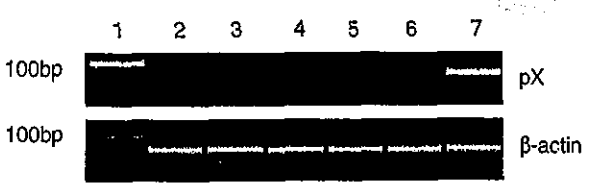

Figure 9 Detection of the *env-pX* transgene by nested polymerase chain reaction (PCR). One thousand lymphocytes accumulating at arthritic joints were dissected from formalin-fixed, paraffin-embedded sections, and then nested PCR was performed according to methods described by Fugo *et al.* (2002). Lane 1: 100 bp molecular marker. Lanes 2 and 3: samples from t/wB/CII rats at 4 weeks after type II collagen (CII) immunization. Lanes 4–6: samples from t/wB/CII rats at 16 weeks after CII immunization. Lane 7: a positive control sample from naturally occurred arthritis in env-pX rats.

weeks after CII immunization. At 16 weeks after immunization, the score of t/wB/CII rats (2.25 ± 1.58) was significantly higher than that of w/tB/CII rats (0.60 ± 0.70) ($P = 0.0181$). There was no significant difference regarding the proliferation of stroma (Figure 7b), fibrosis (Figure 7c) or synovial lining cells (Figure 7d). Representative photographs of arthritic joints at 4 and 16 weeks after CII immunization are shown in Figure 8.

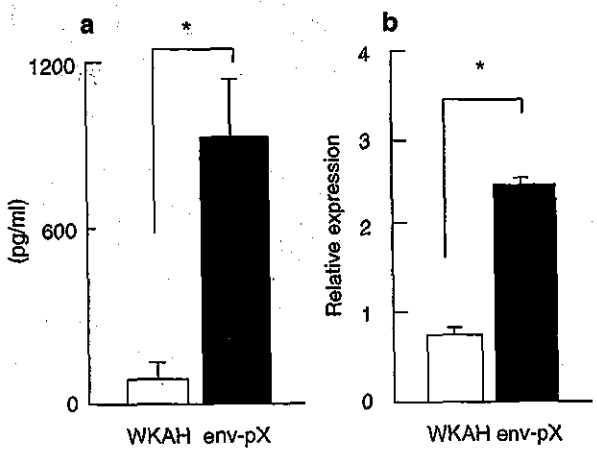

Figure 10 Production of interleukin-6 (IL-6). Rat synovial cells (5×10^5) were cultured in 9 cm dishes. Forty-eight hours later, culture supernatants were harvested, and IL-6 concentration was measured using enzyme-linked immunosorbent assay (ELISA) (a). Expression of IL-6 mRNA in the cells was examined by quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) (b) according to methods described by Ishizu *et al.* (2003). Data are shown as a relative value standardized by the expression of a housekeeping glyceraldehyde 3-phosphate dehydrogenase gene. $^*P < 0.01$.

The env-pX transgene was not detected in lymphocytes accumulating at arthritic joints of t/wB/CII rats

In the BMC transfer experiment, it appears that a few lymphocytes of the recipient remained. Therefore, it is critical to determine whether the lymphocytes accumulating at arthritic joints of t/wB/CII rats were derived from donor WKAH rats or the recipient env-pX rats. Nested PCR showed that the *env-pX* transgene was detected in microdissected lymphocytes of affected joints of t/wB/CII rats at neither 4 nor 16 weeks after CII immunization (Figure 9).

In vitro analysis of cytokine production in synovial cells

Cytokine productivity in synovial cells isolated from env-pX rats before they developed arthritis was examined using ELISA kits. Data were compared with those from synovial cells from WKAH rats. Among the inflammatory cytokines IL-1α, IL-1β, IL-2, IL-6, TNF-α and IFN-γ, the concentration of IL-6 in culture supernatants of env-pX synovial cells was significantly higher than that of WKAH cells (Figure 10a). All other cytokines examined were below the detection level of ELISA kits (data not shown). Quantitative real-time RT-PCR confirmed that the mRNA expression of IL-6 increased in env-pX synovial cells compared with that in WKAH cells (Figure 10b).

Discussion

With CII immunization, env-pX transgenic rats showed a higher response than wildtype WKAH rats. Peripheral lymphocytes of env-pX rats were shown to be readily activated *in vitro* (Nakamaru *et al.* 2001). In line with the findings, lymph node cells from CII-immunized env-pX rats showed significantly higher responses against CII than those from CII-immunized WKAH rats. Thus, we hypothesized that activation-prone lymphocytes carrying the *env-pX* transgene may be critical for the severity and chronicity of arthritis induced by CII immunization in env-pX rats. To test the hypothesis, reciprocal BMC transfers were performed between env-pX rats before manifesting arthritis and WKAH rats, followed by immunization with CII. As expected, a severe degree of arthritis was induced in WKAH rats transplanted with env-pX BMC (w/tB/CII rats). However, inflammatory cells accumulating at arthritic joints decreased time dependently in these rats. On the other hand, inflammatory cell infiltration persisted in env-pX rats transplanted with WKAH BMC (t/wB/CII rats), although the degree was less than that of w/tB/CII rats at an early phase after CII immunization. These findings suggest that BMCs carrying the *env-pX* transgene are associated

with the severity of arthritis, whereas articular tissues rather than the BMCs carrying the transgene may be implicated in the prolongation of arthritis in env-pX rats.

In t/wB/CII experiments, the possibility that radiation-resistant env-pX lymphocytes expanded at arthritic lesions at 16 weeks after CII immunization had to be considered. Using nested PCR, we determined whether env-pX lymphocytes increased in the population of inflammatory cells. The *env-pX* transgene was detected in the microdissected lymphocytes of arthritic joints of t/wB/CII rats at neither 4 nor 16 weeks after CII immunization, whereas the transgene was detected in the positive control samples. These findings suggest that articular tissues carrying the *env-pX* transgene may prolong the infiltration of WKAH lymphocytes than induce alteration of cell populations from WKAH to env-pX lymphocytes.

Joints are composed of various tissues, including synovium, vessels, cartilage and the bones. These tissues are considered to be also involved in the pathogenesis of arthritis (Shiozawa & Tokuhisa 1992). There was no significant difference in growth in tissue-culture dishes between env-pX and WKAH synovial cells (data not shown), which corresponds to *in vivo* findings that proliferation of synovial stroma was not significantly different between w/tB/CII and t/wB/CII rats. However, IL-6 production was selectively detected at a higher level in the culture supernatant of synovial cells that had been isolated from env-pX rats before they developed arthritis than in WKAH-derived cells. IL-6 plays a key role in the pathogenesis of arthritis in various models (Alonzi *et al.* 1998; de Hooge *et al.* 2000) and is also involved in T-cell activation and proliferation, generation of cytotoxic T-cells and leucocyte recruitment at inflammation sites (Romano *et al.* 1997). In addition, Tax encoded by HTLV-I *pX* gene upregulates the expression of IL-6 (Aono *et al.* 1998). Therefore, we suggest that IL-6 derived from env-pX synovial cells may be involved in the prolongation of arthritis in env-pX rats.

Constitutive transcription of the *IL-6* gene was demonstrated in human fibroblast-like synoviocytes derived from a patient with rheumatoid arthritis (Miyazawa *et al.* 1998). The endogenous upregulation of the *IL-6* gene in rheumatoid synoviocytes was mediated by spontaneous activation of transcription factors, including nuclear factor kappa B (NFκB). Although the aetiology of rheumatoid arthritis in humans has not been revealed, activation of NFκB and subsequent upregulation of the *IL-6* gene in the synovial tissues may be critically involved in the pathogenesis. Because HTLV-I Tax activates NFκB (Johnson *et al.* 2001), env-pX rats are suitable models for human rheumatoid arthritis in which NFκB and its downstream IL-6 are activated in synovial tissues.

In summary, env-pX rats seem to be a model of arthritis in which not only lymphocytes but also articular tissues may play critical roles in the pathogenesis of arthritis. The relationship between the chronicity of env-pX arthritis and IL-6 derived from synovial cells carrying the *env-pX* transgene is worthy of further investigation.

Acknowledgements

We thank Ms C. Sudo, Ms M. Tateyama and Mr K. Nakase for technical assistance and Ms M. Ishizaka for secretarial services. This work was supported by grants from the Ministries of Education, Culture, Sports, Science and Technology and Health, Labour and Welfare of Japan.

References

Alonzi T., Fattori E., Lazzaro Z. *et al.* (1998) Interleukin 6 is required for the development of collagen-induced arthritis. *J. Exp. Med.* 187, 461–468.

Aono H., Fujisawa K., Hasumuna T. *et al.* (1998) Extracellular human T cell leukemia virus type 1 tax protein stimulates the proliferation of human synovial cells. *Arthritis Rheum.* 41, 1995–2003.

Arima N. & Tei C. (2001) HTLV-I Tax related dysfunction of cell cycle regulators and oncogenesis of adult T cell leukemia. *Leuk. Lymphoma* 40, 267–278.

de Hooge A.S., van de Loo F.A., Arnts O.J., van den Berg W.B. (2000) Involvement of IL-6, apart from its role in immunity, in mediating a chronic response during experimental arthritis. *Am. J. Pathol.* 157, 2081–2091.

Fugo K., Ishizu A., Ikeda H. *et al.* (2002) The role of the thymus in development of necrotizing arteritis in transgenic rats carrying the env-pX gene of human T-cell leukemia virus type-I. *Am. J. Pathol.* 161, 755–761.

Gessain A., Barin F., Vernant J.C. *et al.* (1985) Antibodies to human T-lymphotropic virus type-I in patients tropical spastic paraparesis. *Lancet* 2, 407–409.

Higuchi M., Ishizu A., Ikeda H. *et al.* (2003) Functional alteration of peripheral $CD25^+CD4^+$ immunoregulatory T cells in a transgenic rat model of autoimmune diseases. *J. Autoimmun.* 20, 43–49.

Ishizu A., Tsuji T., Abe A. *et al.* (2003) Transduction of dominant negative ATF-1 suppresses the pX gene expression in joint fibroblastic cells derived from HTLV-I transgenic rats. *Exp. Mol. Pathol.* 74, 309–313.

Johnson J.M., Harrod R., Franchini G. (2001) Molecular biology and pathogenesis of the human T-cell leukaemia/lymphotropic virus type-1 (HTLV-1). *Int. J. Exp. Med.* 82, 135–147.

Kayashima K., Koga T., Onoue K. (1976) Role of T lymphocytes in adjuvant arthritis. I. Evidence for the regulatory function of thymus-derived cells in the induction of the disease. *J. Immunol.* 117, 1878–1882.

Kikuchi K., Ikeda H., Tsuchikawa T. *et al.* (2002) A novel animal model of thymic tumour: development of epithelial thymoma in transgenic rats carrying human T lymphocyte virus type I pX gene. *Int. J. Exp. Pathol.* 83, 247–255.

