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Efficient Retrovirus-mediated Cytokine-gene Transduction of Primary-cultured Human Glioma Cells for Tumor Vaccination Therapy

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In order to realize a novel vaccination treatment for malignant gliomas using tumor cells genetically modified to express certain cytokines, it is essential to achieve an efficient gene transduction into primary cultured cells. We investigated the feasibility of preparing a glioma vaccine through retrovirus-mediated gene transduction. Glioma cells were cultured primarily from surgically resected tumor tissues of six patients. We obtained more than 1000-fold proliferation of cultures within eight weeks in all six cases. *In vitro* infection with a recombinant retrovirus GKlacZ carrying an *Escherichia coli* β-galactosidase marker gene resulted in over 65% gene transfer to the primary cultured glioma cells. Further enrichment (~90%) of transduced cells was possible by employing repeated infections or using vectors with *neo*^{r} marker gene. Two cytokine genes, granulocyte-macrophage colony-stimulating factor and interleukin-4, were introduced into glioma cells by sequential transduction with two single-expression GK vectors. The transduced glioma cells produced high levels of both cytokines. We also evaluated simultaneous introduction of two genes with double-expression GK vectors containing internal ribosomal entry site (IRES) or internal promoter (PGK). Although the cells transduced with double-expression vectors secreted both cytokines, the level of the gene product following IRES or PGK was 10 times lower than that of the upstream gene product. The transduced cells retained cytokine secretion *in vitro* for 14 days after 100 Gy irradiation. Our data indicate the feasibility of retrovirus-mediated preparation of gene-modified tumor vaccines from clinical glioma materials, which could be useful for potentiating antitumor immunity in glioma patients.

Key words: Glioma — Tumor vaccine — Cytokine — Retrovirus vector — GM-CSF

Glioblastoma is a common malignant brain neoplasm with an invasive and aggressive biological behavior. It is very difficult to cure glioblastoma with current treatment modalities, which include surgery, radiotherapy, and chemotherapy; median survival time of patients with glioblastoma has been reported to be 32 weeks.[1] A novel treatment strategy with curative potential is needed, and a possible approach is gene therapy. Although the systemic immune system recognizes and eliminates tumor cells inadequately in the central nervous system, infiltration and activation of lymphoid cells have been observed in human brain tumors, suggesting that the cellular immune response can reach the brain.[2,3] Products from mutated tumor suppressor genes or rearranged epidermal growth factor receptor genes may serve as tumor-associated antigens recognized by the host immune system.[4,5] These findings support the idea that glioblastoma is also a candidate target of tumor vaccine therapy. Active immunization therapy for brain tumors was studied in murine models, and growth inhibition or rejection of intracerebral tumors was observed.[6–10] These reports support the idea that the central nervous system is less immunologically privileged than had previously been considered. However, clinical trials of vaccine therapy against gliomas using autologous or allogeneic tumor cells failed to provide convincing evidence that the vaccination prolongs survival.[11–13] Therefore, vaccination with increased immunopotentiation is necessary for therapeutic activity against brain tumors.

Use of tumor cells genetically modified to secrete cytokines is a promising strategy for eliciting immunity and eliminating malignant cells *in vivo* (for reviews, see refs. 14 and 15). Several clinical trials of cancer gene therapy that utilize autologous tumor cells transduced with a gene for interleukin (IL)-2, IL-4, interferon-γ, or granulocyte-macrophage colony-stimulating factor (GM-CSF) have already started for renal cell carcinoma and melanoma[16] (for review, see ref. 17). In our previous reports, we described a comparative study on the ability of various cytokines and cell adhesion molecules to induce host-immune responses in murine tumor models.[18,19] We found GM-CSF to be the most potent stimulator of systemic antitumor immunity.[18] To examine whether genetically modified tumor vaccines can eliminate tumor cells in the brain, tumor cells were challenged intracerebrally

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(i.c.) in vaccinated mice.[19] GM-CSF-producing vaccines significantly prolonged survival compared with nontransduced vaccines. To achieve stronger immunopotentiation, we examined the effects of tumor vaccines simultaneously expressing two cytokines. In our intracerebral tumor model, GM-CSF in combination with IL-4 gave increased antitumor effects as compared with GM-CSF alone, leading to the cure of some immunized animals. We proposed that GM-CSF and IL-4 efficiently activate antigen-presenting cells (e.g., dendritic cells), resulting in an induction of T-cell-based tumor immunity. Thus, local subcutaneous vaccine expressing GM-CSF plus IL-4 could enhance systemic antitumor immune responses which reach the brain, suggesting the feasibility of a novel immunological treatment strategy for malignant brain tumors.

Before the clinical application of our experimental results, several *in vitro* studies are required. Firstly, primary cultures of surgical samples should be processed and established. Secondly, efficient and safe genetic transduction should be performed into primary cultured glioma cells. Thirdly, appropriate quantities of cytokines (i.e., GM-CSF plus IL-4) should be produced by the irradiated cell preparations. In this report, we describe a preclinical study to investigate the feasibility of preparing genetically modified tumor vaccine from surgically resected glioma materials.

