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RESEARCH ARTICLE

Efficient gene transfer of VSV-G pseudotyped retroviral vector to human brain tumor

H Lee[1]*, JJ Song[2]*, E Kim[2], C-O Yun[2], J Choi[2], B Lee[1], J Kim[2], JW Chang[3] and J-H Kim[1,4]
[4]*Yonsei Cancer Center,* [2]*Institute for Cancer Research,* [3]*Department of Neurosurgery, Yonsei University College of Medicine, and* [1]*Department of Microbiology, University of Ulsan College of Medicine, Seoul, Korea*

A retroviral vector constructed from the murine leukemia virus (MLV) can only express transgenes in cells undergoing mitosis, indicating its suitability as a delivery vehicle for cancer gene therapy. However, the transduction efficiency (TE) of retroviruses embedding endogenous envelope proteins in human cancer cells was found to be unsatisfactory. Recently, several research groups have demonstrated the feasibility of a retroviral vector pseudotyped with a vesicular stomatitis virus G (VSV-G) protein. In this study, the potential of VSV-G pseudotyped MLV-based retrovirus was examined as a delivery vehicle in a variety of human cancer cells including brain tumor cells in vitro and in vivo. The transduction efficiency of the 293T/G/GP/LacZ retrovirus in cell culture was superior in most cancer cells, particularly in brain tumor cells, compared with that of other retroviruses, such as PA317- or PG13-derived. The relative growth rate and phosphatidylserine expression level on the plasma membrane of target cells mainly influenced the transduction efficiency of VSV-G pseudotyped retrovirus, which suggested that both the relative growth rate and phosphatidylserine expression level were major determinants of TE. Furthermore, 293T/G/GP/LacZ could efficiently transduce human cancer cells regardless of the presence of chemical additives, whereas in other retroviruses, cationic chemical additives such as polybrene or liposomes were essential during virus infection. Finally, an average of 10% gene expression was routinely obtained exclusively in the tumor mass when 293T/G/GP/LacZ concentrated by simple ultracentrifugation was directly administrated to pre-established brain tumors in animal models (U251-N nu/nu mice or C6 Wistar rats). All told, the present study suggests that the VSV-G pseudotyped retrovirus is a suitable vector for brain tumor gene therapy. Gene Therapy (2001) **8,** 268–273.

Keywords: *retroviral vector; transduction efficiency; VSV-G; phosphatidylserine; brain tumor*

As a gene delivery vehicle in cancer gene therapy, a vector with preferential selectivity to cycling cells and feasibility of *in vivo* administration is desirable.[1] Retroviral vectors constructed from the murine leukemia virus (MLV) can only express transgenes inside target cells after chromosome integration of the viral genome. It is essential that the host cells undergo mitosis shortly after virus infection.[2,3] Therefore, quiescent cells, such as normal tissue adjacent to a targeted tumor, would be refractory to therapeutic gene expression and consequently spared from its cytotoxicity, thus indicating the usefulness of retroviral vectors.[4,5] Replication-incompetent MLV-based retroviral vectors can be produced by transfecting or infecting a retroviral packaging cell line providing viral proteins, with a retroviral vector containing a transgene in place of viral coding genes.[6,7] Packaging cells providing an amphotropic envelope protein, such as PA317, are most commonly used to produce retroviral vectors, which infect both murine and human cells.[7,8] However, in most human cancer cells the retrovirus generated from PA317 has suffered from suboptimal transduction efficiency, which has limited therapeutic efficacy, especially in the case of an *in vivo* situation.[1,6,9]

A successful retroviral infection initially depends on the interaction between a viral envelope protein and a specific cell surface receptor protein on the target cell membrane.[10] The retroviral vector, derived from the PG13 packaging cell line bearing the gibbon ape leukemia virus (GALV) envelope protein has shown an improved transduction efficiency in multiple types of human cancer cells, compared with that of PA317.[11,12] However, the PG13-derived retrovirus is readily inactivated both by human serum and by ultracentrifugation.[13–15] Furthermore, retroviruses bearing endogenous retrovirus envelope proteins should be complexed with chemical additives for substantial virus infection.[12] For this reason, polybrene (a polycationic chemical) has been commonly used[9,16–18] and cationic liposomes or other polycations have been recently used as effective alternatives to polybrene.[19–21] Our previous results have demonstrated that polycationic chemical additives, especially Lipofectamine (Gibco-BRL, Gaithersburg, MD, USA), greatly enhance the transduction efficiency of retroviruses derived from both PA317 and PG13, which is consistent with other studies.[12,17–20] However, these chemical additives were found to induce cytotoxicity even *in vitro*, which would ultimately limit their use in a clinical trial.[12,17]

