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Involvement of p38 MAPK signaling pathway in IFN-γ and HTLV-I expression in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis

Naomi Fukushima[a], Yoshihiro Nishiura[b], Tatsufumi Nakamura[a,*], Yasuaki Yamada[c], Shigeru Kohno[a], Katsumi Eguchi[b]

[a]*Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

[b]*First Department of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Japan*

[c]*Department of Laboratory Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Japan*

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Abstract

We analyzed the relationship between the expression of interferon (IFN)-γ and HTLV-I p19 antigen and activation of p38 mitogen-activated protein kinase (p38 MAPK) in two HTLV-I-infected T cell lines derived from two patients (HCT-1 and HCT-4) with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and three HTLV-I-infected T cell lines derived from three patients with adult T cell leukemia (ATL). Expression of phosphorylated (activated)-p38 MAPK was markedly increased concomitant with high levels of both IFN-γ and HTLV-I p19 antigen expression in both HCT-1 and HCT-4 compared with cell lines derived from ATL patients. Treatment with SB203580, a specific inhibitor of p38 MAPK, suppressed IFN-γ and HTLV-I p19 antigen expression levels in HCT-1, HCT-4 and peripheral blood $CD4^+$ T cells of HAM/TSP patients. These findings strongly suggest that activation of p38 MAPK signaling pathway is involved in the up-regulation of IFN-γ expression with high HTLV-I proviral load in HAM/TSP patients.

Keywords: HAM/TSP; HTLV-I; Th1; IFN-γ; p38 MAPK

1. Introduction

Human T lymphotropic virus type I (HTLV-I) is the causative agent for both adult T cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al., 1986; Yoshida, 2001). However, the exact mechanisms underlying the entirely different clinical conditions caused by HTLV-I, such as aggressive lymphoproliferative malignancy and chronic myelitis, are still unknown. In addition, it is still not clear why only a small proportion of HTLV-I-infected individuals develop both HTLV-I-associated diseases.

The main pathological feature of HAM/TSP is chronic inflammation of the spinal cords characterized by perivascular cuffing and parenchymal infiltration of lymphocytes (Iwasaki, 1990). Although the interaction between HTLV-I-infected cells and HTLV-I-specific cytotoxic T cells play an important role in the etiopathomechanism of HAM/TSP (Jacobson, 2002; Osame, 2002), we previously proposed the importance of Th1 immune activation, such as increased expression of interferon (IFN)-γ, in the development of HAM/TSP (Nakamura et al., 2000). Indeed, high HTLV-I proviral load is an important factor in the development of HAM/TSP (Jeffery et al., 1999). Recently, however, a comparative study of intracellular cytokine expression levels in HAM/TSP patients and HTLV-I asymptomatic carriers with a high HTLV-I proviral load equivalent to those of HAM/TSP patients revealed abundance of IFN-γ and

* Corresponding author. Tel.: +81 95 849 7265; fax: +81 95 849 7270.
E-mail address: tatsu@net.nagasaki-u.ac.jp (T. Nakamura).

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tumor necrosis factor (TNF)-α producing cells in the population of HTLV-I tax-expressing cells, but not tax-non-expressing cells, in HAM/TSP patients (Furukawa et al., 2003). These findings suggested that the expression levels of both cytokines in HTLV-I-infected cells influence the development of HAM/TSP with Th1 activation in addition to high HTLV-I proviral load.

Numerous signaling molecules are involved in the regulation of expression of IFN-γ, a well known representative Th1 cytokine (Murphy et al., 2000; Seder and Paul, 1994). The p38 mitogen-activated protein kinase (p38 MAPK) is one of them and is involved in Th1 differentiation with IFN-γ induction through the downstream target, activation transcription factor (ATF)-2 (Dong et al., 2002; Rincón and Pedraza-Alva, 2003; Szabo et al., 2003). The p38 MAPK, which is phosphorylated by activated MAPK kinase, phosphorylates ATF-2 in turn and the phosphorylated-ATF-2 induces transcription of the gene encoding IFN-γ. In addition, the importance of p38 MAPK signaling pathway in the Th1 differentiation, also in signal transducers and activators of transcription-4 or T cell receptor (TCR)-independent manner, has been reported (Yang et al., 2001; Zhang and Kaplan, 2000).

On the other hand, HTLV-I tax protein, which is the gene product of pX region in the 3′ terminal region of HTLV-I genome, plays an important role in transcription of HTLV-I proviral genome from HTLV-I long terminal repeat (LTR) (Yoshida, 2001). However, it is well known that tax binds HTLV-I LTR not directly but through binding of the cAMP response element binding protein and ATF-1 or -2 (CREB/ATF family) (Franklin et al., 1993; Xu et al., 1996; Yoshida, 2001). This implies that the efficient recruitment of CREB and ATF to LTR of HTLV-I provirus is necessary for efficient HTLV-I replication. ATF-2 is the downstream target of p38 MAPK as mentioned above. In addition, it is reported that CREB is also activated by the downstream target of p38 MAPK, MAPKAP kinase-2 (Tan et al., 1996).