MATERIALS AND METHODS

Constructs of recombinant retrovirus vectors Gag-killed retroviral vector plasmid DNA (pGK) was constructed from the pMFG[20] backbone by polymerase chain reaction-mediated mutagenesis. Firstly, two stop codons were introduced at nt450 of MoMLV[21] (i.e., U_{450}UAGAG mutated to U_{450}AAUAG) in the coding region of the cell surface gag protein gPr85gag (glycoGag). Alford *et al.*[22] reported that the RNA sequence between domain 8 and domain 9 (nt435–476 of MoMLV) does not contribute to secondary RNA stem-loop structures and that the RNA sequence of nt455–460 could make a stem structure. According to this analysis, we positioned our mutation introduction at nt450, so that the mutation may cause minimal change in the important secondary structure of the viral packaging signal. By this mutagenesis, the predicted stem of domain 8 could be conserved. Secondly, we replaced the A_{621}UG translation initiation codon of Pr65gag with a termination codon UAG (i.e., GAG AAT **A_{621}TG** GGC CAG ACT of MoMLV was mutated to GAA AAT **T_{621}AG** GGC CAG ACT). It was previously shown that mutagenesis of this type does not significantly decrease viral titer.[23] Thirdly, we removed the residual 87 bp of the *env* sequence (nt7679–nt7765) in pMFG (i.e., ggatccgGA U_{7679}UA GUC CAA UUU GUU AAA GAC AGG AUA UCA GUG GUC CAG GCU CUA GUU UUG ACU CAA CAA UAU CA CAG CUG AAG CCU AUA GAG UAC GAG CCA **U_{7772}AG** was mutated to ggatccgGAG CCA **U_{7772}AG**). This *env* coding sequence is theoretically not necessary for cDNA expression. Moreover, it could be harmful by providing an opportunity to rearrange with the *env* sequence present in the packaging cells by homologous recombination, resulting in helper virus production. The mutation-induced viral sequences were confirmed by sequencing.

The pRx retrovirus vector was derived from pGK, using human cytomegalovirus (CMV) immediate early enhancer (CMVIE) in place of the retroviral enhancer in the 5′-long terminal repeat (LTR) of the pGK. The downstream sequence from the *Xba* I site in the 5′-LTR of the pGK was ligated with the upstream sequence of the CMVIE of pUHD15-1[24] by blunt-end ligation at the *Nco* I site (nt464 of pUHD15-1), resulting in the replacement of the 5′-LTR retroviral enhancer with CMVIE, which supports the transcription from the retroviral promoter. Recombinant retroviruses produced by packaging cells transfected with the pGK vector and the pRx vector are identical.

To construct a LacZ mutant with a nuclear localization signal, an oligonucleotide adaptor encoding the XR30-PK sequence[25] was synthesized. The adaptor sequence (5′-C ATG GAT AAA GCT GAA TTT CTC GAA GCT CCT AAG AAG AAA CGT AAG GTA GAA GAT CCT AGG-3′) was inserted in the *Nco* I/*Eco*R I sites at the 5′-end of the *LacZ* in the pRxLacZ, generating pRx-nZ.

To construct the double expression vectors, internal ribosomal entry site (IRES) fragment of cardiac myoendocarditis virus,[26] and phosphoglycerokinase (PGK) promoter from pPGKneo were used. The *Bam*H I fragment of IRESneo was ligated to the *Bam*H I site of pRx-nZ to generate pRx-nZiN. In this vector, viral LTR is used to express a single polycistronic transcript from which two gene products are translated. The *Xba* I/*Bam*H I fragment of the PGK promoter plus neo was ligated by blunt-end ligation at the *Bam*H I site of pRx-nZ to generate pRx-nZpN. In this vector, the upstream *lac*Z gene is driven by the viral LTR, while the downstream *neo* gene is driven by the PGK promoter.

The cDNAs for human GM-CSF and IL-4 were cloned by reverse transcription-polymerase chain reaction using mRNAs isolated from peripheral blood mononuclear cells stimulated for 48 h with 5 μg/ml concanavalin A. Oligonucleotide primers used were 5′CCGAATTCTAGACCACCATGTGGCTGCAGAGCCTGCTGCTC3′ and 5′CGGATCCTCACTCCTGGACTGGCTCCCAGCA3′ for GM-CSF, and 5′CCGGATCCACCATGGGTCTCACCTCCCAACTGCT3′ and 5′CGGGATCCTCAGCTCGAACACTTTGAATATTTC3′

for IL-4. These primers were designed so that minimal 3′ nontranslated sequences were included in the pGK vectors. The cDNAs were sequenced and confirmed to be identical to the reported sequences in GenBank. The *Xba* I/*Bam*H I fragment of GM-CSF cDNA and *Nco* I/*Bam*H I fragment of IL-4 cDNA were inserted into the retrovirus vector pGK, generating pGKGM and pGKIL4, respectively.

To express two cytokines (i.e., GM-CSF and IL-4) in target cells, we constructed double expression vectors, pRxGMiIL4pN and pRxGMpIL4iN. To generate pRxGMiIL4pN, we first constructed pRxGMpN from pRx-nZpN by replacing the *Xba* I/*Bam*H I fragment of GM-CSF cDNA with nlacZ, and then ligated the ~1.1 kb *Bam*H I fragment of IRES-IL4 to the *Bam*H I site of pRxGMpN. To generate pRxGMpIL4iN, we first inserted *Nco* I (blunt end)/*Bam*H I fragment of IL-4 cDNA into *Pst* I (blunt end)/*Bam*H I sites of pGEM7neo. Next we inserted *Xba* I/*Bam*H I (blunt end) fragment of GM-CSF cDNA into *Xba* I/*Xho* I (blunt end) sites of pGEM7IL4, generating pGMpIL4. We then ligated the ~1.6 kb *Xba* I/*Bam*H I fragment of pGMpIL4 to the *Xba* I/*Bam*H I sites of pRx-nZ, and the ~1.4 kb *Bam*H I fragment of IRESneo to the *Bam*H I site of pRxGMpIL4. The IL-4 gene is driven by viral LTR in pRxGMiIL4pN and by PGK promoter in pRxGMpIL4iN.

