# Transduction of a Drug-Sensitive Toxic Gene into Human Leukemia Cell Lines with a Novel Retroviral Vector (43611)

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*Abstract.* **To investigate the possibility of killing tumor cells by the expression of an exogenously introduced toxic gene, we have constructed a novel retroviral vector (LTRNL) which has the polyA signal deleted herpes simplex virus type 1 thymidine kinase (*HSV1-tk*) gene. The vector becomes toxic by treating cells expressing *HSV1-tk* with the antiherpetic drugs acyclovir or ganciclovir (GCV). Cells of the human leukemia lines (K562, MEG-01) were infected with this vector and two transduced cell lines (K562/LTRNL, MEG-01/LTRNL) were established. Southern blot analysis confirmed the integration of the *HSV1-tk* transgene in these cells and Northern blot analysis exhibited the expression of 4.8-kb viral mRNA containing the *HSV1-tk* gene. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay for the *in vitro* cytotoxic effects of GCV to these cells demonstrated that concentrations of about 2.5 $\mu M$ for K562/LTRNL and 1.25 $\mu M$ for MEG-01/LTRNL cells resulted in 50% inhibition of cell growth after 72 hr. Subcutaneous tumors of MEG-01/LTRNL in KSN nude mice, but not those of uninfected MEG-01 cells, showed durable regressions after exposure of the mice to 40 mg/kg of GCV given subcutaneously once a day for 15 days. This study indicates that the LTRNL-infected human leukemia cells exhibit inducible susceptibility to GCV.** [P.S.E.B.M. 1993, Vol 203]

Retroviral-mediated gene transfer is a promising approach to the genetic modification of mammalian cells. Foreign genes can be transduced functionally and stably into a variety of human cells, including lymphocytes, fibroblasts, keratinocytes, and endothelial cells, with retroviral vectors (1–3). Efficient insertion of a toxic or otherwise therapeutic gene into human cancer cells could eventually provide an approach to an alternative for cancer therapy.

Several toxic or potentially toxic genes are available, including the herpes simplex virus type 1 thymidine kinase (*HSV1-tk*) (4–8). Thymidine kinase is an enzyme that acts in the salvage cycle of DNA synthesis. This enzyme alone is not harmful to cells and, in the case of mammalian thymidine kinase negative ($tk^-$) cells, provides a means for applying a selective pressure for cell survival (9). Nucleoside analogs such as acyclovir (ACV) or ganciclovir (GCV) are phosphorylated by *HSV1-tk* to nucleoside monophosphates (10–14). The ACV or GCV nucleoside monophosphates are phosphorylated by cellular kinases to the diphosphates and finally to the triphosphates that are incorporated into cellular DNA where they inhibit cellular DNA synthesis and lead to cell death (5, 11, 15). The high specificity of these analogs for the viral thymidine kinase and their very low affinity for mammalian thymidine kinase make it possible selectively to kill cells expressing *HSV1-tk*. Preliminary evidence that the *HSV1-tk* can fulfill the role of a chemosensitivity gene has been obtained from a study which demonstrated that murine sarcoma cells transfected with *HSV1-tk* gene could be eradicated by administration of GCV (16). However, there were no reports of *HSV1-tk* gene induction to human leukemic cell lines with retroviral vector.

We have been interested in investigating whether the transduction of the *HSV1-tk* gene with a retroviral

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vector into human leukemia cell lines expressing its endogenous thymidine kinase gene could enable tumor eradication by GCV. We have constructed a novel retroviral vector and have examined the chemosensitivity of *HSV1-tk*$^+$ leukemia cell lines to GCV both *in vitro* and *in vivo*.