Although spontaneous IFN-γ expression is up-regulated with high HTLV-I proviral load in HAM/TSP patients as mentioned above, it is still not clear which mechanisms induces such a status. However, if p38 MAPK signaling is activated in HTLV-I-infected T cells of HAM/TSP patients, such status might be linked to both up-regulated spontaneous IFN-γ expression and high HTLV-I proviral load in HAM/TSP patients. In the present study, we first analyzed activated (phosphorylated)-p38 MAPK expression with regard to both spontaneous IFN-γ production and HTLV-I p19 antigen expression in HTLV-I-infected T cell lines derived from HAM/TSP patients, compared with same cells derived from ATL patients. Second, we analyzed the suppressive effect of a specific pyrinidyl imidazole inhibitor SB203580, which is p38 MAPK specific inhibitor (Cuenda et al., 1995), on both spontaneous IFN-γ production and HTLV-I p19 antigen expression in HTLV-I-infected T cell lines and peripheral blood $CD4^+$ T cells derived from HAM/TSP patients.

2. Subjects and methods

2.1. Cell lines

HTLV-I-infected T cell lines derived from the cerebrospinal fluid of two different HAM/TSP patients (HCT-1 and HCT-4) (Nakamura et al., 1989) and from peripheral blood of three ATL patients (KK-1, SO-4, and KOB) (Yamada et al., 1996) were used. The cell lines were all interleukin (IL)-2-dependent. All cell lines were maintained in RPMI 1640 containing 20% fetal bovine serum (FBS) (HCT-1 and HCT-4) or 10% FBS (KK-1, KOB, and SO-4) supplemented with 100 units/ml of recombinant human IL-2 (kindly provided by Shionogi, Japan).

2.2. Patients

Six patients with HAM/TSP (all women; mean age, 62 years; range, 40–73 years) were recruited for the study. The diagnosis of HAM/TSP was based on the criteria described previously (Osame, 1990). Control subjects comprised 3 anti-HTLV-I-seropositive individuals (1 man and 2 women; average age, 61 years; range, 50–78 years) and 4 anti-HTLV-I-seronegative healthy individuals (1 man and 3 women; average age, 50 years; range, 40–71 years). The three anti-HTLV-I-seropositive individuals comprised patients with spinocerebellar degeneration, lumbar polyradiculopathy, and tension-type headache. None of the patients had been treated with immunomodulatory drugs, including corticosteroids and IFN-α. Informed written consent was obtained from all patients and healthy volunteers.

2.3. Separation of peripheral blood $CD4^+$ T cells

Peripheral blood mononuclear cells were separated from heparinized venous blood by Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). The peripheral blood $CD4^+$-enriched T cells were separated in the negative selection by depletion of $CD8^+$ T cells from macrophage/B cell depleted mononuclear cells using magnetic beads coated with anti-CD8 monoclonal antibody, according to the instructions provided by the manufacturer (Dynal Biotech, Oslo, Norway). $CD4^+$-enriched T cells were washed with phosphate-buffered saline (PBS) and used as $CD4^+$ T cells. Analysis of the purity of $CD4^+$ T cells using anti-CD4, anti-CD20, and anti-CD14 monoclonal antibodies showed more than 90% purity with the presence of less than 1% B cells, and less than 1% monocytes as assessed by flow cytometry (Epics XL system II, Beckman Coulter, Fullerton, CA).

2.4. Cell culture and treatment with SB203580 for HCT-1, HCT-4, and peripheral blood $CD4^+$ T cells

To study IFN-γ production by and HTLV-I p19 antigen expression on HTLV-I-infected T cell lines derived from

HAM/TSP and ATL patients, each cell line was cultured at 1×10^5 cells/ml in the presence of 100 units/ml of IL-2 in 24-well culture plate for 48 h. To study the suppression of both IFN-γ production and HTLV-I p19 antigen expression by p38 MAPK inhibitor SB203580, each cell line was cultured in the presence of dimethyl sulfoxide (DMSO) only or different concentrations of SB203580 (Cuenda et al., 1995; Zhang and Kaplan, 2000) in the same culture condition above. Next, peripheral blood $CD4^+$ T cells were cultured at concentration of 1×10^6 cells/ml in the presence of DMSO only or 10 μM of SB203580 in RPMI 1640 supplemented with 20% FBS in 24-well culture plates for 72 h. The culture medium was then centrifuged at 2500 rpm for 10 min and the supernatant was stored at −40 °C until use.

