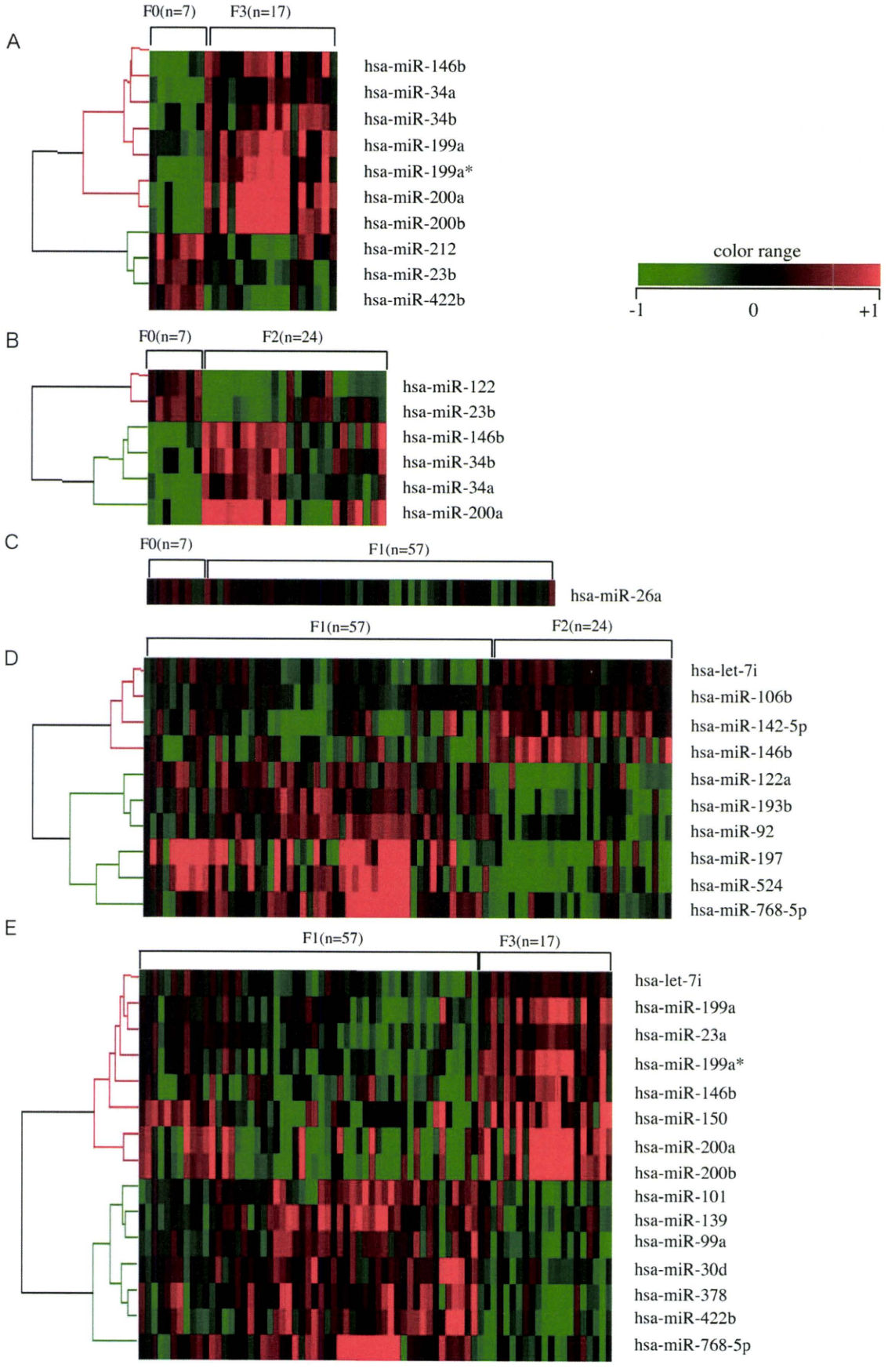
A
F0(n=7)
F3(n=17)
hsa-miR-146b
hsa-miR-34a
hsa-miR-34b
hsa-miR-199a
hsa-miR-199a*
hsa-miR-200a
hsa-miR-200b
hsa-miR-212
hsa-miR-23b
hsa-miR-422b
color range
-1
0
+1
B
F0(n=7)
F2(n=24)
hsa-miR-122
hsa-miR-23b
hsa-miR-146b
hsa-miR-34b
hsa-miR-34a
hsa-miR-200a
C
F0(n=7)
F1(n=57)
hsa-miR-26a
D
F1(n=57)
F2(n=24)
hsa-let-7i
hsa-miR-106b
hsa-miR-142-5p
hsa-miR-146b
hsa-miR-122a
hsa-miR-193b
hsa-miR-92
hsa-miR-197
hsa-miR-524
hsa-miR-768-5p
E
F1(n=57)
F3(n=17)
hsa-let-7i
hsa-miR-199a
hsa-miR-23a
hsa-miR-199a*
hsa-miR-146b
hsa-miR-150
hsa-miR-200a
hsa-miR-200b
hsa-miR-101
hsa-miR-139
hsa-miR-99a
hsa-miR-30d
hsa-miR-378
hsa-miR-422b
hsa-miR-768-5p

Figure 2. Liver fibrosis in human liver biopsy specimen. A. B. C. D. and E. miRNAs whose expression differs significantly between F0 and F3, F0 and F1, F0 and F2, F1 and F2, and F1 and F3, respectively. Relative expression level of each miRNA in human liver biopsy specimen by microarray. Data from microarray were also statistically analyzed using Welch's test and the Bonferroni correction for multiple hypotheses testing. Fold change, p-value are listed in Table S2.
doi:10.1371/journal.pone.0016081.g002

specific E3 ubiquitin protein ligase 2 (SMURF2), both of which play roles in the TGFβ signaling pathway, are candidate targets of miR-199a* and miR-200b, respectively, as determined by the Targetscan algorithm. The expression of miR-199a* was silenced in several proliferating cell lines excluding fibroblasts [21]. Down regulation of miR-199a, miR-199a* and 200a in chronic liver injury tissue was associated with the hepatocarcinogenesis [9]. miR-199a* is also one of the negative regulators of the HCV replication [22]. According to three target search algorithms (Pictar, miRanda, and Targetscan), the miRNAs that may be associated with the liver fibrosis can regulate several fibrosis-related genes (Table S4). Aberrant expression of these miRNAs may be closely related to the progress of the chronic liver disease.

Epithelial-mesenchymal transition (EMT) describes a reversible series of events during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics [23]. Although EMT is not a common event in adults, this process has been implicated in such instances as wound healing and fibrosis. Recent reports showed that the miR-200 family regulated EMT by targeting EMT accelerator ZEB1 and SIP1 [24]. From our observations, overexpression of miR-200a and miR-200b can be connected to the progression of liver fibrosis.

The diagnosis and quantification of fibrosis have traditionally relied on liver biopsy, and this is still true at present. However, there are a number of drawbacks to biopsy, including the invasive nature of the procedure and inter-observer variability. A number of staging systems have been developed to reduce both the inter-observer variability and intra-observer variability, including the METAVIR, the Knodell fibrosis score, and the Scheuer score. However, the reproducibility of hepatic fibrosis and inflammatory activity is not as consistent [25]. In fact, in our study, the degree of fibrosis of the two arbitrary fibrosis groups was classified using the miRNA expression profile with 80% or greater accuracy (data not shown). Thus, miRNA expression can be used for diagnosis of liver fibrosis.

In this study we investigated whether common miRNAs in human and mouse could influence the progression of the liver fibrosis. The signature of miRNAs expression can also serves as a tool for understanding and investigating the mechanism of the onset and progression of liver fibrosis. The miRNA expression profile has the potential to be a novel biomarker of liver fibrosis.

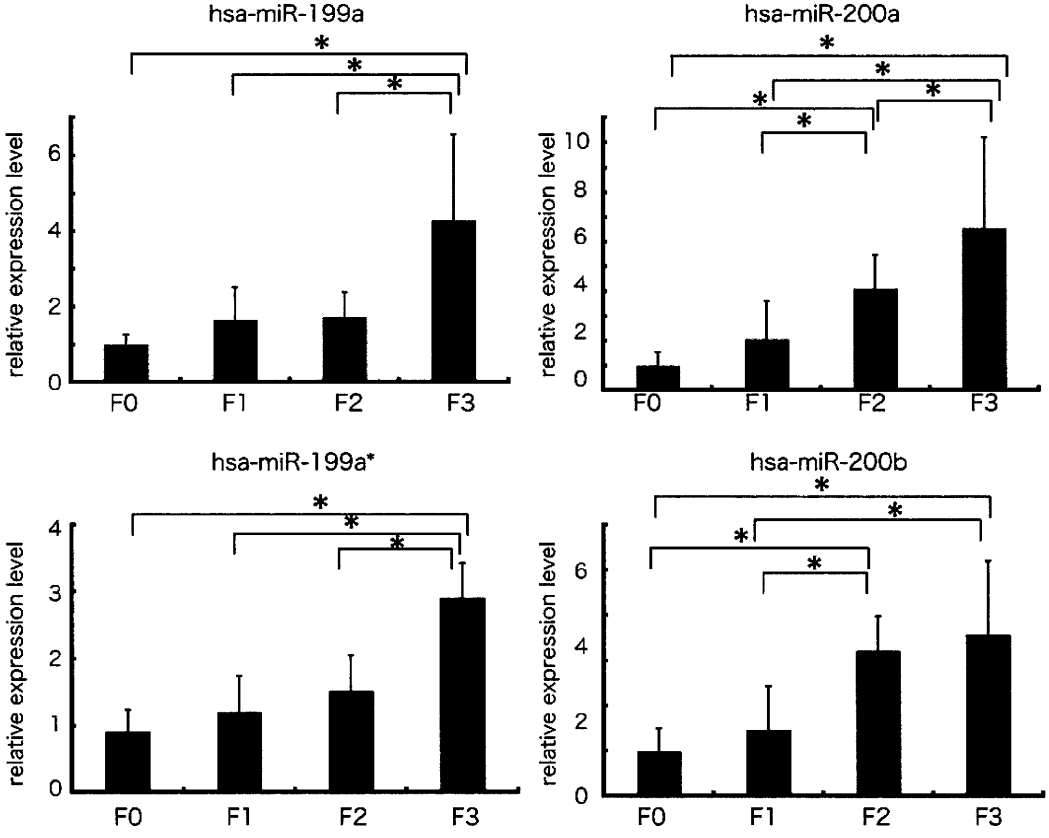

Figure 3. The expression level of miR-199 and 200 families in human liver biopsy specimen by real-time qPCR. Real-time qPCR validation of the 4 miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b). Each column represents the relative amount of miRNAs normalized to the expression level of U18. The data shown are the means+SD of three independent experiments. Asterisks indicates to a significant difference of $p<0.05$ (two-tailed Student-t test), respectively.
doi:10.1371/journal.pone.0016081.g003

