors and the other progressing tumors. The pretreatment status of the tumors is not considered to affect the therapeutic outcome. Instead, the presumable differences in the vector injection site (i.e., the center or periphery of the tumor) would affect the efficacy because the stereotactic coordinates used for the tumor inoculation would not necessarily indicate the center of the established tumor. When used in clinical practice, we can precisely identify the center of the brain tumors by computed tomography-guided stereotactic apparatus or navigation system. Another important factor contributing to the difference in therapeutic efficacy may be the expression level of sialic acids.

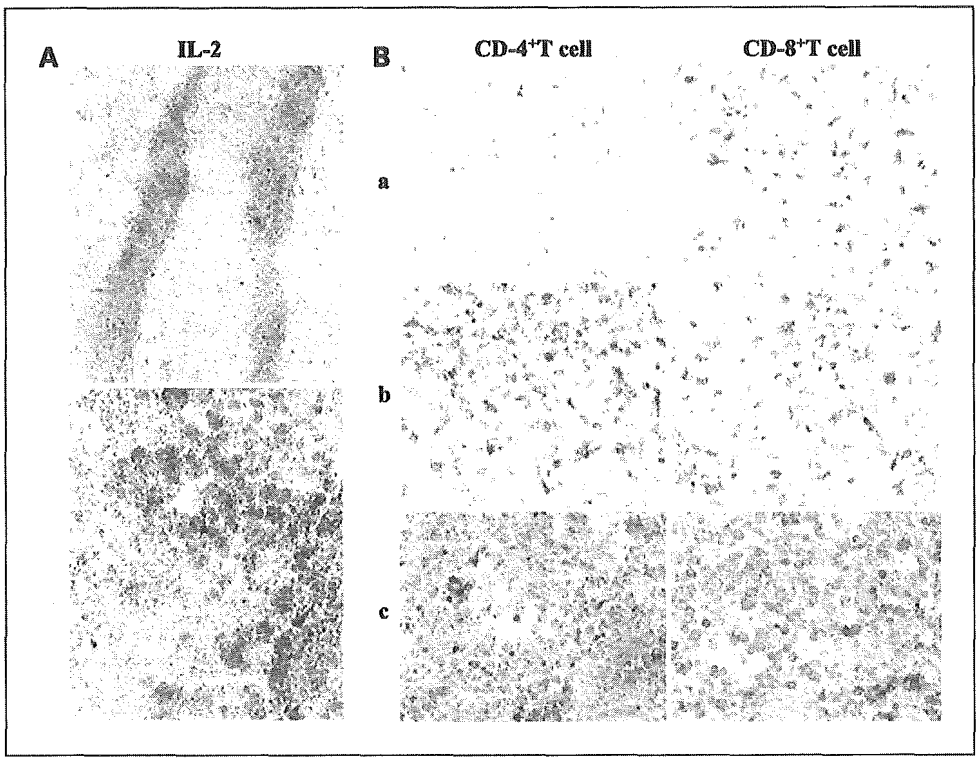

Fig. 6. *A*, immunohistochemical analysis of the expression of IL-2 in the 9L brain tumors treated with i.c. administration of hIL2-SeV/ΔMΔF. IL-2 protein is diffusely expressed. *B*, immunohistochemical analysis of the expression of CD4 and CD8 antigens in rats treated with i.c. administration of lacZ-SeV/ΔMΔF and s.c. vaccination of irradiated 9L cells (*a*), i.c. administration of hIL2-SeV/ΔMΔF alone (*b*), and i.c. administration of hIL2-SeV/ΔMΔF combined with the vaccination (*c*; ×200 magnification). Diffuse and dense infiltrations of $CD4^+$ T cells and $CD8^+$ T cells were observed in the tumors that were treated with i.c. administration of hIL2-SeV/ΔMΔF and the vaccination.

The receptor for SeV is sialic acid bound to gangliosides, which is present on most cell types including the human glia and glioma cells (22, 23). It may be altered by infiltration of the host cells in the xenograft models (24) and this probably affects the infection efficiency of hIL2-SeV/ΔMΔF and resultant efficacy.

Regarding the safety in clinical application, the biological features of SeV, such as the lack of integration into the cellular genome and the lack of homologous recombination between different SeV genomes, are desirable. Furthermore, we utilized both *M* and *F* genes-deleted SeV (SeV/MF) to enhance its safety. We previously constructed an *F* gene-deleted SeV (SeV/F; ref. 13) and an *M* gene-deleted SeV (SeV/M; ref. 14). *F* gene deletion made the SeV vector nontransmissible and *M* gene deletion worked well to make SeV incapable of forming particles from the infected cells. Although simultaneous deletions of these two genes in the same genome resulted in combining both advantages and contributed to increase the safety of the SeV vector, SeV/ΔMΔF still retains high levels of infectivity and gene expression *in vitro* and *in vivo* (i.e., similar to the wild-type SeV; ref. 15). These characteristics of SeV/ΔMΔF are considered suitable for the clinical application in gene therapy. In contrast, a theoretical obstacle for the clinical application could be the presence of antibodies against the human parainfluenza virus type I, which are known to cross-react with SeV HN proteins (4). However, it is currently not known whether a respiratory infection with human parainfluenza virus type I in the past will interfere with the transduction process of SeV vector at distant site, such as the central nervous system.

In conclusion, the present study showed that the nontransmissible recombinant SeV vector provided efficient transduction of the target genes into i.c. glioma cells. The i.c. administration of hIL2-SeV/ΔMΔF could induce a substantial production of IL-2 protein to induce the proliferation and expansion of peripherally activated, tumor-specific T cells. The therapeutic efficacy obtained by sufficient gene transfer with wide distribution and the high-grade safety of the nontransmissible recombinant SeV vector warrants clinical trials to evaluate its usefulness for human glioblastoma.

