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Retrovirus-mediated Gene Transfer Targeted to Malignant Glioma Cells in Murine Brain

Masanobu Yamada,[1] Keiji Shimizu,[2,5] Yasuyoshi Miyao,[2] Toru Hayakawa,[2] Kazuhiro Ikenaka,[3] Kensuke Nakahira,[3] Kazunori Nakajima,[3] Tetsushi Kagawa[3] and Katsuhiko Mikoshiba[4]

[1]Department of Neurosurgery, The Center for Adult Diseases, Osaka, 3-3 Nakamichi 1-chome, Higashinari-ku, Osaka 537, [2]Department of Neurosurgery, Osaka University Medical School, 1-1-50 Fukushima, Fukushima-ku, Osaka 553, [3]Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, 3-2 Yamada-oka, Suita, Osaka 565 and [4]Department of Molecular Neurobiology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108

A murine model for meningeal metastasis of malignant glioma was developed to study selective gene transfer into tumor cells and to establish a reliable means of determining the rate of tumor cell infection. A murine ecotropic retroviral vector was created in which the *Escherichia coli* β-galactosidase gene served as a marker for gene expression from the integrated retrovirus. This retrovirus exhibited a high rate of infectivity in RSV-M mouse glioma cells *in vitro*. The recombinant retrovirus was injected directly into the cisterna magna of the mice. Staining of β-galactosidase showed that the rate of gene integration was high in the disseminated glioma cells. These results suggest the possibility of retrovirus-mediated gene therapy for meningeal dissemination of malignant glioma.

Key words: Glioma — Dissemination — Gene transfer — Retroviral vector

The new molecular technology, retrovirus-mediated gene transfer, is applicable to various types of cancer,[1-6] and in recent years it has been investigated as a treatment for malignant glioma,[4,5] which is the most common malignant tumor of the central nervous system. The brain tumor consists of masses of dividing cells within a background of essentially non-dividing normal brain cells. This metabolic difference provides a significant advantage in terms of gene transfer via retroviral vectors, because retroviruses can integrate only into dividing cells and not into post-mitotic brain cells. A highly efficient *in vivo* gene transfer system is an absolute prerequisite for the clinical application of gene therapy. We will describe highly efficient gene transfer to disseminated glioma cells via a retroviral vector and discuss its potential use against meningeal dissemination of malignant glioma in humans.

The murine model of meningeal metastasis of glioma involved a simple procedure described previously.[7,8] Briefly, cultured RSV-M glioma cells (derived from C3H/HeN mice, kindly provided by Dr. Kumanishi[9]) were harvested and suspended in phosphate-buffered saline (PBS) at a concentration of 1×10^7 cells/100 μl. Under ether anesthesia, the cell suspension was percutaneously transplanted into the cisterna magna of C3H/HeN mice.

In this meningeal dissemination model, 0.1 ml of retroviral supernatant containing 8 μg/ml polybrene was injected into the cisterna magna. The mice were killed 5 days after the viral injection and perfused with a fixative consisting of 4% paraformaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in 0.1 *M* sodium phosphate buffer (pH 7.4). The brains or brain stems were removed with the arachnoid membrane intact and placed in the above-described fixative for 30 min. The brains were then sliced to a 100–200 μm thickness with a microslicer. The sections were rinsed twice in PBS with 1 m*M* $MgCl_2$ and incubated for 4 h at 37°C in an X-gal[6] reaction mixture.

We first attempted to infect retrovirus BAG vector,[10] which produces β-gal in the cytoplasmic region of infected cells, into RSV-M glioma cells inoculated in mouse brain. Although the blue β-gal stain was detected in glioma tissue, some staining was also observed in the control mice, which had been injected only with 8 μg/ml polybrene or saline. The β-gal staining in the control animals was probably due to lysosomal β-gal congregating in inflammatory cells, and this method was obviously unsuitable for estimation of transfer efficiency (data not shown).

To discriminate the nonspecific staining from specific staining produced by the integrated *lacZ*, we created a

[5] To whom correspondence should be addressed.

[6] Abbreviations used: X-gal, 5-bromo-4-chloro-3-indolyl-β-galactoside; neo, neomycin phosphotransferase; β-gal, β-galactosidase; SV40, simian virus 40.