Establishing the packaging cell sublines NIH3T3 murine fibroblast cells, ΨCRIP packaging cell line,[27] and their derivatives were all cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 2 m*M* glutamine.

To establish highly efficient amphotropic packaging cell lines, we subcloned ΨCRIP cells by limiting dilution, and obtained 80 subclones. They were transfected with pMFGlacZ containing the selection marker *lac*Z, and screened by titering virus particles transiently produced. The cell line, designated as P131, which reproducibly produced the highest recombinant viral titers, was used to generate recombinant amphotropic retroviruses.

Generation of recombinant retrovirus producer cells One of the pGK plasmid DNAs (i.e., pGKlacZ, pGKGM, and pGKIL4) was cotransfected with pPGKneo, which contributes to neomycin resistance, into P131 packaging cells by the calcium phosphate precipitation method. Cells were cultured in a selection medium containing G-418 (GIBCO Laboratories, Grand Island, NY, 1 mg/ml) for 7 days. Clones were expanded, and filtered culture supernatants were used for infecting NIH3T3 fibroblasts. Genomic DNAs of the infected cells were isolated and titers of the virus were estimated as copy numbers of integrated provirus, which were determined by Southern blot analysis. The selected clones producing high-titer recombinant viruses were used for later genetic transduction.

Generation of double-expression retrovirus vectors The ecotropic virus packaging cell line BOSC23[28] was obtained from Dr. W. S. Pear of Rockefeller University, New York. The pRx vector plasmid DNAs, pRx-nZpN and pRx-nZiN, containing a neomycin resistance selection marker were transfected into BOSC23 cells by the calcium phosphate precipitation method. The recombinant ecotropic retroviruses transiently produced by transfected BOSC23 cells were used for infecting P131 cells. Infected P131 cells were cultured in 1 mg/ml G418 for 10 days and the resulting cell population was used as an amphotropic retrovirus producer. To express GM-CSF and IL-4 simultaneously in target cells, we constructed double-expression vectors, pRxGMiIL4pN and pRxGMpIL4iN. P131 producers, P131/GKGMiIL4pN and P131/GKGMpIL4iN, were obtained in the same manner with pRx-nZpN and pRx-nZiN.

Helper virus assay Replication competent virus (helper virus) was assayed by the helper-rescue method. NIH3T3 cells were transduced by MFGlacZ infection to express lacZ in 99% of the cells, resulting in the test cell line 3T3/MFGlacZ. Five milliliters of supernatant from producer ΨCRIP cells at confluency was used to infect 1×10^6 3T3/MFGlacZ cells in a 100-mm dish. After three passages, 5 ml of medium from the infected 3T3/MFGlacZ cells was harvested and used to infect 1×10^6 NIH3T3 cells, which were stained for β-galactosidase 48 h later. The assay was repeated three times.

Patient population and primary culture of glioma tissues Surgical specimens were obtained from six patients with a histological diagnosis of glioma (one grade 2 astrocytoma, two anaplastic astrocytomas, and three glioblastomas). All of the tumors were removed as primary resections, and no preoperative adjuvant treatment was given. Informed consent to use these surgical specimens was obtained from patients or their relatives prior to the surgical procedure.

Tumor tissues, weighing 1 to 3 g, were tranferred to sterile serum-free DMEM immediately after resection. They were cut into 2 mm pieces with scissors then enzymatically digested in DMEM containing 0.25% collagenase type I (Sigma Chemical Co., St. Louis, MO) and slowly stirred at 37°C for 1 h. The resulting cell suspensions were passed through nylon meshes with 250 μm and 50 mm pores. The collected cells were washed in Hanks' balanced salt solution three times before plating onto dishes. This process yielded approximately 1×10^6 viable tumor cells from 1.0 g of tumor tissue. Cultures were maintained in DMEM supplemented with 15% fetal bovine serum (culture medium) in a humidified incubator with 5% CO_2 at 37°C. Cells were fed with fresh culture medium every three days. When they reached 80% confluency, they were treated with trypsin/EDTA, counted, and subcultured at a ratio from 1 : 3 to 1 : 5.

The glial nature of the cultured cells was confirmed by immunohistochemical staining for glial fibrillary acidic protein (GFAP) at passage 2 or 3. The immunodetection was performed using anti-GFAP monoclonal antibody (N358, Amersham, Buckinghamshire, UK) at $\times 100$ dilution and a labeled streptavidin biotin kit (Dako, Kyoto) according to the manufacturers' instructions.

