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Serial Transplantation of Adult T Cell Leukemia Cells into Severe Combined Immunodeficient Mice

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The precise mechanism of the neoplastic cell growth of adult T cell leukemia (ATL) still remains unclear. In the present study, we have succeeded in serial transplantation of ATL cells from a patient into severe combined immunodeficient (SCID) mice. In this model, we found that only a leukemic cell clone from an ATL patient could be successively transplanted into SCID mice, although it was difficult to maintain leukemic cell clones *in vitro*, suggesting that the microenvironment provided by SCID mice is suitable for leukemic cell growth. We could not detect human T cell leukemia virus type I (HTLV-I) mRNA or interleukin 2 (IL-2) mRNA in either the tumor cells growing in mice or the original leukemic cells. Thus, it appears that neither HTLV-I viral expression nor the IL-2 autocrine mechanism is directly involved in the neoplastic cell growth of fresh ATL cells as well as HTLV-I-infected cell lines, at least in SCID mice. In addition, we could passage frozen cells and obtain a large number of expanded leukemic cells in this model. Such a serial transplantation model, which can avoid the changes in the nature of leukemic cells that are frequently observed in *in vitro* culture, and which can propagate leukemic cell clones, would be very suitable not only to study the mechanism of neoplastic cell growth, but also to test potential therapeutic agents for ATL.

Key words: Adult T cell leukemia — Human T cell leukemia virus type I — Severe combined immunodeficient mice — Serial transplantation — Interleukin 2

Adult T cell leukemia (ATL) has characteristic clinical features[1,2] and was shown to be caused by a human retrovirus, termed human T cell leukemia virus type I (HTLV-I).[3–5] The clarification of the structure of HTLV-I[6] and extensive studies of the viral gene products have led to a good understanding of the mechanism of the development of ATL, as well as the neoplastic cell growth. It has been shown that Tax, one of the viral gene products, is capable of transactivating many cellular genes, including those of interleukin 2 receptor (IL-2R) α chain (Tac, CD25), IL-2 and c-*fos*.[7–12] Some of these abnormally expressed genes may play a key role in the leukemogenesis of ATL. However, viral gene expression is usually undetectable or at a very low level in fresh ATL cells.[13,14] These data suggest that viral gene products may be involved in polyclonal expansion of HTLV-I-infected cells in an early stage of ATL, but may no longer be involved in the leukemic cell growth of ATL. The precise mechanism of the neoplastic cell growth in the leukemic stage of the disease still remains unclear. Since animal models for the analysis of *in vivo* cell proliferation of ATL cells have not been reported, to our knowledge, and most of the HTLV-I-infected cell lines established from ATL patients *in vitro* are derived from non-leukemic clones,[15] we and others developed an *in vivo* cell proliferation model of leukemic cells from ATL patients using severe combined immunodeficient (SCID) mice,[16,17] and demonstrated that leukemic cell clones could predominantly and selectively proliferate in SCID mice. A study on the cell growth of HTLV-I-infected cell lines in SCID mice revealed that neither HTLV-I viral expression nor the IL-2 autocrine mechanism was directly involved in the neoplastic cell growth of these cell lines in SCID mice.[18] However, we have not yet examined the expression of HTLV-I and IL-2 in fresh leukemic cells proliferating in SCID mice. Since we are concerned about the changes in the nature of HTLV-I-infected cell lines that frequently occur during long-term maintenance *in vitro*, we have been trying to develop a model suitable for serial transplantation of leukemic cells from an ATL patient. Here we present such a model and characterize the cell growth in SCID mice.

MATERIALS AND METHODS

Mice Immune-deficient SCID (CB17scid/scid) mice were obtained from Nihon Clea Inc. (Tokyo). The mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Institute for Virus Research, Kyoto University.