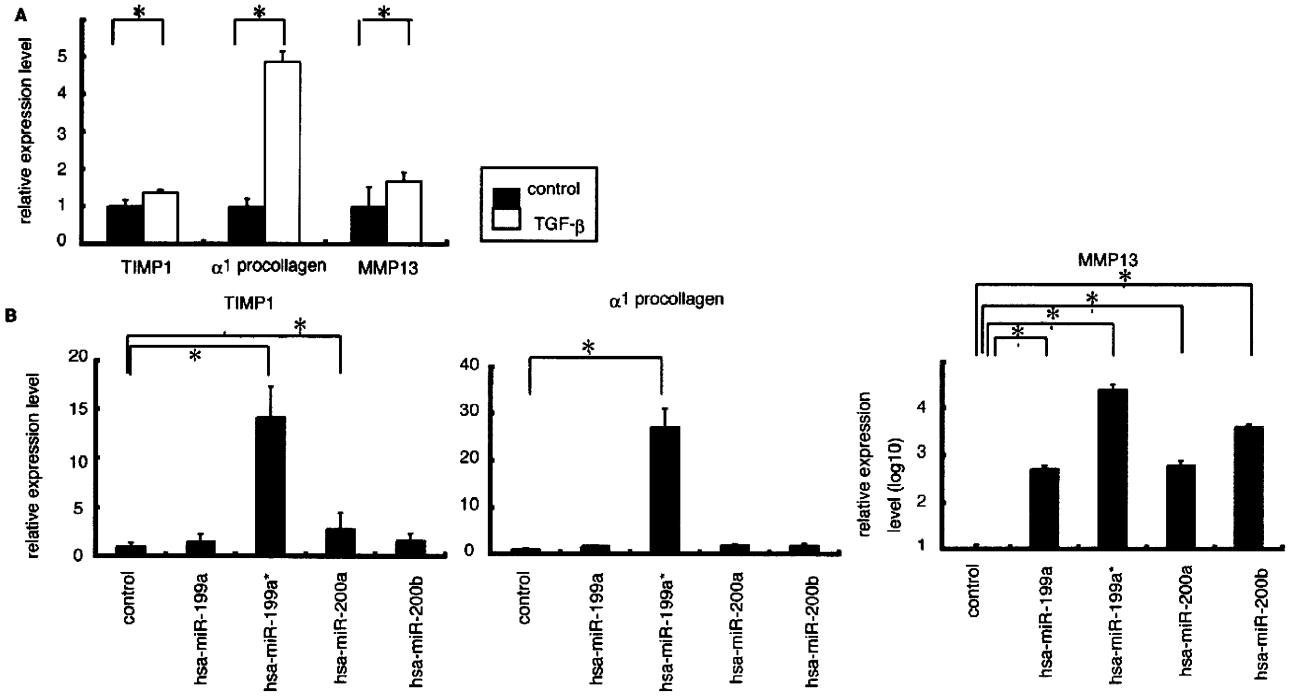

Figure 4. The relationship between expression level of miR-199 and 200 families and expression level of three fibrosis related genes. A. Administration of TGFβ in LX2 cells showed that the expression level of three fibrosis related genes were higher than that in non-treated cells. The data shown are the means+SD of three independent experiments. Asterisk was indicated to the significant difference of $p<0.05$ (two-tailed Student-t test). B. The expression levels of 3 fibrosis related genes in LX2 cells with overexpressing miR-199a, 199a*, 200a, or 200b, respectively were significantly higher than that in cells transfected with control miRNA ($p<0.05$; two-tailed Student t-test).
doi:10.1371/journal.pone.0016081.g004

Moreover miRNA expression profiling has further applications in novel anti-fibrosis therapy in CH.

Materials and Methods

Sample preparation

105 liver tissues samples from chronic hepatitis C patients (genotype 1b) were obtained by fine needle biopsy (Table S1). METAVIR fibrosis stages were F0 in 7 patients, F1 in 57, F2 in 24 and F3 in 17. Patients with autoimmune hepatitis or alcoholic liver injury were excluded. None of the patients were positive for hepatitis B virus associated antigen/ antibody or anti human immunodeficiency virus antibody. No patient received interferon therapy or immunomodulatory therapy prior to the enrollment in this study. We also obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University. All of the patients or their guardians provided written informed consent, and Kyoto University Graduate School and Faculty of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

RNA preparation and miRNA microarray

Total RNA from cell lines or tissue samples was prepared using a *mir*Vana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. miRNA microarrays were manufactured by Agilent Technologies (Santa Clara, CA, USA) and 100 ng of total RNA was labeled and hybridized using the Human microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.5 and Mouse microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.0. Hybridization signals were detected with a DNA microarray scanner G2505B (Agilent Technologies) and the scanned images were analyzed using Agilent feature extraction software (v9.5.3.1). Data were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and normalized as follows: (i) Values below 0.01 were set to 0.01. (ii) In order to compare between one-color expression profile, each measurement was divided by the 75th percentile of all measurements from the same species. The data presented in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16922 (human) and accession number GSE19865 (mouse).

Real-time qPCR for human miRNA

For detection of the miRNA level by real-time qPCR, TaqMan® microRNA assay (Applied Biosystems) was used to quantify the relative expression level of miR-199a (assay ID. 002304), miR-199a* (assay ID. 000499), miR-200a (assay ID. 000502), miR-200b (assay ID. 002251), and U18 (assay ID. 001204) was used as an internal control. cDNA was synthesized using the Taqman miRNA RT Kit (Applied Biosystems). Total RNA (10 ng/ml) in 5ml of nuclease free water was added to 3 ml of 5× RT primer, 10× 1.5μl of reverse transcriptase buffer, 0.15 μl of 100 mM dNTP, 0.19 μl of RNase inhibitor, 4.16 μl of nuclease free water, and 50U of reverse transcriptase in a total volume of 15 μl. The reaction was performed for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. All reactions were run in triplicate. Chromo 4 detector (BIO-RAD) was used to detect miRNA expression.

Animal and Chronic Mouse Liver Injury Model

Each 5 adult (8-week-old) male C57BL/6J mice were given a biweekly intra-peritoneal dose of a 10% solution of CCL_4 in olive oil (0.02 ml/g/ mouse) for the first 4 weeks and then once a week

for the next 4 weeks. At week 4, 6 or 8, the mice were sacrificed. Partial livers were fixed, embedded in paraffin, and processed for histology. Serial liver sections were stained with hematoxylin-eosin, Azan staining, Silver (Ag) staining, and Elastica van Gieson (EVG) staining, respectively. Total RNA from mice liver tissue was prepared as described previously. All animal procedures concerning the analysis of liver injury were performed in following the guidelines of the Kyoto University Animal Research Committee and were approved by the Ethical Committee of the Faculty of Medicine, Kyoto University.

Cell lines and Cell preparation

The human stellate cell lines LX-2, was provided by Scott L. Friedman. LX-2 cells, which viable in serum free media and have high transfectability, were established from human HSC lines [26]. LX-2 cells were maintained in D-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, plated in 60 mm diameter dishes and cultured to 70% confluence. Huh-7 and Hela cells were also maintained in D-MEM with 10% fetal bovine serum. HuS-E/2 immortalized hepatocytes were cultured as described previously [27]. LX-2 cells were then cultured in D-MEM without serum with 0.2% BSA for 48 hours prior to TGFβ1 (Sigma-Aldrich, Suffolk, UK) treatment (2.5 ng/ml for 20 hours). Control cells were cultured in D-MEM without fetal bovine serum.

miRNA transfection

LX-2 cells were plated in 6-well plates the day before transfection and grown to 70% confluence. Cells were transfected with 50 pmol of Silencer® negative control siRNA (Ambion) or double-stranded mature miRNA (Hokkaido System Science, Sapporo, Japan) using lipofectamine RNAiMAX (Invitrogen). Cells were harvested 2 days after transfection.

Real-time qPCR

cDNA was synthesized using the Transcriptor High Fiderity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 10.4 μl of nuclease free water was added to 1 μl of 50mM random hexamer. The denaturing reaction was performed for 10min at 65°C. The denatured RNA mixture was added to 4 μl of 5× reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40U/μl RNase inhibitor, and 1.1 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. The reaction ran for 30 min at 50°C (cDNA synthesis), and five min at 85°C (enzyme denaturation). All reactions were run in triplicate. Chromo 4 detector (BIO-RAD, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows; MMP13 s; 5′-gaggctccgagaaatgcagt-3′, as; 5′-atgccatcgtgaagtctggt-3′, TIMP1 s; 5′-cttggcttctgcactgatgg-3′, as; 5′-acgctggtataaggtggtct-3′, α1-procollagen s; 5′-aacatgaccaaaaaccaaaagtg-3′, as; 5′-cattgtttcctgtgtcttctgg-3′, and β-actin s; 5′-ccactggcatcgtgatggac-3′, as; 5′-tcattgccaatggtgatgacct-3′. Assays were performed in triplicate, and the expression levels of target genes were normalized to expression of the β-actin gene, as quantified using real-time qPCR as internal controls.