Several groups have demonstrated that it is possible to

Correspondence: J-H Kim, Yonsei Cancer Center, Institute for Cancer Research, Yonsei University College of Medicine, 134 Shinchon-Dong Seodae Meen-ku, CPO Box 8044, Seoul 120–752, Korea, South Korea
**Drs H Lee and JJ Song contributed equally to this work*
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produce a retroviral vector pseudotyped with a vesicular stomatitis virus G (VSV-G) protein.[14,15,22] The VSV-G pseudotyped retrovirus appears to have a broad spectrum of susceptible cell types, including mammalian and non-mammalian cells.[23,24] In addition, the VSV-G pseudotyped retrovirus withstands the shearing force encountered during ultracentrifugation when generating highly concentrated virus stocks.[25–27]

To explore the potential of VSV-G pseudotyped retroviral vector as an effective gene delivery vehicle for cancer gene therapy, the transduction efficiency of the virus was investigated in a variety of human cancer cell lines that originated from different organs, such as human hepatocellular carcinoma cells (SK-Hep1, Hep3B), human brain tumor cells (U251-N, U87-MG), human lung cancer cells (H-460), human breast cancer cells (MCF-7), human gastric cancer cells (YCC-1) and human cervical cancer cells (HeLa). For *in vitro* experiments, the cells were infected with retroviruses using *LacZ* as a marker gene and bearing different types of envelope proteins, such as PA317 (amphotropic envelope protein)/LacZ, PG13 (gibbon ape leukemia virus envelope protein)/LacZ. Bing (amphotropic envelope protein)/LacZ, FLYRD18 (cat RD114 envelope protein)/LacZ and 293T/G/GP (VSV-G envelope protein)/LacZ at a multiplicity of infection (MOI) of 10 in the presence of polybrene (4 μg/ml). As shown in Figure 1a, 293T/G/GP/LacZ and PG13/LacZ displayed higher transduction efficiencies, especially in brain tumor cells, compared with the other retroviruses. The transduction efficiencies obtained from 293T/G/GP/LacZ in U251-N and U87-MG were scored as 31 ± 2% and 21 ± 3%, respectively. However, in several other cancer cells, including H-460, MCF-7, YCC-1 and HeLa, all types of retroviruses at the same MOI showed transduction efficiencies of less than 5%. U251-N, SK-Hep1, H-460, MCF-7, Hep3B and YCC-1 were infected with 293T/G/GP/LacZ at various MOIs in order to investigate the effects of the MOIs on the VSV-G pseudotyped retrovirus transduction efficiency, with the results shown in Figure 1b. The transduction efficiency up to a MOI of 100 was enhanced by a substantial proportion, particularly in U251-N, SK-Hep1 and H-460. In contrast, the other retroviruses containing a Bing-derived retrovirus did not show an equivalent enhancement of the transduction efficiency of 293T/G/GP/LacZ by simply infecting the cells at a higher MOI (data not shown). Therefore, these results suggest that the VSV-G pseudotyped retrovirus is superior to other retroviruses in transducing most human cancer cells. Moreover, the enhanced transduction efficiency in some human cancer cells could be achieved by simply infecting the cells with higher MOIs (ie more infectious virus particles).