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Preparation of the cells injected into SCID mice A lymph node (LN) from an ATL patient was obtained with informed consent. The preparation of LN cells (LNC) was performed as previously described.[16]

Injection of the cells into SCID mice LNC prepared as above, that were depleted of B cells and monocytes or that were not treated, were intraperitoneally injected into SCID mice pretreated with 1 mg/mouse of TM-β1, an antimurine IL-2R β chain monoclonal antibody (mAb) (a kind gift from Dr. M. Miyasaka),[19] to facilitate the engraftment of ATL cells by abrogating the function of NK cells.[20] In addition, some mice were intraperitoneally injected with 20 ng/mouse/day of human recombinant IL-2 (a kind gift from Shionogi & Co., Ltd., Osaka) every day from day 1 to day 60 after the inoculation of the cells. Mice were killed and autopsied when they showed signs such as weight loss, lethargy, ruffled fur and a hunched posture. The tumors recovered from the mice were cut into small pieces and resuspended in RPMI 1640 medium containing 10% FCS (Bioproducts, Walkersville, MD). The suspension was passed through a metal mesh for preparing single cell suspensions. Then, the tumor cell suspensions were serially transplanted into SCID mice for passage study. Portions of tumor cell suspensions were resuspended in Cell-Banker (Nippon Zenyaku, Fukushima). Resuspended cells were transferred to cryovials and were precooled in a -80°C freezer overnight. They were then kept frozen in liquid nitrogen until use. After thawing, the cells were resuspended in RPMI 1640 medium containing 10% FCS, and were injected into SCID mice in the same way as fresh tumor cells.

Flow cytometric analysis Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAbs to human CD2 (Leu5b), CD3 (Leu4), CD4 (Leu3a), and CD8 (Leu2a) were purchased from Becton Dickinson (San Jose, CA). The mAb to the α chain of human IL-2 R, anti-Tac,[21,22] was used as FITC-conjugated IgG. Cell suspensions were washed in Hanks' balanced salt solution containing 1% BSA and 0.1% NaN_3 and stained with saturating amounts of mAbs as previously described.[16] The stained cells were analyzed on a FACScan (Becton Dickinson & Co., Mountain View, CA).

Southern blot hybridization High-molecular-weight DNA was prepared from the cells or tumors by proteinase K digestion followed by phenol-chloroform extraction. Five micrograms of DNA was then digested with the restriction enzyme *Eco*R I, *Bam*H I, *Hin*d III, or *Xba* I (Toyobo, Osaka), electrophoresed in a 0.7% agarose gel, and blotted onto a Hybond-N membrane (Amersham International, Buckinghamshire, England). The membranes were hybridized and washed as previously described,[18] and then exposed to X-ray film. Two probes were used in the present study. One was a 0.96 kb *Acc* I/*Sma* I fragment of HTLV-I pX region[23] and the other was a *Hin*d III/*Eco*R I fragment that contained human T cell receptor (TCR) $C\beta_1$ gene.[24]

Reverse transcriptase-polymerase chain reaction (RT-PCR) Single-strand cDNA was synthesized in a volume of 20 μl as previously described.[18] The cDNA preparation was then diluted to 100 μl. Two and a half microliters of cDNA was amplified in a volume of 25 μl in the presence of 800 n*M* 5′ and 3′ primers, 200 μM dNTPs, 1U Taq polymerase (Takara Shuzo, Otsu), and PCR buffer containing 10m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, and 2.5 m*M* $MgCl_2$. The PCR primers specific for IL-2 were purchased from Clontech (Palo Alto, CA). The primers (RPX3 and RPX4) were used to amplify HTLV-I pX (tax/rex) mRNA as previously described.[14] For β-actin amplification, the human-specific primers were used.[18] The amplification was performed in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT) for 25 or 30 cycles. The cycling conditions were 1 min at 94°C for denaturation, 1 min at 60°C for annealing, and 2 min at 72°C for elongation. To rule out false-positive results due to DNA contamination, we ran negative controls without RT. The amplified products were then visualized after electrophoresis through 1.5% agarose gels by staining with ethidium bromide.

Histopathology The tumors were fixed in 10% formalin and processed to 4-mm paraffin wax-embedded sections for staining with hematoxylin and eosin (HE).

RESULTS

Characteristics of the patient with ATL LN from a 65-year-old woman with lymphoma-type ATL was used in the present study. The details of this patient were reported elsewhere.[25]

Histopathologic findings of mice engrafted with ATL cells LNC that were depleted of B cells and monocytes, or that were not treated, were injected into three SCID mice with or without daily injection of IL-2 after the inoculation (Table I). All mice were found to have similar tumors and to show the sign of lethargy within 3 weeks after the inoculation. Histologic examination revealed the infiltration of tumor cells mainly in lymph nodes in all mice. There were no significant differences in the infiltration pattern of tumor cells among these mice.