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *p* values less than 0.05 were considered statistically significant. Microarray data were also statistically analyzed using Welch's test and Bonferroni correction for multiple hypotheses testing.

Supporting Information

Figure S1 Time line of the induction of chronic liver fibrosis. Upward arrow indicated administration of olive oil or CCL_4. Downward arrow indicates when mice were sacrificed.
(TIF)

Figure S2 Comparison of the expression level of miR-199 and 200 familes in several cell lines and human liver tissue. Endogenous expression level of miR-199a, 199a*, 200a, and 200b in normal liver and LX2 cell as determined by microarray analysis (Agilent Technologies). Endogenous expression level of same miRNAs in Hela, Huh-7 and, immortalized hepatocyte: HuS-E/2 by previously analyzed data [9].
(TIF)

Table S1 Clinical characteristics of patients by the grade of fibrosis.
(DOCX)

Table S2 Extracted human miRNAs related to liver fibrosis.
(DOCX)

Table S3 Corresponding human and mouse miRNAs.
(DOCX)

Table S4 Hypothetical miRNA target genes according to in silico analysis.
(DOCX)

Author Contributions

Conceived and designed the experiments: YM KS. Performed the experiments: YM HT YH NK. Analyzed the data: MT MK. Contributed reagents/materials/analysis tools: YM HT YH NK. Wrote the paper: YM MT AT FM NK TO.

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研究成果の刊行に関する一覧表

研究代表者：京都大学ウイルス研究所　松岡　雅雄
研究分担者：琉球大学大学院医学研究科　齊藤　峰輝

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Review Article

Immunogenetics and the Pathological Mechanisms of Human T-Cell Leukemia Virus Type 1- (HTLV-1-)Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

Mineki Saito

Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan

Correspondence should be addressed to Mineki Saito, mineki@med.u-ryukyu.ac.jp

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Human T-cell leukemia virus type 1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease only in a minority of infected individuals: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence suggests that complex virus-host interactions play an important role in determining the risk of HAM/TSP. This review focuses on the role of the immune response in controlling or limiting viral persistence in HAM/TSP patients and the reason why some HTLV-1-infected people develop HAM/TSP whereas the majority remains asymptomatic carriers of the virus.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) infection is of particular interest to the field of immunology as well as microbiology because HTLV-1 is never eliminated from the host in spite of vigorous cellular and humoral immune responses against the virus but causes no disease in vast majority of infected subjects (asymptomatic carriers:ACs). Although only approximately 2%-3% develop adult T cell leukemia (ATL) [1, 2] and another 0.25%–3.8% develop chronic inflammatory diseases involving the central nervous system (HTLV-1-associated myelopathy/tropical spastic paraparesis: HAM/TSP) [3, 4], evaluation of the individual risk for developing diseases in each ACs would certainly be of considerable importance especially in HTLV-1 endemic area such as southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [5]. However, many fundamental questions are remained to be solved. First, how does HTLV-1 persist in the individual host in spite of strong host immune response? Second, why do some HTLV-1-infected people develop consequent diseases such as ATL or HAM/TSP, whereas the majority remains asymptomatic carriers of the virus? Third, how is the inflammatory lesion in HAM/TSP initiated and maintained, and why is the inflammation specifically in thoracic spinal cord? This review summarizes the past and recent works for HAM/TSP attempting to resolve each of these questions.

2. Clinical and Pathological Features of HAM/TSP

HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [6–8]. HTLV-1 can be transmitted through sexual contact [9], injection drug use [8], and breastfeeding from mother to child [10, 11]. Although HTLV-1 infection is associated with a range of nonmalignant chronic inflammatory diseases in the eyes, the lungs, or the skeletal muscles [7], HAM/TSP is the bestrecognized with chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [12]. To date, more

than 3,000 cases of HAM/TSP patients have been reported in HTLV-1 endemic areas. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [13–16].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [17–19]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal bands, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-γ, and an increased intrathecal antibody synthesis specific for HTLV-1 antigens have also been described [20]. Clinical progression of HAM/TSP is associated with increased proviral load in individual patients, and the ratio of proviral loads in CSF cells/in peripheral blood mononuclear cells (PBMCs) is significantly associated with clinically progressive disease [21]. The major histocompatibility complex (MHC) class I tetramer analysis of lymphocytes isolated from the CSF of HAM/TSP patients showed even higher frequencies of HTLV-1 Tax11-19-specific, HLA-A*02-restricted $CD8^+$ lymphocytes compared to those of PBMCs [22]. Therefore, an increased proliferation or migration of HTLV-1-infected and/or HTLV-1-specific lymphocytes to the central nervous system (CNS) might be closely associated with HAM/TSP pathogenesis [23].

3. Risk Factors for HAM/TSP

3.1. Host Genetics. Previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima, HTLV-1 endemic southern Japan, revealed that one of the major risk factors is the HTLV-1 proviral load. The median proviral load was more than ten times higher in HAM/TSP patients than in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Higher proviral load in HAM/TSP patients than in ACs was observed in other endemic area, such as the Caribbean [25], South America [26], and the Middle East [27]. It was suggested that genetic factors such as human leukocyte antigen (HLA) are related to the high proviral load in HAM/TSP patients and genetic relatives. In southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 proviral load and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population [28, 29]. Since the function of class I HLA proteins is to present antigenic peptides to cytotoxic T lymphocytes (CTL), these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may be an important determinant of HTLV-1 proviral load and the risk of HAM/TSP. Further analysis to look at nonHLA host genetic factors revealed that nonHLA gene polymorphism also affects the risk for developing HAM/TSP. For example, the TNF-α promoter −863 A allele [30], and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [31] predisposed to HAM/TSP, whereas IL-10 -592 A [32], Stromal derived factor (SDF)-1 +801A [30] and IL-15 +191 C alleles [30] conferred protection against HAM/TSP. The polymorphisms of MMP-9 and IL-10 promoter each linked to the HTLV-1-encoded transactivator Tax-mediated transcriptional activity of each gene [31, 32].

3.2. HTLV-1 Genotype. Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [33]. The *tax* subgroup A that belongs to cosmopolitan subtype A was more frequently observed in HAM/TSP patients and this effect was independent of protective allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B that belongs to cosmopolitan subtype B but not against *tax* subgroup A in Japanese population [33]. Interestingly, HLA-A*02 appears not to give protection against infection with cosmopolitan subtype A in a population in Iran [27]. These findings suggest that both host genetic factors and HTLV-1 subgroup play a part in determining the risk of HAM/TSP.

4. The Immune Response to HTLV-1

4.1. The Humoral Immune Response. In HTLV-1 infection, anti-HTLV-1 antibody that often includes IgM is detected in all infected individuals, either ACs or patients with HAM/TSP [34]. It has been reported that HAM/TSP patients generally had higher anti-HTLV-1 antibody titer than ACs with the similar HTLV-1 proviral load [34–36]. These data suggest that there was persistent expression of HTLV-1 proteins in vivo and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Interestingly, although antibody responses to the immunodominant epitopes of the HTLV-1 Envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was highest in HAM/TSP (71%–93%) than ATL patients (4%–31%) or ACs (27%–37%) [37]. In 2002, Levin et al. reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous nuclear riboprotein (hnRNP)-A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [38]. However, since the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [39] and is not normally accessible to antibody

attack, it is unlikely that anti-Tax antibody explains the onset or initial tissue damage of HAM/TSP. Rather, anti-Tax antibody might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

4.2. The Natural Killer (NK) Cell Response. Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs, although the results were not normalized with respect to the proviral load [40]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, the synthetic tetramers of HLA-E with the HLA-G signal sequence peptide were used to identify NK cells in HAM/TSP patients [41]. The results clearly showed a lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [40], this reduction in frequency was particularly notable in the $CD3^+$ cells whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [41]. Recent data also suggest that the frequency of invariant NKT cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [42, 43]. These findings indicated that the activity of the NK or NKT cell response was associated with the presence or absence of HAM/TSP. Interestingly, previous uncontrolled preliminary trial of viable *Lactobacillus casei* strain Shirota containing fermented milk for HAM/TSP patients resulted in significant increase of NK cell activity with improvements in clinical symptoms [44]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP.

4.3. The Regulatory T Cells (Tregs). It has been reported that HTLV-1 preferentially and persistently infects $CD4^+CD25^+$ lymphocytes in vivo [45], which contains the majority of the $Foxp3^+$ Tregs [46]. In HAM/TSP patients, the percentage of $Foxp3^+$ Tregs in $CD4^+CD25^+$ cells is lower than that in ACs and uninfected healthy controls [45, 47] whereas the percentage of $Foxp3^+$ cells in the $CD4^+$ population tended to be higher in the HAM/TSP patients than in the ACs [48–50]. As CD25 is induced by HTLV-1 Tax oncoprotein [51], it is most likely that the proportion of $Foxp3^+$ cells falls in the $CD4^+CD25^+$ population, which contain both Tregs and activated nonTregs, in HTLV-1-infected individuals especially HAM/TSP patients. Interestingly, the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells positively correlated with the HTLV-1 proviral load [23, 49] and the CTL activity negatively correlated with the frequency of HTLV-1 negative $Foxp3^+CD4^+$ cells [49]. These data suggest that an increase in HTLV-1 negative $FoxP3^+CD4^+$ Tregs is one of the chief determinants of the efficiency of T cell mediated immune control of HTLV-1. If such Tregs reduce CTL activity, which in turn increases the HTLV-1 proviral load, this activity increases the risk for developing HAM/TSP.