Gene transduction of primary cultured human glioma cells Retrovirus-mediated gene introduction was performed into primary cultured glioma cells. Conditioned medium was harvested from retrovirus producer cells at confluency, and filtered through 0.45 μm pores. Five milliliters of retrovirus supernatants was added to glioma cells of 30 to 40% confluency in a 100-mm dish in the presence of 8 μg/ml Polybrene (Sigma). Cells were cultured for 16 h, then the medium was changed to a culture medium. Infected cells were allowed to grow until 80% confluency, when they were subjected to assay or passage. For vaccine preparation, genetically modified tumor cells were treated with trypsin/EDTA and irradiated with 10,000 rad by a Hitachi MBR-1505R X-ray generator.

Assays for *lacZ* expression and neomycin resistance To determine the efficiency of GK vector-mediated transduction of NIH3T3 fibroblasts and primary human glioma cultures, we used vectors containing the *LacZ* and/or neomycin resistance marker genes. *LacZ* expression was detected as described previously.[29,30] Three days after infection with the retrovirus vector, adherent tumor cells were washed with phosphate-buffered saline (PBS) (pH 7.3) and fixed with 0.5% glutaraldehyde in PBS for 15 min. The cells were washed twice with PBS containing 1 m*M* $MgCl_2$ and immersed in the PBS solution containing the substrate X-gal at a final concentration of 300 mg/ml, 2 m*M* $MgCl_2$, 0.16% potassium ferricyanide and 0.2% potassium ferrocyanide. The cells were incubated at 37°C for 4 to 16 h. Blue precipitates were observed in cytoplasms of cells expressing *lacZ* gene, or in nuclei of cells expressing the *lacZ* gene with the nuclear localization signal. Five high-power fields chosen at random were observed microscopically, and two hundred cells per field (total 1000 cells) were counted. Transduction efficiency was estimated as the percentage of positively stained cells.

Transduction of neomycin-resistant phenotype was determined as described.[31] Retroviral titers were estimated by applying the following formula:

$$\text{viral titer} = N/V \times R \text{ (cfu/ml)}$$

where N represents a number of colonies resistant to G418; V, volume (ml) of virus supernatant used in infection; and R, replication factor, which was 4.0 for NIH3T3.

Assays for human GM-CSF and IL-4 production Human GM-CSF and IL-4 secreted by infected glioma cells were assayed 48 h after plating 1×10^6 cells in 10-cm dishes containing 10 ml of medium using an enzyme-linked immunosorbent assay (ELISA) kit from ENDOGEN. The minimal concentration detected by the ELISA was 5 pg/ml for human GM-CSF and 1 pg/ml for human IL-4 according to the manufacturer.

RESULTS

Generation of recombinant retrovirus vectors Constructs of the retrovirus vectors used in this study are presented in Fig. 1. The pGKlacZ plasmid DNA carrying the *lacZ* marker gene was transfected into the packaging cell line P131, and clones producing high-titer recombinant retrovirus were selected. The GKlacZ viral titer used in this study was 1.0×10^6 infection units/ml as estimated by X-gal staining of the infected NIH3T3 cells. Southern blot analysis of the infected NIH3T3 cells revealed that the viral titer of GKlacZ was 1.5 copies of integrated provirus per cell (data not shown). The viral titer of our most efficient packaging cell line of MFGlacZ was 2×10^6 infection units/ml, which corresponds to three to five copies of proviral integration per cell. P131 packaging cells transfected with the pGKGM or pGKIL4 were screened by Southern blot analysis for clones producing high-titer recombinant viruses. The mean transduction efficiencies into NIH3T3 cells provided by the selected high-titer producers were one copy of proviral integration per cell for GKGM and two copies per cell for GKIL4 (data not shown). These results showed that we have obtained recombinant GK retroviruses with high viral titer ($\sim 10^6$ cfu/ml) comparable to the original MFG. No replication-competent virus was detected from these GK vector-producer cells by helper virus assay (data not shown).

Viral titers of P131/GKnZpN and P131/GKnZiN were determined to be 5×10^5 colony-forming units (cfu)/ml by neomycin resistance assay using NIH3T3 cells. After selection with G418, both NIH3T3/GKnZpN and NIH3T3/GKnZiN were shown to express β-galactosidase in 99% of the cells (data not shown). For expressing two cytokines (i.e., GM-CSF and IL-4) in target cells, we constructed double expression vectors, pRxGMiIL4pN and pRxGMpIL4iN. Neomycin resistance assay determined the viral titers to be 3.0×10^5 for P131/GKGMiIL4pN and 2.9×10^5 cfu/ml for P131/GKGMpIL4iN.

Primary cultures of human gliomas By digesting freshly excised tumor specimens, we usually obtained 1×10^6 viable tumor cells per 1 g of excised tumor. We cultured six glioma samples and evaluated their *in vitro* growth potentials. In all six cases, primary cultures were successfully established. Phase-contrast microscopy of the cultures showed cells with stellate form, a characteristic