Passage studies The tumor cells recovered from one of the mice at the 1st passage were serially transplanted into SCID mice. This procedure was repeated successively to the 14th passage. The histologic features of the tumors hardly changed through the serial passages (Fig. 1). We could passage the tumor cells kept frozen in liquid nitrogen until use, as well as the fresh tumor cells, in SCID mice (data not shown).

Flow cytometric analysis To determine whether the cells

Table I. Transplantation of Fresh Leukemic Cells from a Lymphoma-type ATL Patient into SCID Mice

Cells injected ($\times 10^7$)	Depletion of B cells	Treatment of mice with		Tumor growth/ No. of mice	Survival (days)
		TM-β1	IL-2		
2.2	+	+	+	1/1[a)]	18
2.2	+	+	−	1/1	21
4.8	−	+	+	1/1	21

a) We previously reported the details of this mouse.[16)]

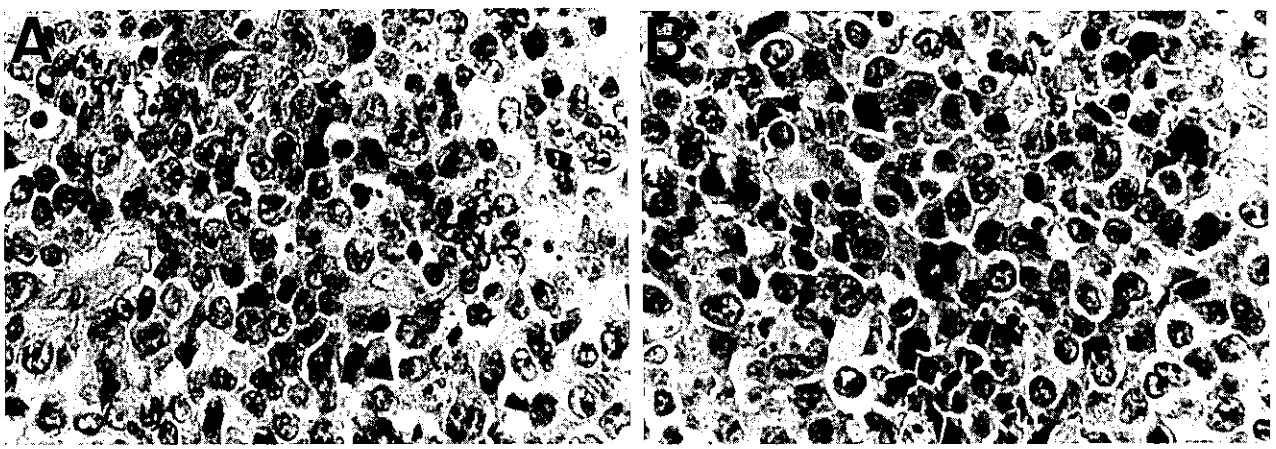

Fig. 1. Histologic findings of a lymph node from the patient and a tumor from a mouse engrafted with ATL cells. A, Section of an original lymph node showing the histologic characteristics of a diffuse large cell lymphoma (original magnification ×40, HE staining). B, Section of a tumor from a mouse at the 9th passage showing the same histologic characteristics as seen in A (original magnification ×40, HE staining).

Table II. Cell Surface Phenotype of Tumor Cells Recovered from the Mice Engrafted with ATL Cells

	% Positive cells						Treatment of mice with	
	CD2	CD3	CD4	CD8	Tac	HLA-DR	IL-2	TM-β1
(primary ATL cells)	93	93	89	3	72	94		
1st passage	96	95	88	2	90	95	+	+
2nd passage	98	NE	96	NE	93	97	+	+
3rd passage	91	89	91	0	79	94	−	+
10th passage	NE	NE	95	0	71	86	−	−
14th passage	99	98	99	0	94	95	−	−

Abbreviation: NE, not examined.

proliferating in the mice were the same clone as the original leukemic cells, we examined the cell surface phenotype of the cells recovered from the tumors. As shown in Table II, the cells obtained from the tumors consisted predominantly of human T cells; the phenotype of these cells was the same as that of the original leukemic cells and did not change through serial passages in SCID mice. The tumor cells were hardly detectable in the peripheral blood from the mice by flow cytometric analysis.