4.4. The $CD4^+$ Helper T Cell Response. It is well known that antiviral $CD4^+$ T cell responses are of central importance in driving B-cell and $CD8^+$ T-cell responses in vivo. The HTLV-1 antigen most commonly recognized by $CD4^+$ T cells is the Env protein [52, 53], in contrast with the immunodominance of Tax in the $CD8^+$ T cell response [54–56]. At a similar proviral load, patients with HAM/TSP had significantly increased frequency of virus-specific $CD4^+$ T-cells compared to that of ACs [53, 57]. The antiviral T helper (Th)1 phenotype is also dominant among HTLV-1-specific $CD4^+$ T cells in both ACs and patients with HAM/TSP [58], and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by $CD4^+$ T cells in patients with HAM/TSP compared to ACs of a similar proviral load [58, 59]. A role for CD4+ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in southern Japan [28, 29] and northeastern Iran [27]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380–394 peptide was used to analyze Env-specific $CD4^+$ T cells directly ex vivo [57]. The results showed that the frequency of $tetramer^+CD4^+$ T cells was significantly higher in HAM/TSP patients than in ACs with similar proviral load. Moreover, direct ex vivo analysis of $tetramer^+CD4^+$ T cells from two unrelated DRB1*0101 positive HAM/TSP patients indicated that certain T cell receptor (TCR) Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These data suggest that the observed increase in virus-specific $CD4^+$ T cells in HAM/TSP patients, which may contribute to $CD4^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected $CD4^+$ T cells but was the result of in vivo selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 $tetramer^+$ $CD8^+$ T cells [60] and muscle infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [61].

4.5. The Cytotoxic T Lymphocyte (CTL) Response. Previous reports indicated that the HTLV-1-specific $CD8^+$ CTL is typically abundant, chronically activated, and mainly targeted to the viral transactivator protein Tax [62]. Also, as already mentioned, the median proviral load in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high proviral load was also associated with an increased risk of progression to disease [24]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower proviral load and a lower risk of HAM/TSP [28, 29], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [63]. These data have raised the hypothesis that the class I-restricted $CD8^+$ CTL response

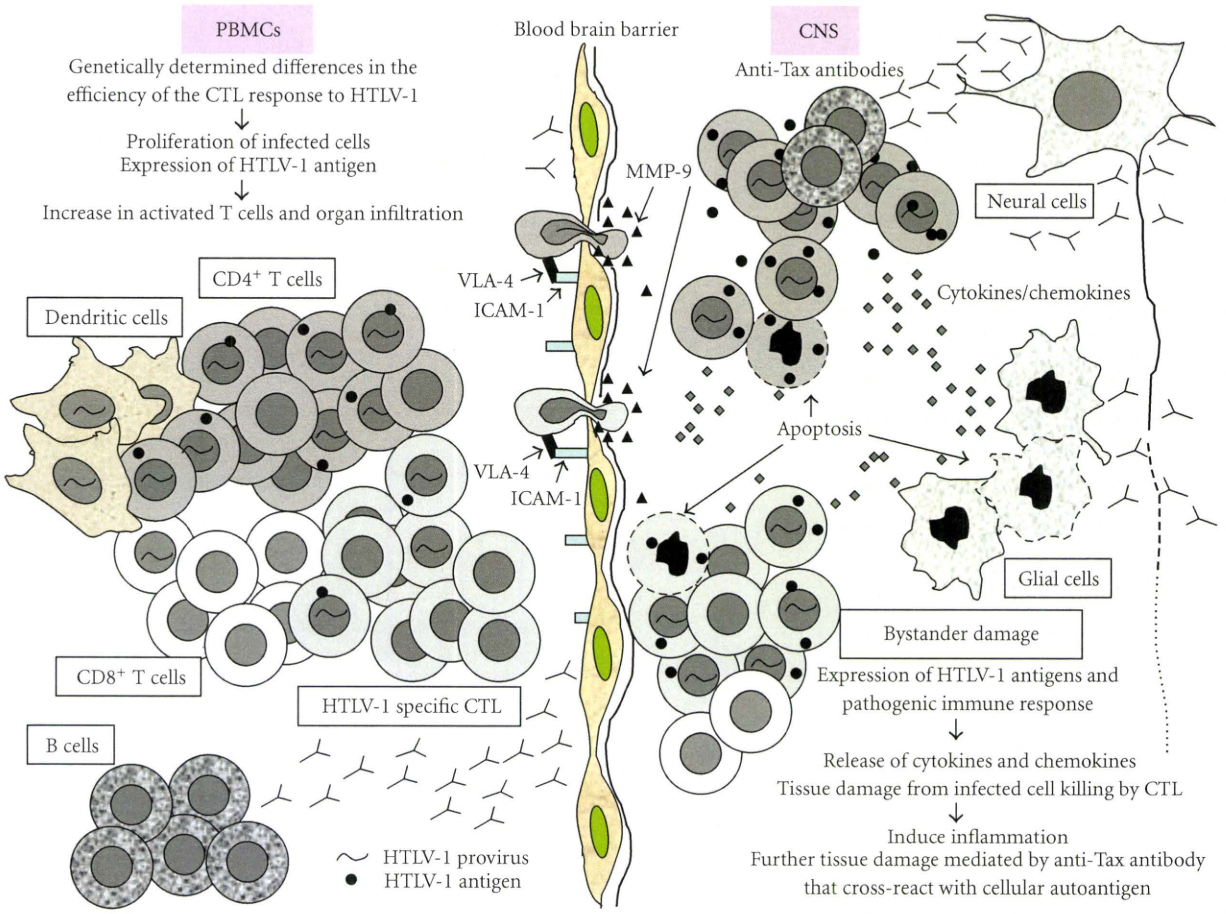

FIGURE 1: *Hypothesis for the pathogenesis of Human T-cell leukemia virus type 1- (HTLV-1-)associated myelopathy/tropical spastic paraparesis (HAM/TSP).* In patients with HAM/TSP, genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression, leading in turn to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-reacts with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

plays a critical part in limiting HTLV-1 replication in vivo and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. However, since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly elevated in HAM/TSP patients than in ACs [64, 65] and these cells have the potential to produce proinflammatory cytokines [66], there is a debate on the role of HTLV-1-specific-$CD8^+$ T cells, that is, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP or whether the dominant effect of such cells in vivo is protective against disease, although these two mechanisms are not mutually exclusive. Recently, Sabouri et al. reported that a frequency of $CD8^+$ T cells that were negative for costimulatory molecules such as CD27, CD28, CD80, CD86, and CD152 was significantly higher in patients with HAM/TSP than in age-matched uninfected controls, but there was no such difference between ACs and uninfected controls [67]. They also found a significantly lower frequency of perforin$^+$ cells and granzyme B^+ cells in the $CD8^+$ T cells in HTLV-1-infected subjects than in uninfected controls, although there was no significant difference between patients with HAM/TSP and ACs. Furthermore, the lytic capacity of HTLV-1-specific CTL between HAM/TSP and ACs estimated by CD107a mobilization assay showed the significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs. These findings suggest that patients with HAM/TSP have a high frequency of HTLV-1-specific $CD8^+$ T cells with poor lytic capacity, whereas ACs has a lower frequency of cells with high lytic capacity.

4.6. Dendritic Cells (DCs). Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [68], and the development of HAM/TSP is associated with rapid maturation of DCs [69]. One of the hallmarks of HTLV-1 infection is the in vitro proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous

lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [70, 71]. Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolishes SPL while supplementing DCs, but not B cells nor macrophages restore proliferation [68]. DC dependent mechanism of SLP was further supported by data showing that antibodies to MHC class II, CD86, and CD58 can block SPL [72]. Recently, Jones et al. had demonstrated that human-derived both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [73]. Furthermore, in contrast to the previous report that $CD4^+CD25^+$ T cells are responsible for the stimulation of Tax-specific $CD8^+$ T cells [74], it was recently demonstrated that, compared to the $CD4^+CD25^+$ T cells, the DCs are the major cell type responsible for the generation and maintenance of the Tax-specific $CD8^+$ T cells both in vitro and in vivo [75]. These findings suggest that the interaction of DCs with HTLV-1 is also crucial for the pathogenesis of HAM/TSP.

4.7. The Other Reservoirs of HTLV-1. Previous studies have indicated that only a small proportion of the monocyte-macrophage lineage cells are infected with HTLV-1 in peripheral blood [76] and that there has been no direct evidence indicating that HTLV-1-infected cells of the monocyte-macrophage lineage cells are present in the CNS [77]. However, monocyte-macrophage lineage cells may also play important roles in the pathogenesis of HAM/TSP, since it has been shown that the activation of macrophage and microglial cells within the CNS closely correlated with the proviral load within the CNS of HAM/TSP patients [78]. Meanwhile, it was also shown that a vast majority of bone marrow cells from HAM/TSP patients are positive for HTLV-1 proviral DNA but negative for viral RNA expression [79], whereas no HTLV-1 proviral DNA positive $CD34^+$ hematopoietic progenitor cells were detected in ATL patients [80]. These results suggest that HTLV-1-infected cells within the bone marrow may be a reservoir of HTLV-1 in HAM/TSP patients and play an important role in the etiology of neuroinflammation observed in HAM/TSP [77].

5. Conclusions

As shown in Figure 1, accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development.

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Activation of the PI3K–Akt pathway by human T cell leukemia virus type 1 (HTLV-1) oncoprotein Tax increases Bcl3 expression, which is associated with enhanced growth of HTLV-1-infected T cells

Kousuke Saito, Mineki Saito *, Naoko Taniura, Takako Okuwa, Yoshiro Ohara

Department of Microbiology, Kanazawa Medical University School of Medicine, 1-1 Daigaku, Uchinada-machi, Ishikawa 920-0293, Japan

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ABSTRACT

Bcl3 is a member of the IκB family that regulates genes involved in cell proliferation and apoptosis. Recent reports indicated that Bcl3 is overexpressed in HTLV-1-infected T cells via Tax-mediated transactivation, and acts as a negative regulator of viral transcription. However, the role of Bcl3 in cellular signal transduction and the growth of HTLV-1-infected T cells have not been reported. In this study, we showed that the knockdown of Bcl3 by short hairpin RNA inhibited the growth of HTLV-1-infected T cells. Although phosphatidylinositol-3 kinase (PI3K) inhibitor reduced Bcl3 expression, inactivation of glycogen synthase kinase 3 (GSK3), an effector kinase of the PI3K/Akt signaling pathway, restored Bcl3 expression in Tax-negative but not in Tax-positive T cells. Our results indicate that the overexpression of Bcl3 in HTLV-1-infected T cells is regulated not only by transcriptional but also by post-transcriptional mechanisms, and is involved in overgrowth of HTLV-1-infected T cells.

