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Infectious Retrovirus Is Inactivated by Serum but Not by Cerebrospinal Fluid or Fluid from Tumor Bed in Patients with Malignant Glioma

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Intravenous gene transfer using recombinant retroviruses tends to suffer from a low infectious viral titer when conducted *in vivo*. This is, in part, caused by complement-mediated proteolytic inactivation of the retrovirus in human serum. However, if the retroviruses were directly injected into the brain, they might not be inactivated. Supernatant from amphotropic retrovirus-producing cells harboring the BAG vectors was incubated with sera or cerebrospinal fluid (CSF) of patients with gliomas or unrelated disorders. The retroviruses were severely inactivated in sera. However, no such inactivation was noted in CSF or fluid from the tumor bed of glioma patients. These data suggest that gene transfer using recombinant retroviruses could be done into the cavity after removal of the tumor in glioma patients.

Key words: Retrovirus-mediated gene therapy — Malignant glioma — Gene transfer

Recombinant retroviruses are one of the most useful vehicles for introducing foreign genes into dividing cells because of the broad host range and the stable inheritance associated with chromosomal integration.[1)] Clinical trials of retroviral-mediated gene therapy have begun in the United States, not only for genetic disorders, but also for malignant diseases.[2–4)] Brain tumors, particularly gliomas, are one of the best candidates among solid malignant tumors because the surrounding differentiated mature central nervous cells are non-proliferative, so suicide genes such as the herpes simplex virus type 1 thymidine kinase (HTK) gene are selectively transferred into malignant glioma cells.[5–7)] To date, *ex vivo* gene therapy for genetic diseases has been the favored approach since disease manifestations disappear if a portion of the targeted cells expresses the wild-type gene. *In vivo* gene transfer, however, should be favorable therapy for malignant diseases because all the malignant cells must be specifically killed. In order to accomplish this type of therapy with recombinant retroviruses, efficient retrovirus-producing cells which can produce a high titer of retrovirus particles are required.[8)] Intravenous gene transfer has an inherently low infectious viral titer and systemic administration increases safety concerns.[9,10)] Largely because of these limitations, gene therapy for brain tumors as proposed by Culver *et al.* uses the direct inoculation of mouse PA-317 cells, which produce recombinant retroviruses expressing the HTK gene, into the brain, followed by intravenous ganciclovir (GCV) administration.[4)] Though clinical results of this application have not yet been evaluated, it is possible that several adverse effects related to host immune response to mouse fibroblasts may occur, even though the brain is a relatively immunologically isolated area.

Thus we propose a clinical application of retroviral-mediated gene therapy for malignant glioma as follows. An Ommaya tube is retained in the tumor bed following surgical resection for operatively accessible tumors, or alternatively, the tube is inserted under stereotactic guidance for operatively inaccessible tumors. Purified retrovirus particles are injected directly from an Ommaya reservoir followed by intravenous GCV injection. Retroviruses are known to be inactivated by human serum, especially by complement, and therefore potential inactivation by the fluid in the tumor residual space or by cerebrospinal fluid (CSF) is a concern in relation to direct retrovirus injection into the brain.

In this study, supernatant from PA-317 amphotropic retrovirus-producing cells transfected with the BAG retrovirus[11)] vector was incubated with serum or fluid from the tumor bed of three glioma patients (two grade 2 astrocytomas and one glioblastoma) and with similar samples from three patients with unrelated disorders

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Table I. Summary of the Patients Whose Serum, CSF or Fluid from Tumor Bed Was Used for Retroviral Titer Assays

Disorder	Diagnosis	Age/Sex	Therapy	Viral assay		Total protein (mg/ml)	CH-50 (U/ml)	S-CH50 (U/ml)
Glioma	Astrocytoma grade 2	42/F	Biopsy, Radiation IFN-β (Ommaya tube)	Fluid from tumor bed	watery clear	2300	<12.0	32.5
	Astrocytoma grade 2 reccurence	55/M	Lobectomy, Radiation IFN-β (Ommaya tube)	Fluid from tumor bed	green viscous bile-like fluid	3681	<12.0	31.6
	Glioblastoma Meningeal dissemination	44/M	Biopsy, Radiation Partial removal	CSF (op)	xanthochromic	58	N.D.	N.D.
Others	Trigeminal neuralgia	54/F	MVD	CSF (op)	watery clear	132.9	<12.0	29.2
	Trigeminal neuralgia	55/F	MVD	CSF (op)	containing blood	194.4	10.2	30.7
	Acoustic neurinoma	61/M	Total removal	CSF (op)	containing blood	N.D.	N.D.	N.D.

