Jpn. J. Cancer Res. 88, 492–500, May 1997

In vivo Retrovirus-mediated Herpes Simplex Virus Thymidine Kinase Gene Therapy Approach for Adult T Cell Leukemia in a Rat Model

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We have previously demonstrated that human T-lymphotropic virus type I (HTLV-I) *tax*-expressing human T cell lines are selectively eliminated in the presence of aciclovir, using a retroviral vector carrying the herpes simplex virus thymidine kinase (HSV TK) gene under the control of the long terminal repeat (LTR) of HTLV-I. Based on these findings *in vitro*, we investigated whether this system could also be effective *in vivo*, using a rat model. Following infection of the HTLV-I-transformed and *tax*-expressing rat T cell line TARS-1 with this retrovirus (LNLTK virus), high levels of HSV TK expression were observed and resulted in increased susceptibility to ganciclovir (GCV). Tumors were generated by subcutaneous injection of TARS-1 in newborn syngeneic WKA/H rats. While the tumors derived from infected TARS-1 cells with control virus, as well as uninfected cells, continued to grow in all the rats with or without administration of GCV, those derived from LNLTK-infected cells exhibited dramatic regression upon GCV treatment. These results indicate that the HTLV-I LTR-HSV TK system also causes selective elimination of HTLV-I-transformed, *tax*-expressing T cells *in vivo*. Therefore, our present study may provide a rationale for clinical gene therapy against adult T cell leukemia.

Key words: HTLV-I *tax* — Adult T cell leukemia — Herpes simplex virus thymidine kinase — Gene therapy

Adult T cell leukemia (ATL), which arises from $CD4^+$ T cells,[1–3] is caused by human T-lymphotropic virus type I (HTLV-I).[4–6] HTLV-I possesses a unique 3′ region in its genome, designated as *pX*, which encodes the viral transactivator tax protein.[7] *tax* can transactivate not only its own long terminal repeat (LTR),[8–10] but also cellular gene promoters including several proto-oncogenes and lymphokines and their receptor genes, such as interleukin 2 (IL-2) and IL-2 receptor α-chain.[11–13] *tax* is capable of immortalizing human primary T cells[14, 15] and rat primary T cells,[16] and of transforming an established rat fibroblast cell line.[17] Although many studies have suggested that *tax* plays a central role in the HTLV-I-associated immortalization and transformation of T cells, the essential molecular mechanism of ATL development remains unclear. In addition, there is no effective treatment for ATL and its prognosis, therefore, is very poor.[18]

Recent advances in the understanding of molecular biology, and the development of gene transfer techniques have resulted in gene therapy approaches to cancer treatment. Among the various paradigms for cancer gene therapy, one promising approach is virus-mediated transduction of tumor cells with suicide genes. To date, the herpes simplex virus thymidine kinase (HSV TK) gene is the most commonly used suicide gene. Cells transduced with the HSV TK gene become sensitive to nucleoside analogs such as ganciclovir (GCV) and aciclovir (ACV). HSV TK can selectively convert these antiviral drugs into phosphorylated forms that act as a chain terminator in DNA synthesis, causing cell death.[19, 20] HSV TK has been used successfully in gene therapy in a variety of animal tumor models. The use of HSV TK is also being evaluated in the treatment of human cancers.[21–26]

We previously reported a retroviral-mediated gene therapy approach to the treatment of ATL based on the concept of the *tax*-targeted selective expression of the HSV TK gene in leukemic cells *in vitro*.[27] Infection of HTLV-I-transformed and *tax*-expressing human T cell lines with the retrovirus carrying the HTLV-I LTR-HSV TK hybrid gene resulted in elimination of these cells in the presence of ACV. Based on these findings *in vitro*, we conducted the current study to investigate whether this

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system could also be effective *in vivo* in an animal model. We demonstrated that HTLV-I LTR-HSV TK worked specifically in HTLV-I, *tax*-expressing T cells and killed such cells following GCV treatment *in vivo*. These results indicate the potential of this approach in clinical gene therapy for ATL.