Introduction

Human T cell leukemia virus type 1 (HTLV-1) (Poiesz et al., 1980; Yoshida et al., 1982) is an exogenous human retrovirus that causes two distinct types of disease: adult T cell leukemia (ATL) (Hinuma et al., 1981; Yoshida et al., 1984) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986). ATL is an aggressive form of leukemia characterized by the malignant proliferation of $CD4^+$ T cells infected with HTLV-1, whereas HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction and mild sensory disturbance in the lower extremities (Nakagawa et al., 1995). HTLV-1 encodes a number of regulatory proteins, i.e. Tax and Rex, as well as accessory proteins, i.e. p12, p13, p30 and HBZ, which participate in the life cycle of the virus (Matsuoka and Jeang, 2007). Of these proteins, the viral oncoprotein Tax has been well characterized as a critical player in both viral and cellular transcription as well as malignant transformation. Tax is a transcriptional activator required for efficient expression of the viral genome through three conserved cyclic AMP response elements (CREs) located in the transcriptional control region of the virus, and also activates a variety of intracellular signal transduction pathways and subsequently deregulates gene expression though interaction with many different cellular proteins, including several transcription factors such as cAMP response element binding protein (CREB), NF-κB, serum response factor (SRF), and the transcriptional inhibitor, IκB (Yoshida, 2001).

B-cell leukemia protein 3 (Bcl3) is a member of the IκB family initially identified as a putative proto-oncogene from chronic B-cell lymphocytic leukemia (McKeithan et al., 1987; Ohno et al., 1990). Increased Bcl3 expression typically results from chromosomal translocations and leads to increased cell survival, proliferation, and malignancy (Courtois and Gilmore, 2006; McKeithan et al., 1987; Ohno et al., 1990) potential tomorigenesis in many cell types including T cells (Palmer and Chen, 2008). Furthermore, it has been reported that respiratory syncytial virus (RSV) infection (Jamaluddin et al., 2005) and viral oncogenic proteins such as Epstein–Barr virus (EBV) latent membrane protein (LMP) (Kung and Raab-Traub, 2008; Nakamura et al., 2008) and Hepatitis B virus X protein (HBX) (Park et al., 2006) upregulate Bcl3 gene expression through activation of the NF-κB signaling pathway. In addition to its possible role in oncogenesis, recent reports also suggest that Bcl3 has an important function in controlling inflammation and autoimmunity mediated by the NF-κB signaling pathway (Schwarz et al., 1997). Namely, Bcl3-deficient mice display defects in secondary lymphoid organs including partial loss of B cells (Franzoso et al., 1997), exhibit severe defects in humoral immune responses, and in protection from *in vivo* pathogenic challenges (Schwarz et al., 1997), and an autoimmune disease-like phenotype including uncontrolled inflammation mediated by aberrant inflammatory cytokine production (Carmody et al., 2007). Bcl3-deficient cells derived from Bcl3-deficient mice are hypersensitive to toll like receptor (TLR) activation and are unable to control responses

* Corresponding author. Present address: Department of Immunology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nakagami-gun, Okinawa 903-0215, Japan. Fax: +81 98 895 1437.
E-mail address: mineki@med.u-ryukyu.ac.jp (M. Saito).

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to lipopolysaccharides because of the absence of the normal Bcl3-mediated blockade of NF-κB p50 degradation (Carmody et al., 2007).

These observations, especially overexpression of Bcl3 in a number of cancers including T cell leukemias, prompted us to investigate the roles of Bcl3 in HTLV-1 infection. In this study, we therefore focus on the molecular mechanism of Bcl3 protein overexpression in HTLV-1 infection and its effect on overgrowth of HTLV-1 infected cells, which may help to generate potential strategies for therapy of ATL.

Results

Bcl3 protein is constitutively expressed in HTLV-1-infected T cell lines

Although recent reports indicated that bcl3 mRNA is induced by HTLV-1 Tax oncoprotein (Hishiki et al., 2007), there is no report whether Bcl-3 protein is also highly expressed and induced by Tax in HTLV-1 infected cells. We therefore first analyzed both bcl3 mRNA and protein expression in six HTLV-1-infected and three uninfected human T cell lines by RT-PCR and Western blot analysis. Bcl3 mRNA was expressed in all the six HTLV-infected T cell lines including MT-1, C5/MJ, HUT102 and MT-4 cells, all of which are not previously tested (Hishiki et al., 2007), but not in all the three HTLV-1-uninfected T cell lines tested (Fig. 1A). Similarly, Bcl3 protein was expressed in all the six HTLV-infected T cell lines, but not in all the three HTLV-1-uninfected T cell lines (Fig. 1B). The Bcl3 protein was abundantly expressed in HUT-102, MT-2, and SLB-1 cells, which express high levels of Tax protein, whereas MT-1 cells, which do not express Tax protein, also express a low level of Bcl3 protein (Fig. 1B). These data suggest that although the level of Bcl3 protein expression is largely dependent on the level of Tax protein expression in most HTLV-1-infected cell lines, Bcl3 protein expression is also maintained in MT-1 cells in a Tax-independent manner. Next, to examine whether Bcl3 protein expression is induced by Tax, we used JPX-9 cells, a Jurkat subclone generated by the stable introduction of a functional Tax expression-plasmid vector, and induced Tax expression by adding $CdCl_2$ into the culture medium (final concentration: 10 μM). Although

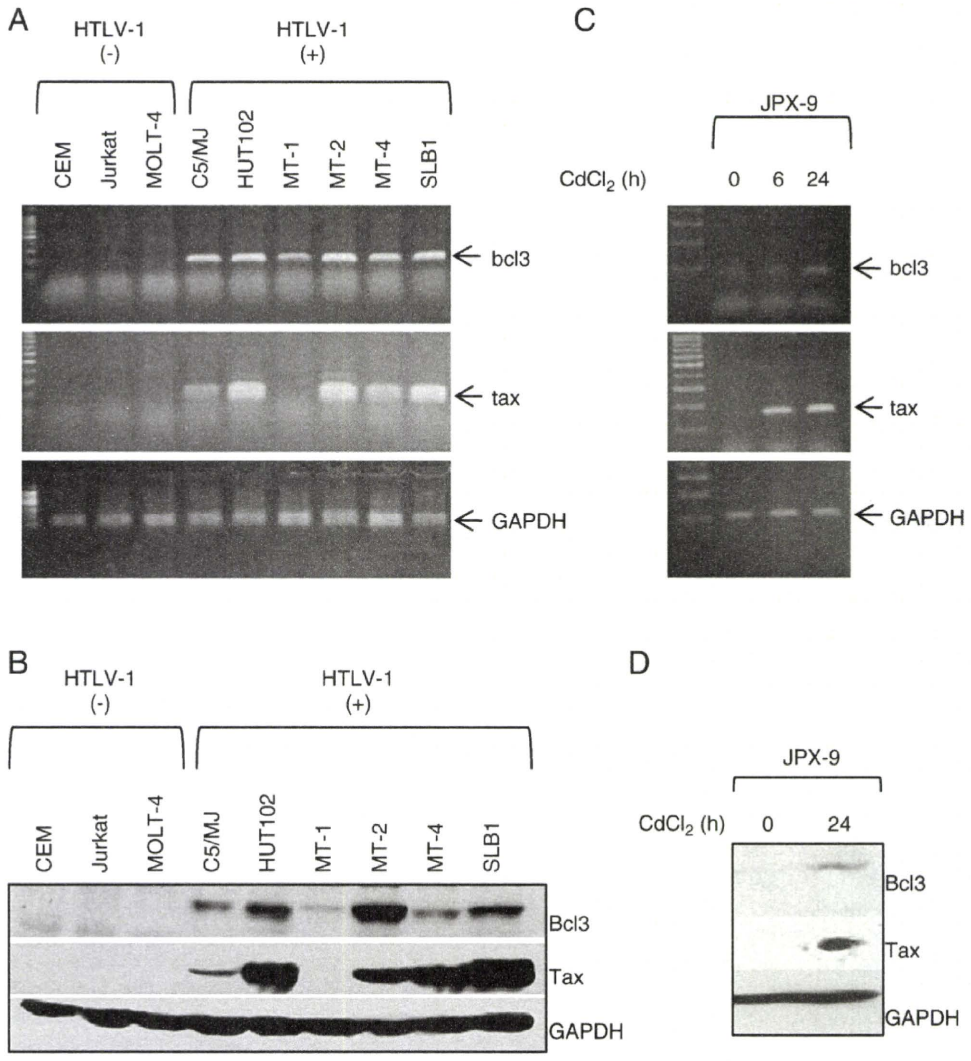

Fig. 1. Overexpression of Bcl3 mRNA and protein in HTLV-1-infected T cell lines and HTLV-1 Tax-mediated induction of Bcl3 mRNA and protein expression. (A) One microgram of total RNA extracted from HTLV-1-infected (C5/MJ, HUT102, MT-1, MT-2, MT-4, and SLB1) and uninfected (CEM, Jurkat and MOLT-4) cell lines were used for reverse transcription. PCR was then performed with the primers for Bcl3, Tax, and GAPDH. The arrowhead indicates the Bcl3 (top panel), Tax (middle panel), and GAPDH mRNA (bottom panel). The analysis of GAPDH mRNA was included as a loading control. (B) Total cell lysates were prepared from HTLV-1-infected (C5/MJ, HUT102, MT-1, MT-2, MT-4, and SLB1) and uninfected (CEM, Jurkat and MOLT-4) T cell lines and applied to Western blot analysis with anti-Bcl3 (top panel) and anti-Tax (middle panel), and anti-GAPDH antibodies (bottom panel). The analysis of GAPDH protein was included as a loading control. (C) JPX-9 cells were treated with 10 μM $CdCl_2$ for the indicated periods. Bcl3, Tax, and GAPDH mRNA levels were examined by RT-PCR. (D) JPX-9 cells were treated with 10 μM $CdCl_2$ for the indicated periods. The expression levels of Bcl3, Tax, and GAPDH protein were examined by Western blot analysis.

bcl3 mRNA was already present before induction of Tax, both RT-PCR and Western blot analysis showed that Bcl3 expression was increased concomitantly with Tax in JPX-9 cells (Figs. 1C and D).