2.5. Western blot analysis of p38 MAPK expression

Each culture of HTLV-I-infected T cells was collected and lysed by the addition of TNE buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 1% Nonidet P-40, and 50 mM NaF) supplemented with 1 mM Na_3VO_4 and complete EDTA-free (Roche, Mannheim, Germany). Insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4 °C and the supernatant was used for western blotting. An identical amount of protein for each lysate (10 μg) was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ATTO, Tokyo). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane. After overnight blocking with PVDF buffer, the PVDF membrane was incubated at room temperature for 2 h in the presence of rabbit polyclonal anti-phosphorylated p38 MAPK antibody (1:2000 dilution, Promega, Madison, WI) or rabbit polyclonal anti-p38 MAPK antibody (1:1000 dilution, Cell Signaling, Beverly, MA). After 1 h incubation with donkey anti-rabbit IgG coupled with horseradish peroxidase (Promega) or sheep anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences, UK), proteins were visualized by using an enhanced chemiluminescence kit (Amersham Biosciences). The blots were stripped and reproved with monoclonal mouse IgG against to β-actin (Sigma) to ensure equal protein loading.

2.6. Measurement of IFN-γ and HTLV-I p19 antigen levels in culture supernatants

IFN-γ level in the culture supernatants was measured using an enzyme-amplified sensitivity immunoassay (EASIA) kit according to the instruction provided by the manufacturer (Medgenix, Fleurus, Belgium). The amount of IFN-γ was determined in duplicate. The minimum measurable level of IFN-γ was 0.03 IU/ml. The suppression ratio of IFN-γ production (SR) in the culture of peripheral blood $CD4^+$ T cells was determined as follows: SR=[(IFN-γ level in culture supernatant of cultured $CD4^+$ T cells in the presence of DMSO only-IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of 10 μM SB203580)/(IFN-γ level in supernatant of cultured $CD4^+$ T cells in the presence of DMSO only)]. The level of HTLV-I p19 antigen in the culture supernatants was measured by using the RETROtek HTLV p19 antigen enzyme-linked immunosorbent assay (ELISA) kit using the instructions provided by the manufacturer (Zeptometrix, Buffalo, NY). The amount of HTLV-I p19 antigen was determined in duplicate. The minimum measurable level of HTLV-I p19 antigen was 25.0 pg/ml.

2.7. Proliferation assay

Each HTLV-I-infected T cell line (2.5×10^3 cells/well) was cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well U-bottom plates. Cells were pulsed for the last 12 h of a 48-h incubation at each HTLV-I-infected T cell line with 0.8 μCi/well of [^{3}H]TdR (Perkin Elmer, Boston, MA) and harvested onto glass-fiber filters. Radioactivity (cpm) was recorded using a liquid scintillation counter (LSC-5100, Aloka, Tokyo). Cultures were studied in triplicate and results were expressed as mean counts per minute.

2.8. MTS assay

HTLV-I-infected T cell line (2×10^4 cells/well) was cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well flat-bottom plates for 48 h. Peripheral blood $CD4^+$ T cells (1×10^5 cells/well) were cultured in the presence of DMSO only or 10 μM SB203580 in 96-well flat-bottom plates for 72 h. The number of viable cells was determined by a modified MTT assay, MTS (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) nonradioactive cell proliferation assay (Promega). Briefly, 20 μl of MTS mixture was added to each individual well, and after 1 h incubation, absorbance at 490 nm was measured using a multiscan plate reader. Cultures were

Table 1
Levels of IFN-γ and HTLV-I p19 antigen in culture supernatants of HTLV-I-infected T cell lines

Cell lines	IFN-γ (IU/ml)	HTLV-I p19 (pg/ml)
HAM		
HCT-1	65.30	16450.0
HCT-4	148.80	43485.0
ATL		
KK-1	0.79	409.0
KOB	0.66	1456.0
SO-4	0.77	<25.0

Each cell line was cultured at 1×10^5 cells/ml in the presence of 100 units/ml of IL-2 for 48 h. Each culture supernatant was collected and the levels of IFN-γ and HTLV-I p19 antigen were measured by ELISA. The minimum measurable level of IFN-γ was 0.03 IU/ml and that of HTLV-I p19 antigen was 25.0 pg/ml.

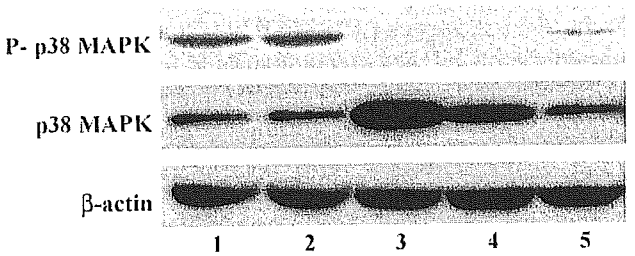

Fig. 1. Western blot analysis of p38 MAPK expression in HTLV-I-infected T cell lines. Lanes 1 and 2: HTLV-I-infected T cell lines derived from HAM patients; HCT-1 and HCT-4, respectively. Lanes 3, 4, and 5: HTLV-I-infected T cell lines derived from ATL patients; KK-1, KOB, and SO-4, respectively. P-p38 MAPK: phosphorylated-p38 MAPK, β-actin: internal control.

studied in triplicate and results were expressed as the mean optical density (OD).