MATERIALS AND METHODS

Cell culture HTLV-I-transformed WKA/H rat T cell lines, TARS-1[28] and W7TM-1,[29] an HTLV-I-transformed human T cell line, ED-40515(−),[30] and an HTLV-I-negative human hematopoietic cell line, HL-60, were cultured in RPMI 1640 supplemented with 10% fetal calf serum.

Animals Inbred WKA/H female rats were purchased from SLC Japan Co., Ltd. (Shizuoka) and maintained in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Plasmid construction The Molony murine leukemia virus (MLV)-based retroviral vectors pLN and pLNCX were kindly provided by Dr. A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA).[31] After removal of the cytomegalovirus promoter from pLNCX, a 2.4-kb fragment of the HTLV-I LTR-HSV TK hybrid gene was inserted downstream of the *neo* gene to generate pLNLTK (Fig. 1).

Production of amphotropic recombinant retroviruses and infection of TARS-1 cells Amphotropic recombinant viruses were produced and harvested as described previously.[27] TARS-1 (1×10^6 cells) cells were incubated for 2 days in 4 ml of medium consisting of 2 ml of virus supernatant, 2 ml of fresh medium, and 4 μg/ml polybrene. The cells were then cultured in complete medium containing 150 μg/ml G418 for 2 weeks. G418-resistant cell populations infected with LNLTK or the control LN virus were subjected to further studies.

Southern blot and western blot analysis DNA isolation, Southern blotting and probe labeling were performed as described previously.[16] Membranes were hybridized with a labeled HSV-TK or *tax* probe.

Western blotting was performed as described previously.[16] The blots were reacted with anti-p40*tax* monoclonal antibody (mAb) Lt-4,[32] then incubated with peroxidase-labeled goat anti-mouse IgG antibodies. The probed proteins were visualized using the ECL system (Amersham, England).

HSV TK analysis Enzyme assay for quantitating HSV TK activity was performed as described.[27] To discriminate HSV thymidine kinase from cellular thymidine kinase, we used ACV as a substrate for HSV TK.

***In vitro* GCV sensitivity** *In vitro* sensitivity to GCV was determined by colorimetric cell proliferation assay. On day 0, cells were plated on 96-well plates at 1×10^4/well in 100 μl of medium containing various concentrations of GCV (Syntex, Palo Alto, CA). On day 3, 100 μl of fresh medium with GCV was added to each well. On day 5, after addition of MTT (MTT assay kit; Chemicon, Temecula, Canada) to each well, the absorbance was measured using an automatic plate reader (Bio-Rad Laboratories, Richmond, CA) at 570 nm. The percentage growth inhibition was calculated as follows: [1-(absorption of GCV-treated wells/absorption of control wells)] $\times100$. The 50%-inhibitory dose (ID_{50}) of GCV was determined by using curve-fitting parameters.

***In vivo* GCV sensitivity** To establish tumors, retrovirus-infected or parental TARS-1 cells (2×10^7 cells suspended in 0.1 ml of RPMI1640 per rat) were injected subcutaneously into the back of newborn WKA/H rats within 24 h after birth. Rats were divided into 4 groups according to the treatment schedules: LNLTK-infected TARS-1 injection and GCV treatment; LNLTK-infected TARS-1 injection without GCV treatment; TARS-1 injection and GCV treatment; TARS-1 injection without