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Review Article

Interferon-β gene therapy for cancer: Basic research to clinical application

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Interferon-β gene therapy for cancer is the first such protocol developed in Japan. Here we describe the development process of our interferon-β gene therapy from basic research to clinical application. Interestingly, the biological and biochemical characteristics of interferon-β gene therapy through transfer of the interferon-β gene into tumor cells by means of cationic liposomes differed from those of conventional interferon-β protein therapy. Interferon-β gene transfer could induce apoptosis in interferon-β protein-resistant tumor cells, such as glioma, melanoma, and renal cell carcinoma. Induction of apoptosis was related to the level of intracellular mRNA of interferon-β, prolongation of the phosphorylation time of molecules in the interferon-β signal transduction pathway, such as JAK1, Trk2, and STAT1, and activation of DNase γ. In our preclinical study we developed lyophilized cationic liposomes containing interferon-β gene (gene drug) for clinical use and confirmed their safety. Thereafter, we performed a pilot clinical trial in patients with malignant glioma and confirmed the safety and effectiveness of this interferon-β gene therapy. In this review we also comment on the status of gene therapy regulation in Japan. Interferon-β gene therapy is expected to become widely available for clinical use in cancer patients, and this new strategy might be extended to molecular targeting therapy, or used in combination with cell therapy or other therapies. (Cancer Sci 2004; 95: 858–865)

Since Watson and Click discovered the double-stranded structure of DNA in 1953, recombinant DNA technology and molecular biology have developed rapidly, and the complete sequence of the human genome, consisting of 3 billion base pairs, has been identified through the human genome project (1990–2003). This has opened the way for a new generation of advanced technology-based medicine, including gene medicine (genetic diagnosis and gene therapy), regenerative medicine, robotic medicine, molecular medicine, and nanomedicine. In particular, gene therapy offers tremendous promise for the future treatment of cancer.

The first gene therapy was initiated on September 14, 1990 in the USA for a patient with severe combined immunodeficiency (adenosine deaminase [ADA] deficiency). Since then, there have been more than 600 trials worldwide, and more than 4000 patients have received some kind of gene therapy. Recently, the target diseases have been extended from congenital metabolic disorders to malignant tumors which cannot be cured by existing treatments, and even chronic benign diseases which result in a poor quality of life.

In Japan, gene therapy for ADA deficiency began in 1995 at Hokkaido University Hospital using the same protocol as in the USA. To date, twenty gene therapy protocols have been developed. Among them, fifteen are related to cancers. Targeted diseases include renal cell carcinoma, lung cancer (non-small cell carcinoma), esophageal cancer, breast cancer, prostate cancer, brain tumor (malignant glioma), leukemia, and colon cancer. In the Department of Neurosurgery and Molecular Neurosurgery, Nagoya University Graduate School of Medicine, we have started a gene therapy protocol of our own since April 2000, an interferon (IFN)-β gene therapy using cationic liposomes. This protocol is the first using made-in-Japan technology. Here we summarize the developmental process of our human IFN-β gene therapy for patients with malignant glioma.

1. Basic studies

Malignant gliomas are too invasive to cure with surgical resection alone. In general, patients with malignant glioma undergo adjuvant therapy, including radiation therapy, chemotherapy, and immunological therapy after surgical resection, but their prognosis remains poor. Gene therapy is a much-awaited new strategy to overcome the misery associated with this neoplasm. However, only a few gene therapies have been directed toward diseases of the central nervous system (CNS), because the CNS is a very complex and critical organ, and access to it is restricted by the blood-brain barrier (BBB). It is therefore important to develop an appropriate delivery system. We currently use a liposomal gene delivery system.

1-1 Liposomes as a gene delivery system

Liposomes are artificial lipid bilayer vesicles considered to be useful as a drug delivery system. They can be morphologically divided into three types; small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), and multilamellar vesicles (MLV) (Fig. 1A). In the 1980s, it was well established that liposomes bearing positive charges on their surface could provide more efficient delivery of their entrapped components into cells, compared to other types of liposomes.[1,2] Our research on cationic liposomes was initiated in 1988. We found, in collaboration with Yagi and his colleagues, that cationic liposomes consisting of *N*-(α-trimethylammonioacetyl)didodecyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidylethanolamine (DOPE) (1:2:2 or 1:2:3, molar ratio) provide high-efficiency DNA entrapment and a high potential for DNA transfer to human glioma cells.[3–8] Moreover, DNA/liposomes are one of the safest delivery modalities to the CNS. Shin *et al.* reported that delivery of genes via DNA/liposome complexes to the brain could be achieved by incorporating antibodies to the transferrin receptor in order to facilitate passage across the BBB.[9]

Recently, various modifications of liposomes, e.g., combination of adeno-associated virus (AAV) vectors, adenovirus vectors, and nanotechnology-based molecules (Fig. 1B),[10–12] have been investigated.