2.9. Statistical analysis

The sign test was used for the statistical analysis. Differences were considered as statistically significant at $p<0.05$.

3. Results

3.1. IFN-γ/HTLV-I p19 antigen expression correlates with phosphorylated-p38 MAPK expression in HTLV-I-infected T cell lines

As shown in Table 1, both IFN-γ and HTLV-I p19 antigen levels were significantly higher in two HTLV-I-infected T cell lines from HAM/TSP patients (HCT-1 and HCT-4) than in three HTLV-I-infected T cell lines from ATL patients (KK-1, KOB, and SO-4). Based on this finding, we next analyzed the expression of phosphorylated-p38 MAPK in each cell line, using western blot. As shown in Fig. 1, the expression of phosphorylated-p38 MAPK was significantly increased in both HCT-1 and HCT-4. On the other hand, although the expression of phosphorylated-p38 MAPK was faintly detected in SO-4, it was not detected in both KK-1 and KOB at all (Fig. 1). These results suggest that high IFN-γ and HTLV-I p19 antigen expression in HTLV-I-infected T cells from HAM/TSP patients depend on activation of p38 MAPK signaling pathway.

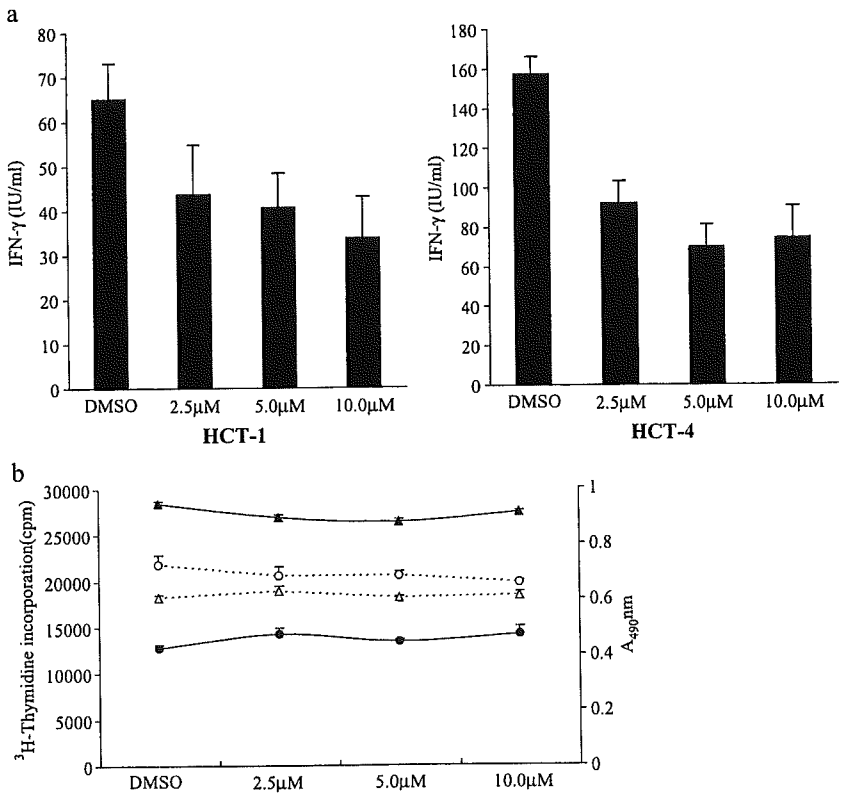

Fig. 2. Effects of SB203580 on HTLV-I-infected T cell lines derived from HAM/TSP patients (HCT-1 and HCT-4). (a) SB203580 suppresses spontaneous IFN-γ production by HCT-1 and HCT-4. HCT-1 or HCT-4 cells were cultured at concentration of 1×10^5 cells/ml in the presence of DMSO only or different concentrations (2.5, 5.0, and 10.0 μM) of SB203580 with 100 units/ml of IL-2 for 48 h, and then the culture supernatants were collected. Levels of IFN-γ in each culture supernatant were measured by ELISA. Data are mean±SEM of triplicate cultures. (b) Cell proliferation and cell viability assays. HCT-1 or HCT-4 cells were cultured in the presence of DMSO only or different concentrations of SB203580 with 100 units/ml of IL-2 in 96-well plates. For cell proliferation assay, cells were pulsed for the last 12 h of a 48-h incubation with [^{3}H]TdR. Data are mean±SEM of triplicate cultures. HCT-1: ●-●, HCT-4: O···O. For cell viability assay, MTS assay was performed according to the instructions provided by the manufacturer. Data represent OD titer at wavelength of 490 nm. Data are mean±SEM of triplicate cultures. HCT-1: ▲-▲, HCT-4: △···△.