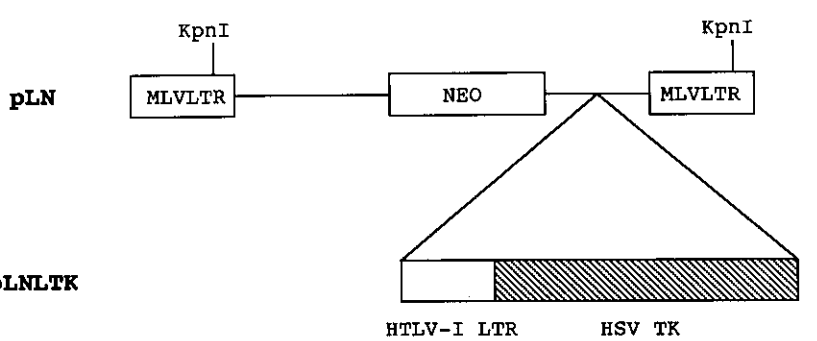

Fig. 1. The structure of the control pLN and pLNLTK (containing the HSV TK gene under the control of the HTLV-I LTR) retroviral vectors. A 2.4-kb fragment of the HTLV-I LTR-HSV TK hybrid gene was inserted into the MLV-based retroviral vector pLN in the forward orientation.

GCV treatment. Each group consisted of 5 rats. Ten days after tumor cell injection, GCV (50 mg/kg) was administered intraperitoneally to the rats once daily for 10 days. The size of the tumor was measured twice weekly with calipers in two dimensions. Tumor volume was estimated by use of the formula $(A^2 \times B)/2$, where B is the smaller dimension, and presented as the mean $\pm$ SD mm^3. The rats were killed at the end of GCV treatment, and histological analysis of tissues at the site of tumor cell inoculation was performed to examine tumor growth.

RESULTS

Infection of TARS-1 cells with recombinant retroviruses carrying the HTLV-I LTR-HSV TK hybrid gene To achieve efficient and selective expression of the HSV TK gene in HTLV-I-infected, *tax*-expressing cells, we generated a hybrid gene in which expression of the HSV TK gene was directed by the HTLV-I LTR and inserted it downstream of the *neo* gene in the pLN retroviral vector (Fig. 1, pLNLTK).

LNLTK and control LN retroviruses prepared from amphotropic producer lines were used for infection of the target cells, TARS-1, an HTLV-I-transformed rat T cell line. The expression of *tax* in TARS-1 cells has been confirmed.[32] After infection with the viruses, the cells were selected in medium with G418. We obtained LNLTK- or LN-infected TARS-1 cells, respectively. Thereafter, genomic DNA was isolated from LNLTK-infected TARS-1, digested with *Kpn* I which cuts the proviral DNA within both MLV LTR, and Southern-blotted with an HSV TK probe. As shown in Fig. 2, a 5.7-kb band, the expected size for the HTLV-I LTR-HSV TK construct, was detected in LNLTK-infected TARS-1 alone. The copy number of this construct was estimated at approximately one copy per cell.

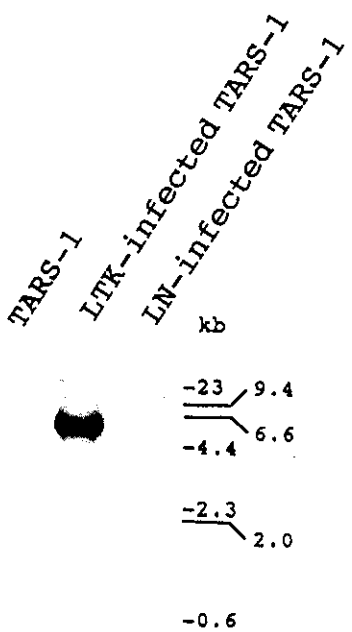

Fig. 2. Southern blot analysis of proviral DNA in the virus-infected TARS-1 lines and the parental TARS-1 line. Genomic DNA (10 μg) was cleaved with *Kpn* I, and Southern blotted with a HSV TK probe.

GCV-mediated selective elimination of LNLTK virus-infected TARS-1 cells *in vitro* To examine HSV TK gene expression in virus-infected TARS-1 cells, we performed an enzyme assay for phosphorylated ^{3}H-ACV. As shown in Fig. 3A, HSV TK activity was hardly detectable in parental TARS-1 cells, as expected. The activity in LNLTK-infected TARS-1 cells was much higher than that in control LN-infected TARS-1 cells. This assay was also performed using ED-40515(−), an HTLV-I-transformed human T cell line. It has been reported that *tax* mRNA is undetectable in cells of this line.[30] As shown in Fig. 3B, HSV TK expression was low in both LNLTK- and LN-infected ED-40515(−) cells, showing that the transduction of the LNLTK retrovirus did not produce TK activity in *tax*-deficient cells. These data demonstrated that *tax*-mediated transactivation of the HTLV-I LTR of HSV TK resulted in a significant increase in HSV TK expression.