Tax physically interacts with endogenous Bcl3 protein in human T cell lines naturally infected with HTLV-1

Although Kim et al. have recently demonstrated that Tax interacts with Bcl3 protein through the ankyrin repeat domain by using an *in vitro* GST-pull down assay and exogenous overexpression system in 293 T cells (Kim et al., 2008), there is no report describing the interaction of Tax with Bcl3 in human T cells naturally infected with HTLV-1. We first confirmed the Bcl3 and Tax interaction by overexpression in 293 T cells. 293 T cells were transfected with pcDNA3–Bcl3 and/or pHβPr–1Tax1 and each lysate was divided into two. Each lysate was immunoprecipitated with anti-Bcl3 or anti-Tax antibody and subjected to Western blot analysis with anti-Tax or anti-Bcl3 antibody, respectively. As expected, Tax and Bcl3 proteins were immunoprecipitated with anti-Bcl3 or Tax antibody, but not with each control antibody (Figs. 2A and B). 1% aliquot of the lysates removed before immunoprecipitation was also analyzed by Western blotting with anti-Tax and anti-Bcl3 antibodies (Fig. 2C). Next, we also investigated whether Tax physically interacts with endogenous Bcl3 protein in HTLV-1-infected T cells. The cell lysates of MT-2 and HUT102, which have high expression of Tax protein, were subjected

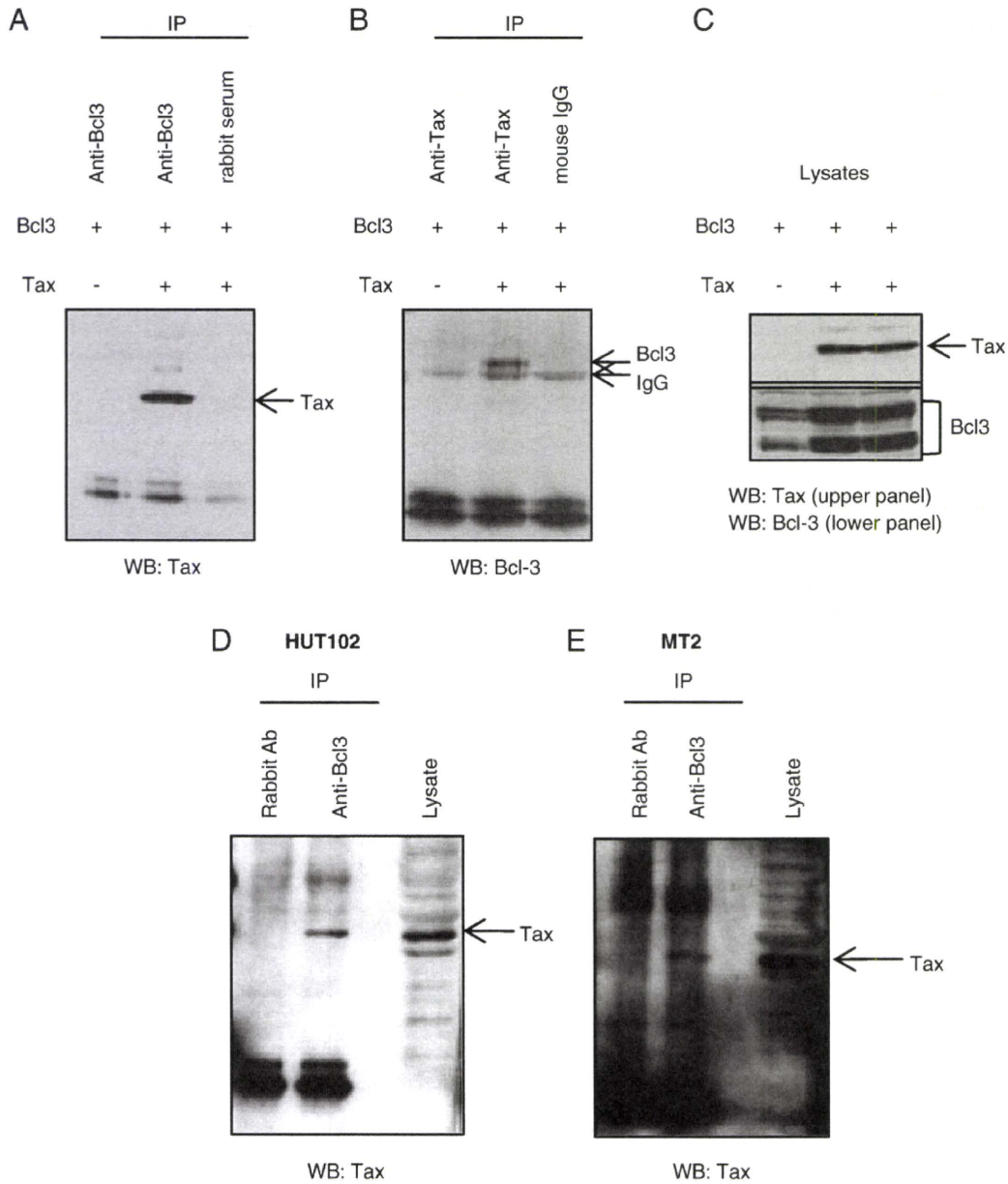

Fig. 2. Tax interacts with Bcl3 protein by using an exogenous overexpression system in 293 T cells. 293 T cells were transfected with 1 μg of pcDNA3–Bcl3 and pHβPr–1Tax1-neo or empty vector. (A) Total cell lysates were prepared and immunoprecipitated with anti-Bcl3 antibody or preimmune rabbit serum, then the immunoprecipitates were resolved by SDS-PAGE (15% polyacrylamide gel) and immunoblotted with anti-Tax antibody. (B) Total cell lysates were prepared and immunoprecipitated with anti-Tax antibody or preimmune mouse IgG, then the immunoprecipitates were resolved by SDS-PAGE (15% polyacrylamide gel) and immunoblotted with anti-Bcl3 antibody. (C) 1% aliquot of the lysates removed before immunoprecipitation was also analyzed by Western blotting with anti-Tax and Bcl3 antibodies. Tax protein physically interacts with endogenous Bcl3 protein in HTLV-1-infected T cells. HTLV-1 infected and Tax-positive T cell lines MT-2 (D) and HUT102 (E) were lysed and immunoprecipitated with anti-Bcl3 or preimmune rabbit serum, and immunoblotted with anti-Tax antibody. Total cell lysates of each T cell lines were prepared separately and included as an indicator for the position of Tax protein in each Western blot.

to immunoprecipitation with anti-Bcl3 antibody, followed by Western blot analysis using anti-Tax antibody. As expected, Tax was immunoprecipitated with anti-Bcl3 antibody both in HUT102 (Fig. 2D) and MT-2 (Fig. 2E) cells, indicating that the physical interaction of Tax with Bcl3 protein is occur naturally in HTLV-1-infected T cells.

Suppression of Bcl3 protein expression inhibits cell growth of HTLV-1-infected T cell lines

It has been reported that the control of cell cycle progression is deregulated in HTLV-1 infected T cell lines (Akagi et al., 1996; Marriott and Semmes, 2005; Suzuki et al., 1996). Also, Bcl3 has been shown to enhance cell cycle progression (Massoumi et al., 2006; Ohno et al., 1990; Rocha et al., 2003; Westerheide et al., 2001). To examine the role of Bcl3 in the growth of HTLV-1-infected T cells, a lentivirus-based RNA interference system was used to knock down Bcl3 expression in three HTLV-1-infected T cell lines (HUT102, C5/MJ and MT-4). Lentiviruses encoding shRNA against Bcl3 together with a puromycin resistance gene were used to transduce each HTLV-1 infected T cell line, and the cells were cultured in the presence of puromycin for 2 days. Western blot analysis showed the suppression of Bcl3 protein expression with a Bcl3 shRNA virus, but not in those treated with a control virus (Fig. 3A). Meanwhile, the cell growth was significantly inhibited in HUT102, C5/MJ and MT-4 cells infected with Bcl3 shRNA lentivirus as compared with those infected with the control lentivirus (Fig. 3B). These results indicated the suppressive role of Bcl3 protein in the growth of HTLV-1-infected T cells.