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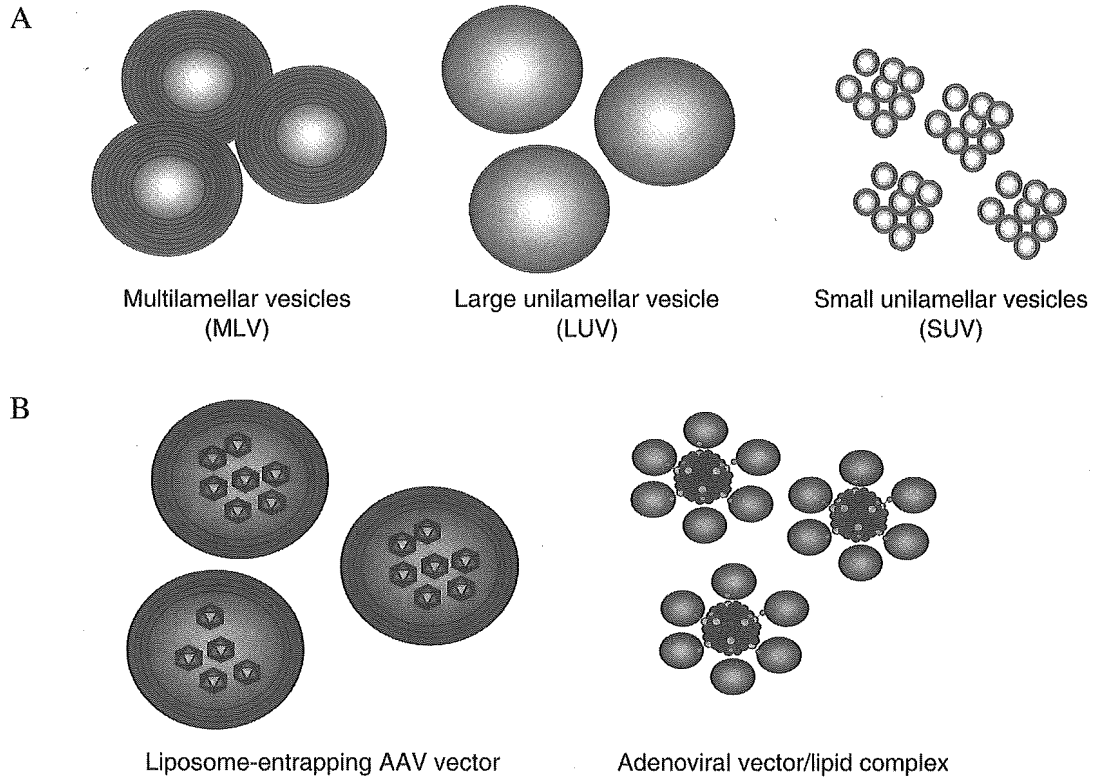

Fig. 1. Cationic liposomes and their application. A. Cationic liposomes can be morphologically divided into three types: small unilamellar vesicles (SUVs); large unilamellar vesicles (LUVs); and multilamellar vesicles (MLVs). SUVs bind the genes to their surfaces, producing DNA-lipid complexes. SUVs have already been applied to clinical cancer gene therapy, especially immuno-gene therapy. LUVs and MLVs generally entrap the genes within the liposomes, rather than on the surfaces. MLVs can retain transfection efficacy for more than 1 year. B. Cationic liposomes have high potential for a variety of modifications. For example, they have a higher potential to combine with other materials such as viral vectors, antibodies, and other therapeutic agents. In our university, we are striving to develop combined therapy with liposomes and adeno-associated virus (AAV) vectors or adenovirus vectors. AAV vector-associated liposomes have more than 10-fold greater transduction efficiency than liposomes containing plasmid DNA and more than 6-fold greater than AAV vector alone. In contrast, adenoviral vector/lipid complex reduces the antigenicity of the adenoviral vector *in vivo* without diminishing the antitumor activity.

1-2 IFN-β

Human IFN-β is thought to be an important factor in the growth of human glioma and melanoma 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 these tumors.[13, 14] In the 1980s, IFN-β protein was clinically used as an anticancer drug in Japan, and it showed a clear growth-inhibitory effect on malignant glioma and melanoma.[15, 16] However, tumor regression was observed in only 10–30% and 15–20% of the patients treated for glioma and melanoma, respectively, and survival prolongation was not attained in either.[17] IFN-β protein also deserves attention as a cell-cycle regulator, inducing aberrant cell-cycle progression, which occurs predominantly as S phase accumulation, and less frequently as other cell-cycle effects, such as G1 arrest, or the entry of tumor cells into a senescent-like state.[18]

1-3 Antitumor mechanisms of IFN-β gene transfer

We investigated the antitumor activity of IFN-β gene therapy in both *in vitro* and *in vivo* experiments. In the *in vivo* experiments, human glioma cells were implanted into the brain of nude mice. One week after implantation, the tumor cells formed a mass 2 mm in diameter. From this time point, we started injection of liposomes containing human IFN-β gene 6 times every other day. One month later, the tumor was eradicated completely, although repeated direct injections of human IFN-β protein (1000 IU) did not suppress the tumor growth at all. We therefore analyzed in detail the mechanisms of this surprising antitumor effect induced by IFN-β gene transfer. From our previous experiments, we speculated that the IFN-β gene has four main anti-tumor effects on glioma cells (Fig. 2). Interestingly, IFN-β gene transfer by means of cationic liposomes induces apoptosis of cultured human glioma cells that are resistant to IFN-β protein.

Fig. 3 summarizes the molecular mechanisms of apoptosis induced by IFN-β gene transfer via cationic liposomes. Susceptibility to extrinsically supplied IFN-β protein correlated closely with the amount of intracellular IFN-β mRNA in cultured human glioma cells, in agreement with the findings of Hanson *et al.* in melanoma cell lines.[19] It was also confirmed that there is a significant prolongation of phosphorylation time of several proteins involved in the intracellular signal transduction pathway of IFN-β, such as JAK1, Tyk2, and STAT-1. This apoptotic process did not involve caspase-3 or 8 activation and cleavage of DFF45/ICAD, but activation of caspase-7 and DNase γ was detected.[20] Besides the activation of DNase γ, the interaction of cell membranes and lipid membranes on cationic liposomes in IFN-β protein-resistant cells was also required (Fig. 3). Accordingly, cell death induced by the IFN-β gene delivered in cationic liposomes probably involves at least two elements, i.e., the intrinsic apoptotic pathway and mitotic catastrophe. The former is a pathway triggered by various extracellular and intracellular stresses, such as hypoxia and DNA damage. The stress signals converge mainly on mitochondria, forming the apoptosome which contains cytochrome *c*, apoptotic protease activating factor-1 (Apaf-1) and caspase 9, and