To test whether the induction of HSV TK expression in LNLTK-infected TARS-1 cells could confer a toxic phenotype on such cells, virus-infected TARS-1 cells were cultured in various concentrations of GCV and the ID_{50} of GCV was determined by MTT assay. It was more than 100 μM in control LN-infected TARS-1 cells, as well as in parental TARS-1 cells. In contrast, a significant increase in the sensitivity of LNLTK-infected TARS-1 cells to GCV was observed ($ID_{50}=2$ μM); this was clearly a consequence of specific induction of susceptibility to GCV in HSV TK-expressing TARS-1 cells. In similar experiments with virus-infected ED-40515(−) cells, GCV exposure did not inhibit cell growth (data not shown).

GCV-mediated selective elimination of LNLTK virus-infected TARS-1 cells *in vivo* Based on the results of the *in vitro* study, we investigated whether the HSV TK/GCV system in HTLV-I-transformed T cells could also be effective *in vivo*. We injected parental or LNLTK-infected TARS-1 cells (2×10^7 cells per rat) subcutaneously into the back of WKA/H newborn syngeneic rats. The rats were divided into two groups. Ten days later, one group, comprising both TARS-1-injected and LNLTK-infected TARS-1-injected rats, received daily

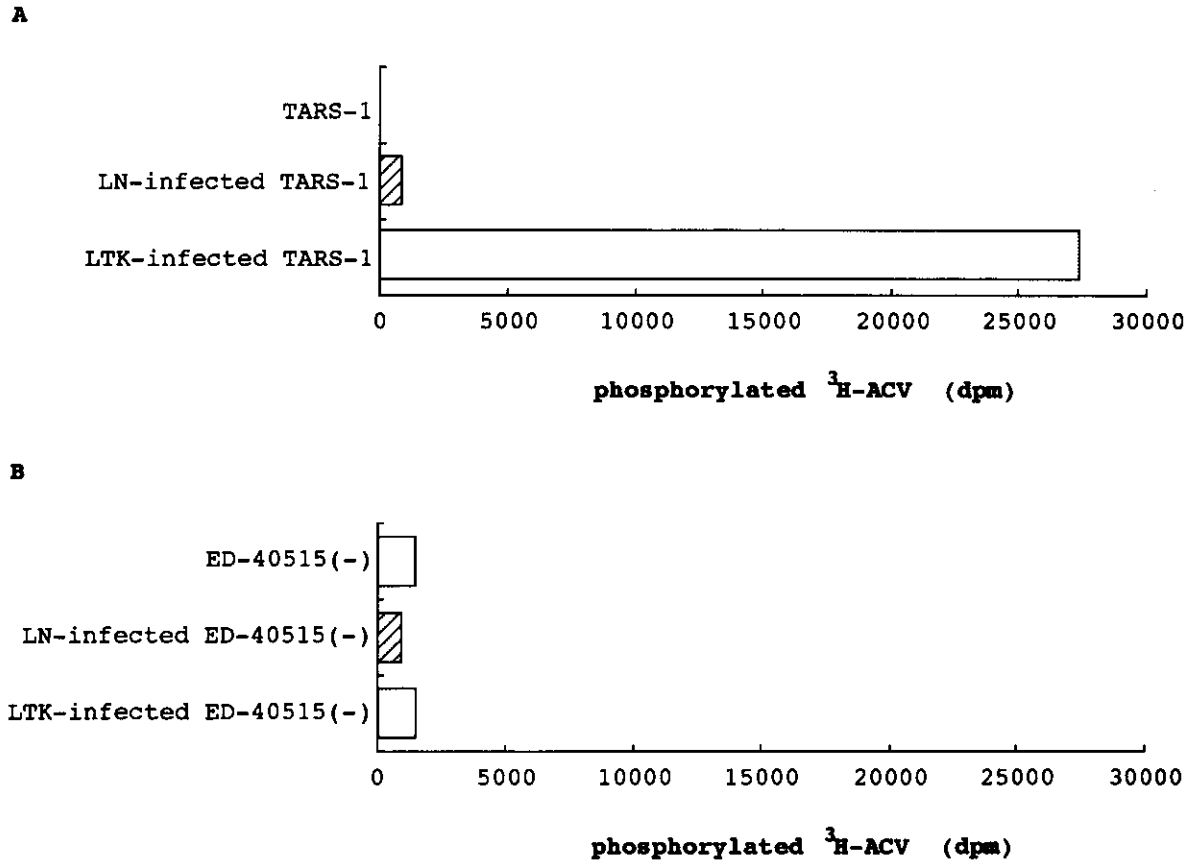

Fig. 3. HSV TK enzymatic assay in parental cells and cells infected with the control virus LN or with the LNLTK virus. The activity was determined using ^{3}H-labeled ACV as a substrate for HSV TK. The amount of phosphorylated ^{3}H-ACV was measured by use of a scintillation counter. A, TARS-1 cells. B, ED-40515(−) cells.