Koizumi F. (1999) Synovitis in rheumatoid arthritis: scoring of characteristic histopathological features. *Pathol. Int.* 49, 298–304.

LaGrenade L., Hanchard B., Fletcher V., Cranston B., Blattner W. (1990) Infective dermatitis of Jamaican children: a marker for HTLV-I infection. *Lancet* 336, 1345–1347.

Miyazawa K., Mori A., Yamamoto K., Okudaira H. (1998) Constitutive transcription of the human interleukin-6 gene by rheumatoid synoviocytes: spontaneous activation of NF-kappaB and CBF1. *Am. J. Pathol.* 152, 641–644.

Mochizuki M., Watanabe T., Yamaguchi K. *et al.* (1992) HTLV-I uveitis: a distinct clinical entity caused by HTLV-I. *Jpn. J. Cancer Res.* 83, 236–239.

Nakamaru Y., Ishizu A., Ikeda H. *et al.* (2001) Immunological hyperresponsiveness in HTLV-I LTR-env-pX transgenic rats: a prototype animal model for collagen vascular and HTLV-I-related inflammatory diseases. *Pathobiology* 69, 11–18.

Nishioka K., Maruyama I., Sato K., Kitajima I., Nakajima Y., Osame M. (1989) Chronic inflammatory arthropathy associated with HTLV-I. *Lancet* 1, 441.

Osame M., Usuku K., Izumo S. *et al.* (1986) HTLV-I associated myelopathy, a new clinical entity. *Lancet* 1, 1031–1032.

Poiesz B.J., Ruscetti F.W., Gazdar A.F., Bunn P.A., Minna J.D., Gallo R.C. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* 77, 7415–7419.

Romano M., Sironi M., Toniatti C. *et al.* (1997) Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* 6, 315–325.

Shiozawa S. & Tokuhisa T. (1992) Contribution of synovial mesenchymal cells to the pathogenesis of rheumatoid arthritis. *Semin. Arthritis Rheum.* 21, 267–273.

Sugaya T., Ishizu A., Ikeda H. *et al.* (2002) Clonotypic analysis of T cells accumulating at arthritic lesions in HTLV-I env-pX transgenic rats. *Exp. Mol. Pathol.* 72, 56–61.

Sugimoto M., Nakashima H., Watanabe S. *et al.* (1987) T-lymphocyte alveolitis in HTLV-I-associated myelopathy. *Lancet* 2, 1220.

Vernant J.C., Buisson G., Magdeleine J. *et al.* (1988) T lymphocyte alveolitis, tropical spastic paraparesis, and Sjögren's syndrome. *Lancet* 1, 177.

Yamada S., Ikeda H., Yamazaki H. *et al.* (1995) Cytokine-producing mammary carcinomas in transgenic rats carrying the pX gene of human T-lymphotropic virus type I. *Cancer Res.* 55, 2524–2527.

Yamazaki H., Ikeda H., Ishizu A. *et al.* (1997) A wide spectrum of collagen vascular and autoimmune diseases in transgenic rats carrying the env-pX gene of human T lymphocyte virus type I. *Int. Immunol.* 9, 339–346.

Yamazaki H., Kunisada T., Ishizu A. *et al.* (1998) Promotion of early osteogenesis and B lymphopoiesis in the bone marrow of transgenic rats with the env-pX gene of human T-cell lymphotropic virus type I. *Oncogene* 17, 2955–2960.

Yoshida M., Miyoshi I., Hinuma Y. (1982) Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA* 79, 2031–2035.

Laboratory Investigation (2004) 84, 245–252

www.laboratoryinvestigation.org

Hematopoietic progenitor cells as possible origins of epithelial thymoma in a human T lymphocyte virus type I *pX* gene transgenic rat model

Takahiro Tsuchikawa[1,2], Hitoshi Ikeda[1], Kazunori Kikuchi[1,*], Takahiro Tsuji[1], Tomohisa Baba[1], Akihiro Ishizu[1], Yuetsu Tanaka[3], Hiroyuki Kato[2] and Takashi Yoshiki[1]

[1]*Department of Pathology/Pathophysiology, Division of Pathophysiological Science;* [2]*Department of Surgical Oncology, Division of Cancer Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan and* [3]*Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Nakahara, Japan*

We earlier reported that Fischer 344/jcl strain (F344) rats carrying a unique *pX* gene of human T lymphocyte virus type I (HTLV-I) under control of a rat lymphocyte-specific protein tyrosine kinase (p56lck) type I promoter (lck-pX rats) spontaneously developed epithelial thymomas from the thymic medulla. To investigate the role of bone marrow cells carrying the HTLV-I *pX* gene in development of thymomas, the bone marrow of normal F344 rats after lethal irradiation was reconstituted by bone marrow mononuclear cells (BMMC) of lck-pX rats. Epithelial thymomas similar to the original thymoma of lck-pX rats frequently developed in the nontransgenic recipients within 5 months after the BMMC transplantation. The thymomas expressed the *pX* gene, thereby indicating the thymoma cells to be of donor BMMC origin. Since the thymoma also developed in nontransgenic recipients reconstituted by BMMC depleted of adherent cells, it is suggested that nonadherent BMMC of donor lck-pX rats may migrate to and lodge in the thymus of recipient nontransgenic rats then transform into thymoma cells with epithelial characteristics. The thymoma cells were shown to bind to *Ulex europaeus* Agglutinin-1 (UEA-1) lectin, which binds epithelial cells in the thymic medulla. It was also shown that the nonadherent BMMC fraction used for bone marrow reconstitution contained a number of UEA-1-positive cells. Taken together, UEA-1 positive BMMC may be progenitor cells of the epithelial thymoma. The epithelial thymoma in lck-pX rats sheds light on epithelial cell development in thymic medulla and for oncogenesis of epithelial thymoma in humans.
Laboratory Investigation (2004) **84,** 245–252, advance online publication, 15 December 2003; doi:10.1038/labinvest.3700028

Keywords: epithelial thymoma; bone marrow cell transfer; transgenic rat; HTLV-I *pX* gene; progenitor cell

Human T lymphocyte virus type I (HTLV-I) is one of the human infectious retroviruses characterized by an etiologic agent of adult T-cell leukemia.[1,2] HTLV-I infection is also implicated in the pathogenesis of myeloneuropathy[3,4] and a number of immunological disorders.[5] Tax protein encoded by a unique *pX* gene of the HTLV-I genome is a potent transcriptional activator or repressor of several host cellular genes,[6,7] and functions as a major pathogenetic molecule of HTLV-I. To investigate the pathogenetic role of the *pX gene*, we established several transgenic rat models carrying the HTLV-I *pX* gene under controls of various promoters.[8–10] Among them, a transgenic rat carrying the *pX* gene under control of a rat lymphocyte-specific protein tyrosine kinase (p56lck) type I promoter (lck-pX rat) is a unique rat model for spontaneously occurring epithelial thymomas in the thymic medulla.[10] The endogenous p56lck type I promoter is specifically active in thymic lymphocytes. However, in our model, mRNA expression of the *pX* transgene was detected in all tissues tested, because the p56lck type I promoter used (−269 to +26) did not include the region (−564 to 433), which contains a sequence to potentially lead cell-specific expression. We concluded that this could be a reason to induce the *pX* transgene expression at significant levels for transformation of thymic medullary epithelial cells. Morphologically, the

Correspondence: Dr T Yoshiki, Department of Pathology/Pathophysiology, Division of Pathophysiological Science, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638, Japan.
E-mail: path1@med.hokudai.ac.jp
*Currently in the Division of Surgical Pathology, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan.
Received 3 August 2003; revised 8 October 2003; accepted 20 October 2003; published online 15 December 2003

thymoma consists of spindle-shaped epithelial cells and expresses p40Tax as a *pX* gene product and cytokeratin as an epithelial marker.

In attempts to determine the origin of thymoma cell in lck-pX rats, we transferred bone marrow mononuclear cells (BMMC) of the lck-pX rat into lethally irradiated nontransgenic Fischer 344/jcl strain (F344) rats and determined if same epithelial thymomas as that of lck-pX rats occur in recipient nontransgenic rats. We further studied to see if BMMC contain possible progenitor cells which express the same molecules as the thymoma cells expressed.

Materials and methods

Animals

Inbred F344 rats were purchased from Clea Japan (Osaka, Japan). We used the lck-pX transgenic rat line, Tg38 which frequently develop thymoma.[10] Rats were maintained at the Institute of Animal Experimentation, Hokkaido University Graduate School of Medicine. All animal experiments were done according to *the Guide for the Care and Use of Laboratory Animals*, Hokkaido University Graduate School of Medicine.

Preparation of BMMC for Reconstitution

After deep anesthetization with sodium pentobarbital, BMMC were prepared from tibias and femurs of 6–8 weeks old male donor rats. Both ends of the bones were cut, and bone marrow was flushed out with 5 ml of phosphate-buffered saline, using a syringe with a needle. Collected bone marrow cells in the tube were dispersed by shaking the syringe then mixed with an equal volume of density separation solution (Lympholyte-rat, CEDERLANE, Hornby, Canada). After centrifugation at 1500 rpm for 30 min, the mononuclear cell layer at the interface was collected as the total BMMC. To obtain nonadherent (nAd)-BMMC, total BMMC were passed through Sephadex G10 (Pharmacia KK, Tokyo, Japan) columns at 37°C to remove adherent cells. Total BMMC were cultured in Petri dishes that contained α-modified minimum essential medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2 incubator for 24 h. After the nonadherent cells had been removed, the adherent cells on the Petri dishes were cultured for 14 days with change of fresh medium at 3 day intervals. Adherent cells as Ad-BMMC were collected after digestion with 0.25% trypsin-EDTA solution for 20 min. Thymuses of all donor rats were histopathologically examined for thymoma.

BMMC Transfer (BMCT) Experiments

Nontransgenic F344 male rats at age from 5 to 9 weeks of age were used as recipients of BMCT. All recipient rats were given a lethal dose of radiation (12 Gy) before BMCT. About 10^7 cells of unseparated total, nAd- or Ad-BMMC were injected intravenously in each experiment. Three experimental groups were designed. One group was given an intravenous injection of unseparated total BMMC from lck-pX donor rats, another group was given nAd-BMMC from lck-pX rats, and the third group was given Ad-BMMC from lck-pX rats mixed with 10^7 of total BMMC from nontransgenic F344 male rats. As a control group, recipient rats were given total BMMC from nontransgenic F344 male rats. All recipient rats of the experimental groups were histopathologically examined when symptoms indicating development of thymoma occurred, such as thoracic enlargement, tachypnea or dyspnea, were observed, or when rats reached at 45 weeks after the BMCT. The control group was examined at about 20 weeks after the BMCT. In addition, total BMMC of nontransgenic rats were transferred into lck-pX rats for a comparative group, by the same procedure described above. Recipient rats that had died within 2 weeks after the BMCT were discarded from the count as a technical failure.