The constitutive expression of Bcl3 protein is maintained by the posttranslational regulation through PI3K/Akt signaling pathway in HTLV-1-infected T cell lines

Since previous reports have shown that the activation of PI3K/Akt signaling pathway inhibits the Bcl3 protein degradation (Viatour et al., 2004) and PI3K/Akt signaling pathway is constitutively activated in HTLV-1-infected T cells (Ikezoe et al., 2007; Jeong et al., 2005; Tomita et al., 2006), we examined whether overexpression of Bcl3 protein in HTLV-1-infected T cells is maintained by the posttranslational regulation via activation of PI3K/Akt signaling pathway, as well as the Tax-mediated transcriptional activation of Bcl3 gene (Hishiki et al., 2007; Kim et al., 2008). Both Tax-negative HTLV-1-infected T cell line MT-1 and Tax-positive HTLV-1-infected T cell line MT-2 were treated with 20 μM of PI3K inhibitor LY294002 or its solvent DMSO for 6 h in the presence of 50 μg/ml cycloheximide (CHX) to block new protein synthesis. MG132, an inhibitor of 20 S proteasome mediated ubiquitin-dependent protein degradation, was used as a negative control of the Bcl3 protein degradation in the presence of CHX. In Tax-positive MT-2 cells, although Bcl3 protein expression was significantly reduced in the presence of 20 μM LY294002, such degradation was prevented by the MG132 treatment (Fig. 4A, the top). In contrast, Bcl3 protein expression was not significantly changed in the presence of 20 μM LY294002 but increased in the presence of 20 μM MG132 in Tax-negative MT-1 cells. Meanwhile, in Tax-positive MT-2 cells, phosphorylation of both Ser473 and Thr308 residues in Akt protein was inhibited by LY294002 (Fig. 4A, second and third from the top) whereas the Tax protein expression was not significantly changed by the LY294002 and

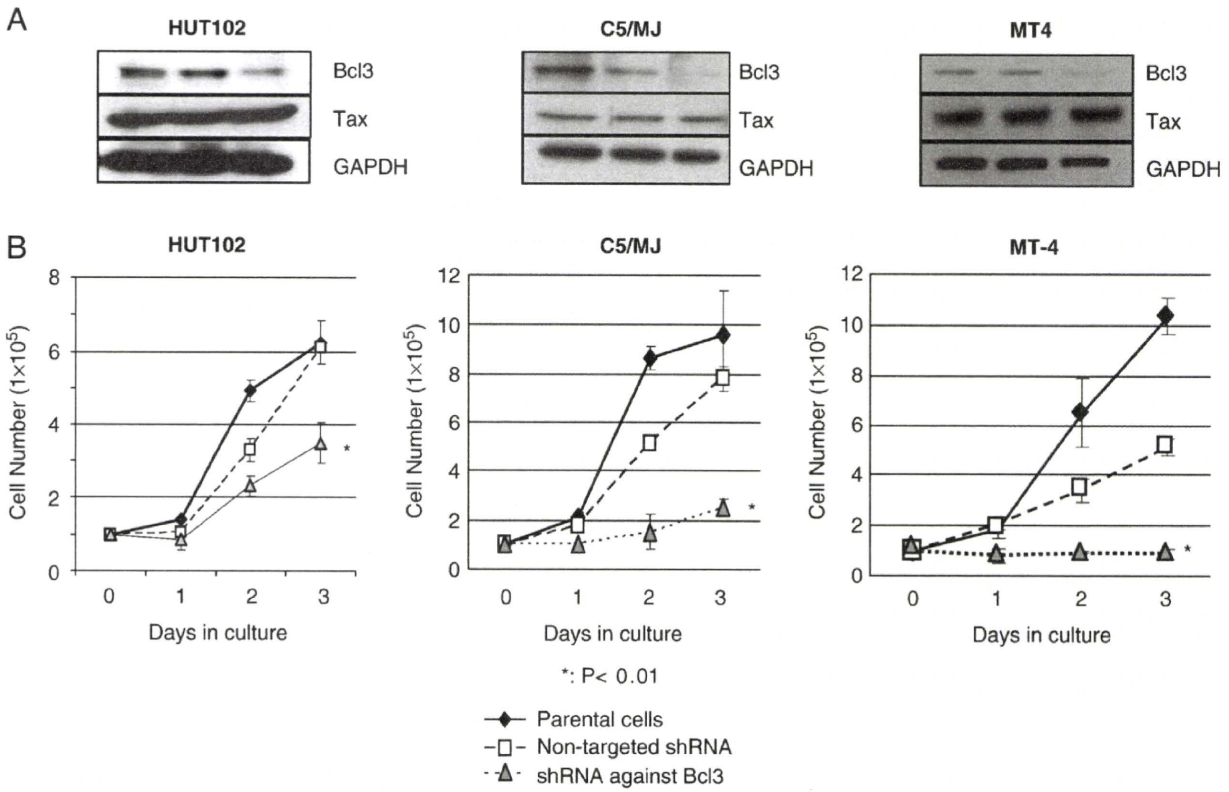

Fig. 3. Knock-down of Bcl3 expression suppressed cell growth of HTLV-1-infected T cells. (A) HUT-102, C5/MJ or MT-4 cells were infected with lentivirus encoding shRNA against human Bcl3 or control shRNA, or remained uninfected as a control of parental cells, then cultured for 2 days. The infected cells were further cultured in the presence of 1 μg of puromycin for another 2 days other than the control of parental cells. After the selection with puromycin, total cell lysates were prepared and applied to Western Blotting with anti-Bcl3 (top) and anti-Tax (middle), and anti-GAPDH (bottom) antibodies. (B) The effect of shRNA on cell growth was examined by counting the viable cell number in triplicate by the trypan blue dye exclusion method. Error bars indicate standard deviations of the results of two independent experiments performed in triplicate. Parental: uninfected HUT102, C5/MJ or MT-4 cells. Non-targeted: HUT102, C5/MJ or MT-4 cells infected with lentivirus encoding control shRNA. Bcl3 KD (knockdown): HUT102, C5/MJ or MT-4 cells infected with lentivirus encoding shRNA against human Bcl3. *: $P<0.01$.

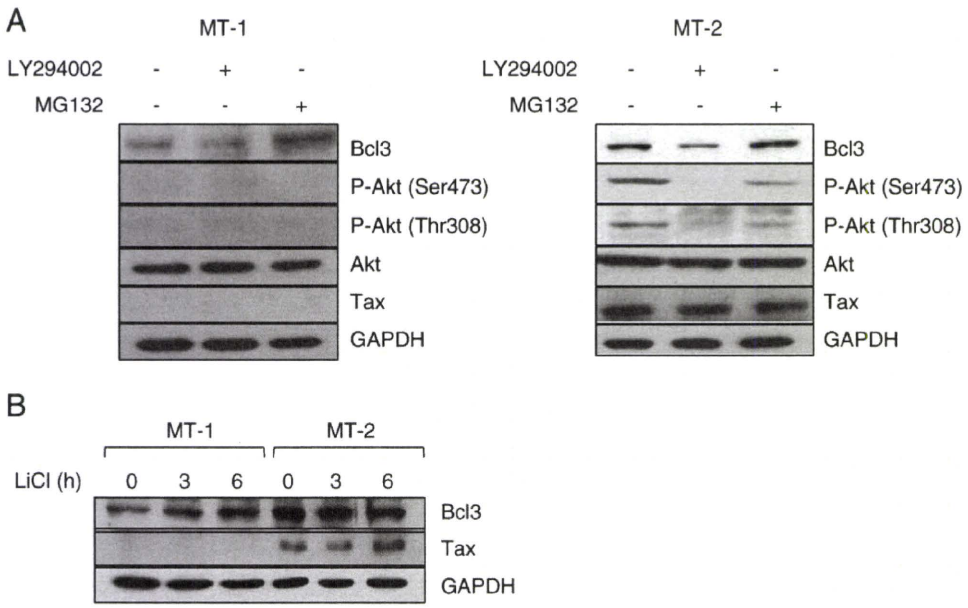

Fig. 4. The constitutive expression of Bcl3 protein is regulated by the activation of PI3K/Akt signaling pathway and Tax enhances inhibition of Bcl3 protein degradation in HTLV-1-infected T cells. (A) MT-1 and MT-2 cells were treated with 20 μM each of PI3K inhibitor LY294002 or proteasome inhibitor MG132, or DMSO in the presence of 50 μg/ml CHX for 6 h. Total cell lysates were analyzed by Western blot analysis with anti-Bcl3, anti-phosphorylated Akt (Ser473), anti-phosphorylated Akt (Thr308), anti-Akt, anti-Tax, and anti-GAPDH antibodies, as indicated. (B) MT-1 and MT-2 cells were treated with 10 mM LiCl for 6 h and total cell lysates were analyzed by Western blot analysis with anti-Bcl3, anti-Tax, and anti-GAPDH antibodies, as indicated.

MG132 treatments (Fig. 4A, fifth panel from the top). In Tax-negative MT-1 cells, phosphorylation of both Ser473 and Thr308 residues in Akt protein was not detected. These data suggest that the over-expression of Bcl3 protein is maintained by the activation of PI3K/Akt signaling pathway in Tax-positive but not in Tax-negative HTLV-1 infected T cell lines.

Previous reports indicated that the glucose synthase kinase 3 (GSK3) mediates the Bcl3 protein degradation (Viatour et al., 2004), and that GSK3 remains activated in Tax-negative HTLV-1-infected T cell line MT-1 but is fully inactivated in the Tax-positive HTLV-1-infected T cell line MT-2 (Tomita et al., 2006). Therefore, to examine the effect of Tax for the Bcl3 protein degradation, we compared the degree of Bcl3 protein degradation mediated by GSK3 between Tax-negative MT-1 and Tax-positive MT-2 cells. When the cells were treated by lithium chloride (LiCl), which acts as a specific inhibitor of GSK3, we observed the accumulation of Bcl3 protein in Tax-negative MT-1 cells in a time-dependent manner, whereas the level of Bcl3 protein was not significantly changed in the Tax-positive MT-2 cells (Fig. 4B, top panel). These results indicated that LiCl increases Bcl3 protein levels in cells with activated GSK3, but not in cells with inactive GSK3, because of constitutive activation of PI3K/Akt signaling pathway by Tax (Fig. 5).