intraperitoneal injections of GCV at 50 mg/kg for 10 consecutive days, whereas the other group was left untreated as a control. As shown in Fig. 4, all the rats without GCV treatment formed progressively growing tumors by 20 days after cell inoculation. The growth of the tumor derived from LNLTK-infected TARS-1 was completely suppressed in 3 of 5 rats by GCV treatment, and the remaining 2 rats appeared to have few residual tumor cells present, whereas all TARS-1 tumors increased in size even after GCV treatment. Similar experiments were performed using the LN-infected TARS-1 line. LN-infected TARS-1 cell growth did not differ from parental TARS-1 cell growth, and administration of GCV had no effect (data not shown). Twenty days after the initial tumor cell inoculation, the rats were killed and examined histologically. Tumors derived from LNLTK-infected TARS-1 without GCV treatment showed a massive proliferation of large lymphoblastic cells morphologically similar to TARS-1 cells (Fig. 5, upper panel). In contrast, the residual LNLTK-infected TARS-1 tumors after GCV treatment consisted of necrotic tissue with inflammatory infiltrates occurring where a few residual tumor cells were present (Fig. 5, lower panel). These findings demonstrated that HTLV-I LTR-HSV TK works specifically in HTLV-I-transformed, *tax*-expressing T cells and kills such cells following GCV treatment *in vivo* as well as *in vitro*.

Characterization of tumors derived from LNLTK virus-infected TARS-1 cells To compare the features of established tumors derived from LNLTK-infected TARS-1 cells *in vivo* with those of these cells growing *in vitro*, we first examined whether the cells proliferating in the rats were the same as the original LNLTK-infected TARS-1 cells by Southern blotting. Genomic DNA was isolated from four LNLTK-infected TARS-1 tumors established independently, digested with *Eco*R I, which cleaves the flanking region of the HTLV-I genome, and Southern-blotted with a *tax* probe. As demonstrated in Fig. 6, exactly the same bands were observed in the four tumors and the original LNLTK-infected TARS-1 cells, indicat-