Separation of Thymocytes and Thymic Adherent Cells

Thymus or thymoma of rats were removed aseptically and cut into small fragments with scissors in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS, 5×10^{-5} M 2-mercaptoethanol and 50 μg/ml streptomycin. Two or three pieces of the fragments were put into each well of a 24-well flat-bottomed culture plate and cultivated with 200 μl of DMEM with 50% FBS in a 5% CO_2 incubator at 37°C. Without discarding the medium, 100 μl of DMEM with 10% FBS was added to the wells at 3-day intervals. After incubation for 2 weeks, nonadherent cells were collected as thymic lymphocytes. Adherent cells were washed with PBS, removed with trypsin-EDTA solution and seeded into 10 cm Petri dishes with 10 ml of DMEM with 10% FBS for a subculture. After 2 weeks with changing the medium at 3-day intervals, the adherent cells were collected as thymic adherent cells.

Histopathology and Immunohisto(cyto)chemistry

Thymomas of nontransgenic F344 rats given nAd-BMMC of lck-pX rats were fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin blocks. Each 4 μm of sections was stained with hematoxylin and eosin. For immunohisto(cyto)-chemical analysis, mouse monoclonal anti-cytokeratin (MNF116, DAKO, Glostrup, Denmark) and anti-Tax (Lt-4),[11] for detecting the product of the *pX* transgene, antibodies and biotinylated Ulex Europaeus Agglutinin-1 (UEA-1) lectin (Vector Laboratories, Burlingame, CA, USA) were used as first detecting reagents to histological tissue sections of

thymus and thymoma of nontransgenic F344 rats given nAd-BMMC of lck-pX rats and to BMMC of lck-pX rats fixed on a glass slide. To develop these first reactants, an avidin–biotin immunoperoxidase kit (DAKO) was used. After immunostaining, tissue sections or fixed BMMC were counter-stained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Karyotype Analysis

Chromosomes of separated thymoma cells from an original thymoma of lck-pX rats and a thymoma developed in nontransgenic rats with BMCT from lck-pX rats were analyzed, using a standard G-bands by trypsin using Giemsa method.[12] In all, 17 cells for original thymoma and 10 cells for BMCT thymoma were analyzed.

Polymerase Chain Reaction (PCR) and Reverse Transcriptase (RT)-PCR

Total DNAs were extracted from either cell pellets of about 1×10^6 of separated thymocytes, thymic adherent cells, or thymoma cells, and PCR amplification was done to detect integration of the *pX* transgene according to methods described elsewhere.[8] Expression of the *pX* transgene was detected using RT-PCR.[8,9] Briefly, total RNAs were extracted from separated thymoma cells and the thymoma tissues using RNA extraction kits (ISOGEN, Nippon Gene, Toyama, Japan) and treated with DNase, then they were reversely transcribed with RT and the cDNAs were amplified using a pX specific primer pair to detect the transgene and a rat β-actin primer pair[13] as an internal control.

Quantitative RT-PCR

Expression of the winged-helix-nude (*whn*) gene in the thymoma was quantitatively measured, using real-time RT-PCR. After cutting small pieces of tissues, the lck-pX thymoma, and nontransgenic thymocyte-depleted thymus and spleen as controls, total RNAs were extracted using ISOGEN. After DNase treatment, total RNAs were reversely transcribed with RT and a random hexamer primer mixture. *whn* gene expression levels in 1 ng of the resulting cDNAs were quantified using a QuantiTect STBR Green PCR kit (Qiagen GmbH, Hilden, Germany) and ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was done with primers 5′-CCCAAGCTTTGGACAATGGT-3′ for sense and 5′-CTGATGAAAGGTGGGCTGAGA-3′ for antisense[14] (Genbank accession number S80120) according to methods described in the manufacturer's handbook. For standardization, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, one of the house-keeping genes, was quantified in each sample, using primers 5′-GGGAGTTGCTGTTGAAGTCA-3′ for sense and 5′-CCGAGGGCCCACTAAAGG-3′ for antisense[15] (Genbank accession number M17701). Four lck-pX thymomas, three nontransgenic thymuses and one nontransgenic spleen were used and all experiments were done triplicate and average amounts of *whn* expression were indicated by a ratio against those of GAPDH expression in each sample.

Flow Cytometry

BMMC were reacted with fluorescence thiocyanate-conjugated UEA-1 (Vector Laboratories), then were analyzed using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Results

Development. of Epithelial Thymomas in Normal F344 Rats Reconstituted by BMMC from lck-pX Rats

To examine the role of the HTLV-I *pX* gene in BMMC, about 10^7 cells of total BMMC from lck-pX rats were transferred into lethally irradiated nontransgenic F344 rats. By reconstructing bone marrow by lck-pX rats, thymoma developed in 18 of 19 recipient normal rats within 5 months after the BMCT. BMMC from donor rats with or without primary thymoma did not affect the outcome (Table 1 and Figure 1a). The tumor, which had medullary growth, consisted of spindle-shaped cells expressing cytokeratin as an epithelial marker, similar to the original thymomas in lck-pX rats (Figure 1b and c). The p40Tax protein expression as an HTLV-I pX gene product was immunohistochemically detected

Table 1 Development of thymomas in normal F344 rats received BMMC from lck-pX rats

Combination[a]	*Cells transferred*	*Thymoma in donors*[b]	*No. thymoma*[c]*/ no. recipient rats*
lck-pX to N	Total BMMC	+	14/14
	Total BMMC	–	4/5
	nAd-BMMC	+	2/3
	Ad-BMMC	+	0/17[d]
N to lck-pX	Total BMMC	–	3/3
N to N	Total BMMC	–	0/5[e]

[a]BMCT experiments were done on lck-pX rats to nontransgenic rats (lck-pX to N) or nontransgenic rats to lck-pX rats (N to lck-pX). As a negative control, BMMC of nontransgenic rats were transferred to nontransgenic rats (N to N).
[b]Development of thymomas in donor rats was histopathologically examined when BMMC were prepared.
[c]All thymomas developed in recipient rats were shown to have histopathological features similar to the original thymomas in lck-pX rats.
[d]All recipient rats were histopathologically examined at about 45 weeks after the BMCT.
[e]No neoplastic change was found in thymuses of all recipients at about 20 weeks after the BMCT.

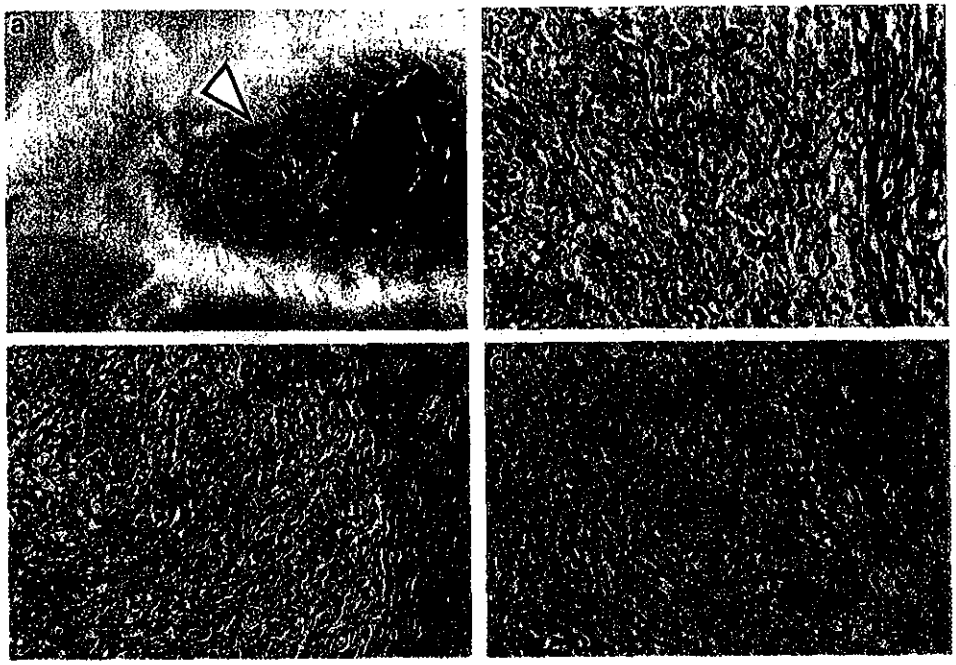

Figure 1 Development of thymomas in normal F344 (nontransgenic recipient) rats after being given total BMMC of lck-pX rats. A large tumor in the anterior mediastinum of a recipient rat at 20 weeks after the BMCT is shown (**a** arrowhead). Microscopically, the tumor, similar to the lck-pX thymoma, is composed predominantly spindle-shaped cells (**b**) (HE staining, original magnification: × 125). Immunohistochemically, spindle-shaped tumor cells are uniformly stained by anti-cytokeratin (**c**) and anti-Tax monoclonal antibodies (**d**) (original magnification: × 125).

in the tumor (Figure 1d). Karyotype analysis showed that the same 42XY karyotype, 20 pairs of autosome and a sex chromosome pair, as the standard karyotype of rat was found in both separated thymoma cells from original thymoma and thymoma developed in BMCT rats (data not shown). The reconstitution experiments using separated Ad- and nAd-BMMC from lck-pX rats showed similar thymoma developed in two of three recipient nontransgenic rats given nAd-BMMC of lck-pX rats within 40 weeks after the BMCT (Table 1). Development of thymoma was never evident macroscopically and histologically in all 17 recipient rats given Ad-BMMC of lck-pX rats at about 45 weeks after the BMCT. Therefore, the nAd-BMMC fraction of lck-pX donor rats may contain progenitor cells of epithelial thymoma. On the other hand, thymomas developed in all three irradiated lck-pX rats with total BMMC transfer from nontransgenic rats within 4 months after the BMCT (Table 1).

Detection of pX Gene in Thymoma Cells of Recipient Rats

PCR and RT-PCR methods were used to determine the presence and expression of the *pX* gene in thymoma cells of recipient rats. After separation of thymic lymphocytes and adherent cells, the *pX* gene was evident in both lymphocytes and adherent cells in the recipient rats, regardless of thymoma occurrence (Figure 2a). pX mRNA expression was also evident in lymphocyte-depleted adherent thymoma cells of recipient rats (Figure 2b), but it was out of the detection levels in thymic adherent cells in recipient rats before the development of epithelial thymoma (data not shown).