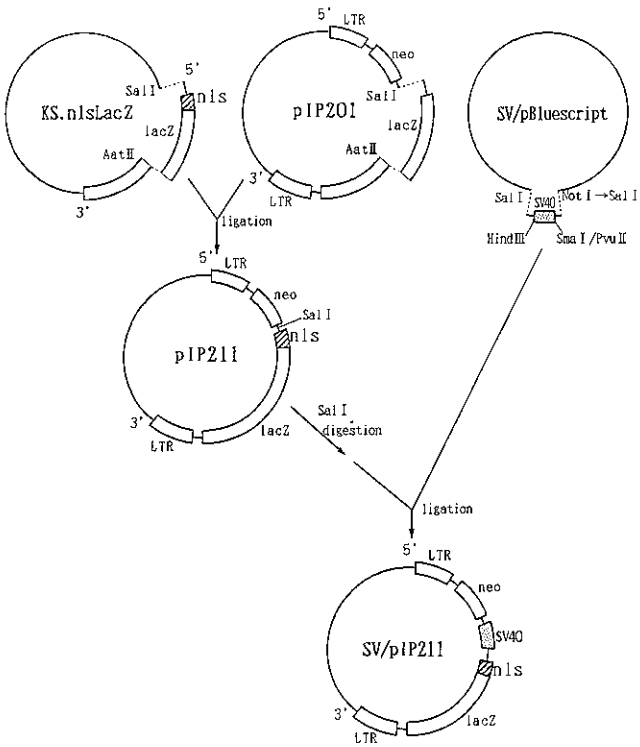

Fig. 1. Construction of the SV/pIP211 plasmid. A *Sal*I-*Aat*II region of pIP201 was exchanged with *Sal*I-*Aat*II fragment of KS.nlsLacZ plasmid to obtain pIP211 plasmid. The pBluescript vector, which was modified to contain an additional *Sal*I linker (GGTCGACC), was digested with *Sma*I and *Hin*dIII, and ligated with the SV40 early promoter fragment derived from pCH110 plasmid to construct the SV/pBluescript plasmid. The SV/pBluescript was digested with *Sal*I, and the *Sal*I-*Sal*I fragment containing SV40 early promoter was inserted into the *Sal*I site of the pIP211 to obtain the SV/pIP211 plasmid.

retrovirus vector, SV/pIP211, which contains a nuclear locating signal at the *N*-terminal of β-gal, to localize the β-gal stain in the cell nuclei.

KS.nlsLacZ plasmid (kindly provided by J.-P. Changeux), which contains a nuclear location signal (nls) in front of the 5′-end of the *lacZ* gene, was digested with *Sal*I and *Aat*II, yielding a fragment consisting of the nls and the 5′-side region of the *lacZ*. This fragment was used to exchange the corresponding *Sal*I-*Aat*II region of pIP201 (the same as pIP200, which was described previously,[11] except for deletion of the *Sal*I-*Sal*I region of the 3′-side of the *neo* gene, which had no apparent influence on enzymatic activity) to obtain the pIP211 plasmid.

A fragment containing SV40 early promoter was obtained by digesting the pCH110 plasmid (Pharmacia) with *Pvu*II and *Hin*dIII. The pBluescript vector (Stratagene), which was modified to contain an additional *Sal*I linker (GGTCGACC) at its *Not*I site after blunting with Klenow enzyme, was digested with *Sma*I and *Hin*dIII, and ligated with the SV40 early promoter fragment to construct the SV/pBluescript plasmid. The SV/pBluescript was digested with *Sal*I, and the *Sal*I-*Sal*I fragment containing SV40 early promoter was inserted into the *Sal*I site of the pIP211 to obtain the SV/pIP211 plasmid (Fig. 1).

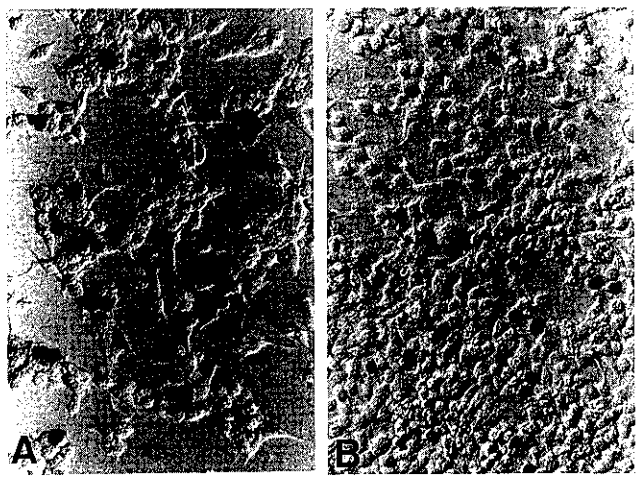

Fig. 2. Retroviral gene transfer to RSV-M glioma cells *in vitro*. RSV-M glioma cell line was highly susceptible to the retrovirus BAG vector (A). The transfer efficiency of SV/pIP211 into RSV-M glioma was the same as that of the BAG vector, and X-gal staining was confined to the cell nuclei (B).