## Materials and Methods

**Cell Lines and Culture.** All cells were grown in Iscove's modified Dulbecco's medium with 10% (v/v) fetal bovine serum in a 5% $CO_2$ atmosphere at 37°C. The ecotropic retrovirus packaging cell line $\psi$-2 and the amphotropic packaging cell line PA317 have been described previously (17, 18). MEG-01 derived from a patient with chronic myelogenous leukemia in blast crisis, which exhibits the characteristics of the immature megakaryocyte, have been established previously in our laboratory (19). K562 derived from a patient with chronic myelogenous leukemia in blast crisis displays characteristics common to myeloid, megakaryocytic, and erythroid cells (20). Neomycin-resistant cells were selected in medium containing the neomycin analog G418 (600 $\mu$g/ml for $\psi$-2 and PA317 and 1 mg/ml for K562 and MEG-01).

**Oligonucleotide-Directed Mutagenesis and Construction of Recombinant Retrovirus.** Oligodeoxyribonucleotides were synthesized on an Applied Biosystems model 391 DNA synthesizer by using phosphoramidite chemistry. Oligonucleotides were purified on 20% polyacrylamide gels. A 1.5-kb *Eco*RI restriction fragment containing the full-length coding region of *HSV1-tk* (21) gene was cloned into Bluescript SK+ (Strategene) and specific nucleotide change was introduced by oligonucleotide-directed mutagenesis. The mutagenic oligonucleotide was 5′-CTCTaGaTTATG-3′ (Fig. 1). The lower case letter identifies nucleotide change that was introduced to make a *Bgl*II site. Oligonucleotide mutagenesis was done using a kit (Amersham RPN, 1523), following the manufacturer's recommendations. After mutagenesis, the *Bgl*II fragment of *HSV1-tk* gene that did not contain the polyA signal sequence was ligated into the *Bam*HI site of retroviral vector pLRNL (22) downstream from the Molony murine leukemia virus (MoMLV) LTR in the same transcriptional orientation as the viral transcriptional unit to form construct pLTRNL. The MoMLV-based retroviral vector pLRNL contains the Neo-resistance (Neo$^R$) gene under control of the promoter of the Rous sarcoma virus between LTRs. DNA sequence analysis of this plasmid confirmed the mutagenesis and checked for any undesired mutations in the *HSV1-tk* gene. The other retroviral vector pLTwRNL was constructed by inserting a 1.5-kb wild-type *HSV1-tk* fragment, which had polyA signal, into the blunt-ended *Bam*HI site of pLTRNL.

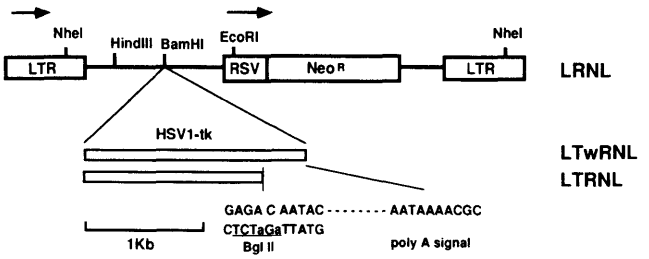


**Figure 1.** Structures of pLTwRNL and pLTRNL retroviral vectors, and oligonucleotide-directed mutagenesis. LRNL vector carrying the G418 neomycin phosphotransferase gene was derived from the Molony murine leukemia virus. The full-length 1.5-kb *HSV1-tk* cDNA or polyA signal-removed 1.3-kb *HSV1-tk* gene were ligated into the *Bam*HI site of the LRNL, creating 5.0-kb and 4.8-kb vectors. *Eco*RI, *Hin*dIII, and *Nhe*I restriction sites are shown. To remove the polyA signal from *HSV1-tk* gene, the mutagenic oligonucleotide was applied to make a *Bgl*II site between 3′-end of coding region and polyA signal.

**Transfection and Infection.** To produce transmissible virus, 20 $\mu$g of vector DNA were transfected into $5 \times 10^5$ $\psi$-2 cells using the calcium phosphate coprecipitation method (23) in a 10-cm plate. One day after transfection, culture medium was collected, filtered through a 0.22-$\mu$m membrane filter, and applied to amphotropic PA317 cells in the presence of polybrene (Sigma; 5 $\mu$g/ml). Infected cells were selected in medium containing G418 and colonies were picked 14 days after infection. To titer the virus, serial dilutions of viral stocks were applied to 208F cells (24), and selected in G418-containing medium as described above. Neo$^R$ colonies were scored 14 days after infection. K562 and MEG-01 cell lines were infected by incubation of cells with cell-free viral supernatant from the overnight culture of amphotropic producer line in the presence of polybrene.