Since Figure 1 indicates a significantly improved transduction efficiency of the VSV-G pseudotyped retrovirus in brain tumor cells, the transduction efficiency of 293T/G/GP/LacZ was further examined in a variety of brain tumor cells. As expected, Figure 2a indicates that the 293T/G/GP/LacZ retrovirus can efficiently transduce most brain tumor cells. At the highest (MOI of 100), an average transduction efficiency of 78 ± 7% by the 293T/G/GP/LacZ retrovirus was obtained in U251-N, U343, U87-MG, TE671 and C6 cells. Figure 2b shows that the transduction efficiency by 293T/G/GP/LacZ in U251-N cells was 85 ± 8% at a MOI of 100, whereas that by Bing/LacZ was only 8 ± 1%. The transduction

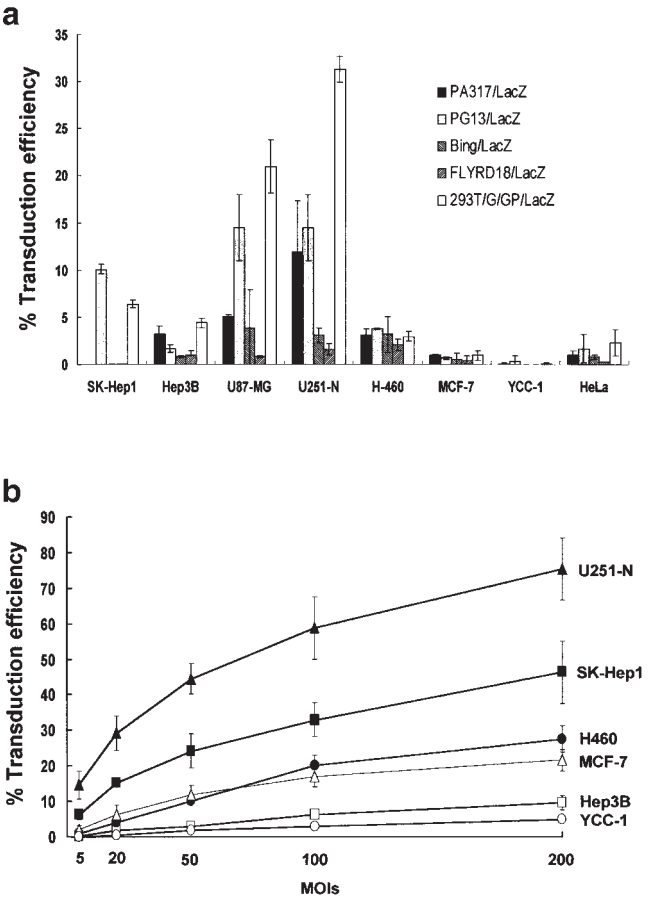

Figure 1 *Transduction efficiency of VSV-G pseudotyped retrovirus in a variety of human cancer cell lines. The values are the averages of at least three independent experiments, and the error bars indicate the standard errors of the means, if not specifically described in the figures throughout this manuscript. (a) Effects on transduction efficiency of retroviruses containing different envelope proteins. All the cell lines were maintained as described previously*[12] *and retroviral vector pBabe/puro was as is described elsewhere.*[12,28] *PA317/LacZ, PG13/LacZ, Bing/LacZ and FLYRD18/LacZ were generated by transfection of pBabe/puro/LacZ to its corresponding packaging cell lines and established as described previously.*[12] *293T/G/GP/LacZ retrovirus was generated by employing plasmids, pBCtTA, pMDtetG and pMDgagpol based on the tetracycline-regulated gene expression system of Gossen and Bujard.*[29] *To determine virus titer (number of infectious particles per ml of virus supernatant), 293T cells were infected with serial dilutions of each virus soup containing 4 μg/ml of polybrene (Sigma, St Louis, MO, USA) to estimate the degree of X-gal staining or resistance to puromycin (1 μg/ml). Human cancer cells of* 2.5×10^4 *cells per well in 48-well plates were infected at a MOI of 10 with various retroviruses, and transduction efficiency was determined by X-gal staining 48 h after infection.*[12] *For X-gal staining in vitro, cells were fixed in fixing solution (0.2% formaldehyde and 0.2% glutaraldehyde in* dH_2O, *and then incubated with staining solution (0.4 mg/ml X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM* $MgCl_2$ *in PBS) at 37°C for 4 to 16 h. Transduction efficiency (designated as a transduction unit) was defined as the average number of blue cells per ×100 field multiplied by magnification factors, plate size and dilution of the infectious stock. (b) Effects of 293T/G/GP/LacZ at different MOIs on transduction efficiency. Differences on the transduction efficiency of 293T/G/GP/LacZ at various MOIs was investigated by infecting the cells at indicated MOIs of 293T/G/GP/LacZ virus and measuring the degree of X-gal staining.*