Characteristics of Epithelial Thymoma Developed in the Medulla of lck-pX Rats

Expression of the *whn* gene, which is a forkhead-winged helix transcriptional factor (reviewed in Kaufmann and Knöchel[16]) the mutation of which disrupts thymus development and normal hair growth,[17] was examined in the thymoma of lck-pX rats to determine if the thymoma cells have the same characteristics as thymic epithelial cells originating from the third branchial cleft and pharyngeal pouch, using quantitative real-time RT-PCR. Only minimal levels of the *whn* gene expression were detected in the thymoma tissues compared with that in the normal thymus (Figure 3). Since the thymoma tissues seem to contain resident stromal cells of the thymus, it appears that thymoma cells do not express the *whn* gene. The thymoma cells were positively stained for UEA-1 (Figure 4), a kind of fucose-binding lectins and is known to specifically bind to epithelial cells in the medulla of the thymus,[18] indicating that the thymoma of lck-pX rats originates in UEA-1-positive epithelial cells in the thymic medulla. This observation is consistent with histopathological findings of early lesions of

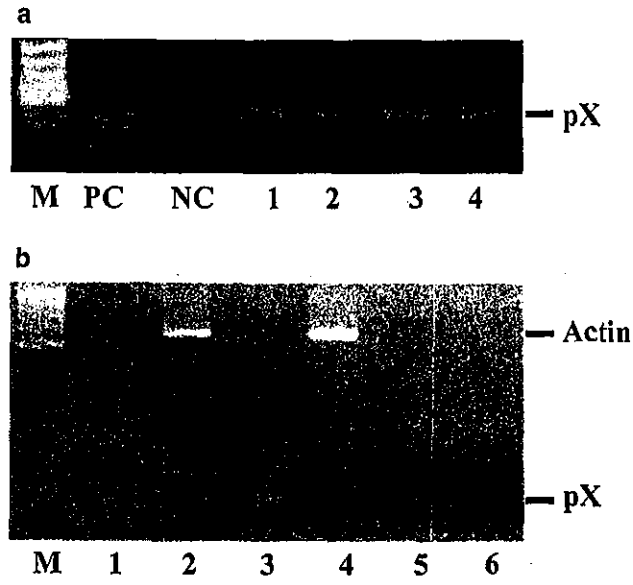

Figure 2 (a) Detection of the pX transgene in thymoma cells isolated from nontransgenic recipient rats received BMMC of lck-pX rats. The *pX* transgene was detected in both thymic adherent stromal cells (lane 1) and thymocytes (lane 2) isolated from the thymus of a recipient rat before developing thymoma and in both thymoma cells (lane 3) and thymocytes (lane 4) of a recipient rat with a developed thymoma, by PCR. NC indicates thymic adherent stromal cells isolated from an untreated nontransgenic rat as a negative control and PC is thymic adherent stromal cells isolated from an lck-pX rat thymus as a positive control. M is a *Hae* III digested ϕX174 DNA size marker. (b) RT-PCR detection of the pX mRNA in thymoma cells isolated from recipient nontransgenic rats received BMMC of lck-pX rats. Total RNAs were prepared from adherent thymoma cells isolated from a recipient rat (lanes 1, 2 and 5) and an original thymoma tissue as a positive control (lanes 3, 4 and 6). Lanes 1 and 3 are results of RT-PCR using a pX specific primer pair. Lanes 2 and 4 are using a β-actin (Actin) primer pair as internal control. Lanes 5 and 6 are results of a pX PCR amplification without RT. M is a 100 bp DNA ladder size marker.

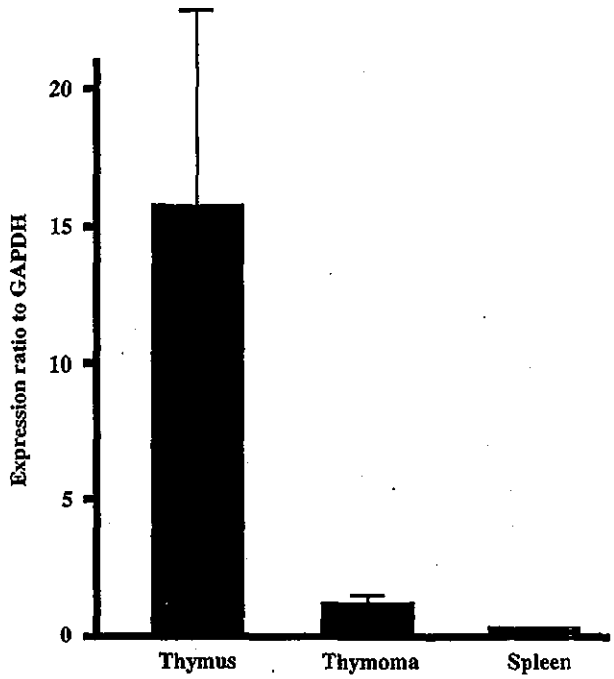

Figure 3 Quantification of whn mRNA expression levels in thymomas. A measure of 1 ng of each cDNA from total RNAs of each tissue was amplified with a *whn* gene specific primer pair using quantitative real-time PCR. Results are shown as expression ratios against the level of *GAPDH expression in each tissue*. Thymus means the result of nontransgenic lymphocyte-depleted thymus for a positive control, Thymoma means that of lck-pX original thymomas, and Spleen means that of nontransgenic spleen for a negative control. All experiments were done by triplicate. The average ratio with standard deviation in each experiment group is shown.

the primary thymoma which apparently originated in the thymic medulla.[10] Next, we asked if UEA-1-positive cells can be observed in BMMC of lck-pX rats, using flow cytometry, even though it is known that megakaryocytes are positive for UEA-1 in humans.[19] BMMC of the lck-pX rats contained about 7.5% cells strongly positive for UEA-1 and these were morphologically small mononuclear round-type cells which differed from megakaryocytes (Figure 5). Cells positive for cytokeratin were not evident in the BMMC fractions (data not shown).

Discussion

In this study, the transfer of BMMC into irradiated nontransgenic recipient rats from lck-pX rats frequently induced development of thymoma in the host, similar to the original epithelial thymomas spontaneously developed in the lck-pX rats. Since the thymoma cells expressed p40Tax as the *pX* transgene product, the epithelial thymoma cells in the recipient rats would likely have derived from progenitor cells present in the BMMC of donor lck-pX rats.

It is generally believed that the epithelial tumor cells in primary thymomas develop from proper epithelial cells which existed constitutively in the cortex or medulla of the thymus, although many bone marrow-derived cells, such as thymocytes and dendritic cells, are principal constituents of thymus. It is generally considered that thymic epithelial cells in the cortex and medulla are derived from ectoderm of the third branchial cleft and endoderm of the third pharyngeal pouch, respectively. The multipotentiality of cells derived from bone marrow, hematopoietic stem cells, was demonstrated in various models, including transdifferentiations into cardiomyocytes,[20] hepatic cells,[21,22] existence of precursor cells for bone, cartilage and lung,[23] and nerve cells.[24] Donor bone marrow cells could migrate into the recipient thymus and participate in the positive selection of thymocytes as thymic stromal cells by bone marrow transplantation plus bone graft in mice.[25] In our model, epithelial thymomas with donor characteristics develop in the recipient thymus by nAd-BMMC transfer, suggesting that nAd-BMMC may contain progenitor

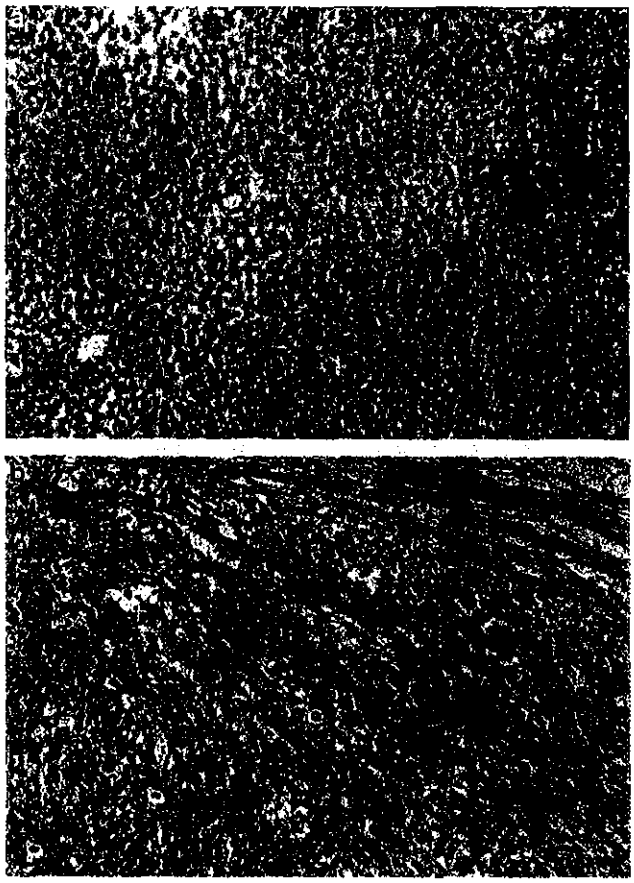

Figure 4 Distribution of the UEA-1-positive cells in the lck-pX rat thymus and the lck-pX rat thymoma. UEA-1-positive cells were localized in the medulla of lck-pX rat thymus before thymoma development (**a**) (original magnification: ×60). Thymoma of lck-pX rat was positive for UEA-1 (**b**) (original magnification: ×75).

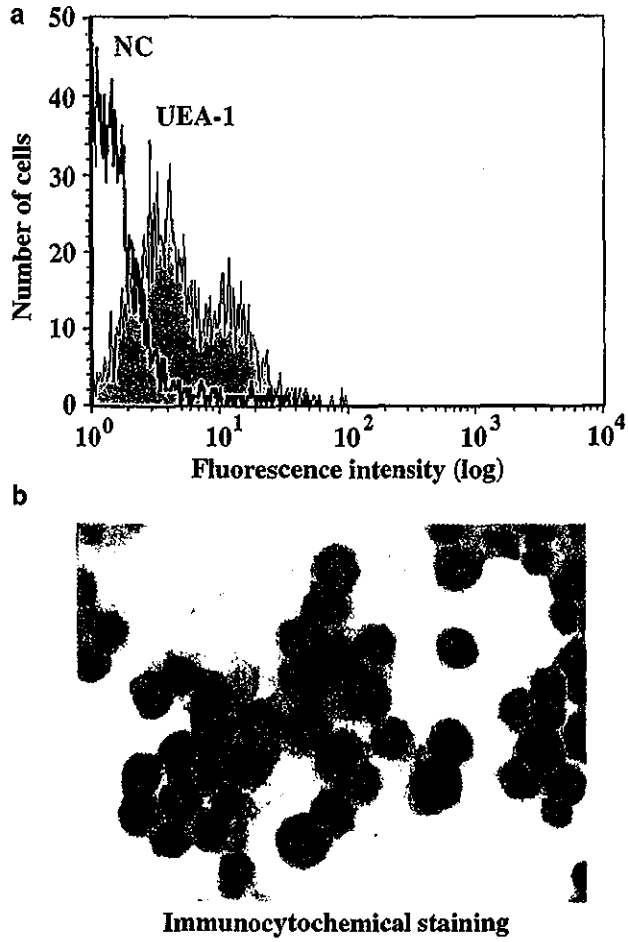

Figure 5 UEA-1 expression of the BMMC in the lck-pX rat. Flow cytometry revealed two peaks of the curve as for the positive intensity of UEA-1 (UEA-1). The population strongly stained with UEA-1 was about 7.5% of the lck-pX BMMC (**a**). NC means BMMC without fluorescence thiocyanate-conjugated UEA-1 staining as a negative control. Immunocytochemistry revealed UEA-1-positive cells (brown) to be small round mononuclear ones (**b**).

cells with the potential to transdifferentiate into a kind of epithelial cells in the thymic medulla. In the thymus, three distinct stromal and epithelial cell types related to T-cell differentiation, cortical and medullary epithelial cells and bone marrow-derived dendritic cells, are known.[26] Except for dendritic cells, epithelial cells in both areas specifically express not only cytokeratin but also the *whn* gene a transcriptional factor expressed in epithelial cells of thymic primordium and adult thymus.[27] *whn* expression was evident in all the thymomas examined including medullary thymomas in humans.[28] Therefore, it is reasonable to expect that epithelial thymomas developed from thymic medulla of lck-pX rats would express the *whn* gene if the thymoma develops from epithelial cells in thymic medulla derived from endoderm of the third pharyngeal pouch. However, expression of the *whn* gene was minimal in the thymoma of lck-pX rats, which means that the thymoma may not develop from thymic epithelial cells derived from the pharyngeal pouch. It was clearly shown that the epithelial thymomas carrying the *pX* transgene of lck-pX rats developed in thymus of nontransgenic recipient rats by transfer of BMMC from lck-pX rats. The collective evidence indicates that certain medullary epithelial cells of thymus in rats may derive from hematopoietic progenitor cells.