**Cell Growth Assay.** K562 or MEG-01 cells stably transformed with *HSV1-tk* by LTRNL were seeded at a concentration of $5 \times 10^4$ cells/ml on 96-well flat-bottomed microplates in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, and various concentrations ACV or GCV were added to the culture. After a total of 72 hr of incubation, the viable cells were detected by a colorimetric assay using tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, as MTT assay described previously (25). The results were read on a multiwell scanning spectrophotometer (SLT 210; SLT-LAB Instruments, Salzburg, Austria) and showed a high degree of precision. This assay detects living cells, but not dead cells, and the signal generated is dependent upon the degree of activation of the cells.

**DNA and RNA Analysis.** Genomic DNA was prepared and digested with appropriate restriction enzymes, as described. The resulting DNA was electrophoresed in 0.8% agarose gels and transferred to nylon filters (Amersham Japan, Tokyo) (26). Southern hy-

bridization was carried out at 65°C using a $^{32}$P-labeled DNA probe containing the *HSV1-tk* gene.

Total RNA was isolated by the guanidine isothiocyanate method (27) and quantitated by absorbance of 260 nm. About 15 μg of each total RNA were denatured, separated in 1.2% agarose formaldehyde gels, and blotted to nylon filters. Northern hybridization was performed at 42°C with radiolabeled DNA probe containing the *HSV1-tk* gene. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (28) was used as a control for differences in the amount of RNA loaded on the gel.

***In Vivo* Study with Mice.** Tumors were induced in syngeneic KSN nude mice (29) by subcutaneous injection of $2 \times 10^7$ MEG-01 or MEG-01/LTRNL cells. Each cell line was injected into six mice. Tumors first became palpable 1–2 weeks later. Subcutaneous tumor formation was seen in all mice after 2 weeks, but MEG-01/LTRNL tumors grew more slowly than MEG-01 tumors. Each group of mice was divided into two subgroups, with or without GCV (GCV(+) or GCV (−)). From 20 days after the cell lines had been injected, when the tumors had grown to adequate size, approximately 200 mm$^3$, we injected mice bearing MEG-01 or MEG-01/LTRNL tumors with GCV once a day. GCV was freshly prepared as 2.4 mg/ml solution in sterile water. Each mouse was injected subcutaneously with GCV at a dose of 40 mg/kg daily for 15 days. Mice of GCV(−) groups were injected with physiologic saline by the same procedure as GCV(+) groups. Tumor size was measured by cross-sectional diameter every 5 days.

## Results

The retroviral vectors pLTwRNL and pLTRNL were used to transfect ecotropic ψ-2 cells. One day after transfection, 5 ml of harvest were applied to the amphotropic packaging cell line PA317 to make stable producer lines. The highest titer virus producers with each construct were named PA317/LTwRNL and PA317/LTRNL cells that produced $7 \times 10^2$ colony forming units/ml and $4 \times 10^4$ colony forming units/ml virus assayed on 208F cells, respectively. Thus, the PA317/LTRNL producer cells were used for further study.

K562 and MEG-01 cell lines were infected with the LTRNL virus by incubation with viral supernatant from the overnight culture of PA317/LTRNL. After G418 (1 mg/ml) selection, the K562/LTRNL and MEG-01/LTRNL cell lines were isolated.