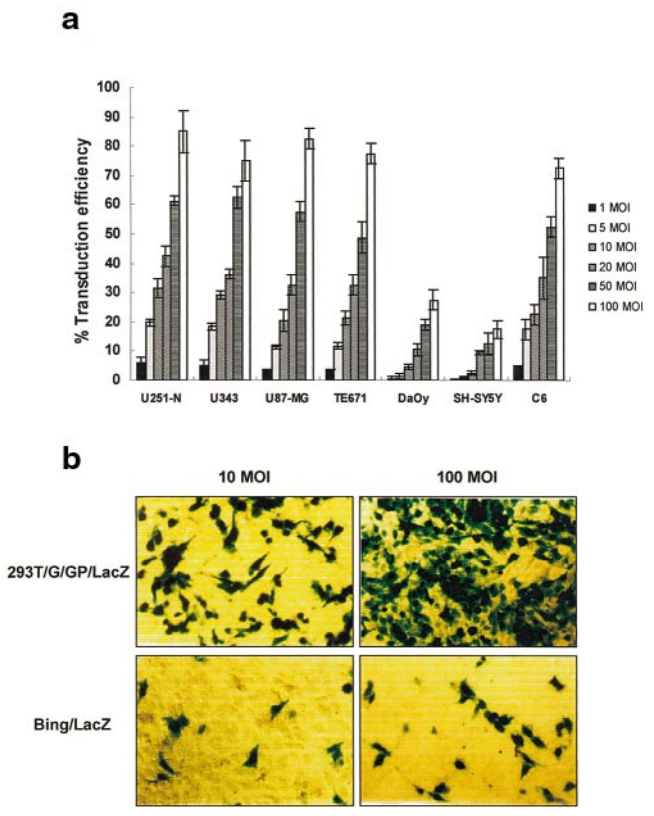

Figure 2 *Enhanced transduction efficiencies in a variety of brain tumor cells by VSV-G pseudotyped retrovirus. (a) Transduction efficiency in brain tumor cells by 293T/G/GP/LacZ at different MOIs. Transduction efficiency was determined after infecting the cells with 293T/G/GP/LacZ at various MOIs. (b) Comparison of transduction efficiency by 293T/G/GP/LacZ and Bing/LacZ. Transduction efficiency of 293T/G/GP/LacZ or Bing/LacZ virus at MOIs of 10 or 100 was investigated by infecting U251-N cells.*

a

Cell line	PS Content	Growth Rate	TE
U251-N	0.76 ± 0.04	0.80 ± 0.04	**31.3 ± 1.5**
U87-MG	0.65 ± 0.14	0.65 ± 0.01	**20.5 ± 2.8**
U343	0.70 ± 0.02	0.65 ± 0.05	**29.0 ± 1.4**
TE671	0.54 ± 0.03	0.72 ± 0.05	**21.3 ± 1.8**
Daoy	1.00 ± 0.05	0.39 ± 0.01	**4.5 ± 1.5**
SH-SY5Y	0.17 ± 0.05	0.46 ± 0.03	**2.5 ± 0.7**
C6	0.66 ± 0.03	1.00 ± 0.10	**22.5 ± 3.5**
SK-Hep1	0.25 ± 0.01	0.78 ± 0.05	**6.4 ± 0.4**
Hep3B	0.29 ± 0.02	0.40 ± 0.01	**4.4 ± 0.5**
H-460	0.75 ± 0.10	0.98 ± 0.07	**3.0 ± 0.5**
MCF-7	0.05 ± 0.05	0.65 ± 0.10	**1.0 ± 0.5**
HeLa	0.32 ± 0.05	0.52 ± 0.02	**2.3 ± 1.4**
YCC-1	0.65 ± 0.05	0.46 ± 0.05	**0.2 ± 0.1**