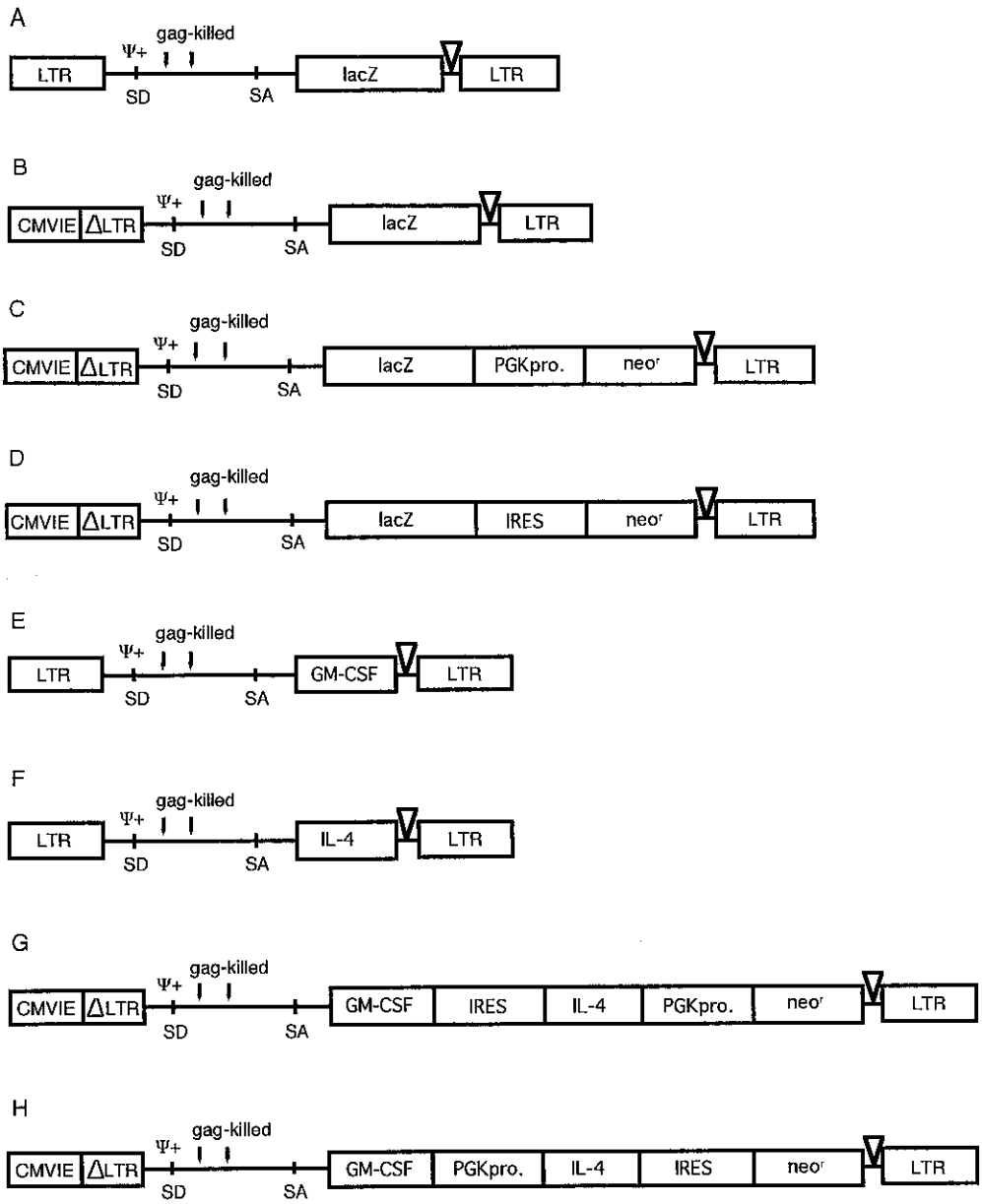

Fig. 1. Schematic representation of constructs of retrovirus vectors used in this study. A, pGKlacZ; B, pRxlacZ; C, pRxZpN; D, pRxZiN; E, pGKGM; F, pGKIL4; G, pRxGMiIL4pN; H, pRxGMpIL4iN; LTR, long terminal repeat; SD, splice donor site; SA, splice acceptor site. Arrows indicate the mutation sites introduced into the *gag* gene.

feature of astrocytic cells (data not shown). Initially, endothelial cells and fibroblasts were present as contaminants with glial cells. To verify that cultured cells are tumor cells of glial origin and not other contaminating cells such as fibroblasts or endothelial cells, we performed immunocytochemical staining for GFAP, a specific marker of glial cell lineage. At the second passage, where glial cells start to grow exponentially and other contaminants tend to disappear, we performed GFAP immunohistochemistry and obtained 75 to 100% positivity of the cells in all six cultures (data not shown), indicating that most of the cell populations were of glial origin. During passage two to passage six, glial cells proliferate rapidly with doubling times of three to four days (Fig. 2). Under

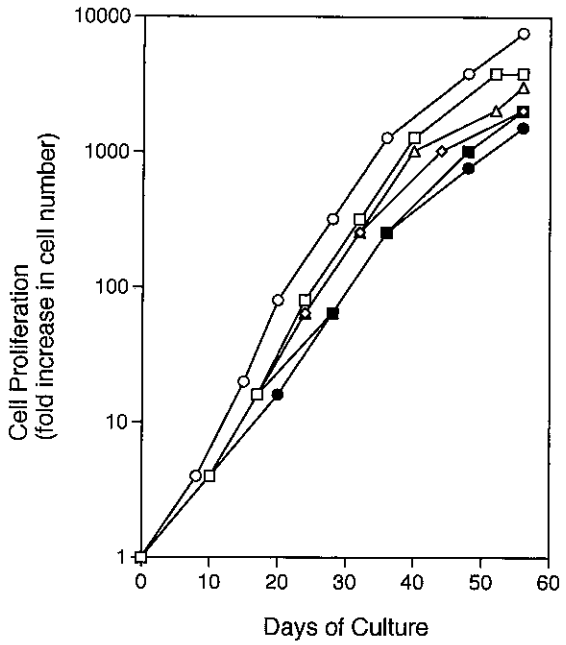

Fig. 2. Growth curves of six primary cultures of human gliomas. Cell culture was initiated on day 0 and terminated when cell proliferation was arrested. □ case 1; ◇ case 2; ○ case 3; △ case 4; ■ case 5; ● case 6.

our culture conditions, 1000-fold or greater expansion of the tumor cell population, reaching 1×10^9 cells or more, was reproducibly attained in 6.5 to 8 weeks.

