Tat-induces apoptosis in $CD4^+$ T cells. Katsikis et al. suggested in their report that some mechanism other than Fas/FasL system is responsible for the Tat induced apoptosis of $CD4^+$ T cells [17]. Another mechanism may also be important in the apoptosis and depletion of $CD4^+$ T cells in patients with an HIV infection.

RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) is a type II membrane protein that forms homo-oligomers through the C-terminal coiled-coil structures. RCAS1 can also be secreted, and both the secreted and the transmembrane forms induce growth arrest and apoptosis in RCAS1 receptor-positive cells [18]. RCAS1 is similar to tumor necrosis factor-related apoptosis-induced ligand (TRAIL) in that both the membrane-bound and soluble forms are biologically active, while it is different from Fas ligand (FasL) in that membrane-bound FasL is much more active than soluble FasL. Several reports have shown RCAS1 to be expressed in various kinds of cancers [19,20] and its putative receptor is expressed in the cells involved with the immune system including T cells, B cells, and NK cells especially when they are activated [21]. Furthermore, these reports suggested that RCAS1 could help cancer cells to survive or avoid immunosurveillance. We previously reported the elevated serum levels of RCAS1 to be associated with a poor immunological prognosis in HIV-1-infected patients, thus suggesting that RCAS1 were also associated with the apoptosis of $CD4^+$ T cells in HIV infection.

We herein investigated the effect of Tat on the RCAS1 expression and our findings demonstrated Tat to increase the RCAS1 expression in $CD4^+$ T cells and monocytes. We also demonstrated the effect of RCAS1 on Tat-induced apoptosis, thus indicating that RCAS1-induced apoptosis is one of the mechanisms that induces the death of $CD4^+$ T cells in HIV-1 infection.

2. Materials and methods

2.1. Reagents

HIV-Tat protein, HIV-TAT BH10 [1–86] was purchased from ZEPTAGEN (Napoli, Italy), and it is a chemically synthesized and HPLC purified protein. According to the manufacturer's data sheet, the purity of the Tat protein is over 95%, and it has also been found to preserve its biological activity.

2.2. Cells and cell culture

The human leukemia Jurkat T cell line, MOLT4 T cell line and promonocytic U937 cell line were obtained from American type Culture Collection. MOLT4/IIIB cells, which are MOLT4 cells persistently infected with the HIV-IIIB strain, was kindly provided by Dr. T. Kaneda (Nagoya Medical Center, Aichi, Japan). As shown in Fig. 1, MOLT4/IIIB cells express HIV-Tat mRNA. Human $CD4^+$ T cells and monocyes were isolated from the PBMC of

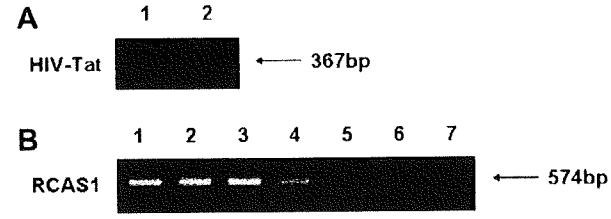

Fig. 1. (A) Analysis of the Tat mRNA expression by RT-PCR. Lane 1, MOLT4 cells; lane 2, MOLT4/IIIB cells. (B) Analysis of the RCAS1 mRNA expression by RT-PCR. Lane 1, MOLT4 cells; lane 2, MOLT4/IIIB cells; lane 3, Jurkat cells; lane 4, U937 cells; lane 5, monocytes from HIV-uninfected healthy donor; lane 6, $CD4^+$ T cells from HIV-uninfected healthy donor; lane 7, negative control, distilled water.

healthy donors by negative selection using Stem-Sep magnetic separation column (StemCell Technologies, Vancouver, Canada). Cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS).