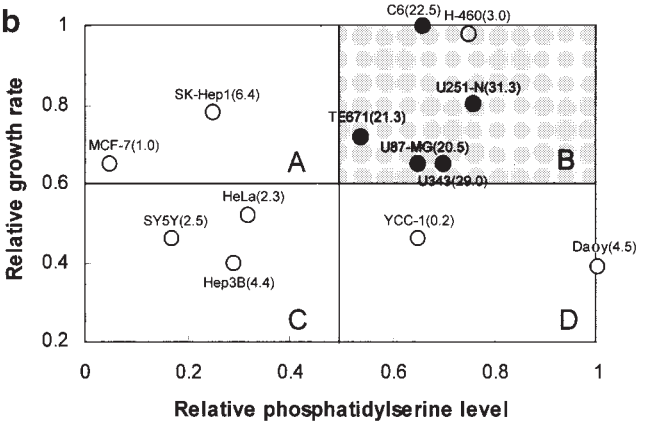

Figure 3 *Effects of the relative growth rates and phosphatidylserine levels on the transduction efficiency of VSV-G pseudotyped retrovirus. (a) Relative growth rate, phosphatidylserine level and transduction efficiency of 293T/G/GP/LacZ in a variety of cancer cells. To estimate the level of phosphatidylserine in a variety of cancer cells, cells in 25T flasks were dissociated, resuspended in binding buffer and then incubated with 5 μl of FITC-labeled annexin V (Clontech, Palo Alto, CA, USA) as supplied by the manufacturer at room temperature for 10 min in the dark. FACS analysis was performed to the manufacturer's instructions and the relative phosphatidylserine level was determined by the following equation. Relative phosphatidylserine level = the level of FITC-positive cells in the sample/the level of FITC-positive Daoy cells. To calculate the relative cell growth rate, 1×10^3 cells were inoculated into 48-well culture plates, harvested and counted at 48 h intervals. The relative cell growth rate was determined by the following equation. Relative cell growth rate = doubling time of C6 cells/doubling time of the cells under test. Transduction efficiency was determined by infecting the cells at a MOI of 10 in the presence of polybrene. (b) Correlation of transduction efficiency with cell growth rate and phosphatidylserine level. Transduction efficiencies of each cell line by VSV-G pseudotyped retrovirus at a MOI of 10 are indicated in parenthesis.*

efficiency by 293T/G/GP/LacZ in U251-N and U87-MG were even superior to that obtained by a recombinant adenovirus (data not shown). When compared with Figure 1, it suggests that the VSV-G pseudotyped retrovirus can transduce brain tumor cells most efficiently over any other type of retrovirus or a recombinant adenovirus. Yet, the VSV-G pseudotyped retrovirus still could not enhance the transduction efficiency in some cancer cell lines, including YCC-1, Hep3B, and HeLa cells, which have previously shown an extremely low transduction efficiency with other retroviruses.[12] Many research groups including this one have demonstrated that the efficient gene transfer by MLV-based retrovirus initially relies on the subsequent mitosis of host cells shortly after virus infection.[2,3,12] Based on this, the relative growth rate of these cells was determined by counting the cells[30] (Figure 3a) or by performing a modified MTT assay (data not shown; Promega, Madison, WI, USA). Figure 3a shows that Daoy, Hep3B, YCC-1, HeLa and SH-SY5Y (panels C and D) have slow growth rate (below $0.52 \pm 0.02\%$ in relative growth rate), compared with the other cells including MCF-7 and SK-Hep1 in panel A, H-460, C6, U251-N, U87-MG, TE671 and U343 in panel B (over $0.65 \pm 0.01\%$). Despite the fast growth rate in the case of H-460, SK-Hep1 and MCF-7, these cancer cells suffered from a low transduction efficiency (below 6.4% ± 0.4% at a MOI of 10). However, all the cells with a high transduction efficiency (C6, TE671, U87-MG, U343, U251-N in panel B) had a fast growth rate. This suggests that the mitosis of target cell shortly after virus infection is necessary for efficient transgene expression by a VSV-G pseudotyped retrovirus, but on its own is not a sufficient condition.