Southern blots of DNA from infected or uninfected cells hybridized with *HSV1-tk* are shown in Figure 2. *HSV1-tk* genes were seen in infected cells as a 1.3-kb band after digestion of cellular DNA with both *Eco*RI and *Hin*dIII, and as a 4.5-kb band after digestion with *Nhe*I. By Northern blot hybridization, a 4.8-kb band was detected in infected cells indicating the expression

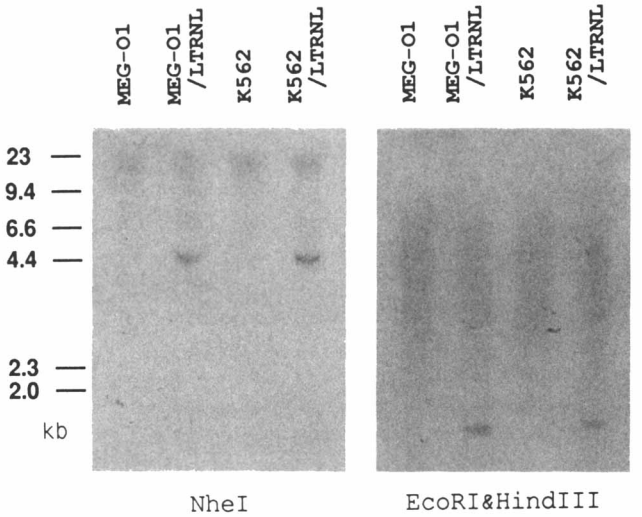


**Figure 2.** Southern blot analysis of uninfected and infected K562 or MEG-01 cells using the *HSV1-tk* probe. Equal amounts (10 μg) of DNA from these cells were digested with restriction enzymes *Nhe*I or both *Eco*RI and *Hin*dIII. The digested DNA were electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with $^{32}$P-labeled *HSV1-tk* probe.

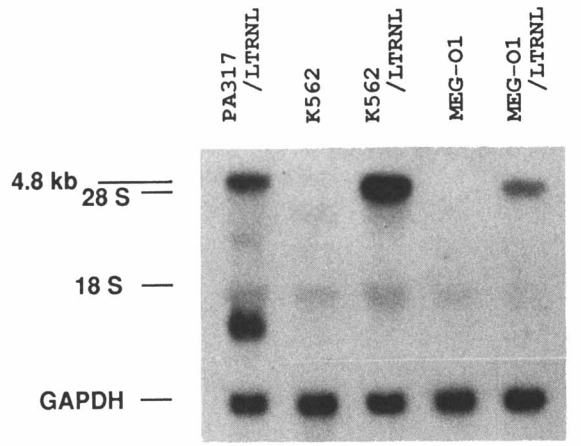


**Figure 3.** Northern blot analysis of PA317/LTRNL, uninfected and infected K562 or MEG-01. Equal amounts (15 μg) of each RNA sample were assayed for *HSV1-tk* expression. Virus mRNA were seen about 4.8-kb band. The same blot was then assayed for GAPDH expression as a 1.2-kb band.

of integrated virus RNA containing *HSV1-tk* gene (Fig. 3). The PA317/LTRNL lane exhibits another hybridizing band because the PA317 cell was transferred to another *tk* gene in the course of establishing a packaging cell line. These results indicate that provirus DNA containing *HSV1-tk* and the Neo-resistance genes were stably inserted into cellular DNA, and virus mRNA containing the *HSV1-tk* gene were expressed in infected cells.

The growth curves shows the cytotoxic effects of GCV or ACV upon K562, K562/LTRNL, MEG-01, and MEG-01/LTRNL (Fig. 4). The surviving fractions of K562/LTRNL were decreased in a dose-dependent manner when compared with nontransduced K562