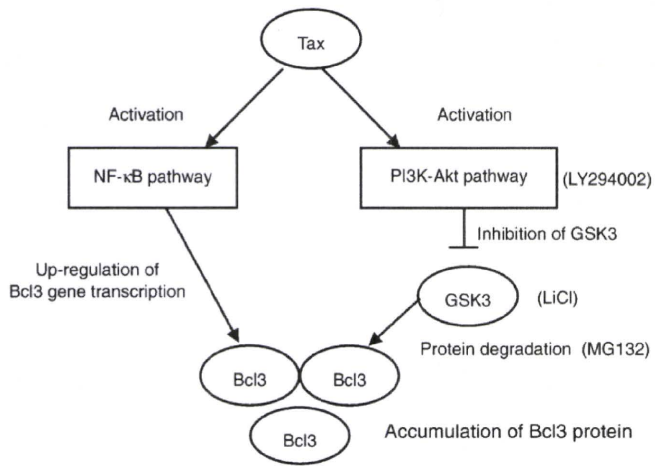

Fig. 5. Schematic representation of the mechanisms of Bcl3 protein accumulation in HTLV-1 infected T cells. Tax transactivates Bcl3 gene transcription through NF-κB pathway. Tax also enhances the inhibition of Bcl3 protein degradation through activating PI3/Akt signaling pathway and subsequent inactivation of GSK3. Inactivation of GSK-3 prevents proteosomal degradation of Bcl3. The words in parentheses are the names of the inhibitors for each site of action (see Fig. 4).

Discussion

Recently, Hishiki et al. reported that bcl3 mRNA is constitutively expressed in three HTLV-1 infected T cell lines (MT-2, C91-PL and SLB-1) but not in two HTLV-1-uninfected human leukemic T cell lines (Jurkat and JPX-9). They also reported that bcl3 mRNA expression was induced in JPX-9 cell line treated with $CdCl_2$ along with the induction of viral transactivator Tax (Hishiki et al., 2007). Since one NF-κB site within intron 2 acts as a transcriptional enhancer of Bcl3 gene in Jurkat T cells stimulated with phorbol myristate acetate (PMA) and ionomycin, which is mimicking T cell receptor activation (Ge et al., 2003), it is likely that bcl3 overexpression is maintained by Tax-dependent mechanism. Indeed, Kim et al. have recently demonstrated that Tax mediates Bcl3 induction by intronic enhancers through activation of NF-κB signaling pathway in Jurkat T cells (Kim et al., 2008). In this study, we first confirmed Bcl3 overexpression by using different cell lines both mRNA and protein levels. As expected, both Bcl3 mRNA and protein were overexpressed in five Tax-positive and one Tax-negative HTLV-1 infected T cell lines but not in three HTLV-1 uninfected T cell lines, and both Bcl3 mRNA and protein expression were induced along with the induction of Tax in JPX-9 cells.

The identification of Tax interacting proteins has been extensively performed, and numerous cellular proteins including transcriptional factors and cell signaling mediators have been reported (Sun and Yamaoka, 2005). It is therefore believed that the interaction of Tax with those cellular proteins has critical roles in Tax-mediated cell immortalization and transformation as well as tomorigenesis (Giam

and Jeang, 2007). Since previous study showed interaction between Tax and Bcl3 by artificial experimental systems employing over-expression in 293 T cells or GST-pull down assay (Kim et al., 2008) but not in human T cells naturally infected with HTLV-1, we demonstrated that Tax physically interacts with Bcl3 protein also in HTLV-1-infected T cell lines. It has been reported that the G1 to S transition in the cell cycle is enhanced by transcriptional upregulation of G1 cyclins including Cyclin D1 and D2 in HTLV-1 infected T cells (Neuveut and Jeang, 2002), and the activation of NF-κB signaling pathway is critical for upregulation of those G1 cyclins and cell growth promotion in HTLV-1 infected T cells (Sun and Yamaoka, 2005). Moreover, Mori et al. reported that both Cyclin D1 and D2 mRNAs were expressed at markedly higher levels in all HTLV-I-infected T cell lines (MT-2, MT-4, C5/MJ, SLB-1, and HUT102) than in any uninfected T cell lines (Jurkat, MOLT4, and CCRF-CEM), and Tax activated Cyclin D1 and D2 promoters mainly through NF-κB. Interestingly, expression of Cyclin D1 and D2 proteins concomitantly increased in JPX-9 cells after treatment with $CdCl_2$, whereas the expression of Tax was not associated with any concomitant change in Cyclin D3 expression. Since Bcl3 has an important role in cell cycle progression by inducing Cyclin D1 gene expression (Westerheide et al., 2001), and also in tumorigenesis through NF-κB signaling pathway (Courtois and Gilmore, 2006; Kerr et al., 1992; Massoumi et al., 2006; Ohno et al., 1990), these observations suggest that constitutive expression of Bcl3 may promote cell cycle progression via upregulation of G1 cyclins through NF-κB signaling pathway in HTLV-1 infected T cells, although further studies are needed to clarify this point.

Previous report indicated that the GSK3-mediated Bcl3 protein degradation is induced by inhibition of *de novo* protein synthesis with cycloheximide (CHX), and this degradation is inhibited by the activation of PI3K/Akt signaling pathway in 293 T cells (Viatour et al., 2004). On the other hand, previous report also indicated that Tax enhances the inactivation of GSK3 to inhibit β-catenin protein degradation through PI3K/Akt signaling pathway in HTLV-1-infected T cell line (Tomita et al., 2006). We therefore compared the effects of PI3K/Akt inhibitor LY294002 and GSK3 inhibitor LiCl on both Tax-negative (MT-1) and Tax-positive (MT-2) HTLV-1 infected human T cell lines. As a result, consistent with these observations, our data indicated that PI3K/Akt inhibitor LY294002 induced the Bcl3 protein degradation in Tax-positive but not Tax-negative HTLV-1 infected T cell lines whereas treatment of GSK3 inhibitor LiCl enhanced the accumulation of Bcl3 protein in Tax-negative (i.e. GSK3 active) but not in Tax-positive (i.e. GSK3 inactive) HTLV-1-infected T cells. Namely, as well as the Tax-mediated transcriptional activation of Bcl3 gene (Hishiki et al., 2007; Kim et al., 2008), overexpression of Bcl3 protein in HTLV-1-infected T cells is also maintained by the posttranslational regulation via inhibition of Bcl3 degradation through activation of PI3K/Akt signaling pathway and inactivation of GSK3 in Tax-positive but not Tax-negative HTLV-1-infected T cells. Since our data showed that the levels of Bcl3 protein in HTLV-1 infected T cells do not correlate with the expression levels of Tax protein, and a low level of Bcl3 protein was expressed also in Tax-negative MT-1 cells, Bcl3 protein expression is maintained in MT-1 cells in a Tax-independent manner. Recently, Kim et al. reported that Tax interacts with Bcl3 protein and that the ankyrin repeat domain is necessary and sufficient for inhibition of HTLV-1 LTR promoter activation (Kim et al., 2008), whereas Hishiki et al. demonstrated that the interaction of Bcl3 with transducer of regulated CREB-binding proteins-3 (TORC-3) through the ankyrin repeat domain represses the transactivation of HTLV-1 LTR in a Tax-independent manner (Hishiki et al., 2007). These results indicate that Bcl3 functions as a repressor of HTLV-1 LTR through the ankyrin repeat domain both in Tax-dependent and independent manners. In this study, we further showed that an overexpression of Bcl3 protein in HTLV-1-infected T cells is also maintained by the posttranslational regulation via inhibition of Bcl3 degradation through activation of PI3K/Akt signaling pathway and subsequent inactivation of GSK3 in Tax-positive HTLV-1-infected T cells. These data suggest that the mechanisms of Bcl3 upregulation and its stabilization are complex and different in each HTLV-1 infected T cell line, therefore the levels of Bcl3 protein do not simply correlate with the levels of Tax protein.

In summary, Bcl3 is constitutively expressed in HTLV-1 infected T cells by at least two Tax-mediated mechanisms, i.e. transactivation of Bcl3 gene expression (Kim et al., 2008) and posttranslational upregulation of Bcl3 protein through the activation of PI3K/Akt signaling pathway. Although the precise mechanism remains unclear, the interaction of Tax with Bcl3 may contribute to the formation of the ternary complex among Bcl3, coactivators and transcription factors, and thereby regulate the genes for cell cycle progression in HTLV-1 infected cells.

Materials and methods

Reagents

The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 and 20 S proteasome inhibitor MG132 were purchased from Calbiochem (San Diego, CA). Cyclohexamide (CHX) was purchased from Sigma-Aldrich (St. Louis, MO). Lithium chloride (LiCl) and cadmium chloride ($CdCl_2$) were purchased from WAKO (Osaka, Japan).

Antibodies

Rabbit polyclonal anti-Bcl3 antibody (C-14) was purchased from Santa Cruz Biotechnology (San Diego, CA). Mouse monoclonal anti-GAPDH antibody was purchased from Millipore (Billerica, MA). Rabbit anti-Akt and anti-phosphorylated Akt (Ser473, Thr308) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti-hemagglutinin (HA) antibody (HA-7) was purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-Tax antibody (Lt-4) was kindly provided by Prof. Y. Tanaka (University of the Ryukyus, Okinawa, Japan).

Cell culture

Six HTLV-1 infected cell lines (C5/MJ, HUT-102, MT-1, MT-2, MT-4, and SLB-1) and three HTLV-1-uninfected T cell lines (CEM, MOLT-4, and Jurkat) were used in this study. MT-2, MT-4, C5/MJ, and SLB-1 cell lines are HTLV-1-transformed T cell lines established by an *in vitro* coculture protocol. MT-2, MT-4 and C5/MJ are umbilical cord blood T cell lines, whereas SLB-1 is an adult peripheral blood T cell line. HUT-102 was established from a patient with ATL, but it is unclear whether HUT-102 cells represent an actual tumor clone from the donor ATL patient. MT-1 is a leukemic T cell line derived from a patient with ATL. The Tax-inducible JPX-9 cell line is a derivative of the Jurkat cell line, which expresses biologically active Tax protein under the control of the metallothionein promoter (Nagata et al., 1989). These cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Wako) at 37 °C in 5% CO_2. 293 T cell is a human embryonic kidney cell line, and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% CO_2.

Reverse transcriptase PCR (RT-PCR)

Total cellular RNA was extracted from human leukemic T cell lines and HTLV-1-infected T cell lines by using RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using TaqMan Reverse Transcription kit (Applied Biosystems, Tokyo, Japan). For cDNA