The VSV-G protein primarily recognizes the phospholipid membrane ubiquitously present, such as phosphatidylserine, instead of a specific cell surface protein.[23,31,32] To investigate the correlation between the presence of phosphatidylserine (a major binding target on cell membrane for VSV-G protein) with the transduction efficiency by the VSV-G pseudotyped retrovirus in cancer cells, the

level of phosphatidylserine on cancer cell surface was measured using FITC-labeled annexin V. Annexin V, a vascular protein with strong anticoagulant properties, binds preferentially to a negatively charged phospholipid, such as phosphatidylserine.[33–35] It was found that the phosphatidylserine level in a variety of cancer cells was rather diverse (Figure 3a). C6, U251-N, TE671, U87-MG, U343, YCC-1, H-460 and Daoy (panels B and D) embedded a substantial level of phosphatidylserine on the cell surface (over 0.54 ± 0.03%), whereas HeLa, SK-Hep1, Hep3B, MCF-7 and SH-SY5Y (panels A and C) expressed a significantly lower level (below 0.32 ± 0.05%). Additionally, Figure 3 indicates that brain tumor cell lines with a significantly high transduction efficiency (78 ± 7% at a MOI of 100), except SH-SY5Y and Daoy (17 ± 3%, 27 ± 4%, respectively, at the same MOI), are located in panel B. These cell lines experienced a significantly faster growth rate (from 1.00 ± 0.10% to 0.65 ± 0.50%) and higher phosphatidylserine expression (from near 0.76 ± 0.04 to 0.54 ± 0.03%). Thus, this supports the idea that both the cell growth rate and phosphatidylserine expression level are major determinants of the VSV-G pseudotyped MLV-based retrovirus transduction efficiency. All told, the results suggest that the low phosphatidylserine expression level or poor human cancer cell growth rate may limit efficient gene transfer by the VSV-G pseudotyped retrovirus. The only exception was H-460 (less than 3.0 ± 0.5% in transduction efficiency at a MOI of 10), which expressed the high phosphatidylserine level (0.75 ± 0.10 in relative phosphatidylserine level) and a fast growth rate (0.98 ± 0.07 in relative growth rate). Unfortunately, the reason for the poor H-460 transduction efficiency cannot be explained in our studies. However, the poor transduction efficiency of H-460 could be influenced by the lack of undefined host cellular factor(s) or other unknown reasons, which influence the ultimate transduction efficiency by the MLV-derived retrovirus.[6,7,9]

In previous studies, other research groups including this one have consistently reported the improved transduction efficiency of various retroviral vectors complexed with chemical additives, including cationic liposomes.[12,19–21] Based on this, the effects of chemical additives on the transduction efficiency in human brain tumor cells by 293T/G/GP/LacZ were studied in the presence of various polycationic reagents, including polybrene, protamine sulfate and cationic liposomes (Figure 4). Surprisingly, 293T/G/GP/LacZ efficiently transduced the brain tumor cells in the absence of cationic chemical additives (Figure 4a), whereas in PG13/LacZ or other retroviral vectors, chemical additives were essential. To determine whether the ratio of retrovirus and liposome concentration can influence the transduction efficiency, a fixed number of 293T/G/GP/LacZ were incubated with various concentrations of Lipofectamine and the resulting transduction efficiency was determined (Figure 4b). The figure shows that transduction efficiency did not increase significantly in 293T/G/GP/LacZ complexed with Lipofectamine, with only a 1.7 ± 0.5-fold increase (U87-MG) and 1.4 ± 0.1 (U251-N) at 0.1× of relative Lipofectamine ratio. Furthermore, a consistent decrease in the transduction efficiency in most concentrations of Lipofectamine was observed in all the cells tested. A similar degree of reduced transduction efficiency was found under identical conditions when using other kinds of liposome, such