It is also known that there is heterogeneity in epithelial cells of the thymic medulla in rats similar to those in humans and mice.[18,29,30] UEA-1 high reactivity is only evident in the medulla and can divide epithelial cells of the medulla into UEA-1 positive or negative. We showed that UEA-1-positive cells are clearly localized in the medulla of the rat thymus and the thymomas were positive for UEA-1. About 7.5% UEA-1-positive cells were evident in nAd-BMMC fraction used for our BMMC transfer experiments, suggesting the possibility that thymomas in the medulla of nontransgenic recipient rats are derived from the transferred BMMC with UEA-1 reactivity, although cells positive for cytokeratin were nil in BMMC fractions.

Recently, Terada *et al*[31] and Ying *et al*[32] independently suggested that spontaneous cell fusion not transdifferentiation leads to changed phenotypes of neural or bone marrow stem cells to other adoptive cells. However, the possibility of cell fusion in our BMCT model can be negated, since the thymoma developed in BMCT rats had the same morphological and biological features to the original thymoma and no additional chromosome by cell fusion was evident in separated thymoma cells from thymoma developed in BMCT rats.

The thymic organogenesis, including thymic epithelial cells, is still controversial (reviewed in Manley and Blackburn[33]). Recent reports suggested that thymic epithelial cells might derive from one progenitor cell in the thymic primordium. Each epithelial islet in the thymic medulla was shown to consist of cells from a single progenitor.[34] It was also suggested that all functional epithelial cell types of both cortex and medulla might derive from a common progenitor cell with expression of the MTS24 cell surface glycoprotein in the thymic primordium.[35] Cell fractions containing the progenitor cells were sufficient to fully reconstitute the complex thymic epithelial microenvironment.[36] However, our results suggest the possibility that bone marrow can supply progenitor cells which migrate and transdifferentiate into epithelial cells in the thymic medulla.

Acknowledgement

We thank Tsutomu Osanai and the entire staff of the Institute of Animal Experimentation, Hokkaido University Graduate School of Medicine, for maintenance of transgenic rats, Ken-ichi Nakase, Chisato Sudo and Masayo Tateyama for technical assistance and Mariko Ohara (Fukuoka, Japan) for language assistance. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1 Poiesz BJ, Ruscetti FW, Gazder AF, *et al.* Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc Natl Acad Sci USA 1980;77: 7415–7419.
2 Yoshida M, Miyoshi I, Hinuma Y. Isolation and characterization of retrovirus from cell lines of human adults T-cell leukemia and its implication in the disease. Proc Natl Acad Sci USA 1982;79:2031–2035.
3 Gessain A, Barin F, Vernant JC, *et al.* Antibodies to the human T cell lymphotropic virus type I in patients with tropical spastic paraparesis. Lancet 1985;8452: 407–410.
4 Osame M, Usuku K, Izumo S, *et al.* HTLV-I associated myelopathy, a new clinical entity. Lancet 1986;p8488: 1031–1032.
5 Hollsberg P, Hafler DA. Pathogenesis of diseases induced by human lymphotropic virus type I infection. N Engl J Med 1993;328:1173–1182.
6 Sodroski JG, Rosen CA, Haseltine WA. A transcriptional protein encoded by the x-lor region of the human T cell leukemia virus. Science 1984;228: 1430–1434.
7 Fujisawa J, Seiki M, Kiyokawa T, *et al.* Functional activation of the long terminal repeat of human T cell leukemia virus type I by a trans-acting factor. Proc Natl Acad Sci USA 1985;82:2277–2281.
8 Yamada S, Ikeda H, Yamazaki H, *et al.* Cytokine-producing mammary carcinomas in transgenic rats carrying the pX gene of human T-lymphotropic virus type-I. Cancer Res 1995;55:2524–2527.
9 Yamazaki H, Ikeda H, Ishizu A, *et al.* A wide spectrum of collagen vascular and autoimmune diseases in transgenic rats carrying the env-pX gene of human T lymphocyte virus type I. Int Immunol 1997;9:339–346.
10 Kikuchi K, Ikeda H, Tsuchikawa T, *et al.* A novel animal model of thymic tumor: development of epithelial thymoma in transgenic rats carrying human T lymphocyte virus type I pX gene. Int J Exp Pathol 2002;83:247–255.
11 Tanaka Y, Yoshida A, Tazawa H, *et al.* Production of a recombinant human T-cell leukemia virus type-I trans-activator (tax1) antigen and its utilization for generation of monoclonal antibodies against various epitopes on the tax1 antigen. Int J Cancer 1991;48: 623–630.
12 Wang HC, Fedoroff S. Banding in human chromosomes treated with trypsin. Nat New Biol 1972;235:52–54.
13 Tomaru U, Ikeda H, Ohya O, *et al.* Human T lymphocyte virus type I-induced myeloneuropathy in rats: implication of local activation of the *pX* and tumor necrosis factor-α genes in pathogenesis. J Infect Dis 1996;174:318–323.
14 Segre J, Nemhauser JL, Taylor BA, *et al.* Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. Genomics 1995;28:549–559.
15 Tso JY, Sun XH, Kao TH, *et al.* Isolation and characterization of rat and human glyceraldehydes-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res 1985;13:2485–2502.
16 Kaufmann E, Knöchel W. Five years on the wings of fork head. Mech Dev 1996;57:3–20.
17 Nehls M, Pfeifer D, Schorpp M, *et al.* New member of the wing-helix protein family disrupt in mouse and rat *nude* mutations. Nature 1994;372:103–107.
18 Farr AG, Anderson SK. Epithelial heterogeneity in the murine thymus: fucose-specific lectins bind medullary epithelial cells. J Immunol 1985;134: 2971–2977.
19 Liu SM, Li CY. Immunohistochemical study of *Ulex europaeus* agglutinin 1 (UEA-1) binding of megakaryocytes in bone marrow biopsy specimens: demonstration of heterogeneity in staining pattern reflecting the stages of differentiation. Hematopathol Mol Hematol 1996;10:99–109.
20 Makino S, Fukuda K, Miyoshi S, *et al.* Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J Clin Invest 1999;103:697–705.
21 Petersen BE, Bowen WC, Patrene KD, *et al.* Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168–1170.

22 Lagasse E, Connors H, Dhalimy ML, *et al.* Purified hematopoietic stem cells can differentiate into hepatocyte *in vivo*. Nat Med 2000;16:1282–1286.
23 Pereira RF, Halford KW, O'Hara MD, *et al.* Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA 1995;92: 4857–4861.
24 Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 1999;96:10711–10716.
25 Li Y, Hisha H, Inaba M, *et al.* Evidence for migration of donor bone marrow stromal cells into recipient thymus after bone marrow transplantation plus bone grafts: a role of stromal cells in positive selection. Exp Hematol 2000;28:950–960.
26 Lo D, Reilly CR, Burkly LC, *et al.* Thymic stromal cell specialization and the T-cell receptor repertoire. Immunol Res 1997;16:3–14.
27 Nehls M, Kyewski B, Messerle M, *et al.* Two genetically separable steps in the differentiation of thymic epithelium. Science 1996;272:886–889.
28 Gattenlöhner S, Müller-Hermelink HK, Marx A. Transcription of the nude gene (WHN) in human normal organs and mediastinal and pulmonary tumors. Pathol Res Prac 1999;195:571–574.
29 Waal EJ, Rademakers LH. Heterogeneity of epithelial cells in the rat thymus. Microsc Res Tech 1997;38: 227–236.
30 Such CD, Gao EK, Kosaka H, *et al.* Two subsets of epithelial cells in the thymic medulla. J Exp Med 1992;176:495–505.
31 Terada N, Hamazaki T, Oka M, *et al.* Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002;416:542–545.
32 Ying QL, Nichois J, Evans EP, *et al.* Changing potency by spontaneous fusion. Nature 2002;416:545–548.
33 Manley NR, Blackburn CC. A developmental look at thymus organogenesis: where do the non-hematopoietic cells in the thymus come from? Curr Opin Immunol 2003;15:225–232.
34 Rodewarld HR, Paul S, Haller C, *et al.* Thymus medulla consisting of epithelial islets each derived from a single progenitor. Nature 2001;414:763–768.
35 Bennett AR, Fartey A, Blair NF, *et al.* Identification and characterization of thymic epithelial progenitor cells. Immunity 2002;16:803–814.
36 Gill J, Malin M, Holländer GA, *et al.* Generation of a complete thymic microenvironment by MTS24$^+$ thymic epithelial cells. Nat Immunol 2002;3:635–642.