3.2. SB203580 suppresses IFN-γ production by HCT-1 and HCT-4

To confirm whether this p38 MAPK signaling pathway is functionally activated for IFN-γ induction in HCT-1 and HCT-4, we analyzed the effect of p38 MAPK specific inhibitor, SB203580, on spontaneous IFN-γ production by both cell lines. As shown in Fig. 2a, SB203580 suppressed dose-dependently, by up to about 50%, IFN-γ production by both cell lines. To determine whether the SB203580-induced suppression of IFN-γ production was dependent on inhibition of cell proliferation, we checked the changes in cell proliferation of both HCT-1 and HCT-4 treated with SB203580. However, as shown in Fig. 2b, SB203580 did not affect cell proliferation. In addition, MTS assay revealed that this treatment also did not affect cell viability (Fig. 2b). These results indicate that the suppression of spontaneous IFN-γ production by treatment with SB203580 was based on inhibition of p38 MAPK signaling pathway in IFN-γ induction, but not suppression of cell proliferation.

3.3. SB203580 suppresses IFN-γ production by peripheral blood $CD4^+$ T cells

SB203580 induced about 24–79% suppression of IFN-γ production by peripheral blood $CD4^+$ T cells of all HAM/TSP patients (Fig. 3, p=0.0156). However, SB203580 produced only about 20% suppression of IFN-γ production by peripheral blood $CD4^+$ T cells of all HTLV-I carriers and HTLV-I-seronegative controls (Fig. 3). Similar to HTLV-I-infected T cell lines, SB203580 did not affect the viability of peripheral blood $CD4^+$ T cells (data not shown). These findings suggest that p38 MAPK signaling pathway is preferentially involved in spontaneous IFN-γ production by peripheral blood $CD4^+$ T cells in HAM/TSP patients.

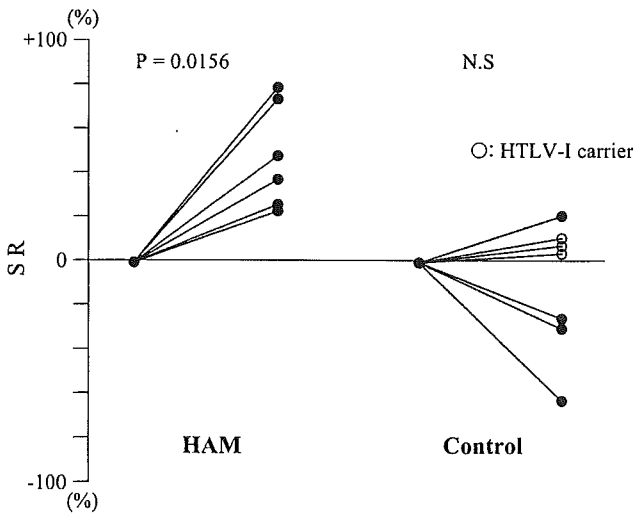

Fig. 3. SB203580 suppresses IFN-γ production by peripheral blood $CD4^+$ T cells. Peripheral blood $CD4^+$ T cells were cultured at concentration of 1×10^6 cells/ml in the presence of DMSO only or 10 μM SB203580 for 72 h, and the culture supernatants were collected. Levels of IFN-γ in each culture supernatant were measured by ELISA. The suppression ratio of IFN-γ production (SR) was determined as described in the text. Open circle: an HTLV-I-seropositive carrier, N.S.: not significant.