In two patients with astrocytoma, fluids were removed through the Ommaya tube at the outer clinic before injection of IFN-β. Both fluids contained a high concentration of protein. CSF was obtained from the glioblastoma patient intraoperatively from the basal cistern; it contained tumor cells (165/3), but the protein level was within the normal range. From three non-glioma patients, CSF was obtained from the infratentorial cistern intraoperatively and used for retroviral titer assays. CSF, cerebral spinal fluid; op, intraoperative specimen; MVD, microvascular decompression; CH50, complement level of CSF or fluid from tumor bed (SRL, Tokyo); S-CH50, serum level of complement; N.D., not done.

(two trigeminal neuralgia and one acoustic neurinoma). Profiles of the patients are summarized in Table I. A 42-year-old female (case 1) was diagnosed with a grade 2 astrocytoma 12 months previously by open biopsy. An Ommaya tube was retained in the tumor bed and interferon-β was injected through the tube; no recurrence has been observed so far. Watery clear fluid removed from the Ommaya reservoir contained 2300 mg/dl of protein and 15 mg/ml of glucose. The recovered fluid and the patient's serum were used in the retrovirus titer assay. Similar specimens were also obtained from a 55-year-old male (case 2; astrocytoma grade 2). The fluid obtained through the Ommaya tube was a green viscous bile-like fluid containing 3681.3 mg/dl protein. Complement levels (CH50) of the fluid from tumor bed of these two patients were under 12.0 U/ml (normal complement level in serum is between 30 and 40 U/ml: SRL, Tokyo). Fluid was also removed from a 44-year-old man (case 3; glioblastoma with meningeal dissemination) at surgery for recurrent tumor. This fluid was xanthochromic and contained tumor cells, but contained a normal protein concentration. CSF and serum were also obtained intraoperatively from patients with other disorders. All specimens contained a small amount of blood, a slightly high protein concentration, and a normal glucose concentration.

NIH-3T3 fibroblasts (2×10^4/2 cm^2 well) were seeded the day before titer assays. Supernatant from PA-317 cells producing BAG retroviruses (PA-317/BAG) (1×10^6 colony forming units /ml) was incubated with the same volume of CSF, fluid from the tumor bed, or serum at 37°C for 0, 1, 2, 3, 4, 6 and 18 h. Each fluid type (100 μl) was added to the culture media of NIH-3T3 fibroblasts with 8 μg/ml polybrene at 37°C for 5 h. Fifty μl of PA-317/BAG supernatant was also added to a control specimen. Following incubation, the culture medium was replaced with fresh medium and incubation was continued for an additional 48 h at 37°C. Finally, cells infected with retroviruses were fixed in 5% glutaraldehyde and stained with X-gal as previously described.[12] The viral titer was estimated by scoring the number of blue-stained cells. The relative viral titer compared to that after 0 h of incubation was calculated (Fig. 1).

The infectious retroviral titer spontaneously declined to 50% of the initial titer after a 6 h incubation in the absence of body fluids. Viral titers were reduced by serum to 40%, 20%, 15% and 0% after 1, 2, 3, and 6 h incubation, respectively. On the other hand, the viral titer after incubation with fluid from the tumor bed, which generally contained a higher protein concentration than did controls, was always higher than that in the supernatant exposed to serum. Fluid from the tumor bed caused reduction to 85%, 75%, 60%, 50% and 0% after 1, 2, 3, 6 and 18 h incubation, respectively, which was essentially the same as the spontaneous reduction in the retrovirus titer (Fig. 1A). In specimens from control patients, retroviral inactivation was very similar to that observed in glioma patients, although the titer was reduced to 25% after a 6 h incubation compared to the spontaneous reduction to 35% (Fig. 1B). In both groups, retroviral inactivation by serum was much greater than that by tumor bed fluid or CSF. Complement levels in the