The SV/pIP211 plasmid was transfected to the packaging cell line, Psi-2 (a generous gift of R. C. Mulligan[12]), by the calcium phosphate method.[13] Stable transformants were obtained by selection with G418 (Gibco, 1 to 1.5 mg/ml). Independent colonies were isolated and expanded. The culture supernatant without G418 was used as a retroviral supernatant.

The transfer efficiency of SV/pIP211 and BAG *in vitro* was determined in RSV-M glioma cells. The murine glioma cell line RSV-M was grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Infection was accomplished by incubating the cells in the viral supernatant plus 8 μg of polybrene (Aldrich) at 37°C for 3 to 6 h, after which the medium was replaced with a fresh virus-free medium. The cells were further incubated overnight at 37°C. The cells were then fixed and processed for the X-gal stainings as described previously.[11] RSV-M was highly susceptible to both retrovirus vectors *in vitro*. Approximately 20 to 30% of cultured cells displayed β-gal activities (Fig. 2A, 2B), as reflected by blue staining. As predicted, X-gal staining was confined to the cell nuclei in the cells infected with SV/pIP211 (Fig. 2B).

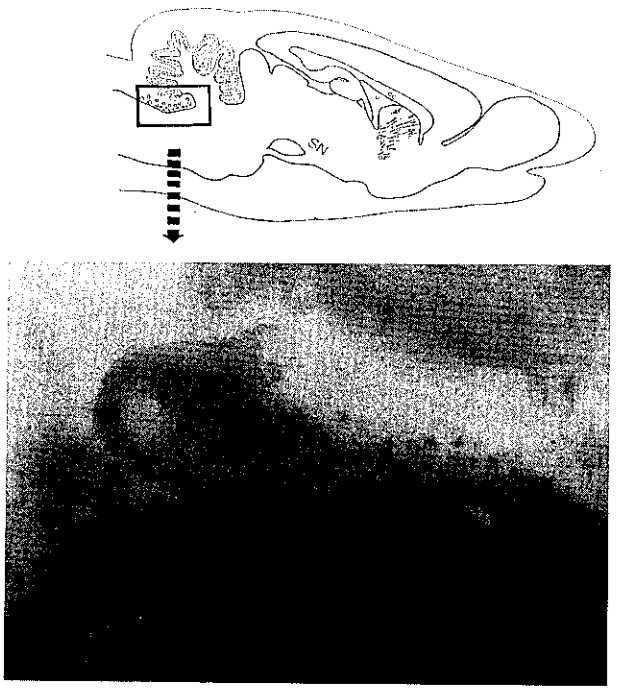

Fig. 3. Highly efficient gene transfer to disseminated glioma cells *in vivo*. Many β-gal-positive cells were distributed in the subarachnoid space on the dorsal aspect of the brain stem indicated by the box. Approximately 25% of tumor cells were stained, and the stain was localized in their nuclei. No staining was seen in the adjacent cerebellum or brain stem. Magnification $\times 50$.

To determine the extent of gene transfer into glioma cells *in vivo*, we used the meningeal dissemination model again. In all mice, tumor cell layers were observed mainly at the dorsal aspect of the brain stem and also in the subarachnoid space around the cerebral hemispheres. Many of the tumor cells in the cisterna magna, the site of viral injection, were positive for β-gal staining (Fig. 3). We assessed the gene transfer rate by staining the cell nuclei of whole injected cells with propidium iodide. The transfer rate was thus calculated to be approximately 25–30%. The most important finding was that the β-gal staining was observed only in the tumor cell nuclei and was not seen in normal brain parenchymal cells, vascular endothelial cells, or any other non-neoplastic cells.

In humans with malignant glioma, the incidence of meningeal dissemination via the cerebrospinal fluid is approximately 10–20%, and it tends to increase as survival time lengthens. These metastases resist conventional treatment and are nearly always fatal, despite administration of multimodal therapy.[7,8] A new therapeutic strategy against meningeal dissemination is essential for improvement of long-term survival. Our meningeal dissemination model provides an excellent *in vivo* system of gene delivery to malignant glioma cells.