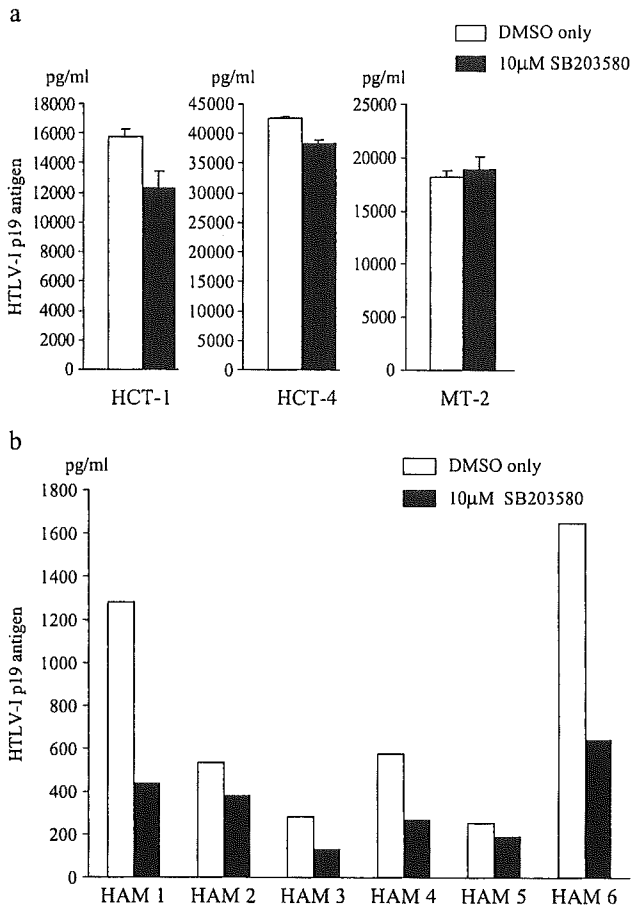

Fig. 4. SB203580 reduces HTLV-I p19 antigen expression in HCT-1, HCT-4, and peripheral blood CD4+ T cells of HAM/TSP patients. (a) HCT-1 or HCT-4 cells were cultured at concentration of 1×10^5 cells/ml in the presence of DMSO only or 10 μM SB203580 with 100 units/ml of IL-2 for 48 h. As control, MT-2 cells were cultured without IL-2 for 48 h in the same condition. The culture supernatants were collected and the levels of HTLV-I p19 antigen were measured by ELISA. Data are mean±SEM of triplicate cultures. Open bars: DMSO only, solid bars: 10 μM SB203580. (b) Peripheral blood $CD4^+$ T cells of HAM/TSP patients were cultured at 1×10^6 cells/ml in the presence of DMSO only or 10 μM SB203580 for 72 h. The culture supernatants were collected and the levels of HTLV-I p19 antigen in the culture supernatant were measured by ELISA. Open bars: DMSO only, solid bars: 10 μM SB203580.

3.4. SB203580 inhibits HTLV-I p19 antigen expression in HCT-1, HCT-4, and $CD4^+$ T cells of HAM/TSP patients

Treatment of HCT-1 and HCT-4 with SB203580 suppressed HTLV-1 p19 antigen expression by about 22% and 10%, respectively (Fig. 4a). However, the same treatment did not reduce HTLV-I p19 antigen expression in HTLV-I-transformed T cell line, MT-2 cells (Miyoshi et al., 1981) (Fig. 4a), which produce low amounts of IFN-γ without the activation of p38 MAPK expression (data not shown). SB203580 produced about 24–66% reduction of HTLV-I

p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients (Fig. 4b).

4. Discussion

This is the first report that examined the involvement of p38 MAPK signaling pathway in both IFN-γ induction and HTLV-I expression in HTLV-I infection. Spontaneous IFN-γ expression is up-regulated by the high HTLV-I proviral load in HAM/TSP patients. However, it is not clear how such status is induced in patients with HAM/TSP. Although it is well known that cytokine expression is regulated by various signaling molecules, we focussed on the involvement of p38 MAPK signaling pathway for IFN-γ expression in HAM/TSP patients because p38 MAPK signaling plays an important role in IFN-γ induction in TCR-independent condition (Yang et al., 2001). Analysis of activated p38 MAPK expression in relation to IFN-γ production in HTLV-I-infected T cell lines derived from HAM/TSP patients, compared with same cells derived from ATL patients, revealed that spontaneous IFN-γ production correlated with activation of p38 MAPK. We also confirmed the activation of p38 MAPK signaling pathway in IFN-γ expression in HTLV-I-infected T cell lines of HAM/TSP patients by treatment of these cells with SB203580, a p38 MAPK specific inhibitor. Since SB203580 did not completely inhibit IFN-γ production by these cells, other signaling pathways such as nuclear factor kappaB (NF-κB) and c-Jun N-terminal kinase (Murphy et al., 2000; Rincón and Pedraza-Alva, 2003) or IFN-γ promoter activation by HTLV-I tax itself (Brown et al., 1991), might also contribute to IFN-γ induction in these cell lines. Although these signaling pathways were not analyzed in our experiment, activation of p38 MAPK pathway seems to be certainly an important signaling pathway involved in the up-regulation of spontaneous IFN-γ expression in these cell lines.

To confirm the involvement of the p38 MAPK signaling pathway, we also investigated the effect of SB203580 on IFN-γ production by peripheral blood $CD4^+$ T cells of HAM/TSP patients. SB203580 significantly reduced spontaneous IFN-γ production by peripheral blood $CD4^+$ T cells of HAM/TSP patients but not those of control patients including HTLV-I carriers. These results suggest that up-regulation of spontaneous IFN-γ expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients is also based on the activation of p38 MAPK pathway in TCR-independent manner. Although it is not clear in our experiment whether increased IFN-γ production from peripheral blood $CD4^+$ T cells of HAM/TSP patients is derived from HTLV-I-infected T cells only, TCR-independent IFN-γ induction occurs over a relatively long period compared with TCR-dependent IFN-γ induction (Yang et al., 2001). Therefore, activation of p38 MAPK signaling and the related up-regulation of IFN-γ expression seem consistent with the clinical course of HAM/TSP with chronic inflammatory status, such as slow progression.