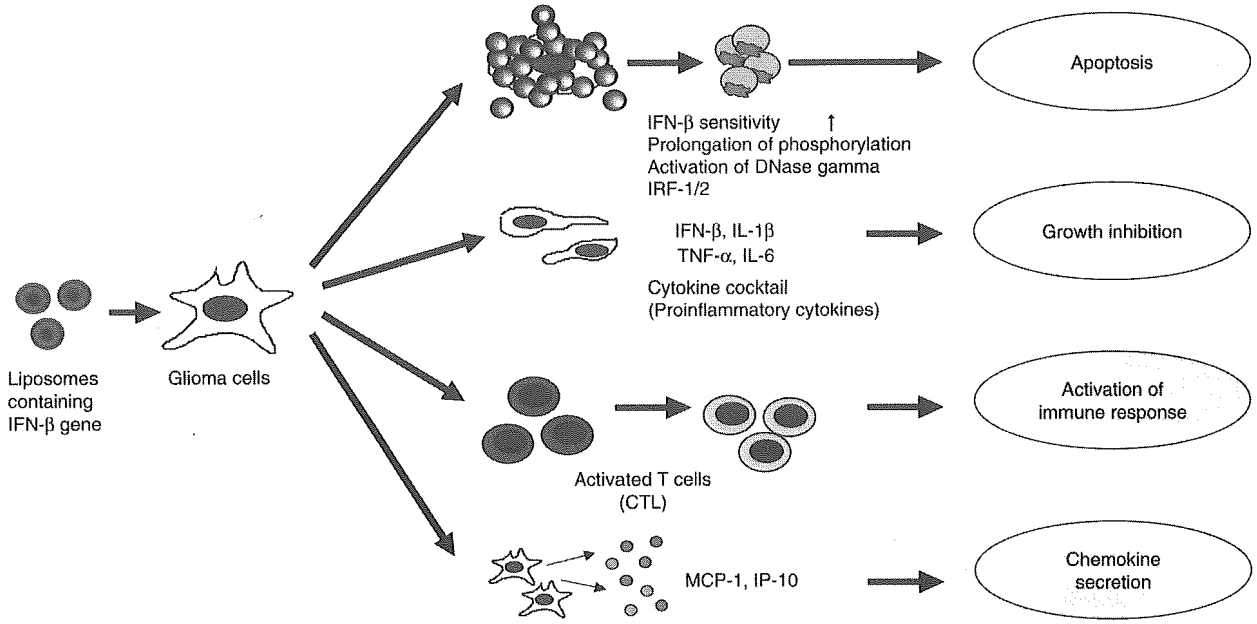

Fig. 2. Summary of antitumor mechanisms of IFN-β gene therapy for malignant glioma. There are at least four antitumor mechanisms of IFN-β gene therapy for malignant glioma; induction of apoptosis. IFN-β gene transfer by means of cationic liposomes can induce apoptosis in IFN-β protein-resistant cells; IFN-β gene transfer to glioma cells produces some cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in addition to IFN-β. The mixture of these cytokines exerted a strong antitumor effect on glioma cells; IFN-β gene transfer activates systemic immune responses and facilitates immune cell infiltration into a brain tumor, although the brain is an immunologically privileged site. Tumor-infiltrating cells are mainly CD8-positive cytotoxic T lymphocytes (CTLs) and macrophages; IFN-β gene transfer to glioma cells also produces some chemotactic factors such as monocyte chemotactic protein (MCP)-1 and IP-10.

leading to activation of caspase-7, followed by activation of DNase γ. The latter results in mitotic catastrophe, a pathway triggering mammalian cell death through aberrant mitosis. In fact, IFN-β gene transfer by means of cationic liposomes increased the number of multinucleated giant cells, i.e., the rate of abnormality of chromosome segregation, and subsequently induced apoptosis in cultured human glioma cells. Accordingly, we speculate that IFN-β gene transfer could be relevant to mitotic catastrophe in human glioma cells.

In addition to apoptosis, glioma cells transduced by the IFN-β gene produced interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, IFN-γ-inducible protein-10 (IP-10), and heat shock protein (HSP) in addition to IFN-β. The mixture of these cytokines exerted a strong antitumor effect on glioma cells. Duguay *et al.* reported similar results, namely that IFN regulatory factor (IRF)-3 gene transfer, which has been shown to activate type I IFN genes, can mediate important antitumor responses, increasing the inducibility of mRNAs for cytokines such as IFN-β, TNF-α, IL-6, MIP-1α, RANTES, and IP-10.[21] Moreover, IFN-β gene transfer activated systemic immune responses and facilitated immune cell infiltration of the brain tumor, despite the fact that the brain is an immunologically privileged site. Tumor infiltrating cells were mainly CD8-positive cytotoxic T lymphocytes (CTLs) and macrophages. According to Zhang *et al.*, IFN-β gene therapy for human prostate cancer stimulated expression of inducible nitric oxide synthase, down-regulated transforming growth factor (TGF)-β and IL-8, reduced microvessel density, and resulted in apoptosis of endothelial cells in the lesions. These data suggest that macrophages may play an important role in IFN-β gene therapy.[22] Additionally, we found that dendritic cells (DCs) or macrophages which had been injected into the brain moved to the cervical lymph node. This finding suggests that macrophages may transport antigen information to the cervical lymph node. We also found that subcutaneous injection into the neck of plasmacytoid DCs, which are CD11c(+) and B220(+), effectively promoted the infiltration of CTLs in a mouse experimental glioma, while subcutaneous injection into the neck of myeloid DCs, which are CD11c(+) and B220(−), only slightly promoted it. From these findings, we speculate that there may be an "immune circuit" for brain tumors, which can be activated by the IFN-β gene delivered in cationic liposomes, as shown in Fig. 4.

2. Pre-clinical studies

In pre-clinical studies, we investigated how to make cationic liposomes containing IFN-β gene for clinical use and also how to assess safety. We have conducted careful, repeated safety tests on the liposomal products (Table 1). We did not encounter any problems in animal studies using several species, including mice, rats, dogs, and monkeys. Animal studies revealed that cationic liposomes containing the IFN-β gene are most effective when administered by a regimen that will maintain a constant low concentration of IFN-β protein for a definite time in treated tumors, rather than a single bolus administration.

We have developed freeze-dried (lyophilized) cationic liposomes, which retain therapeutic activity for more than 1 year. The lyophilization technique has made it possible to transport this gene drug (cationic liposomes containing IFN-β gene) to other institutes collaborating in this work. In 2004, we sent our gene drug to Shinshu University Hospital to begin clinical research on IFN-β gene therapy for malignant melanoma.