Tohoku J. Exp. Med., 2004, **204**, 289-298

Increased mRNA Expression of Th1-Cytokine Signaling Molecules in Patients with HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis

YOSHIHIRO NISHIURA, TATSUFUMI NAKAMURA,[1] NAOMI FUKUSHIMA,[1] RYOZO MORIUCHI,[1] SHIGERU KATAMINE[1] and KATSUMI EGUCHI

First Department of Internal Medicine, [1]Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

NISHIURA, Y., NAKAMURA, T., FUKUSHIMA, N., MORIUCHI, R., KATAMINE, S. and EGUCHI, K. *Increased mRNA Expression of Th1-Cytokine Signaling Molecules in Patients with HTLV-I-Associated Myelopathy/Tropical Spastic Paraparesis.* Tohoku J. Exp. Med., 2004, **204** (4), 289-298 —— Expression of inflammatory cytokines derived from Th1 cell population is increased in patients with human T-lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP). It has been shown that cytokine signaling molecules, including transcription factors T-bet and GATA-3, interleukin-12 receptor β2 (IL-12Rβ2) and suppressors of cytokine signaling (SOCS), such as SOCS1, are important in differentiation of naive T cells into Th1 helper T cells. To assess the immunological status from the standpoint of cytokine signaling in patients with HAM/TSP, we analyzed mRNA expression of these cytokine signaling molecules in peripheral blood mononuclear cells using quantitative RT-PCR. Twenty-eight HAM/TSP patients, nine HTLV-I-infected individuals without HAM/TSP and twenty-two HTLV-I-uninfected individuals were included in this study. Expression of T-bet, GATA-3, IL-12Rβ2 and SOCS1 was significantly increased in HAM/TSP patients in comparison with HTLV-I-uninfected individuals. In contrast, expression of SOCS3, a marker for Th2 cells, was significantly decreased in HTLV-I-infected individuals. These results indicate that HAM/TSP patients are associated with increased Th1 and decreased Th2 cytokine signaling activities. ——— HAM/TSP; HTLV-I; cytokine signaling; Th1; Th2

Received July 12, 2004; revision accepted for publication October 20, 2004.
Address for reprints: Yoshihiro Nishiura, First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
e-mail: yoshihironishiura@hotmail.com

Human T-lymphotropic virus type I (HTLV-I) is the etiological agent of a rapidly progressive lymphoproliferative malignancy, known as adult T-cell leukemia (ATL) (Yoshida 2001), and of a chronic spastic myelopathy, known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al. 1986). HTLV-I infection has also been associated with other inflammatory diseases, such as arthropathy, uveitis, polymyositis and alveolitis (Hollsberg 1999). Of these HTLV-I-related inflammatory diseases, HAM/TSP is the paradigm. It is characterized by perivascular cuffing accompanied by parenchymal lymphocytic infiltration in the spinal cord (Akizuki et al. 1987). While greater insights have been gained into the pathological mechanisms operative in HAM/TSP (Osame 2002), it is still unclear why only a minor proportion of HTLV-I-infected individuals develop HAM/TSP and other HTLV-I-associated inflammatory diseases.

Numerous immunological abnormalities are known to exist in patients with HAM/TSP (Nakamura 2000a). Previously, cytokine studies in HAM/TSP patients have revealed elevated serum, cerebrospinal fluid (CSF) and $CD4^+$ cell culture supernatant levels of interferon-γ (IFN-γ) (Kuroda and Matsui 1993; Nishiura et al. 1996; Furuya et al. 1999) and high intracellular IFN-γ^+/interleukin-4^+ (IL-4^+) cell ratio in $CD4^+$ T cells (Horiuchi et al. 2000), suggesting that the immunological state of HAM/TSP patients is under Th1 activation (Nakamura et al. 2000b). On the other hand, HTLV-I infection also causes production of Th2 cytokines (Mogensen and Paludan 2001). Thus, the immunological state of HTLV-I-infected individuals is still obscure, with data showing up-regulation of both Th1 and Th2 cytokine production (Carvalho et al. 2001) and production of only Th1 cytokine in HTLV-I-infected cells (Hanon et al. 2001).

In differentiation of naive T cells into Th1 or Th2 helper T cells, cytokine signaling plays an

TABLE 1. *Oligonucleotide primer sequences and sizes of PCR products*

	Sequence	Size
β2m	forward, CCAGCAGAGAATGGAAAGTC reverse, GATGCTGCTTACATGTCTCG	391 bp
IL-12Rβ2	forward, TTCACCCACTCACCTTCTCC reverse, CCAGTTCCTAAGACTCCCCC	243 bp
T-bet	forward, CCCCAAGGAATTGACAGTTG reverse, GGGAAACTAAAGCTCACAAAC	317 bp
GATA-3	forward, CTACGGAAACTCGGTCAGG reverse, CTGGTACTTGAGGCACTCTT	372 bp
SOCS1	forward, AGACCCCTTCTCACCTCTTG reverse, CTGCACAGCAGAAAATAAAGC	245 bp
SOCS3	forward, CTTCAGCATCTCTGTCGGAAGA reverse, GCATCGTACTGGTCCAGGAACT	104 bp
HTLV-I tax	forward, AAACAGCCCTGCAGATACAAAGT reverse, ACTGTAGAGCTGAGCCGATAACG	247 bp

important role. Interleukin-12 receptor/signal transducers and activators of transcription 4 (IL-12R/STAT4) and IL-4R/STAT6 signaling are involved in Th1 and Th2 differentiation, respectively (Murphy et al. 2000). In addition, T-bet, a member of the T-box family, was recently cloned as a Th1-specific transcription factor (Szabo et al. 2000). Therefore, both IL-12R/STAT4 signaling and T-bet work cooperatively in Th1 differentiation, and IL-4R/STAT6 signaling induces GATA-3 activation, one of the Th2-specific transcription factors, in Th2 differentiation (Zheng and Flavell 1997).

Recently, the importance of negative regulation of cytokine signal transduction in the differentiation and homeostasis of the immune system has become somewhat clearer (Yasukawa et al. 2000). The suppressor of cytokine signaling (SOCS) family is a representative of the negative regulators which act through a feedback mechanism or an inhibitory signal to the Janus kinases (JAK)/STAT systems in cytokine signaling (Krebs and Hilton 2001). In this system, SOCS1 is induced by IFN-γ receptor (IFN-γR)/STAT1 signaling and inhibits IL-4R/STAT6 signaling concomitant with the negative feedback to its own JAK/STAT1 signaling (Losman et al. 1999; Yasukawa et al. 2000). Although SOCS3 is induced by a number of humoral factors, such as IL-2, IL-6, IL-10, growth hormone, prolactin and leptin, with simultaneous negative feed back to their own JAK/STATs (Alexander 2002), recent data indicate that SOCS3 also inhibits IL-12R/STAT4 signaling (Egwuagu et al. 2002).

These findings suggest that up-regulation of SOCS1 expression, based on activation of IFN-γ signaling, inhibits Th2 differentiation by blocking the IL-4R/STAT6 signaling pathway, and that down-regulation of SOCS3 expression leads to Th1 differentiation. Indeed, Egwuagu et al. (2002) have reported that Th1 cells express significantly higher levels of SOCS1 than SOCS3 and expression levels of each SOCS protein are reversed inTh2 cells, indicating that either SOCS1 or SOCS3 protein is a marker for Th1 cells or Th2 cells, respectively. In addition, very recently, Seki

TABLE 2. *PCR cycling conditions*

	Denaturation	Aannealing	Extensio	Cycles
β2m	95°C, 15 s	58°C, 5 s	72°C, 15 s	32
IL-12Rβ2	95°C, 15 s	65°C, 5 s	72°C, 12 s	45
T-bet	95°C, 15 s	62°C, 5 s	72°C, 15 s	45
GATA-3	95°C, 15 s	62°C, 5 s	72°C, 15 s	45
SOCS1	95°C, 15 s	62°C, 5 s	72°C, 10 s	45
SOCS3	95°C, 15 s	60°C, 5 s	72°C, 10 s	45
HTLV-I tax	95°C, 15 s	55°C, 5 s	72°C, 10 s	40

s, seconds.

TABLE 3. *mRNA expression of cytokine signaling molecules*

	HAM/TSP patints	HTLV-I-infected individuals without HAM/TSP	HTLV-I-uninfected individuals
T-bet	0.260 ± 0.043	0.241 ± 0.100	0.118 ± 0.028
GATA-3	0.167 ± 0.040	0.115 ± 0.051	0.022 ± 0.005
IL-12Rβ2	0.321 ± 0.126	0.288 ± 0.104	0.114 ± 0.023
SOCS1	0.338 ± 0.062	0.147 ± 0.038	0.035 ± 0.007
SOCS3	4.860 ± 0.818	6.714 ± 1.467	13.234 ± 1.385

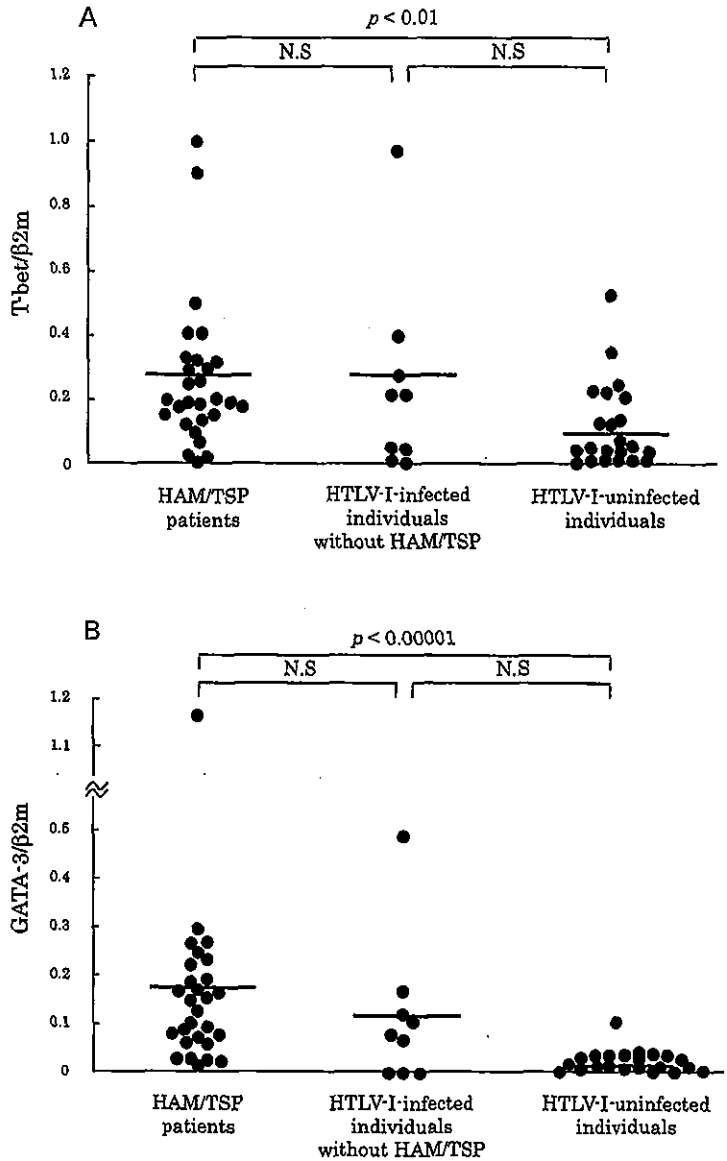

Fig. 1. Comparison of mRNA expression of T-bet and GATA-3 in PBMC. mRNA expression of both T-bet (A) and GATA-3 (B) in PBMC was significantly increased in HAM/TSP patients compared to HTLV-I-uninfected individuals. Although mRNA expression of T-bet and GATA-3 appeared to be higher in HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals, this difference was not statistically significant. Mann-Whitney's U-test was used for statistical analysis.

et al. (2003) have demonstrated that SOCS3 is predominantly expressed in Th2 cells.