On the other hand, HTLV-I p19 antigen expression also correlated with activation of p38 MAPK expression in our study using HTLV-I-infected T cell lines. It is already known that HTLV-I tax binds HTLV-I LTR, which is the promoter of HTLV-I itself, not directly, but in concert with CREB/ATF family (Franklin et al., 1993; Yoshida, 2001). These transcription factors are the downstream target of activated p38 MAPK (Dong et al., 2002; Tan et al., 1996). Therefore, we postulate that activation of p38 MAPK is also involved in HTLV-I expression in HTLV-I-infected T cell lines derived from HAM/TSP patients. Indeed, SB203580 treatment down-regulated production of HTLV-I p19 antigen from HTLV-I-infected T cell lines of HAM/TSP patients. This action was probably mediated by interruption of efficient recruitment of CREB/ATF-2 to HTLV-I LTR. Interestingly, HTLV-I p19 antigen expression in peripheral blood $CD4^+$ T cells of HAM/TSP patients was also suppressed about 24–66% by SB203580, suggesting that activation of p38 MAPK signaling pathway plays a crucial role in HTLV-I expression in HAM/TSP patients. These facts imply that high HTLV-I proviral load in HAM/TSP patients might be based on activated p38 MAPK signaling in HTLV-I-infected T cells.

In conclusion, we have demonstrated in the present study that activation of p38 MAPK signaling pathway is involved in the up-regulation of IFN-γ expression in HAM/TSP patients with high HTLV-I proviral load. Although it is not clear how p38 MAPK signaling is activated in HAM/TSP patients, perpetuation of activation of p38 MAPK signaling pathway in HTLV-I-infected state might strongly contribute to the development of HAM/TSP. In addition, it is also still obscure why only a minor proportion of HTLV-I-infected individuals develop HAM/TSP. However, p38 MAPK might be a potential target in the treatment of HAM/TSP.

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Detection of the soluble form of Fas ligand (sFasL) and sFas in the saliva from patients with Sjögren's syndrome

Sirs,

We examined the possible involvement of the soluble form of Fas ligand (sFasL) and sFas in the saliva from Sjögren's syndrome (SS) patients in the process of salivary gland destruction.

Sera were obtained from 40 primary SS at our hospital. All the patients fulfilled the revised European criteria for the diagnosis of SS (1). Anti-SS-A and anti-SS-B seropositivity were 54.6% and 19.4%, respectively. Saliva was obtained from 25 primary SS patients by catheter, which was inserted 1-2 cm into the Stensen's duct as previously described (2). 20 subjects (for sera examination) and 12 subjects (for saliva examination), who were age-matched normal controls, also enrolled. Informed consent was obtained, and the study was conducted in accordance with the human experimental guidelines of our institution.

Protein concentrations of sFasL and sFas were measured by a sandwich enzyme-linked immunosorbent assay kit using anti-human FasL monoclonal antibodies (mAbs) or anti-Fas mAbs (MBL, Nagoya, Japan). Sialography of the parotid gland was carried out to assess the radiographic grading of glandular destruction in patients with SS. Radiographic grading of glandular destruction was determined according to Rubin & Holt (from stage 0; normal to stage IV; destructive) (3). Statistical analysis was performed using Student *t*-test.

sFasL was detected in all the sera; however, their concentrations were not different between the SS patients and control subjects (mean ± SD; SS patients: 0.16 ± 0.07 ng/ml, control subjects: 0.16 ± 0.08 ng/ml, p = 0.83) (Fig.1A). sFas in sera was also detected in all the SS patients (mean ± SD; 3.94± 1.20 ng/ml), which was higher than the control subjects (mean± SD; 2.12 ± 0.59 ng/ml, p< 0.01) (Fig.1B). sFasL protein concentration in saliva was significantly higher in the SS patients than the control subjects (Fig. 1C). Interestingly, sFasL in saliva in SS was higher than that of sera (compare Fig. 1C to Fig. 1A). sFas in saliva from SS patients was also higher than the control subjects. However, sFas in saliva was significantly less than in sera concentration (compare Fig. 1D to Fig. 1B).

Parotid gland sialography was examined in 22 SS patients, all of whom were also evaluated for protein concentration of sFasL and sFas in saliva. We classified 22 SS patients into 2 groups according to Rubin & Holt radiographic grading, as 11 early-stage patients (stage 0 and stage I) and 11 advanced-stage patients (stage II to IV). A notable data shown in Fig. 2 was that sFasL protein concentration was markedly high in advanced-stage SS patients as compared with early stage SS patients. In contrast to sFasL, sFas protein concentration was not different between early-stage patients and advanced-stage patients.