3. Clinical studies

3-1 Regulation of gene therapy (Fig. 5)

The regulation of gene therapy is intended to ensure not only proper assessment of risks, but also a suitable safety margin. Currently, any clinical trial should follow Good Clinical Practice (GCP) in compliance with worldwide consolidated GCP guidelines.

In our hospital, a gene therapy committee was formed prior to our clinical study. As the first step, we selected candidates

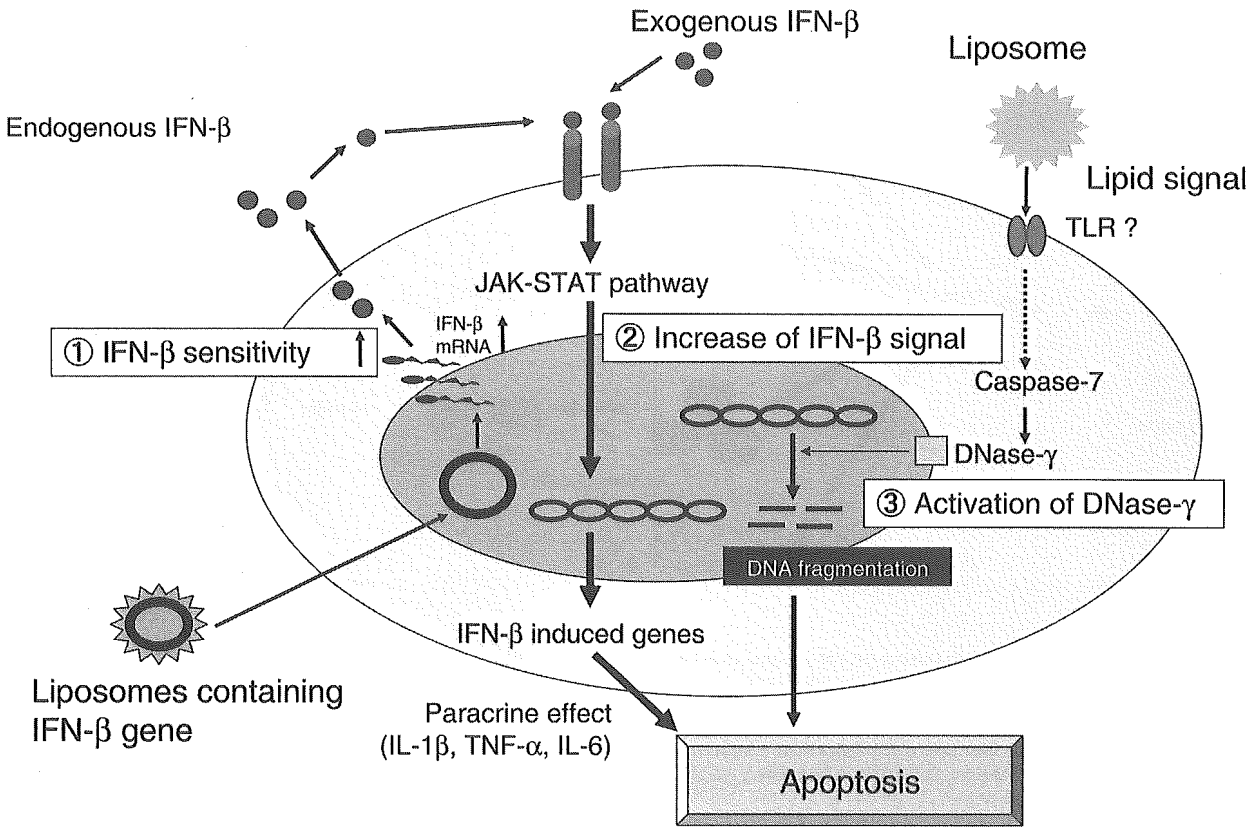

Fig. 3. Molecular mechanisms of apoptosis induced by IFN-β gene transfer by means of cationic liposomes. There are at least three molecular pathways of apoptosis induced by IFN-β gene transfer by means of cationic liposomes; the susceptibility to extrinsically supplied IFN-β protein correlates closely with the amount of intracellular IFN-β mRNA in cultured human glioma cells; IFN-β gene transfer causes a significant prolongation of phosphorylation time of several proteins involved in the intracellular signal transduction pathway of IFN-β, such as JAK1, Tyk2, and STAT-1; IFN-β gene transfer activates DNase γ through the interaction of cell membrane and lipid membrane on cationic liposomes even in IFN-β protein-resistant cells.