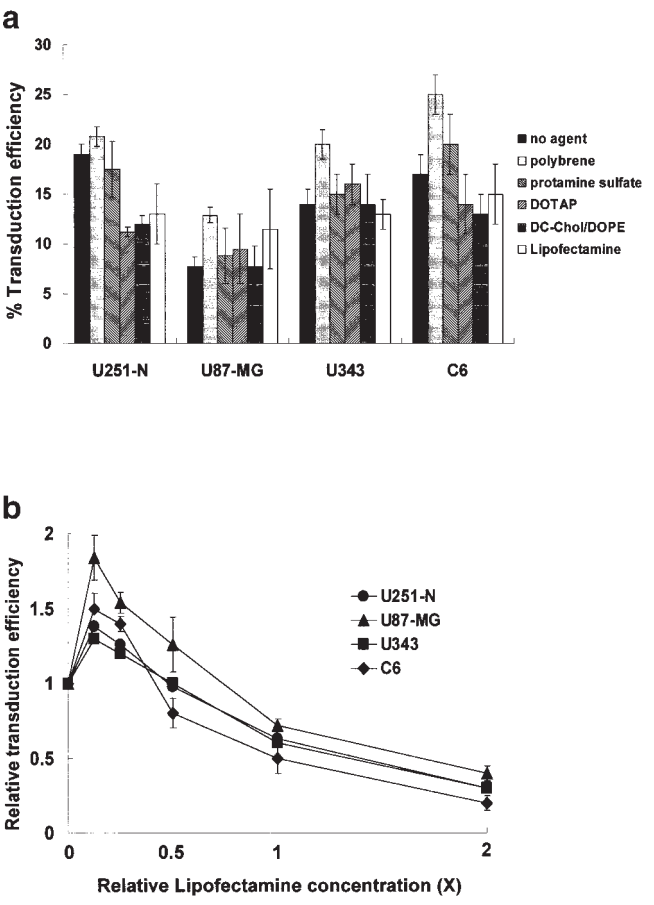

Figure 4 *Effects of cationic chemical additives on the transduction efficiency of VSV-G pseudotyped retrovirus. (a) Effects of chemical additives on the transduction efficiency of 293T/G/GP/LacZ in brain tumor cells. 293T/G/GP/LacZ at a MOI of 5 was pre-incubated with no agent, polybrene, protamine sulfate, DOTAP, DC-Chol/DOPE or Lipofectamine. For liposome treatment, equal volumes of virus soup were mixed with liposome solution at the designated concentration, 200 μl of the mixture was added to the cells for 4 h, and the cells were then refed with fresh media. Protamine sulfate (Sigma) was diluted to 10 μg/ml and treated in the same manner as polybrene, as described previously.[12] Transduction efficiency was determined by X-gal staining 2 days after infection, as described in Figure 1. (b) Relative transduction efficiency of 293T/G/GP/LacZ complexed with Lipofectamine. Values indicate the ratios of the relative transduction efficiency of Lipofectamine treated/the transduction efficiency in the absence of chemical additives.*

as DC-Chol/DOPE or DOTAP (data not shown). Conversely, the concentration initially used (1×) was optimal for PG13/LacZ and a relative transduction efficiency by PG13/LacZ increased up to 126 ± 8-fold (U87-MG) and 124 ± 16-fold (U251-N), when compared with that in the absence of chemical additives (data not shown). Consistently, a similar pattern in the transduction efficiency of PG13/LacZ was observed with other types of retroviral vectors (data not shown). Burns *et al*[25] demonstrated that a complete omission of polycationic additives during VSV-G pseudotyped retrovirus infection resulted in a 100-fold reduction in the number of normal cells, such as the MDCK cell (canine kidney cell). However, Costello *et al*[27] recently reported that the VSV-G pseudotyped HIV-1 vector using polybrene only enhanced the transduction efficiency of human T lymphocytes by approximately two times. Furthermore, in all the human cancer cell lines

tested in this study, subsequently enhanced gene transfer was not detected when using different kinds of cationic chemicals, including polybrene and different cationic liposomes. Overall, these observations suggest that unlike other retroviruses, the VSV-G pseudotyped retrovirus does not require chemical additives for efficient transduction in brain tumor cells.