DNA analysis To confirm that the cells proliferating in SCID mice were the same clone as the original leukemic cells, Southern blot hybridization analysis using HTLV-I pX and $C\beta_1$ of human TCR as probes was performed. As

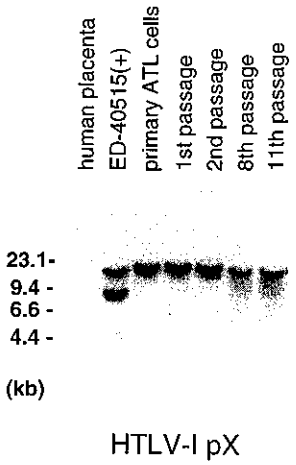

Fig. 2. Southern blot analysis of HTLV-I proviral DNA in original leukemic cells and the tumor cells recovered from the mice. High-molecular-weight DNA extracted from the cells was digested with *Eco*R I, and the blots were hybridized with HTLV-I pX probe. The positive control for detection of HTLV-I viral integration was ED-40515 (+), an HTLV-I-infected cell line from an ATL patient.

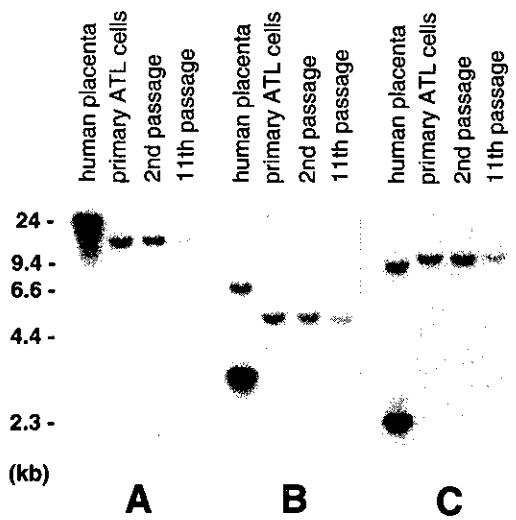

Fig. 3. Southern blot analysis of human TCR β chain gene rearrangement in original leukemic cells and the tumor cells recovered from the mice. High-molecular-weight DNA extracted from the cells was digested with *Bam*H I (A), *Hin*d III (B), or *Xba* I (C), and the blots were hybridized with $C\beta_1$ of human TCR probe.

shown in Fig. 2 and Fig. 3, the clear bands were detected at the same positions both in the tumor cell and the original cell DNA, confirming their clonal identity and

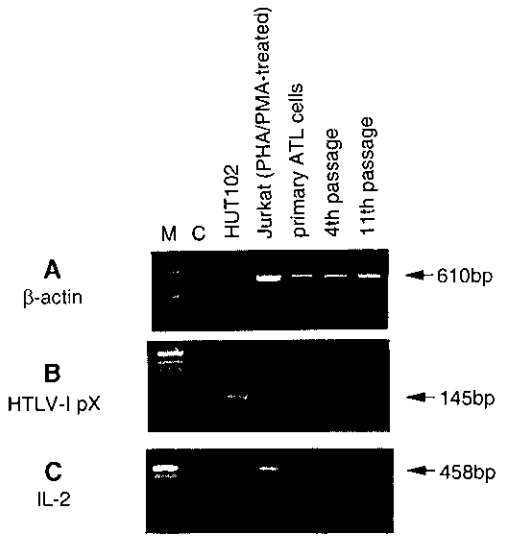

Fig. 4. Determination of the expression of HTLV-I and IL-2 mRNAs by RT-PCR analysis in original leukemic cells and tumor cells recovered from the mice. PCR for 25, 30, or 30 cycles was performed for the detection of human β-actin, HTLV-I pX (tax/rex), and IL-2 mRNAs, respectively. C indicates the negative control (RT reaction without RNA). As molecular weight markers (M), $\phi\chi174$ DNA digested with *Hinf* I (A) and pUC9 DNA digested with *Hpa* II (B and C) were used.

that only the cells of leukemic origin were successively transplanted into SCID mice.