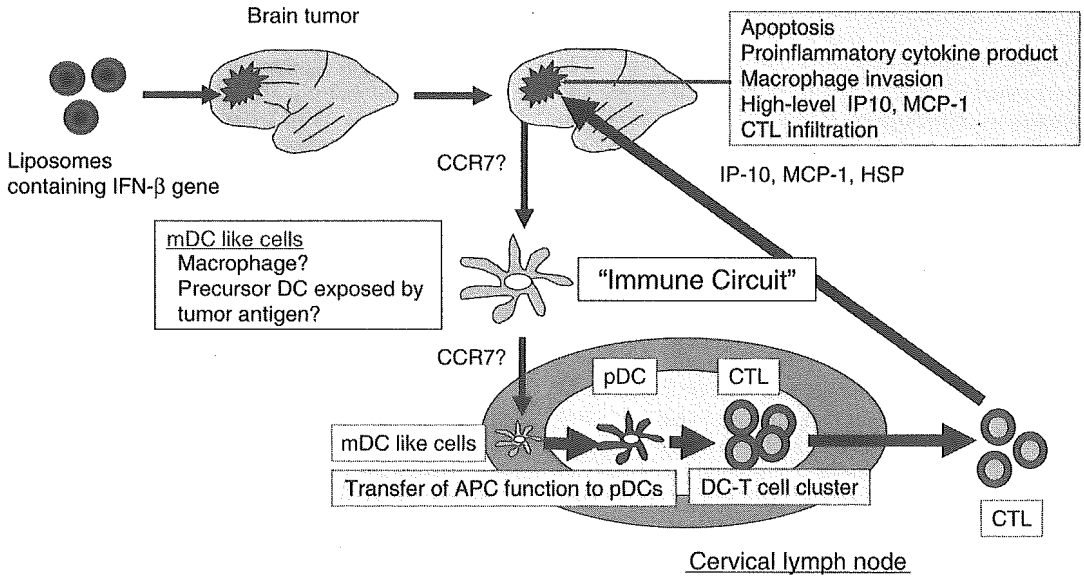

Fig. 4. Hypothetical pathway of cytotoxic T lymphocyte (CTL) activation in the brain induced by IFN-β gene therapy. We speculate that macrophages or plasmacytoid DCs (pDCs) probably transport antigen information to cervical lymph nodes, forming DC-T cell clusters, and then producing activated CTLs. The CTLs are guided to the brain tumor by IP-10, MCP-1, and HSP, then attack the tumor cells. mDC, myeloid dendritic cells; CCR, C-C chemokine receptor; APC, antigen-presenting cells; IP-10, IFN-γ-inducible protein-10; MCP-1, monocyte chemotactic protein-1; HSP, heat shock protein.

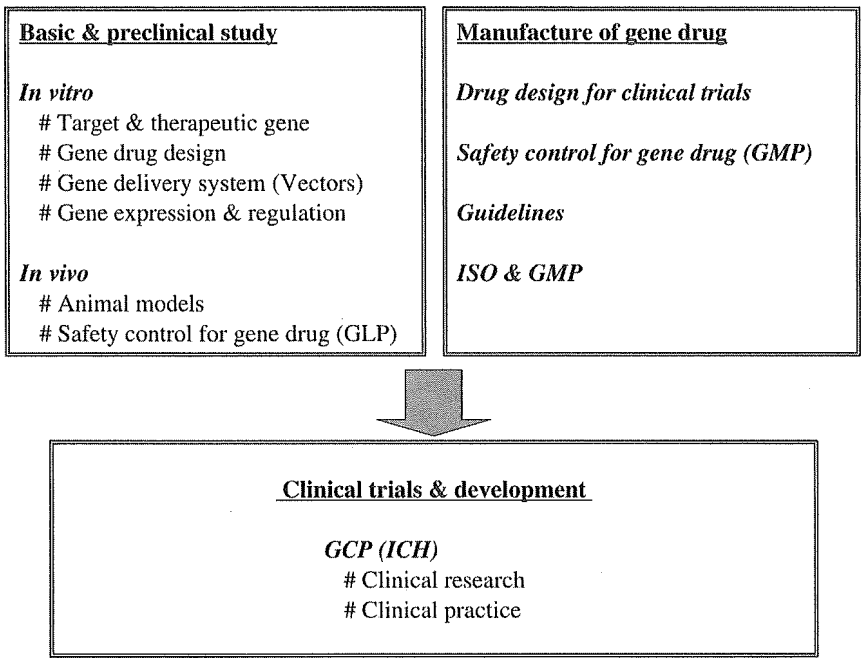

Fig. 5. Regulation and implementation of gene therapy. GLP, good laboratory practice; GMP, good manufacturing practice; ISO, international organization for standardization; ICH, international conference on harmonization.

Table 1. Safety control for gene drug (liposomes containing human interferon-β gene)

1. Single injection toxicity test (Intracranial and intravenous injection in rats and monkeys)
2. Repeated injection toxicity test (30-day repeat injection toxicity test for rats and monkeys)
3. Reproductive toxicity test
4. Deformity test
5. Antibody measurement
6. Toxicokinetics
7. Test for fever-producing activity in rabbits

No problems were detected in animal studies in several species including mice, rats, dogs, and monkeys.

who met the criteria for our gene therapy. Next, a subcommittee composed of several medical doctors, called the subcommittee for judging safety, efficacy, and indications, assessed the candidates using clinical data, and decided whether each patient would be a suitable candidate for our gene therapy. After a positive decision, a human gene therapy advisory board, composed of doctors, nurses, ethical specialists, legal professionals, and an outsider carefully reviewed the selection process again, to finally confirm the suitability of the candidate.

The manufacture and distribution of the gene drug were also of critical importance. Especially in the preparation of a gene drug, strict adherence to good manufacturing practice (GMP) is mandatory. In accordance with the regulations, our clinical-grade gene drug was produced in the Human Gene Therapy Vector Producing Facility at Nagoya University Hospital, where a fully documented quality management system is implemented. This system is similar to the management system of the International Organization for Standardization (ISO). In the near future, the development of advanced medicines will require ISO and GMP approval in Japan.

3-2 Clinical protocol

The patient received open surgery for tumor resection, followed by stereotactic injection of the gene drug. Treatment consists of reoperation and injection of liposomes containing human IFN-β gene on days 0, 14, 17, 21, 24, and 28 (first case) or 0, 14, 21, and 28 (other cases). The surgical margin of the cavity after tumor removal was infiltrated with 1 ml of the gene drug at a concentration of 30 μg DNA/ml, evenly distributed at multiple sites. From injections 2 to 6, the procedure was repeated stereotactically under local anesthesia. After the 28th day of treatment, patients entered a follow-up period and were evaluated 3 months after the first injection, then every 3 months through the third year, and then annually until the study was terminated at the patients' death.

The clinical end points were evaluatation of the safety of this gene drug and determination of the efficacy of this gene therapy.