To further clarify the molecular basis of the Th1/Th2 state from the stand-point of cytokine signaling in HTLV-I-infected individuals with and without HAM/TSP, we investigated mRNA expression of T-bet, GATA-3, IL-12Rβ2, SOCS1 and SOCS3 in peripheral blood mononuclear cells (PBMC) by quantitative RT-PCR. In addition, we analyzed the relationship between cytokine signaling molecules and HTLV-I tax mRNA expression in patients with HAM/TSP, compared to HTLV-I-infected individuals without HAM/TSP.

Materials and Methods

Subjects

Twenty-eight HAM/TSP patients (6 men and 22 women; mean age, 58.5 years; age range, 29-78 years) were included in the study. The diagnosis of HAM/TSP was based on previously described criteria (Osame 1990). Control subjects comprised of 9 HTLV-I-infected individuals without HAM/TSP (2 men and 7 women; mean age, 60.4 years; age range, 26-69 years) and 22 HTLV-I-uninfected individuals (8 men and 14 women; mean age, 54.5 years; age range, 26-75 years). The 9 HTLV-I-infected individuals without HAM/TSP included patients with cerebrovascular accident ($n = 2$), essential tremor ($n = 2$), tension-type headache ($n = 1$), spinocerebellar degeneration ($n = 1$), amyotrophic lateral sclerosis ($n = 1$), cervical spondylosis ($n = 1$), diabetic polyneuropathy ($n = 1$), whereas the 22 HTLV-I-uninfected individuals included patients with cerebrovascular accident ($n = 5$), tension-type headache ($n = 3$), Parkinson disease ($n = 2$), spinocerebellar degeneration ($n = 2$), multiple sclerosis (remission phase, $n = 2$), migraine ($n = 2$), cervical spondylosis ($n = 2$), essential tremor ($n = 1$), epilepsy ($n = 1$), Bell's palsy ($n = 1$), and brain tumor ($n = 1$). None of the patients had been treated with immunomodulatory drugs, including corticosteroids and interferon-α during the last one year. Informed written consent was obtained from all patients in the study. This research was approved by the review boards of Graduate School of Biomedical Sciences, Nagasaki University.

RNA isolation and quantitative RT-PCR analysis

PBMC were separated by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Total cellular RNA was extracted from PBMC by Sepasol-RNA I super (NACALAI TESQUE, INC., Kyoto). cDNA was synthesized from 2.0 μg RNA treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) using Oligo (dT) 12-18 primer (Promega). M-MLV reverse transcriptase (1 μl) (Promega) and 25 units of Ribonuclease inhibitor (Promega) were used in a total volume of 25 μl. For quantitative analysis of IL-12Rβ2, T-bet, GATA-3, SOCS1, SOCS3, HTLV-I Tax and β2-microglobulin (β2m) in

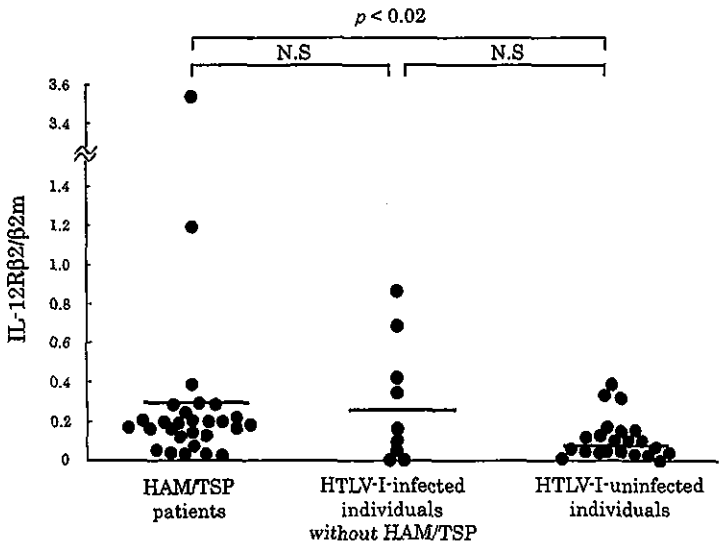

Fig. 2. Comparison of mRNA expression of IL-12Rβ2 in PBMC. mRNA expression of IL-12Rβ2 in PBMC was significantly higher in HAM/TSP patients than in HTLV-I-uninfected individuals. Although mRNA expression of IL-12Rβ2 seemed to be higher in HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals, this difference was not statistically significant. Mann-Whitney's U-test was used for statistical analysis.

PBMC, real-time quantitative RT-PCR was performed in a Light-Cycler FastStart DNA Master (Roche Diagnostics, Mannheim, Germany) based on general fluorescence detection with SYBR Green. The primer sequences and the sizes of the PCR products are shown in Table 1; and PCR conditions are shown in Table 2. For quantitative RT-PCR, bulk cDNA derived from cell lines were used as standards. HCT-1, derived from CSF cells from a patient with HAM/TSP, was used for IL-12Rβ2, SOCS1 and SOCS3; MT-2 was used for β2m, T-bet and HTLV-I Tax; and Jurkat was used for GATA-3. Each mRNA expression was evaluated by normalization to β2m.

Statistical analysis

Results were expressed as mean ± standard error of the mean. Differences between groups were tested for statistical significance by use of the nonparametric Mann-Whitney's U-test. Correlation analyses were performed by use of nonparametric Spearman's rank correlation test. The level of significance was set at $p = 0.05$.

Results

mRNA expression of each cytokine signaling molecules in PBMC was measured by real-time quantitative RT-PCR. Each mRNA expression was evaluated by normalization to β2m. The value of each mRNA expression was shown in Table 3. As shown in Fig. 1A and 1B, mRNA expression of both T-bet and GATA-3 was significantly increased in PBMC of HAM/TSP patients compared to HTLV-I-uninfected individuals. In addition, mRNA expression of IL-12Rβ2 was significantly higher in HAM/TSP patients than in HTLV-I-uninfected individuals (Fig. 2). Although mRNA expression of T-bet, GATA-3 and IL-12Rβ2 seemed to be higher in HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals, these differences were not statistically significant. In addition, no significant differences in mRNA expression were found among HTLV-I-infected individuals with or without HAM/TSP.

mRNA expression of SOCS1 in PBMC was significantly increased in HAM/TSP patients and

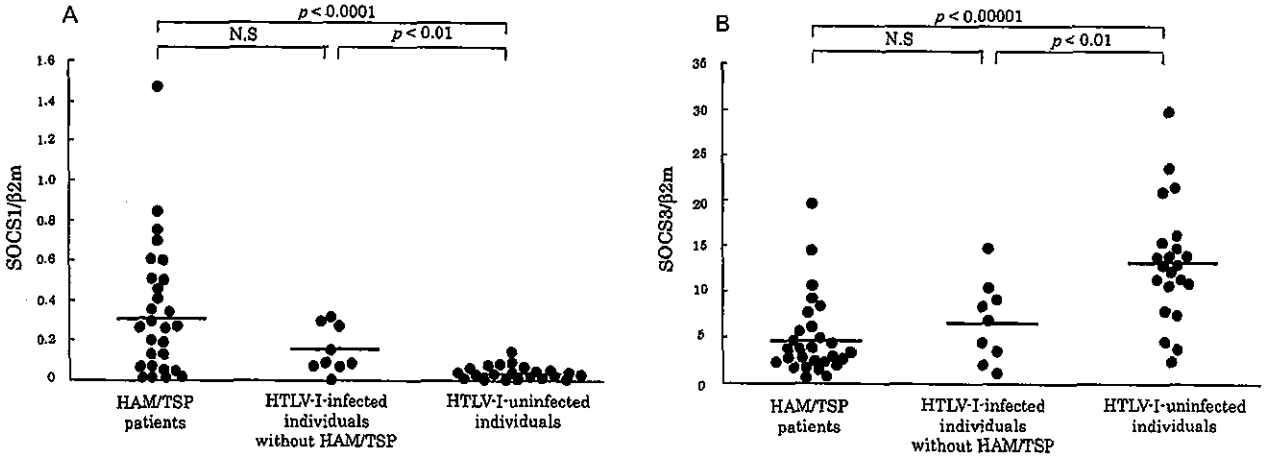

Fig. 3. Comparison of mRNA expression of SOCS1 and SOCS3 in PBMC. mRNA expression of SOCS1 in PBMC (A) was significantly higher in HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP than in HTLV-I-uninfected individuals. Although mRNA expression of SOCS1 seemed to be higher in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP, this difference was not statistically significant. Conversely, mRNA expression of SOCS3 in PBMC (B) was significantly decreased in both groups of HTLV-I-infected individuals compared to HTLV-I-uninfected individuals. There was no significant difference in mRNA expression of SOCS3 between HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP. Mann-Whitney's U-test was used for statistical analysis.

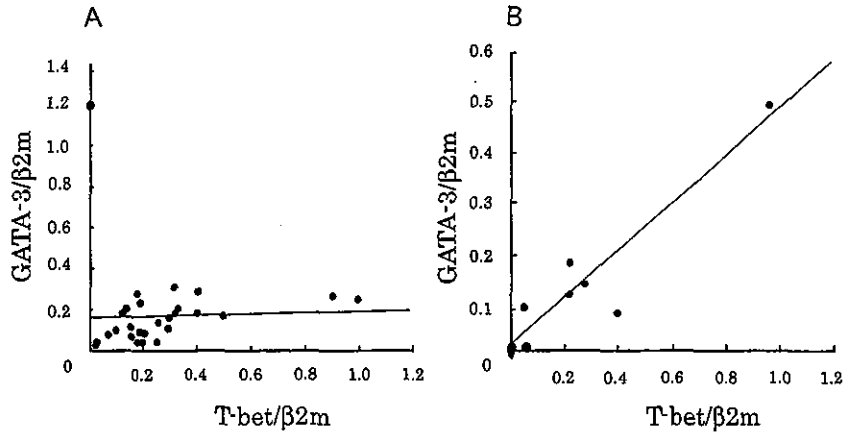

Fig. 4. Correlation between T-bet and GATA-3 mRNA expression among HTLV-I-infected individuals. mRNA expression of both transcription factors showed moderately positive correlation in HAM/TSP patients and strongly positive correlation in HTLV-I-infected individuals without HAM/TSP, respectively. Nonparametric Spearman's rank correlation test was used for statistical analysis.
A: HAM/TSP patients (rs = 0.393, $p = 0.041$), B: HTLV-I-infected individuals without HAM/TSP (rs = 0.754, $p = 0.033$).