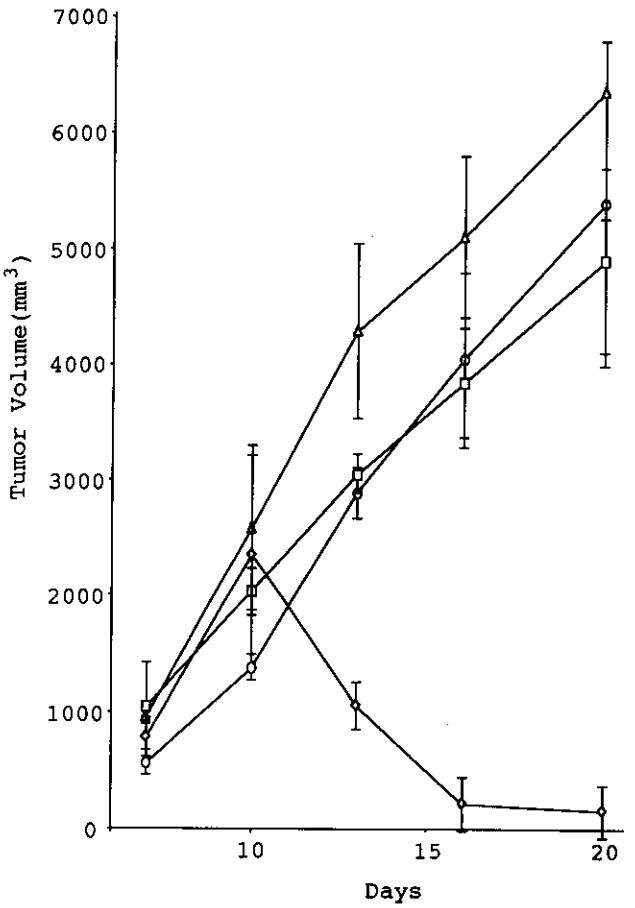

Fig. 4. *In vivo* effect of GCV on the growth of established tumors derived from parental or LNLTK-infected TARS-1 cells in WKA/H rats. The cells were injected subcutaneously into newborn syngeneic rats (2×10^7 per rat). Ten days later, the rats injected with each cell line were treated with GCV at 50 mg/kg once daily for 10 days. ○: TARS-1 and GCV(−); □: TARS-1 and GCV(−); △: LNLTK-infected TARS-1 and GCV(−); ◇: LNLTK-infected TARS-1 and GCV(+). Points, mean; bars, SD.

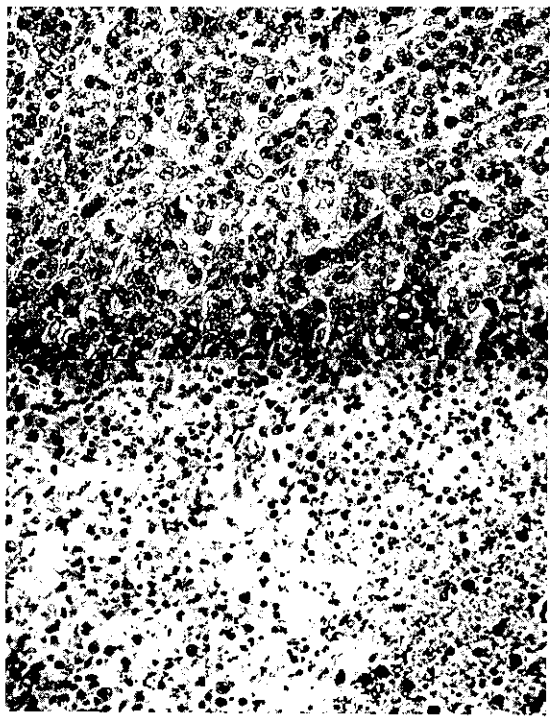

Fig. 5. Histological features of LNLTK-infected TARS-1 tumors in WKA/H rats after completion of GCV treatment. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin/eosin. A section of the tumor without GCV treatment is shown in the upper panel: tumor cells exhibiting marked atypical large lymphoblastic cells grew compactly. A section of the tumor after GCV treatment is shown in the lower panel: there were a few remaining tumor cells, which were largely necrotic, with infiltration of inflammatory cells. Magnification, ×400.

ing that cells in tumors established by injection of LNLTK-infected TARS-1 were identical to the original line. The copy numbers of the *tax* gene for established tumors 1, 2, 3, and 4 were estimated by using a densitometer to be approximately 0.83, 0.91, 0.86, and 0.67 per cell, respectively. The reason for the difference in copy number between LNLTK-infected TARS-1 cells and the four tumors derived from them is unclear. However, this may be because of the sensitivity of Southern blotting.