Genetic transduction of glioma cells We investigated the efficiency of retrovirus-mediated gene transduction of glioma cells by *in vitro* infection with retrovirus vector GKlacZ. This was performed at passage three when glioma cells started to proliferate exponentially. More than 65% of the cells were positively stained in all six glioma cultures, demonstrating an efficient gene transfer to the primary cultured glioma cells (Fig. 3A). The mean transduction rate was 71.8±4.1%, and there was no significant difference in transduction efficiency among the 6 cases studied (Table I). Our result is comparable with or better than the previous reports for melanoma and other carcinoma cells.[32, 33] We next performed repeated infections with GKlacZ to increase transduction efficiency. The rates of transduction were raised to 89.3% after the second infection (Table I, Fig. 3B) and to 95.0% after the third infection. These results indicate that the viral titer is the principal factor for determining the transduction rate and that a retrovirus-resistant cell population did not exist in the cultures. Retrovirus vectors GKnZiN and GKnZpN, which contain both *lacZ* and neomycin resistance genes as selectable markers, were used to infect glioma cells, followed by a drug selection with 500 μg/ml G418 for 10 days. Successful enrichment of transduced cells was possible with these vectors, leading to a transduction efficiency of 95 to 99% (Fig. 3, C and D).

Retrovirus vector-mediated expression of two cytokine genes Recently, we reported on the therapeutic activity of tumor vaccine expressing both GM-CSF and IL-4 in a murine intracerebral tumor model.[19] Our experimental findings implied that full immunopotentiation provided by the vaccine secreting the double cytokines is required for treatment of intracerebral tumors. Therefore, we performed a study of double-gene transduction to investigate how to express simultaneously two transgenes in human glioma cells. We constructed several GK-based vectors for expressing human GM-CSF and IL-4, and made glioma cells secrete the cytokines in two ways: 1) sequential transduction with two single-expression GK vectors; 2) transduction with double-expression GK vectors, which contain IRES or internal promoter (PGK). Two single-expression GK vectors, GKGM and GKIL4, were sequentially used to generate double cytokine-producing glioma cells designated as glioma/GKGM+GKIL4. The double-expression GK vectors, GKGMiIL4pN and GKGMpIL4iN, were used to generate double-cytokine-expressing glioma cells. These were designated as glioma/GKGMiIL4pN and glioma/GKGMpIL4iN and were cultured in the presence of G418 for 10 days following infection. ELISA revealed that all these genetically modified gliomas secreted significant levels of both cytokines, while no unmodified gliomas secreted detectable levels of GM-CSF or IL-4. As shown in Fig. 4A, glioma/GKGM+GKIL4 expressed high levels of both cytokines: 90.5±33.2 ng/10^6 cells/48 h (mean+SD) for GM-CSF and 66.0±25.0 for IL-4. The expression levels of the gene products examined were comparable to those reported in previous studies.[32, 33] These results indicate that GK vectors permit highly efficient gene transduction of human glioma cells. We also demonstrated simultaneous expression of GM-CSF and IL-4 by transducing glioma cells with our double-expression vectors, GKGMiIL4pN and GKGMpIL4iN, in all six cultures. However, a difference of expression level was observed between the upstream gene and the downstream gene (Fig. 4, B and C). Glioma/GKGMiIL4pN produced 132±43.9 ng of GM-CSF and 7.2±2.9 ng of IL-4; glioma/GKGMpIL4iN produced 32.2±12.4 ng of GM-CSF and 2.0±0.5 ng of IL-4. Double-expression vectors used in this study provided high-level production of upstream gene GM-CSF from transduced glioma cells, while the level of downstream gene IL-4 production was about 10-fold lower.

Preparation of tumor vaccines by irradiation Glioma cultures were transduced with GKGM, and the genetically modified tumor cells were X-ray irradiated