3-3 Case reports

So far we have performed the therapy on five patients. A brief summary is shown in Table 2. The tumor of patient 1 had shown rapid progression before gene therapy, with the volume increasing about 13-fold in only 4 weeks (3.0 ml to 38.7 ml). After gene therapy, tumor growth ceased, as measured by outlining the enhanced area in MRI, and there was little change in size for the following 10 weeks. Growth then resumed, and the patient died approximately 3 months later. Patients 2 and 3 each had a partial response (PR). Patient 4 could not be evaluated because no viable tumor cells could be confirmed to exist in the enhanced mass, after γ-knife therapy. Patient 5 had stable disease during the 10 weeks following the first injection. The general and neurologic condition of all patients was unchanged or improved 3 months after starting therapy, except for patient 5. Before the therapy, patients had various neurological deficits such as hemiparesis (patients 1 and 5), aphasia (patients 1 and 5), and memory disturbance (patients 2 and 4). In particular, patient 1 could not walk or speak before the therapy because of severe right hemiparesis and motor aphasia. However, at her discharge from the hospital 3 months later, she could walk by herself and talk with her family. Patients 2 and 3 had long survivals of 29 and 26 months after tumor recurrence, respectively.[23]

Transduced gene product in the fluid collected was assessed by EIA. Samples obtained from all patients before injection contained no detectable IFN-β protein. After injection, IFN-β protein was detected by EIA in fluid from the tumor bed in

cases 1, 2, and 5. The highest concentration, 23 IU/ml, was seen in patient 1 10 days after the first injection. IL-1β was detected in patients 1 and 5, and TNF-α was detected in patients 1, 2, and 5. Each protein was detected a few days after injection, reached maximum concentration 10 days later, then decreased gradually. IFN-β mRNA was also detected in tumor-rich tissues (patients 1, 2, 3, and 5) obtained by microdissection and examined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). In contrast, mRNA for TNF-α was detected in tissues containing many macrophages (patients 1 and 2), but not in tumor-rich tissues. In addition, we confirmed the dramatic induction of immune response in the treated tumor tissues. After therapy, tumor tissues showed dramatic changes in all patients. Many tumor cells showed shrinkage or picnosis of the nuclei, reflecting apoptosis or necrosis. Simultaneously, MIB-1-positive cells were notably decreased. These alterations were observed over an area a few centimeters in diameter. Immunohistochemistry identified many CD8(+) lymphocytes and macrophages infiltrating into the tumor and surrounding brain, while few CD4(+) lymphocytes or B lymphocytes were present.[23] These findings were the same as reported by Brown, i.e., the antitumor response mediated by IFN-β gene delivery relied on CD8(+) T cells, but was completely independent of CD4(+) T cells.[24] Notable cell infiltration was detected at 2 weeks after injection in all patients; infiltrates then gradually increased, persisting for at least 1 month after the first injection.

Based on these results, we compared the antitumor mechanisms observed in the basic and clinical studies. We confirmed that these were almost the same in all patients treated with IFN-β gene therapy, except patient 4 (Fig. 6).

4. Expansion of clinical indications to other malignancies

4-1 Melanoma

The incidence of malignant melanoma has been increasing by 5% per year for the last 40 years in Caucasian and other populations. Patients with this neoplasm have a poor prognosis. Unfortunately, there is no effective treatment when melanoma

Table 2. Clinical results of IFN-β gene therapy for recurrent malignant gliomas

Case	Gene therapy (pDRSV-IFN-β)	Results			
		Tumor size (MRI) (3 months after Tx)	Histology	TTP (month)	D (month)
1	30 μg×1 15 μg×5	Stable/progression	Tumor cell death Immune response	3	D (6)
2	30 μg×4	Partial response	Tumor cell death Immune response	16	D (29)
3	30 μg×1	Partial response	Tumor cell death Immune response	15	D (26)
4	30 μg×2	Stable	Tumor cell death Immune response	6	D (13)
5	30 μg×4	Stable	Tumor cell death Immune response	5	D (11)

TTP, time to pregression; D, dead; Tx, treatment.

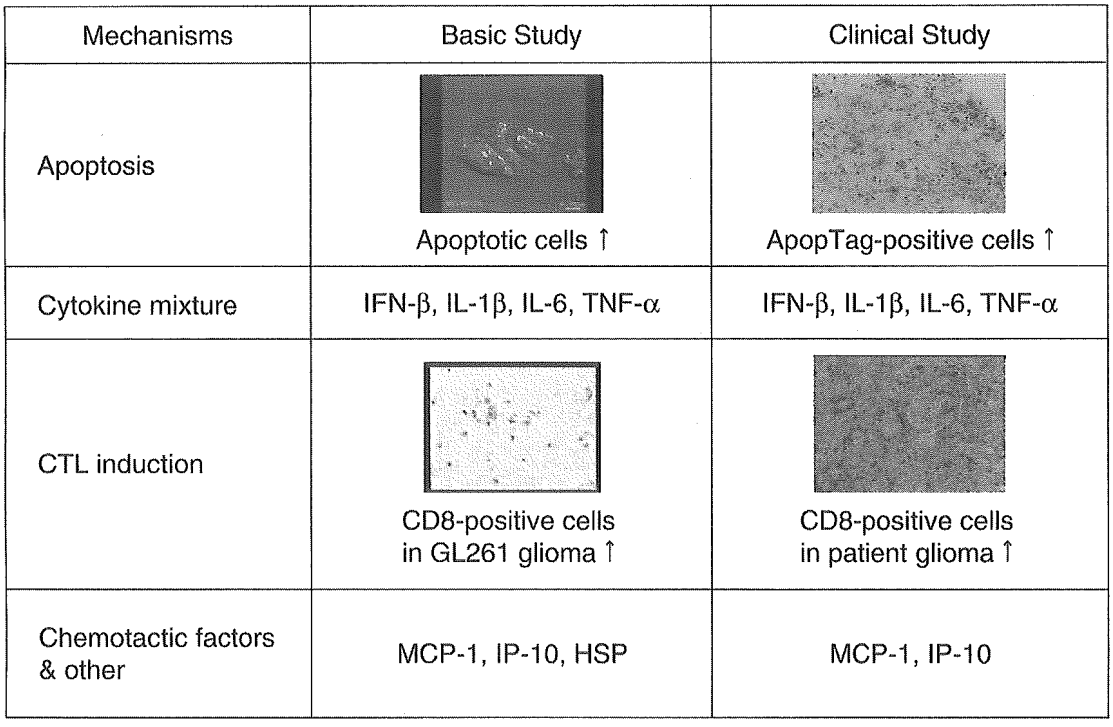

Fig. 6. Comparison of antitumor mechanisms of IFN-β gene therapy found in basic and clinical studies. Basic studies on the antitumor mechanisms of IFN-β gene therapy were consistent with clinical findings. HSP, heat shock protein.