We next compared the expression levels of *tax* protein in these four tumors with those in the original LNLTK-infected TARS-1 by western blotting (Fig. 7). When we adjusted the loading dose of lysates for each sample on the basis of total protein amount, similar band intensities for *tax* protein (40 kD) were detected in both the samples from the four tumors and the original line. These results suggest that expression of the *tax* gene in LNLTK-infected TARS-1 *in vitro* was not modulated in the tumors derived from LNLTK-infected TARS-1 *in vivo*.

DISCUSSION

The aim of this study was to evaluate the effects of a *tax*-targeted gene therapy approach to the treatment of ATL *in vivo* as well as *in vitro*. For this purpose, we chose TARS-1, an HTLV-I-transformed WKA/H rat T cell line, as the target cells for infection with the retrovirus carrying the HSV TK gene under the control of the HTLV-I LTR.[27] It has been reported that this line is transplantable into newborn syngeneic rats.[28] In our *in*

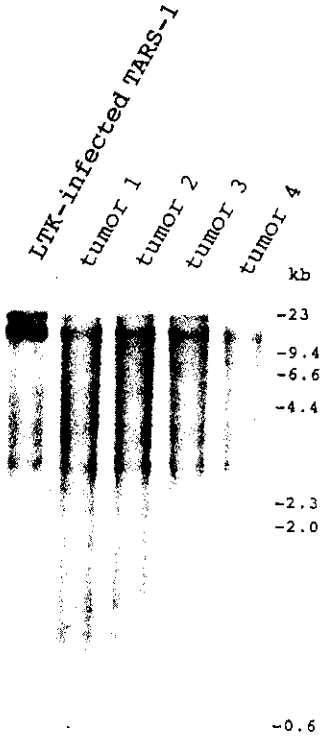

Fig. 6. Southern blot analysis of HTLV-I proviral DNA in LNLTK-infected TARS-1 growing *in vitro* and the four tumors derived from this line *in vivo*. Genomic DNA (10 μg) was digested with *Eco*R I and Southern-blotted with a *tax* probe.

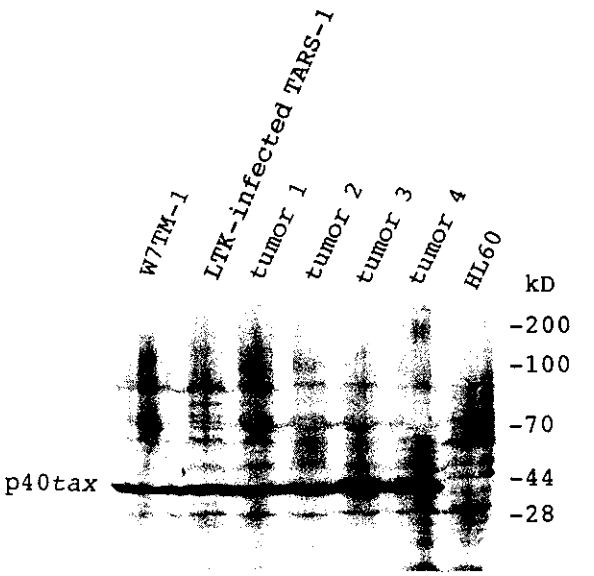

Fig. 7. Western blot analysis of tax protein in LNLTK-infected TARS-1 growing *in vitro* and the four tumors derived from this line *in vivo*. Cell lysates adjusted to equal protein amounts were analyzed with mAb for *tax*. Cell lysates from W7TM-1 and HL60 cells were used as positive and negative controls, respectively.

vitro study, LNLTK virus-infected TARS-1 cells showed high levels of HSV TK expression, but LN virus-infected cells did not show TK activity (Fig. 3A). Furthermore, a significant increase in susceptibility to GCV was found only in LNLTK-infected TARS-1 cells. Selective high levels of HSV TK expression in *tax*-expressing cells were achieved by utilizing the HTLV-I LTR coupled to the HSV TK gene. Several studies have succeeded in the tissue-specific expression of suicide genes by using the promoter element of genes activated only in tumor cells.[26, 33–35] Since the expression of cytotoxic genes should be limited to target cells, gene therapy using tumor-specific promoters is an advantageous approach to cancer treatment.