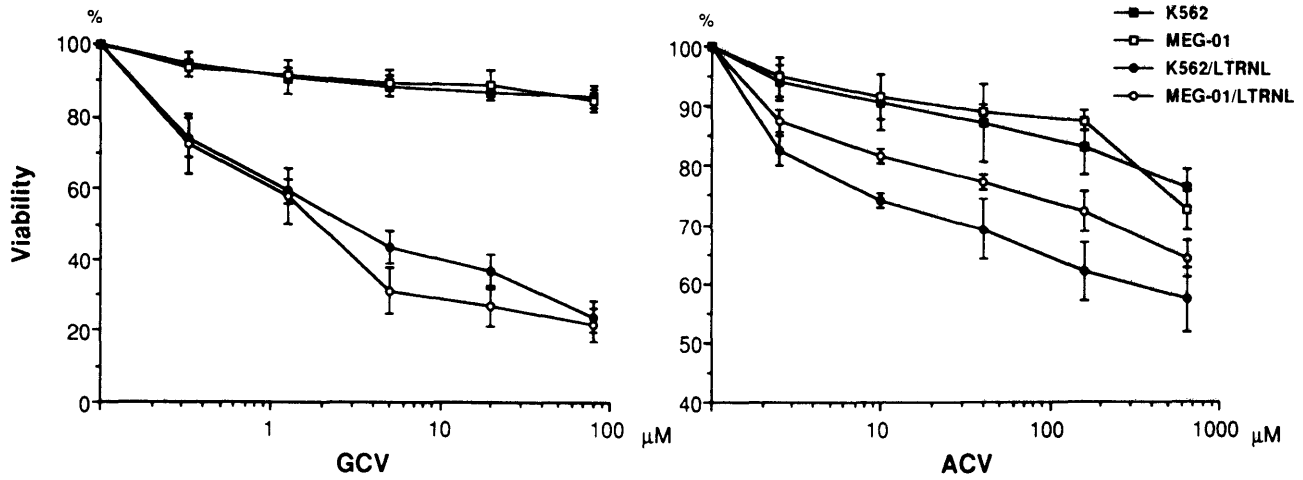


**Figure 4.** Cytotoxic effect of GCV or ACV to human leukemia cell lines with or without expression of the *HSV1-tk* gene: K562 (■), MEG-01 (□), K562/LTRNL (●), and MEG-01/LTRNL (○). After 72 hr of incubation with various concentrations of GCV or ACV, viable cells were counted by the MTT assay. Data represent the mean ± SD from three independent experiments.

cells. At a concentration range of 0–80 $\mu M$, GCV showed no significant toxicity to wild-type cells while demonstrating toxicity to cells expressing *HSV1-tk*. The same tendency was observed with MEG-01 and MEG-01/LTRNL cells. After 72 hr of incubation with GCV, the concentrations of GCV required to achieve 50% inhibition of cell growth were 2.5 $\mu M$ for K562/LTRNL and 1.25 $\mu M$ for MEG-01/LTRNL cells. Greater than 80% of cells expressing *HSV1-tk* were killed with exposure to 80 $\mu M$ GCV for 3 days. In contrast, ACV seems to be less effective because the cytotoxic effect of ACV to induced cells is weak, and at higher concentrations ACV exerted some toxicity to wild-type cells. At a concentration of over 640 $\mu M$, ACV appeared to be considerably toxic to wild-type cells.

The *in vivo* study was performed with syngeneic KSN mice by subcutaneous injection of $2 \times 10^7$ MEG-01 or MEG-01/LTRNL cells. We divided each group into two subgroups: with or without GCV treatment. The recipient animals were divided into four groups of three mice each, including groups injected with MEG-01 with or without GCV. In the absence of GCV, tumors of MEG-01 and MEG-01/LTRNL cells grew at indistinguishable rates. After the GCV injections were begun, tumor size was determined (Fig. 5). Tumors in the MEG-01/LTRNL GCV(+) group showed no increase in size, whereas GCV did not affect the growth of untransduced MEG-01 tumors. MEG-01/LTRNL tumors after 15 days of GCV therapy also remained at almost the same size over the next 5 days, but regrew after 20 days.