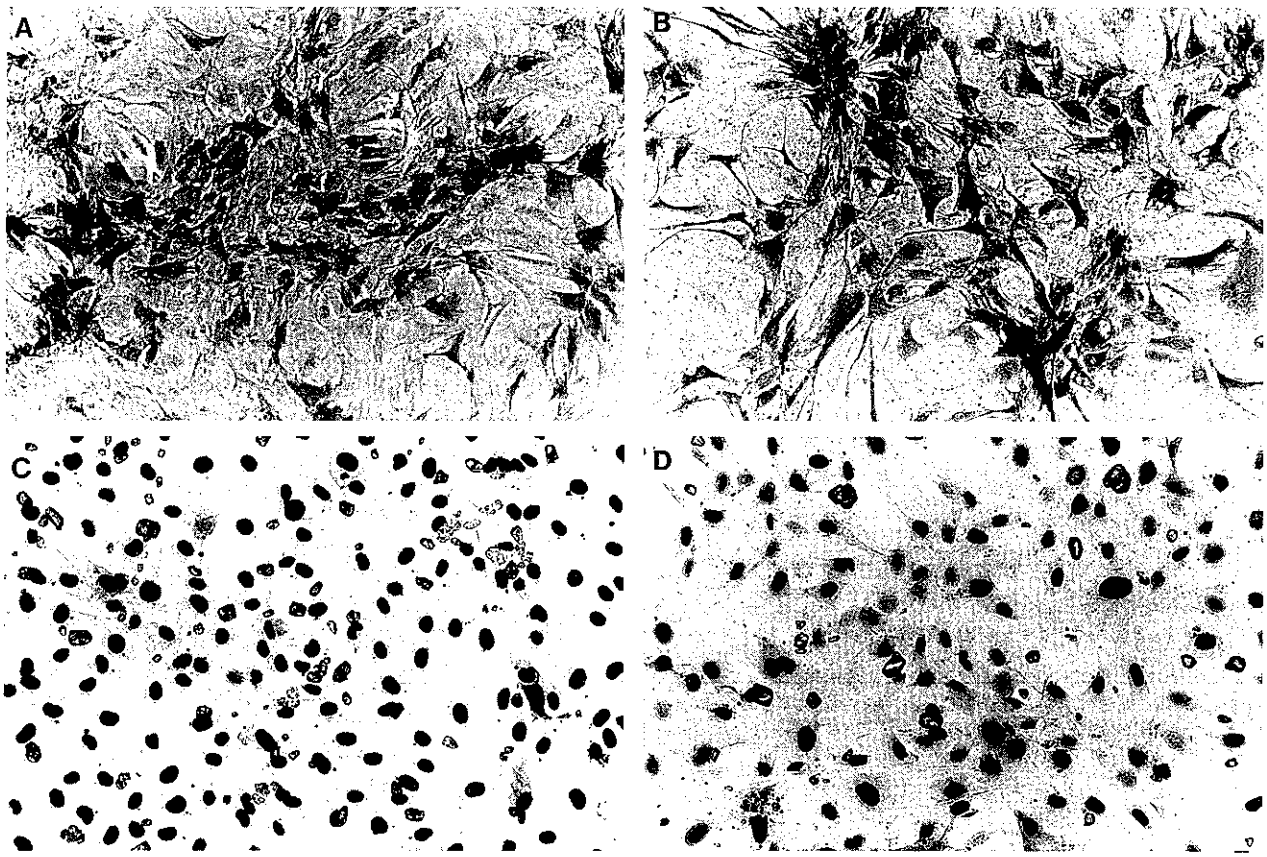

Fig. 3. Retrovirus-mediated gene transfer into primary cultured glioma cells. Expression of *E. coli* β-galactosidase was demonstrated by X-gal staining. A, glioma cells transduced by a single infection with GKlacZ (case 4, passage 4); B, glioma cells transduced by repeated infection (two infections) with GKlacZ (case 4, passage 5); C and D, enrichment of gene-transduced cells by using retrovirus vectors containing *neo*^{r} marker gene. Cells were cultured for 10 days in the medium containing G418 following infection. Expression of β-galactosidase is clearly demonstrated in nuclei (case 3, passage 6). C, GKnZpN. D, GKnZiN. Original magnification ×200.

Table I. Efficiency of GKlacZ-mediated Gene Transduction to Primary Cultured Human Glioma Cells

Case	Transduction efficiency (% positivity)	
	1st[a)]	2nd
1	73.2±4.8[b)]	90.2±3.9
2	65.2±2.9	nd[c)]
3	76.6±2.7	91.3±4.5
4	75.3±4.4	86.3±3.6
5	69.8±5.9	nd
6	70.9±4.6	nd

a) Number of retrovirus infections.
b) Transduced cells were stained for β-galactosidase, and five high-power fields chosen at random were observed microscopically. Cells with cytoplasm containing blue precipitates were judged as positive. Two hundred cells per field (total 1000 cells) were counted. Data are represented by means ±SD.
c) Not done.

with 10,000 rad for use in tumor vaccination preparations. The irradiation completely destroyed the replication ability of the glioma cells, while the cells *in vitro* continued to secrete the transduced cytokine for at least 14 days as measured by ELISA (Fig. 5). A similar time course of cytokine secretion was observed in GKIL4 (data not shown).

DISCUSSION

Patients with malignant glioma do generate a cellular immune response to the tumor.[2,3)] Mononuclear cell-dominant immune cell infiltrates are usually observed around small vessels in glioma lesions. Kuppner *et al.*[2)] reported, from an analysis of tumor-infiltrating lymphocytes of glioma, that these $CD3^+$ lymphocytes selectively lysed autologous glioma cells. These findings suggest that