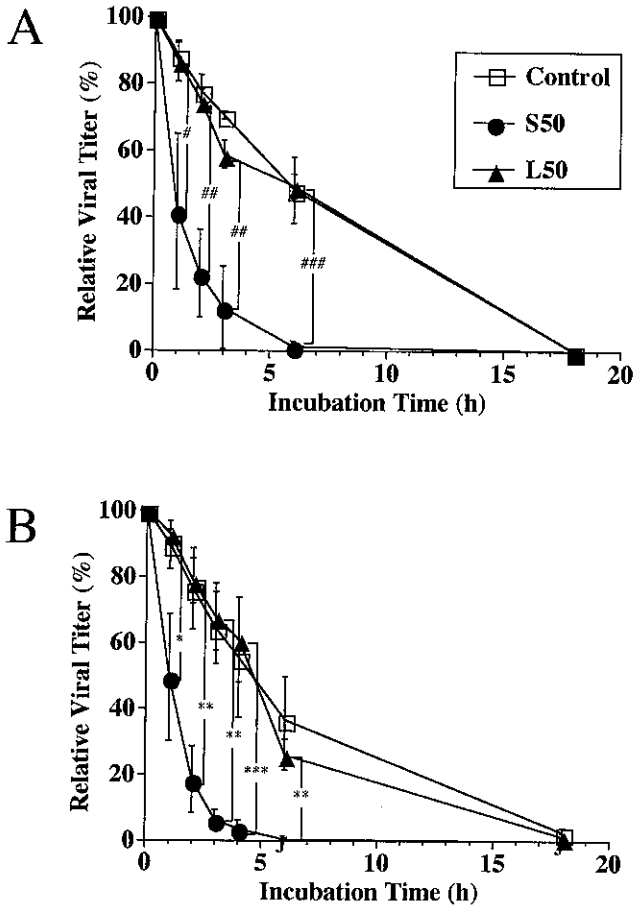

Fig. 1. NIH-3T3 fibroblasts were seeded onto 2 cm^2 culture dishes (Iwaki Glass 3820-024) at the concentration of 1×10^4 cells/cm^2 on the day before the retrovirus titer assay. Supernatant from PA-317 cells producing BAG retroviruses (1×10^6 colony forming units/ml) was collected and passed through a 0.44 μm filter, and the filtrate was used as the BAG retrovirus source. One hundred microliters of BAG filtrate was incubated with the same amount of CSF, tumor bed fluid, or serum from patients with glioma (A) or unrelated diseases (B) at 37°C for 0, 1, 2, 3, 4, 6 and 18 h. Fifty microliters of fluid from each time point was added to the culture medium of NIH-3T3 fibroblasts with 8 μg/ml polybrene at 37°C for 5 h. Fifty microliters of PA-317/BAG supernatant which had been preincubated at 37°C for 0, 1, 2, 3, 4, 6 and 18 h was added as described above as a control to observe spontaneous inactivation of retroviruses. Following incubation, the culture medium was replaced with fresh virus-free medium and incubated for an additional 48 h at 37°C. Finally, retrovirus-infected cells were fixed in 5% glutaraldehyde and stained with X-gal solution as described previously.[12] Viral titer was estimated from the number of blue-stained cells. The viral titer relative to the initial culture supernatant was calculated. Each assay was performed twice per patient specimen, and the average percentage was plotted. Open squares indicate the percentage of initial retrovirus titer remaining after incubation without CSF or serum, i.e., the spontaneous inactivation rate of the retrovirus. Solid circles indicate the serum inactivation rate of BAG retroviruses which were incubated with the same amount of a patient's serum. Solid triangles indicate the rate of retrovirus inactivation by CSF or tumor bed fluid. Vertical bars indicate the average of standard deviation. #, $P=0.0714$; ##, $P<0.01$; ###, $P<0.0001$; *, $P<0.02$; **, $P<0.001$; ***, $P<0.005$.

fluid from tumor bed of glioma patients (cases 1 and 2) were less than 12 U/ml, and no inactivation of retrovirus by the fluid was observed, in spite of the higher protein level of CSF in the same patients (Table I). The normal range of serum complement is between 30 and 40 U/ml. This serum inactivation of retrovirus infectious titer disappeared after heat inactivation at 56°C for 20 min (unpublished data). Thus, complement may play an important part in reduction of retroviral infectious titer. These data indicate that retrovirus-mediated *in vivo* gene transfer is potentially feasible for intrathecal injection or direct instillation into the residual tumor space in glioma patients.

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