2.3. Polymerase chain reaction (PCR) analysis for Tat and RCAS1

PCR was performed with GeneAmp PCR System 9600 (Perkin-Elmer Co., Norwalk, CT) using TaKaRa Taq (TaKaRa). The primer pairs used for amplification were as follows: RCAS forward; 5′-ATCACTCAGTTTCGGTTATT-3′, and reverse, 5′-CCATTTTCTTCCTCTGTTGT-3′; TAT forward; 5′-ATAGGCATAATTCGACAGAGGA-3′, and reverse, 5′-TCGACCCAGATAATTGCTAAGAATC-3′.

2.4. Real-time PCR

Total RNA was extracted using QIAamp RNA Blood Mini kit (Qiagen, Tokyo, Japan), followed by treatment with RNase-free DNase (Qiagen). cDNA was synthesized from 500 ng of RNA, using AMV Reverse Transcriptase XL according to the manufacturer's instructions (TaKaRa, Siga, Japan). Real-time PCR was conducted with the Line-Gene33 (BioFlux, Tokyo, Japan) using SYBR Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). The primers used for amplification were as follows: RCAS1 forward, 5′-ACGGCTAATGAAGAAGGAAC-3′, and reverse, 5′-TACTTTGGGAGAATCGTGG-3′; β-actin forward, 5-C TGGAACGGTGAAGGTG-3, and reverse, 5′-TTTAGGA TGGCAAGGGAC-3′. Standards were obtained by amplification of a control sample in a PCR reaction, using the same primers. The expression was normalized to the housekeeping gene (β-actin) and to unstimulated wild type controls.

2.5. ELISA for RCAS1

The amount of soluble RCAS1 in the culture medium was measured by ELISA, using the RCAS1 ELISA Kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's instructions. Briefly,

culture media were collected after 48 h culture and incubated in anti-RCAS1 antibody-coated polystyrene wells. The wells were washed and incubated with a biotin-labeled anti-RCAS1 antibody and then with a streptavidin/horseradish peroxidase conjugate. Horseradish peroxidase was detected by incubation with tetramethylbenzidine and H_2O_2, the reaction was stopped, and the intensity of color produced was measured at 450 nm on a microplate reader. Both standards and samples were assayed in duplicate.

2.6. Apoptosis assay

Apoptosis was determined by detection of denatured DNA in apoptotic cells with monoclonal antibody to single-stranded DNA, using ssDNA ApoptosisELISA Kit (CHEMICON, Temecula CA) according to the manufacturer's instructions. Briefly, 10,000 cells cultured with or without Tat in 96-well plate were fixed with 80% methanol in PBS, and then were incubated with Formamide at room temperature. The plate was heated to 75 °C in an oven for 20 min, and then was cooled in a refrigerator for 5 min. After removing formamide, the wells were incubated with 3% nonfat dry-milk to block non-specific binding sites. The wells were washed and incubated with an antibody mixture included with primary monoclonal antibody to ssDNA and horseradish peroxidase-labeled anti-mouse IgM. The horseradish peroxidase was detected by incubation with a solution containing 2′,2-AZINO-bis[3-ethylbenziazoline-6-sulfonic acid], then the reaction was stopped with HCl solution, and the intensity of color produced was measured at 405 nm on a microplate reader. Both standards and samples were assayed in duplicate.

2.7. Caspase 3 activity assay

The caspase 3 activity was assayed, using Caspase-3 Colorimetric Activity Assay Kit (CHEMICON, Temecula CA) according to the manufacturer's instructions. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA. 2×10^6 cells treated with or without HIV-Tat were lysed by chilled Cell Lysis Buffer, and assayed the protein concentration for each sample. Two hundred micrograms protein was used for each assay. The assay mixture was prepared in a 96-well plate according to the manufacturer's protocol. After incubating for 2 h, the samples were read at 405 nm in a microplate reader. The fold-increase in caspase 3 activity was determined by comparing the absorbance from each sample with untreated MOLT 4 cells.

2.8. Short interfering RNA transfection

siRNA duplexes were synthesized and purified by Ambion (Austin, TX). The siRNA sequence for targeting RCAS1 mRNA was 5′-GCAGCCGAACAACAAAGG ATT-3′. A scrambled sequence was used as a negative control (Ambion). The transfection of siRNAs was done using RCAS1 siRNA or scrambled siRNAs, siRNA transfection medium (sc-36868, Santa Cruz Biotechnology, Santa Cruz, CA), SilenceMag (OZ Biosciences, Marseille, France) and CombiMag (OZ Biosciences) according to the manufacturers' instructions. A real-time PCR analysis was performed to assess any changes in the RCAS1 gene expression.