The above data may indicate that sFasL in saliva appears to be involved in the salivary gland destruction of SS. Since sFasL concentration in sera from the SS patients was not different from the normal subjects, the increased amount of sFasL in saliva from SS patients appears to be associated with the microenvironment of the salivary glands from SS patients. Furthermore, it is interesting to note that sFasL concentration of saliva was clearly increased in the radiographic advanced-stage of SS patients. sFasL is cleaved from membrane-bound (mFasL) by MMP-like enzyme, and also can induce Fas-mediated apoptosis (4, 5). Although the lymphocyte infiltrates may also influence apoptotic cell death of salivary epithelial cells (6), we speculate that, salivary "cytokine-rich" microenvironment of SS patients facilitates the release of sFasL in saliva from acinar cells, and are closely involved in the apoptotic cell death of ductal epithelial cells.

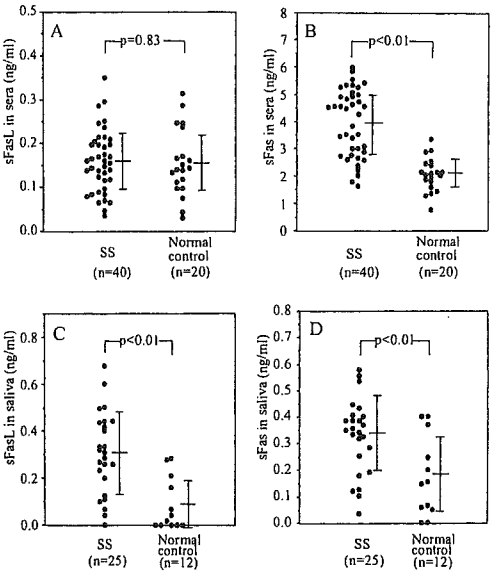

Fig. 1. Levels of sFasL and sFas in the sera and saliva from SS patients determined by ELISA.
A: Levels of sFasL in sera of SS patients (n = 40) determined by ELISA compared with normal subjects (n = 20). No significant difference was observed between SS and normal subjects (p = 0.83). **B**: Levels of sFas in sera of SS patients (n = 40) determined by ELISA, which were higher than those of normal subjects (n = 20) (p < 0.01). **C**: Levels of sFasL in saliva of SS patients (n = 25) were significantly elevated compared to normal subjects (n = 12) (p < 0.01). In SS patients, sFasL in saliva was higher than that in sera (compare Fig. 1C to Fig. 1A). **D**: Levels of sFas of saliva in SS patients (n = 25) were also higher than normal subjects (n = 12) (p < 0.01). However, sFas in saliva was significantly less than in sera concentration (compare Fig. 1D to Fig. 1B). Error bar indicated mean ± SD.

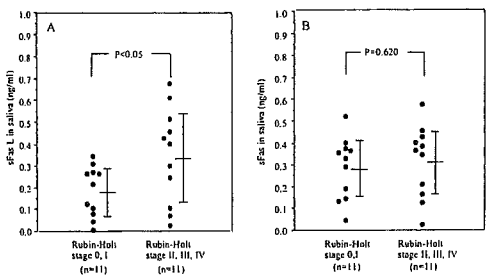

Fig. 2. Relationship between the protein concentrations of sFasL/sFas in saliva of SS patients and radiographic grading of glandular destruction. Although no significant difference was determined in sFas concentration of saliva between radiographic early-stage SS patients and advanced-stage SS patients (**B**, p = 0.62), sFasL concentration in radiographic advanced-stage SS patients was significantly higher than in early-stage patients (**A**, p < 0.05). Error bar indicates mean ± SD.

H. NAKAMURA, A. KAWAKAMI, M. IZUMI[4], T. NAKASHIMA[1], Y. TAKAGI[2], H. IDA, T. NAKAMURA[3], T. NAKAMURA[2], K. EGUCHI

First Dept. of Internal Medicine, [3]Dept. of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, [1]Dept. of Hospital Pharmacy, School of Medicine, Nagasaki University, [2]Dept. of Radiology & Cancer Biology, Nagasaki University School of Dentistry, Nagasaki; [4]Dept. of Oral and Maxillofacial Radiology, Aichi-Gakuin University School of Dentistry, Nagoya, Japan.

Address correspondence to: Hideki Nakamura, MD, First Dept. of Internal Medicine, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: nakamura_hideki911@yahoo.co.jp

Abbreviations: ELISA: enzyme-linked immunosorbent assay; MMP: matrix metalloprotease; SS: Sjögren's syndrome; sFasL: soluble form of Fas ligand; mFasL: membrane-bound Fas ligand; mAbs: monoclonal antibodies.

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