is recurrent and/or at an advanced stage. Gene therapy has therefore received particular attention as a promising treatment modality for melanoma. Many investigators have performed new strategic gene therapies including IL-2, human leucocyte antigen (HLA)-B7β2m, granulocyte-macrophage colony-stimulating factor (GM-CSF), gp100, and MART-1. Recently, cationic liposomes have been used as a safer alternative to virus vectors in experimental and/or clinical trials on melanoma.[25, 26] We also confirmed that melanoma is susceptible to IFN-β protein, like glioma, and we assessed the growth-inhibitory effect of IFN-β gene transferred in cationic liposomes in *in vitro* and *in vivo* experiments. As expected, cationic liposome-mediated IFN-β gene therapy was effective against melanoma, inducing direct cell death and stimulating the host immune system.[27, 28] Thus, in experiments using an experimental human melanoma implanted subcutaneously in nude mice, we found extensive apoptotic tissue and a significant decrease of Ki67-positive cells after IFN-β gene transfer.[27] In experiments using subcutaneous mouse melanoma in immunocompetent mice, liposomes containing the murine IFN-β gene, but not recombinant murine IFN-β, induced dramatic apoptosis, including nuclear condensation, shrinkage of cells, bleb formation, and ballooning. Immunocytochemical analysis demonstrated that a larger number of natural killer cells infiltrated the tumor following the gene treatment as compared with the controls. *In vivo* depletion of NK cells using anti-asialoGM1 antibody reduced the efficacy of liposomes containing the murine IFN-β gene treatment. Taken together, our data demonstrated that cationic liposome-mediated IFN-β gene therapy could be effective against melanoma by inducing direct cell death and by stimulating NK cells.[28] In this experiment, we initially expected that IFN-β gene transfer would activate CTLs in melanoma as well as glioma. However, activated immune cells were not CTLs, but NK cells. Thereafter, it became clear that major histocompatibility complex (MHC) class I expression was small in the murine melanoma model (B16F1) used in this experiment. Moreover, we found that combined therapy of cationic liposome-mediated murine IFN-β gene therapy and DCs effectively induced CTLs because a co-culture of murine melanoma (B16F1), DCs, and naïve T lymphocytes produced large amounts of IFN-β and IL-12, and IFN-β increased MHC class I expression on the surface of the melanoma cells.

In any case, liposomes containing the murine IFN-β gene played an important role in activating immune responses. We found that DCs pulsed with tumor extract-cationic liposomes complex increased the induction of CTLs in mouse brain tumor.[29]

4-2 Renal cell carcinoma

We examined the feasibility of IFN-β gene therapy for renal cell carcinoma and confirmed that it was effective. Cationic liposomes containing IFN-β gene significantly induced apoptosis, although recombinant human IFN-β protein failed to do so, suggesting clinical applicability of gene therapy for renal cell carcinoma.[30]

5. Future directions

The success of gene therapy for cancer depends on a combination of applied bioengineering and so-called translational research. In the development of previous gene therapies for cancer, the biological principles were sound, but it proved difficult or impossible to translate these principles into reality. Although suitable systems with potential for cancer gene therapy have been known for a long time, efforts have generally remained at the preclinical stage, especially in Japan. In other countries, herpes simplex virus-thymidine kinase (HSV-tk)-based suicide gene therapy has just completed phase III trials. Therefore, our home-produced technology for IFN-β gene therapy is an important step forward.

Although the search for new vectors (viral and non-viral) continues, cationic liposomes are among the most fascinating vectors for cancer gene therapy because they are non-infective, have low immunogenicity, low toxicity, and high stability, and are not expensive to manufacture. The cost-benefit relationship is important, especially in the development of advanced medicines. Moreover, this protocol needs to be combined with other advanced medicines. Indeed, lessons learnt in developmental studies of cancer gene therapy may contribute to the development of other advanced medicines, such as molecular targeting therapy, regenerative medicine, cell therapy, and organ transplantation. The understanding of antitumor mechanisms in cancer gene therapy helps us to identify candidate target molecules for molecular targeting therapy and also leads to new approaches, such as the combination of gene therapy and chemotherapy or antibody therapy. The former approach is exemplified by the dramatic results with STI-571 (Gleevec) in chronic myelogenous leukemia, and the latter by the use of anti-vascular endothelial growth factor (Avastin) and anti-epidermal growth factor (Cituximab) monoclonal antibodies in the management of advanced colorectal cancer. An improved understanding of antitumor mechanisms in cancer gene therapy will have many spin-offs.

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Practice of Interferon Therapy
—Brain tumor—

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Interferon Therapy for Hairy Cell Leukemia

HCL is characterized by the development of cells with many hairy cytoplasmic projections. Since it becomes chronic, it is classified as a chronic leukemia. Typical hairy cells in Western countries are positive for tartrate-resistant acid phosphatase staining. There is a subtype called "Japanese type HCL", and its hairy cells show only weak tartrate-resistant acid phosphatase staining. Both types of HCL often cause splenomegaly.

HCL is treated with IFN therapy, splenectomy, and purine analogues. Recently, the efficacy of anti-CD20 antibody, rituximab, has been reported. The standard therapy for HCL should be started with splenectomy and, if the disease progresses, treatment with IFN or purine analogues should be considered.

The IFN therapy for HCL is based on the administration of 3 to 5 million units/day of IFN-α consecutively 3 times a week. The response rate of this therapy ranges from 50 to 90%.[6] Treatment with IFN improves blood cell abnormalities and reduces splenomegaly. Smaller doses of 0.2 to 0.6 million units of IFN will reduce the adverse effects, but will also reduce the efficacy rate. It is generally considered that the efficacy of IFN therapy for Japanese type HCL is lower than that for the European/American type.

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