2.9. Statistical analysis

We used Student's *t* test for a statistical analysis. *p* values less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Cell lines, $CD4^+$ T cells, and monocytes in the peripheral blood express RCAS1

We first examined the expression of RCAS1 of Tat-unstimulated Jurkat cells, MOLT4 cells, MOLT4/IIIB cells, U937 cells, $CD4^+$ Tcells, and monocytes in the peripheral blood of HIV-1 uninfected individuals, using RT-PCR method. As shown in Fig. 1B, RCAS1 mRNA was detected in all cell lines, $CD4^+$ T cells, and monocytes . MOLT4/IIIB cells, which are MOLT4 cells persistently infected with the HIV-IIIB strain, continuously produce Tat mRNA (Fig. 1A).

3.2. HIV-Tat increased RCAS1 mRNA expression on MOLT4 cells and MOLT4/IIIB cells

According to previous reports, Tat has been shown to induce some apoptosis-associated proteins, such as FasL and TRAIL. We therefore examined the effects of Tat on the induction of RCAS1 in MOLT4 cells and MOLT4/IIIB cells. The effect of Tat on RCAS1 production was examined by ELISA analysis of the secreted soluble forms of RCAS1 (sRCAS1) in 48 h cultured medium with various concentrations of Tat. Tat up-regulated the release of sRCAS1 from MOLT4 cells and MOLT4/IIIB cells with all concentrations we used, but as the maximum effect was obtained with 0.01 μg/ml of Tat, further examinations were made using 0.01 μg/ml of Tat. HIV-Tat inhibited the growth of MOLT4 cells, MOLT4/IIIB cells, Jurkat cells, $CD4^+$ T cells, and monocytes, so the difference between the amount of soluble RCAS1 in the culture medium of the Tat treated and untreated cells was more evident, when the results were normalized for the cell numbers. As shown in Fig. 2B, treatment with Tat induced RCAS1 mRNA expression rapidly, reaching a peak at 4–8 h after treatment. Since Tat is easily oxidized, RCAS1 mRNA expression dropped off after 16 h. Both the release of sRCAS and the expression of RCAS1 mRNA were higher in MOLT4/IIIB cells than in MOLT4 cells, and one of the reasons for this may be due to Tat released from the MOLT4/IIIB cells themselves.