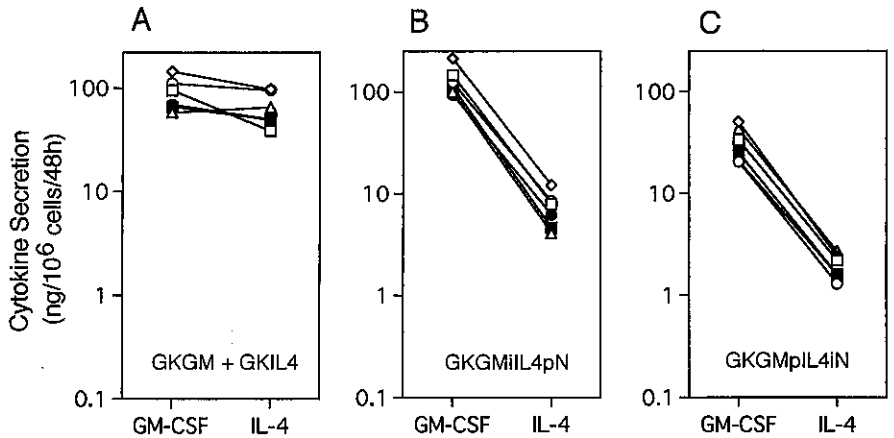

Fig. 4. Cytokine secretion from cultured glioma cells transduced with retrovirus vectors. One million cells were cultured in a 100-mm dish with 10 ml of medium for 48 h until harvest of the medium. Data are the means of duplicate determinations. □ case 1; ◇ case 2; ○ case 3; △ case 4; ■ case 5; ● case 6.

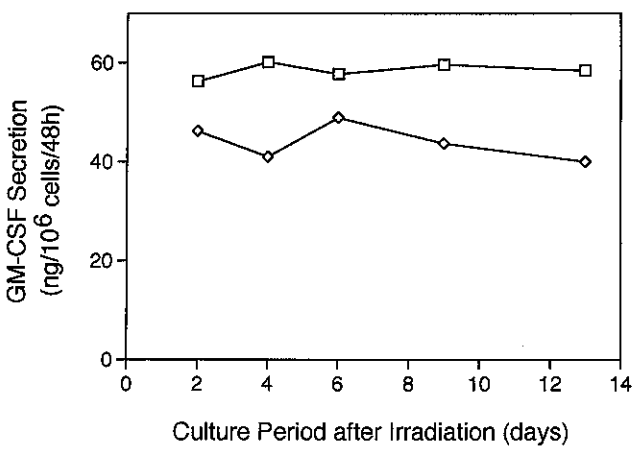

Fig. 5. Time course of cytokine secretion from irradiated glioma cells. Cells were transduced with GKGM, irradiated with 10,000 rad, and cultured. Medium was harvested every two days and used for assay. The amounts of cytokines represent those produced during the indicated period. Data are the means of duplicate determinations. □ case 5; ◇ case 6.

the host immune system recognizes glioma cells and that glioma antigen-specific cytotoxic T lymphocytes could be induced. Therefore, we considered gliomas to be one of the target malignancies of cytokine gene-modified tumor vaccine therapy, which aims at augmenting host systemic immunity.

There are two crucial points for successful clinical application of tumor vaccine therapy. Firstly, primary autologous tumor cells, usually obtained by surgical excision, must be cultured and expanded *in vitro*. We reproducibly succeeded in expanding cultures by 1000-fold within eight weeks. Usually more than 10^9 cells are required to prepare tumor vaccine for a clinical study,[16] which should be started as early as possible (usually within a few months after the surgical treatment). Culturing bulk populations of tumor cells is desirable to maintain antigenic heterogeneity. The culture period should be as short as possible, because long-term cultures may lose or alter tumor antigenicities, resulting in a failure of induction of specific tumor immunity. Secondly, tumor cells must be efficiently and safely transduced to express appropriate amounts of cytokines. Since a retrovirus vector can provide stable and long-term expression of the transgene in infected cells, it is a suitable tool for generating tumor vaccine. We have demonstrated that primary cultured glioma cells were efficiently transduced by using high-titer retrovirus vector. We could attain efficient gene transfer into fresh human gliomas with a mean transduction rate of 72%. Further, the transduction efficiency could be increased by repeated infections. The high transduction efficiency obtained with GK eliminates the need for drug selection of transduced cells. This results in preserved heterogeneity of tumor antigens and shortened culture periods. With regard to safety, the helper virus was not detected in the supernatant of virus-producer cells transfected with GK vector plasmid DNAs. Thus, GK vector is a candidate mode of gene delivery for clinical usage.

Our study on double gene expression from glioma cells provides basic data for clinical studies involving double cytokine-producing cells. Sequential transduction with two single-expression GK vectors, GKGM and GKIL4, resulted in a high-level expression of each transgene.

Furthermore, the expression level of the downstream gene was 10 times lower than that of the upstream gene when a double-expression vector carrying IRES or PGK was used. These results suggest that sequential transduction with the two single-expression GK vectors is the more reliable method for obtaining high-level expression of both products. Further, the levels of transgene products can be controlled by adjusting the multiplicity of infection (moi) of each virus.

Our data indicate the feasibility of retrovirus-mediated preparation of gene-modified tumor vaccines from clinical glioma materials, which could be useful to potentiate antitumor immunity in glioma patients. We are planning a phase I study employing GM-CSF plus IL-4 gene-modified tumor vaccine for treating patients with malignant gliomas. The study will evaluate adverse effects or toxicity associated with vaccine cells and locally produced cytokines. Therapeutic efficacy will be evaluated by radiological monitoring of tumor size. T lymphocytes from peripheral blood mononuclear cells and vaccine-draining lymph nodes will be used to study induction of the specific antitumor immune response. An analysis of the results should enable us to determine the optimum cell dosage, cytokine concentration, and schedule of vaccine administration for glioma patients.

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