Recently, Culver *et al.* reported an excellent outcome in the treatment of experimental brain tumors by *in vivo* gene transfer with a recombinant retroviral vector carrying herpes thymidine kinase gene.[14] They injected retrovirus-producing cells directly into a mass of 9L glioma previously implanted in the right cerebral hemisphere. When the mice were treated with gancyclovir, the tumor cells were eliminated efficiently. However, the injection of retrovirus-producing cells involves various risks in clinical application for the treatment of brain tumors in humans. The histoincompatible implantation of cells engineered to produce actively recombinant retroviral particles is immunologically disadvantageous for the recipient brain, although the brain is a relatively immunologically privileged site. In other cases, intracerebral injection of such proliferating cells may lead to uncontrollable growth and virus production. These considerations strongly indicate that direct injection of viral particles would be more favorable for treatment of brain tumors in humans. However, this treatment is considered to be less efficient for delivering the exogenous genes into tumor cells than direct injection of producing cells themselves. This prompted us to investigate the infection rate in brain tumor cells *in vivo*. The rate of gene transfer in the meningeal dissemination model was approximately 20–30%, which was higher than that in solid tumors inoculated into host parenchyma (less than 10%[4]), and equal to that in RSV-M cells in culture. In solid tumors, it is apparently very difficult for the viral particles to penetrate all of the dividing cells within the tumor mass. In contrast, disseminated glioma cells are as accessible as cultured cells *in vitro*. Moreover, the infection rate can be maximized by repeated administration of viral particles, although a single injection was administered in this study.

In the meningeal dissemination model, β-gal staining was observed exclusively in glioma cells and not in normal brain cells, including parenchymal and endothelial cells, and reactive astrocytes. The retroviral genome can be integrated into a wide range of proliferating cells, though in the adult brain, it selectively integrated into neoplastic cells rather than into the normal, nondividing cells. This strongly suggests that retroviral vectors would be an efficient means of gene transfer into glioma cells in the clinical setting.

Our murine model is simple and can be widely applied in the study of gene therapy for malignant glioma. The thymidine kinase gene (*tk*) of the herpes simplex type 1 virus (HSV-1) is promising as a toxic, or "suicide" gene to be carried by a retrovirus.[5,6,14] The experimental trials of gene therapy using *tk* gene in our meningeal dissemination model are ongoing.

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REFERENCES

1) Kuriyama, S., Yoshikawa, M., Ishizaka, S., Tsujii, T., Ikenaka, K., Kagawa, T., Morita, N. and Mikoshiba, K. A potential approach for gene therapy targeting hepatoma using a liver-specific promoter on a retroviral vector. *Cell Struct. Funct.*, **16**, 503–510 (1991).
2) Huber, B. E., Richard, C. A. and Krenitsky, T. A. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc. Natl. Acad. Sci. USA*, **88**, 8039–8043 (1991).
3) Moolten, F. L., Wells, J. M., Heyman, R. A. and Evans, R. M. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. *Human Gene Ther.*, **1**, 125–134 (1990).
4) Short, M. P., Choi, B. C., Lee, J. K., Malick, A., Breakefield, X. O. and Martuza, R. L. Gene delivery to glioma cells in rat brain by grafting of a retrovirus packaging cell line. *J. Neurosci. Res.*, **27**, 427–433 (1990).
5) Ezzeddine, Z. D., Martuza, R. L., Platika, D., Short, M. P., Malick, A., Choi, B. and Breakefield, X. O. Selective killing of glioma cells in culture and *in vivo* by retrovirus transfer of the herpes simplex virus thymidine kinase gene. *New Biologist*, **3**, 608–614 (1991).
6) Plautz, G., Nabel, E. G. and Nabel, G. J. Selective elimination of recombinant genes *in vivo* with a suicide retroviral vector. *New Biologist*, **3**, 709–715 (1991).
7) Yoshida, T., Shimizu, K., Ushio, Y., Hayakawa, T., Arita, N. and Mogami, H. Development of experimental meningeal gliomatosis models in rats. *J. Neurosurg.*, **65**, 503–507 (1986).
8) Yamada, M., Shimizu, K., Tamura, K., Moriuchi, S., Mabuchi, E., Park, K., Miyao, Y. and Hayakawa, T. Murine model of leptomeningeal dissemination using human medulloblastoma cells. *Neurol. Med. Chir.*, **31**, 763–767 (1991).
9) Kumanishi, T., Ikuta, F. and Yamamoto, T. Brain tumors induced by Rous sarcoma virus, Schmidt-Ruppin strain. III. Morphology of brain tumors induced in adult mice. *J. Natl. Cancer Inst.*, **46**, 539–559 (1971).
10) Price, J., Turner, D. and Cepko, C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA*, **84**, 156–160 (1987).
11) Ikenaka, K., Nakahira, K., Nakajima, K., Fujimoto, I., Kagawa, T., Ogawa, M. and Mikoshiba, K. Detection of brain specific gene expression in brain cells in primary culture: a novel promoter assay based on the use of a retrovirus vector. *New Biologist*, **4**, 53–60 (1992).
12) Mann, R., Mulligan, R. C. and Baltimore, D. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell*, **33**, 153–159 (1983).
13) Gorman, C. "DNA Cloning," Vol. 2, ed. D. M. Glover, pp. 143–190 (1985). IRL Press, Oxford.
14) Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H. and Blaese, R. M. *In vivo* gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science*, **256**, 1550–1552 (1992).