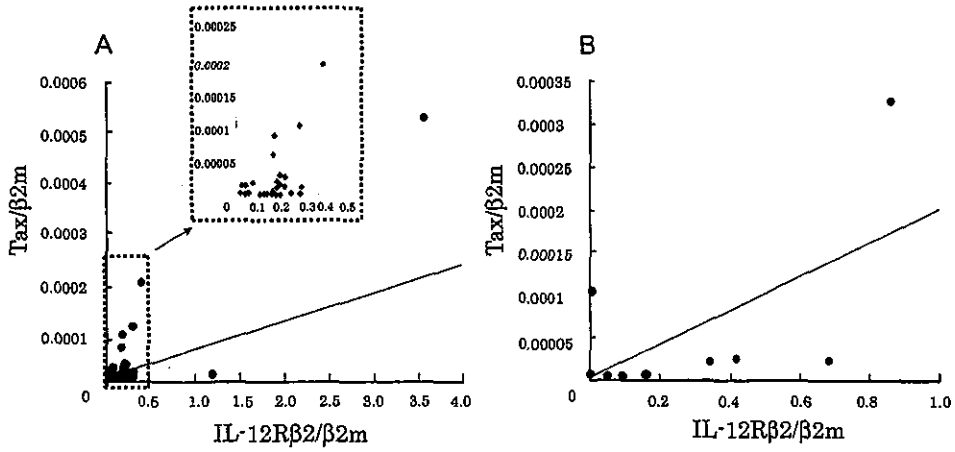

Fig. 5. Correlation between IL-12Rβ2 and HTLV-I tax mRNA expression among HTLV-I-infected individuals. There was moderately positive correlation in only HAM/TSP patients, but not HTLV-I-infected individuals without HAM/TSP. Nonparametric Spearman's rank correlation test was used for statistical analysis.
A: HAM/TSP patients (rs = 0.380, $p = 0.048$), B: HTLV-I-infected individuals without HAM/TSP (rs = 0.197, $p = 0.367$).

HTLV-I-infected individuals without HAM/TSP, compared to HTLV-I-uninfected individuals (Fig. 3A). Conversely, mRNA expression of SOCS3 was significantly decreased in HTLV-I-infected individuals with HAM/TSP and without HAM/TSP, compared to HTLV-I-uninfected individuals (Fig. 3B). However, no significant difference in mRNA expression of SOCS3 was found between HTLV-I-infected individuals with or without HAM/TSP.

In HTLV-I-infected individuals, increased mRNA expression of SOCS1 in PBMC indicated up-regulation of IFN-γ signaling, and decreased mRNA expression of SOCS3 in PBMC indicated down-regulation of IL-4 signaling. Moreover, GATA-3 mRNA expression seemed to also be up-

regulated in HTLV-I-infected individuals. Therefore, we analyzed the correlation between T-bet and GATA-3 mRNA expression in HTLV-I-infected individuals. As shown in Fig. 4, mRNA expression of both transcription factors showed moderately positive and strongly positive correlation in HAM/TSP patients (rs = 0.393; $p = 0.041$) and in HTLV-I-infected individuals without HAM/TSP (rs = 0.754; $p = 0.033$), respectively.

Next, we analyzed the correlation between either T-bet, SOCS1 or IL-12Rβ2 and HTLV-I tax mRNA expression. There were no correlations between either T-bet or SOCS1 and HTLV-I tax mRNA expression in both HAM/TSP patients (T-bet vs. HTLV-I tax: rs = 0.050; $p = 0.398$, SOCS1 vs. HTLV-I tax: rs = 0.080; $p = 0.678$) and HTLV-I-infected individuals without HAM/TSP (T-bet vs. HTLV-I tax: rs = 0.029; $p = 0.467$, SOCS1 vs. HTLV-I tax: rs = −0.067; $p = 0.850$) (data not shown). However, as shown in Fig. 5, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients (rs = 0.380; $p = 0.048$), but not in HTLV-I-infected individuals without HAM/TSP (rs = 0.197; $p = 0.367$).

Discussion

This is the first ex vivo analysis of Th1/Th2-related cytokine signaling molecules in HTLV-I-infected individuals. Firstly, to clarify the molecular mechanism of Th1 activation in HTLV-I-infected individuals, we focused mRNA expression of T-bet which are master switches in Th1 differentiation (Grogan and Locksley 2002; O'Shea and Paul 2002). Expression of T-bet mRNA in PBMC was increased in HAM/TSP patients than in HTLV-I-uninfected individuals.

Secondly, we showed significantly increased SOCS1 mRNA expression concomitant with the decreased SOCS3 mRNA expression in PBMC of HTLV-I-infected individuals compared to HTLV-I-uninfected controls. Increased SOCS1 mRNA expression strongly suggests that both IFN-γ and IL-12 signaling are up-regulated, and conversely, decreased SOCS3 mRNA expression suggests IL-4 signaling is down-regulated in HTLV-I-infected individuals. Alternatively, there might exist more Th1 cells and fewer Th2 cells in PBMC of HTLV-I-infected individuals compared to HTLV-I-uninfected individuals, if the distinct pattern of SOCS expression indicates Th lineage (Egwuagu et al. 2002; Seki et al. 2003). From the point of view of SOCS expression, our data revealed that the Th1/Th2 balance is toward Th1 immune activation in HTLV-I-infected individuals. We previously reported the importance of Th1 immune deviation in the development of HAM/TSP (Nakamura et al. 2000b). However, as far as the pattern of SOCS mRNA expression is concerned, there was no significant difference between HAM/TSP patients and HTLV-I-infected individuals without HAM/TSP. Nevertheless, the pattern of SOCS1 or SOCS3 mRNA expression showed the tendency that both IFN-γ and IL-12 signaling are more up-regulated with more down-regulated IL-4 signaling in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP.

From these results, we thought that the expression of GATA-3, located downstream of IL-4R, might be down-regulated in HTLV-I-infected individuals because of down-regulation of IL-4 signaling. However, unexpectedly, GATA-3 mRNA expression was also up-regulated in HAM/TSP patients than in HTLV-I-uninfected individuals. We are unable to precisely explain the reasons for this phenomenon. One possible explanation, however, is that auto-activation of GATA-3 itself might be involved (Ouyang et al. 2000). In this regard, there was moderate to strong correlation between T-bet and GATA-3 mRNA expression among HTLV-I-infected individuals with and without HAM/TSP. Therefore, Th1/Th2 balance in HTLV-I-infected individuals is under a compensatory control.

Thirdly, we analyzed mRNA expression of IL-12Rβ2, which is located up-stream of STAT4 signaling and plays an important role in Th1 differentiation (Grogan and Locksley 2002). IL-12Rβ2 mRNA expression in PBMC was significantly

higher in HAM/TSP patients than in HTLV-I-uninfected individuals, strongly indicating that Th1 cells are increased in HAM/TSP patients. IL-12Rβ2 is induced by STAT4 signaling following the stimulation of IL-12 itself (Rogge et al. 1997). Therefore, down-regulation of SOCS3 mRNA expression in HAM/TSP patients might be involved in this phenomenon although the precise mechanisms are unclear.

Although mRNA expression of T-bet, GATA-3, IL-12Rβ2 and SOCS1 seemed to be higher, and mRNA expression of SOCS3 seemed to be lower in HAM/TSP patients than in HTLV-I-infected individuals without HAM/TSP, the differences were not statistically significant. Therefore, we, next, analyzed the correlation between these Th1-related cytokine signaling molecules and HTLV-I tax mRNA expression in both groups. Neither T-bet nor SOCS1 mRNA expression correlated with HTLV-I tax mRNA expression in HTLV-I-infected individuals with or without HAM/TSP, suggesting that HTLV-I tax expression is not directly related to expression of these signaling molecules. Interestingly, however, there was a moderately positive correlation between IL-12Rβ2 and HTLV-I tax mRNA expression in HAM/TSP patients. Therefore, in patients with HAM/TSP, HTLV-I tax might be cooperatively involved in IL-12R/STAT4 signaling in HTLV-I-infected cells and contribute to Th1 activation. Recently, Furukawa et al. (2003) demonstrated that IFN-γ producing cells in the population of HTLV-I tax-expressing cells are increased in HAM/TSP patients, compared to HTLV-I asymptomatic carriers with a high HTLV-I proviral load comparable to that in HAM/TSP patients. Further studies are needed to investigate at which step HTLV-I tax up-regulates IL-12R/STAT4 signaling pathway in patients with HAM/TSP.

Acknowledgments

Special thanks to Dr. Richard Yanagihara, University of Hawai'i at Manoa, for critical review of the manuscript.

This work was supported in part by a Research Grant for Neuroimmunological Diseases from the Ministry of Health, Labor and Welfare, Japan.

References

Akizuki, S., Nakazato, O., Higuchi, Y., Tanabe, K., Setoguchi, M., Yoshida, S., Miyazaki, Y., Yamamoto, S., Sudou, S., Sannomiya, K. & Okajima, T. (1987) Necropsy findings in HTLV-I associated myelopathy. *Lancet*, **1**, 156-157.

Alexander, W.S. (2002) Suppressors of cytokine signaling (SOCS) in the immune system. *Nat. Rev. Immunol.*, **2**, 410-416.

Carvalho, E.M., Bacellar, O., Porto, A.F., Braga, S., Galvao-Castro, B. & Neva, F. (2001) Cytokine profile and immunomodulation in asymptomatic human T-lymphotropic virus type 1-infected blood donors. *J. Acquir. Immune. Defic. Syndr.*, **27**, 1-6.

Egwuagu, C.E., Yu, C.-R., Zhang, M., Mahdi, R.M., Kim, S.J. & Gery, I. (2002) Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance. *J. Immunol.*, **168**, 3181-3187.

Furukawa, Y., Saito, M., Matsumoto, W., Usuku, K., Tanaka, Y., Izumo, S. & Osame, M. (2003) Different cytokine production in Tax-expressing cells between patients with human T cell lymphotropic virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis and asymptomatic HTLV-I carriers. *J. Infect. Dis.*, **187**, 1116-1125.

Furuya, T., Nakamura, T., Fujimoto, T., Nakane, S., Kambara, C., Shirabe, S., Hamasaki, S., Motomura, M. & Eguchi, K. (1999) Elevated levels of interleukin-12 and interferon-gamma in patients with human T lymphotropic virus type I-associated myelopathy. *J. Neuroimmunol.*, **95**, 185-189.

Grogan, J.L. & Locksley, R.M. (2002) T helper cell differentiation: on again, off again. *Curr. Opin. Immunol.*, **14**, 366-372.

Hanon, E., Goon, P., Taylor, G.P., Hasegawa, H., Tanaka, Y., Weber, J.N. & Bangham, C.R. (2001) High production of interferon γ but not interleukin-2 by human T-lymphotropic virus type I-infected peripheral blood mononuclear cells. *Blood*, **98**, 721-726.

Hollsberg, P. (1999) Mechanisms of T-cell activation by human T-cell lymphotropic virus type I. *Microbial. Mol. Biol. Rev.*, **63**, 308-333.