The major advantage of the VSV-G pseudotyped retrovirus is its resistance to ultracentrifugation and human serum.[25,26,36] It was also observed that 293T/G/GP/LacZ could be readily concentrated by simple centrifugation with little reduction in viability, which concurs with other reports. In this study, approximately 1 to 2×10^7 infectious 293T/G/GP/LacZ particles/ml could be easily recovered from 293T/G/GP/LacZ producer cells. Furthermore, the 293T/G/GP/LacZ virus could be concentrated up to 500-fold, reaching up to 1×10^{10} infectious particles/ml with less than $10 \pm 5\%$ loss of initial infectivity (data not shown). When 293T/G/GP/LacZ was incubated with human serum at 37°C for 3 h, the 293T/G/GP/LacZ retained about $40 \pm 10\%$ of its initial virus infectivity, whereas the PA317/LacZ virus retained only $5 \pm 1\%$ (data not shown). These characteristics may allow the VSV-G retroviral vector to become a suitable delivery vehicle *in vivo*. In investigating whether the 293T/G/ GP/LacZ virus can transduce human brain tumor cells efficiently *in vivo*, a U251-N pre-existing abdominal tumor in *nu/nu* mice or a C6 brain tumor stereotaxically implanted into a Wistar rat were intratumorally injected with 293T/G/GP/LacZ virus. Figure 5 shows that the transduction efficiency of 293T/G/GP/LacZ *in vivo* reached an average of around 10% in both cases, indicating that efficient transduction by the VSV-G pseudotyped retrovirus can be readily achieved by a direct intratumoral injection. Recently, Galipeau *et al*[37] reported that the VSV-G pseudotyped retrovirus, containing thymidine kinase as a therapeutic gene, led to significant GCV sensitization in a C6 glioma tumor model. Extremely few human malignant gliomas are curable with the conventional modality, including surgery, radiation therapy, and chemotherapy.[38,39] In this regard, gene transfer strategy has been intensively investigated recently as a new concept for alternative glioma tumor treatment.[40,41] This investigation, taken together with Galipeau's study, demonstrates the feasibility of the VSV-G pseudotyped MLV-based retrovirus for cancer gene therapy, especially for brain tumors *in vivo*.

In summary, the transduction efficiency of VSV-G pseudotyped retrovirus in a variety of human cancer cells and the effects of chemical additives on its transduction efficiency *in vitro* and *in vivo* was examined. It was demonstrated that the VSV-G pseudotyped retrovirus could efficiently transduce a variety of cancer cells, especially brain tumor cells *in vivo* as well as *in vitro*, in the absence of chemical additives. These results suggest the possibility of the VSV-G pseudotyped retrovirus vector as a suitable vector for direct *in vivo* gene transfer in brain tumors.

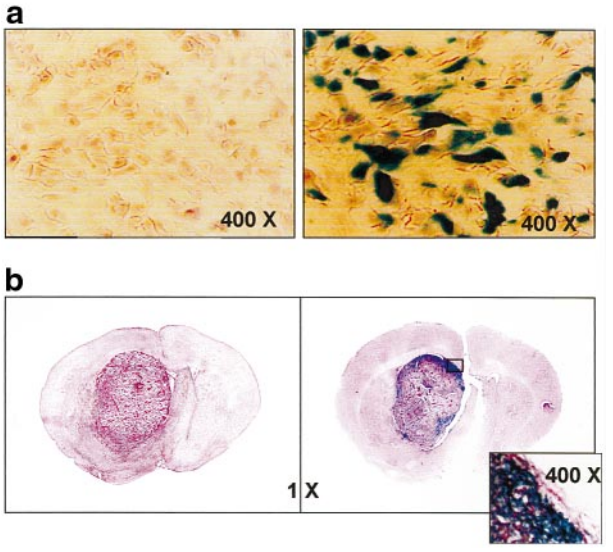

Figure 5 *In vivo gene transfer efficiency of VSV pseudotyped retrovirus. Male nu/nu mice or Wistar rats were obtained from the Korea Research Institute of Chemical Technology, housed and handled in accordance with the Animal Research Committee Guidelines at Yonsei University. Cryostat or paraffin-embedded sections were prepared, fixed in fixing solution and processed using the similar procedure as that used for X-gal staining as described in Figure 1. Sections were then counterstained with hematoxylin and eosin. More than four animals were used in each experimental group and the figures shown are representative of each group. (a) The expression of β-galactosidase in the nu/nu model. 1×10^7 of U251-N cells in 100 μl were implanted subcutaneously into the abdominal walls of mice. When the tumor sizes reached 0.5 to 0.8 cm in diameter, 100 μl of concentrated 293T/G/GP/LacZ virus (1×10^9 ml) was injected intratumorally daily for 2 consecutive days. After 3 days of viral injection, mice were anesthesized and tumor tissues were removed. (b) The expression of β-galactosidase in the brain tumor model. 1×10^5 of C6 cells in 5 μl were implanted stereotactically into the right caudate-putamen of Wistar rat brains. Ten days after implantation, 5 μl of 1×10^7/μl 293T/G/GP/LacZ virus was injected into the tumor. Three to 7 days after viral injection, mice were anesthesized and brain tissues removed.*

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