Expression of HTLV-I and IL-2 mRNAs To test the possible involvement of HTLV-I or the IL-2/IL-2R system in the *in vivo* proliferation, we studied the expression of HTLV-I and IL-2 mRNAs in the tumor cells and original leukemic cells. RT-PCR analysis showed that neither HTLV-I pX (tax/rex) nor IL-2 mRNA was detected in the tumor cells growing *in vivo*, as was also the case in the original leukemic cells (Fig. 4). As for the sensitivity of RT-PCR in this study, both tax/rex mRNA in HUT102 cell RNA and IL-2 mRNA in stimulated Jurkat cell RNA were still detectable at a 100-fold dilution with negative control cell RNA (data not shown).

DISCUSSION

In the present study, we have established a serial transplantation model of leukemic cells from a lymphoma-type ATL using SCID mice. We have many HTLV-I-infected cell lines available for the study of HTLV-I and related diseases. However, most of them have been demonstrated to be derived from non-leukemic cell clones.[15] Moreover, it was reported that changes of the cellular genes and cell surface molecules occurred

during maintenance *in vitro*, such as upregulation of HTLV-I viral expression,[26, 27] and loss of IL-2R γ chain[28] and some adhesion molecules (unpublished data). Although the *in vitro* studies using such cell lines have provided useful information concerning cell growth characteristics and phenotype, it is difficult to analyze the interaction between leukemic cells and their microenvironment *in vivo* by means of *in vitro* studies. The development of successive transplantation of leukemic cells into SCID mice would enable us easily to address at least some of these issues and would facilitate studies on the mechanism of the growth of leukemic cells from ATL patients.

The histologic features, the HTLV-I provirus integration site and the cell surface phenotype of tumor cells passaged in SCID mice remained consistent as determined by histopathologic analysis, Southern blot analysis, and flow cytometric analysis. Furthermore, the tumorigenicity of cells successively transplanted into SCID mice does not appear to have changed at least up to the 14th passage. These results suggest that the microenvironment provided by SCID mice is suitable for leukemic cell growth, and is capable of preventing the changes in the nature of leukemic cells that are frequently observed in *in vitro* culture.

On the other hand, HTLV-I mRNA could not be detected in the original leukemic cells or the tumor cells growing in mice, in contrast to its upregulation during *in vitro* short-term culture.[26, 27] Our results strongly indicate that HTLV-I viral expression is not needed for the neoplastic cell growth of fresh ATL cells in SCID mice. This is consistent with our previous data on HTLV-I-infected cell lines.[18] With regard to the involvement of the IL-2/IL-2R system, we previously showed that the IL-2 autocrine mechanism was not directly involved in the neoplastic cell growth of HTLV-I-infected cell lines in SCID mice.[18] However, it has been unclear whether IL-2 is required for the proliferation of fresh ATL cells in SCID mice. In the present study, the leukemic cells could be successively engrafted in SCID mice without the supply of exogenous IL-2, and we could not detect the expression of IL-2 mRNA in the tumor cells proliferating in such mice or in the original leukemic cells. The tumor cells recovered from the 1st to 5th passages could not be maintained *in vitro* even in the presence of IL-2 (unpublished data). In addition, murine IL-2 cannot promote cell growth of human T cells. Overall, it is suggested that the IL-2 autocrine mechanism is not directly involved in the neoplastic cell growth of fresh ATL cells, at least in SCID mice. Since we found no direct involvement of the IL-2 autocrine mechanism in tumor cell growth in SCID mice, we have not been treating mice with IL-2 in this model.

Finally, we could passage not only fresh tumor cells, but also tumor cells kept frozen in liquid nitrogen until use in SCID mice, and easily obtain a large number of expanded leukemic cells growing *in vivo*. In view of the other advantages of an *in vivo* cell proliferation model, such as the feasibility of testing tissue toxicity and the pharmacokinetics of therapeutic agents, the model presented here is expected to be useful not only to study the mechanism of neoplastic cell growth, but also to develop an effective treatment for ATL.

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