We observed dramatic regression of the established tumors derived from LNLTK virus-infected TARS-1 cells after GCV treatment of the rats. In 3 of the 5 rats treated, complete regression of the tumor was observed. The other rats had a few remaining tumor cells in the region of the tumor, which was largely necrotic, and they were surrounded by inflammatory cells. This observation suggests that *in vivo* GCV-induced killing of HSV TK-expressing tumor cells is not only the result of a direct effect of GCV on susceptible cells, but also involves host antitumor immune response. Chen *et al.*[36] reported that combination therapy with HSV TK and mouse IL-2 genes in a liver metastasis model of colon carcinoma resulted in significant tumor regression, as well as induction of antitumoral immunity. Santodonato *et al.*[37] also reported a gene therapy for established tumors involving combined treatment with tumor cells expressing both interferon-α and HSV TK. Although we did not design a genetic approach involving cytokine genes in this study, combination gene therapy, as indicated by these reports, may be more useful for tumor eradication than suicide gene therapy alone.

We then examined the properties of established tumors derived from LNLTK-infected TARS-1 cells *in vivo* by comparison with those of these cells growing *in vitro*. Southern blot analysis revealed that the cells were identical (Fig. 6). In addition, *tax* gene expression was not modulated in the tumors (Fig. 7), suggesting that sufficient *in vivo* expression of the *tax* gene induced successful tumor regression. Previously, Oka *et al.*[38] reported the

establishment of a cell line with enhanced expression of the *tax* gene from peripheral blood mononuclear cells of a rat in which lymphoma/leukemia-like disease had been induced by injection of TARS-1 cells. The nature of the difference in the levels of *in vivo tax* expression between their and our results is unknown at present. However, it is possible that this difference is related to the areas in which tumor cells proliferate *in vivo*. It is likely that, following systemic administration of tumor cells, gene(s) expression of the injected cells is influenced by various factors during circulation in the body. A change in gene expression, like that observed by Oka and his colleagues, may thus allow further transformation in tumor cells. However, additional study would be required to prove this.

Although we designed an approach to *tax*-targeted gene therapy for ATL, expression levels of the *tax* gene have been reported to be generally limited in peripheral ATL cells.[39] Ohshima *et al.*[40] have detected *tax* mRNA in 10–30% of nuclear cells of the lymph nodes of ATL patients, using reverse transcriptase polymerase chain reaction *in situ* hybridization. Since more cycling ATL cells are found in lymph nodes than in peripheral blood,[41] this finding suggests that *tax*-mediated elimination of ATL cells may occur mostly in the lymph nodes. Alternatively, several lines of evidence support the idea that *tax* is expressed in the early stage of ATL development.[42,43] An attractive strategy for therapeutic intervention against ATL would be to eliminate the initial pool of HTLV-I-infected T cells and thus prevent disease progression. To evaluate the potential of this strategy by using our HTLV-I LTR-HSV TK *in vivo*, the development of vector systems that can produce high titers of viruses capable of efficient expression in target cells is naturally indispensable. The efficacy of such an approach could be tested in a suitable animal model, such as *tax* carrier rats.[16]

In conclusion, we have demonstrated that the HTLV-I LTR-HSV TK system can cause selective elimination of HTLV-I-transformed and *tax*-expressing T cells *in vivo*. Thus, our study may provide a rationale for clinical gene therapy against ATL.

ACKNOWLEDGMENTS

We thank Dr. A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) for generously providing materials.

(Received December 24, 1996/Accepted February 21, 1997)

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