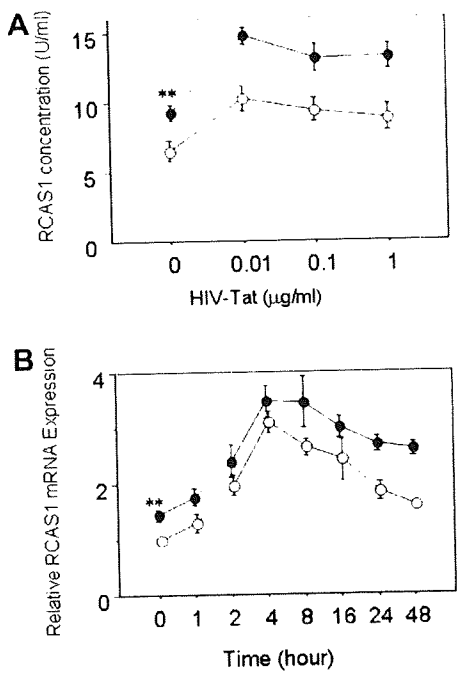

Fig. 2. HIV-Tat induced RCAS1 in MOLT4 cells and MOLT4/IIIB cells. (A) ELISA of conditioned medium from MOLT4 and MOLT4/IIIB cells treated with Tat. MOLT4 (○) and MOLT4/IIIB (●) cells (1×10^5/ml) were incubated with or without Tat (0.01–1 μg/ml) for 48 h. The cells were collected by centrifugation, and aliquot of the cell-free supernatant was analyzed using ELISA. Data are the mean values with SEs of three independent experiments. (B) mRNA expression of MOLT4 and MOLT4/IIIB cells treated with Tat Total RNA from MOLT4 (○) and MOLT4/IIIB (●) cells were isolated and subjected to RT-PCR after 0, 1, 2, 4, 8, 16, 24 and 48 h of incubation in the presence of Tat (0.01 μg/ml). The mRNA expression was measured with real-time PCR method. The results have been normalized to β-actin mRNA and to unstimulated controls (MOLT cells). Data are the mean values with SEs of three independent experiments. (A and B) $^{**}p < 0.05$ between MOLT4 and MOLT4/IIIB cells.

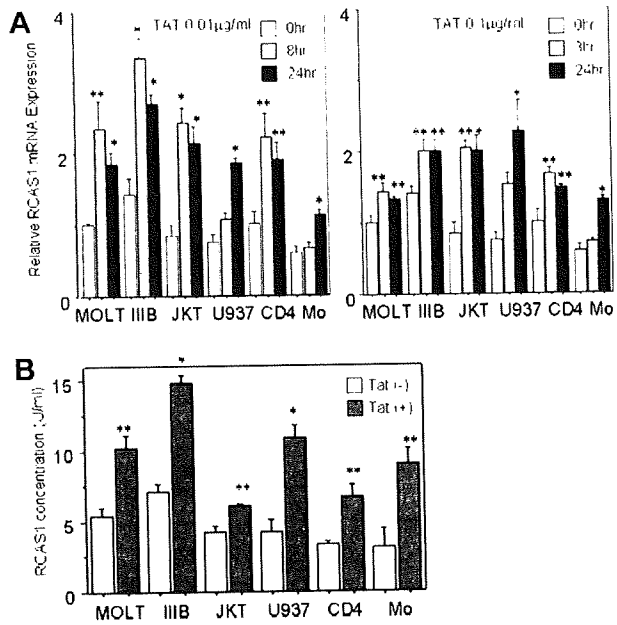

Fig. 3. HIV-Tat induced RCAS1 in Jurkat cells, U937 cells, $CD4^+$ T cells and monocytes from peripheral blood of healthy donors. (A) mRNA expression of MOLT4 cells, MOLT4/IIIB cells, Jurkat cells, U937 cells, $CD4^+$ T cells, and monocytes treated with Tat. Cells were incubated with or without Tat (0.01 μg/ml and 0.1 μg/ml). After 8 and 24 h culture, cells were isolated and subjected to RT-PCR. mRNA expression was measured with real-time PCR method. The results have been normalized to β-actin mRNA and to unstimulated MOLT cells. (B) ELISA of conditioned medium from MOLT4 cells, MOLT4/IIIB cells, Jurkat cells, U937 cells, $CD4^+$ T cells, and monocytes treated with Tat. Cells (1×10^5/ml) were incubated with or without Tat (0.01 μg/ml for MOLT4 cells, MOLT4/IIIB cells, Jurkat cells and $CD4^+$ T cells, and 0.1 μg/ml for U937 cells and monocytes) for 48 h. The cells were collected by centrifugation, and an aliquot of the cell-free supernatant was analyzed using ELISA. (A,B) Data are the mean values with SEs of three independent experiments. MOLT: MOLT4 cell, IIIB: MOLT4/IIIB cell, JKT: Jurkat cell, U937: U937 cell, CD4: $CD4^+$ T cell from peripheral blood of healthy donors , Mo: monocyte from peripheral blood of healthy donors. $^*p < 0.01$ between each untreated ant Tat-treated cells. $^{**}p < 0.05$ between each untreated ant Tat-treated cells.