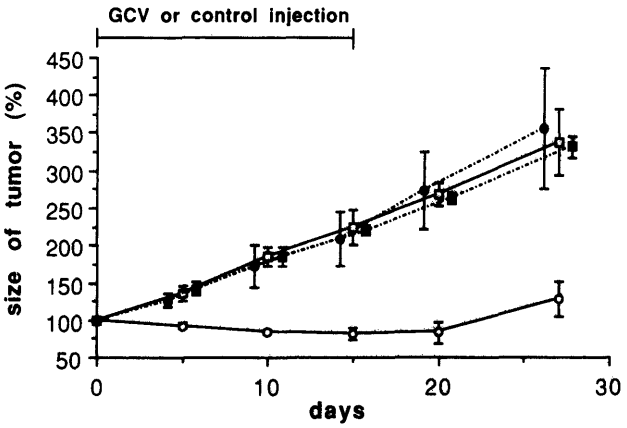


**Figure 5.** Effect of GCV on *HSV1-tk*$^+$ and *HSV1-tk*$^-$ tumors. GCV (40 mg/kg) was injected subcutaneously daily for 15 days. The average tumor size of each group before GCV injection was defined as 100%. Tumors enlarged at approximately the same rate among the MEG-01 GCV(−) (■), MEG-01 GCV(+) (□), and MEG-01/LTRNL GCV(−) (●) groups. But tumors of the MEG-01/LTRNL GCV(+) (○) group shrank or remained unchanged in size for the period of GCV injection and next 5 days. Points represent the mean size of tumors from each three mice; bars, SD.

## Discussion

To our knowledge, this paper appears to be the first experiment that showed the toxic gene transfer to human leukemia cell using a retroviral vector. We used novel retroviral vector pLTRNL containing *HSV1-tk* insert without polyA signal. The titer of pLTRNL producer cell line is about 50-fold higher than that of pLTwRNL producer cell line with polyA signal. Using the retroviral vector pLTRNL, we have estabished human leukemia cell lines, MEG-01/LTRNL and K562/LTRNL, that have transduced *HSV1-tk* gene.

The expression of the *HSV1-tk* gene alone appears to have no beneficial or deleterious effects on cultured cells. The toxic effects of this gene product in this study result from the ability of this enzyme to convert ACV, GCV, and related compounds to toxic nucleotide intermediates. In our study, using modified *HSV1-tk* gene,

we have examined the possibility of conditional ablation with the antiherpetic drug ACV, GCV in human leukemia cell lines. The toxic effect of GCV to *HSV1-tk*-expressing cells was more selective and sensitive than that of ACV, efficiently killing the *HSV1-tk* transduced cells without significantly injuring wild-type cells. The concentration of GCV required to achieve 50% inhibition of cell growth was about 1.25 $\mu M$ for MEG-01/LTRNL and 2.5 $\mu M$ for K562/LTRNL. It is known that the plasma concentration of GCV is about 40 $\mu M$ after 1 hr of intravenous injection of 5 mg/kg of GCV into humans, so this concentration may be applicable to tumor eradication *in vivo*. Moolten and Wells (30) reported that 50% inhibitory doses of GCV to murine cell lines expressing *HSV1-tk* for 3 days of culture were about 0.02 $\mu M$ to 0.04 $\mu M$. Of the human leukemia cell lines, the concentration of GCV required to achieve 50% inhibition of cell growth was about 20–50 times higher than that required for murine cells. This may be due to differences of the promoter activities of *HSV1-tk* gene and those of the transduced cells between these studies.

In our *in vivo* study, GCV-treated tumors bearing the *HSV1-tk* gene showed no increase in size, but complete regression of tumors was not seen. This may due to mutational loss or epigenetic shutdown of the *tk* gene (31), poor tumor vascularization, or the limited time of exposure of tumor cells to GCV.

It is clear that the success of such therapy will depend upon the development of efficient and selective means of delivering retroviral vector to tumor cells. Many ideas for cell targeting have been described, such as antibody bridging between virus envelope and target cells (32) or virus envelope modification (24). The use of tumor-specific promoter may become an another approach. For example, an $\alpha$-fetoprotein promoter could be used to express the toxic gene only in hepatocellular carcinoma (33). Recently another approach of *HSV-tk* gene-mediated cytotoxicity has been reported by Culver *et al.* (34). They showed the *in vivo* gene transfer with retroviral vector producer cells for the treatment of rat cerebral glioma and treatment of GCV showed complete tumor regression by the "bystander effect." The bystander effect might be the host immune response to tumor cells. Taken together, we think these results show that the potent clinical use of our vector is promising.

Intensive work on recombinant virus has produced vehicles capable of efficient transduction of foreign gene into cells. Such defective recombinant viruses may be applicable to the delivery of toxic gene to accessible populations of cancer cells.

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