We next examined the effect of Tat on RCAS1 expression, using Jurkat cells, U937 cells, $CD4^+$ Tcells, and monocytes in the peripheral blood of HIV-1 uninfected individuals. Tat increased the RCAS1 mRNA expression and the release of sRCAS1 from all cells we examined (Fig. 3). The optimum concentration of Tat and the optimum incubation time with Tat were variable between cell types. U937 and monocytes required higher concentration (0.1 μg/ml), and longer incubation time (24 h) for the induction of RCAS1 expression. Considering that HIV-Tat is easily oxidized, it has been suggested that HIV-Tat is taken up by cells and activates some intermediate factors rapidly, which mediate the expression of RCAS1. A previous report demonstrated that the Tat treatment of monocytes enhanced the gene expression of some cytokines which are rapidly regulated by HIV-Tat, and some changes in the monocyte function induced by HIV-Tat were caused by the production of these cytokines [8].

The relative amounts of Tat in the sera from HIV-1 infected patients have been reported to range from 0.1 to 1 ng/ml [13]. In our experiment, Tat is detected in some sera of the HIV-1 infected individuals, which significantly increased the RCAS1 mRNA expression in $CD4^+$ T cells in comparison to the sera of the HIV-1 Tat negative controls (data not shown). These data suggest that effective Tat concentrations might also be sufficient to induce RCAS1 and apoptosis in $CD4^+$ T cells as well as in peripheral blood. However, the maximum effects of exogenous Tat in this study were observed at a concentration of more than 0.01 μg/ ml for $CD4^+$ T cells, and more than 0.1 μg/ml for monocytes. In previous reports, the concentration of Tat required either to activate or to directly induce the apoptosis of $CD4^+$ T cells varies from 1 to 2 μM in different experiments [12,13,22]. The concentrations of Tat needed to induce RCAS1 in monocytes in our study was almost as high as that for cytokine production (10 ng/ml) [8], and that needed to induce TRAIL (100 ng/ml) in monocytes [15]. Although these concentrations were considerably higher

than the estimated physiologic levels in the peripheral blood, it is possible that higher quantities of Tat may be found in the lymph nodes of HIV-1 infected patients, in which HIV-1 infected cells are much more numerous and T cell depletion is most evident [23].

In a previous report [18], the expression of RCAS1 receptor on cells was analyzed, using GST-RCAS fusion protein. Since GST-RCAS fusion protein is unavailable at the present, we could not examine the expression of RCAS1 receptor on cells. However, the expression of RCAS1 receptor has already been reported to increase when T cells are activated [18]. Given that HIV-1 infection is characterized by a state of chronic T cell activation [24], and given that this activation is driven in part by HIV-Tat [9,10,25], it is possible that the expression of RCAS1 receptor may thus increase after the addition of HIV-Tat.

3.3. RCAS1 induced by Tat are involved in Tat-mediated apoptosis

We investigated the effects of siRNA targeting RCAS1 (siRNA-RCAS1) on RCAS1 mRNA expression, RCAS1 secretion and apoptosis of MOLT4 or MOLT4/IIIB cells. siRNA-RCAS1 reduced RCAS1 mRNA expression by 53.9% in MOLT4 cells and by 58.9% in MOLT4/IIIB cells at 24 h after treatment with siRNA-RCAS1. We then incubated these cells with or without Tat for 16 h. Tat-induced RCAS1 mRNA expression was inhibited by 38.3% in MOLT4 cells treated with siRNA-RCAS1 (MOLT4-siRNA) and by 42.6% in MOLT4/IIIB cells treated with siRNA-RCAS1 (MOLT4/IIIB-siRNA), compared with the cells untreated with siRNA. The release of sRCAS1 was also inhibited by siRNA-RCAS1 (Fig. 4B).

We next analyzed Tat-induced apoptosis in MOLT4 cells and MOLT4/IIIB cells, using ssDNA ApoptosisELISA Kit that detects denatured DNA. We found Tat-induced apoptosis in MOLT4 cells more than in MOLT4/IIIB cells (Fig. 4C). MOLT4/IIIB cells were relatively resistant to the apoptotic effects of exogenous Tat. This is compatible with the previous report that showed uninfected T cells are more susceptible to Tat-induced apoptosis than are HIV-infected T cells [12,15,26]. Certainly, in the process of making stable-transfected cells, the cells often obtain cell death resistance, but previous reports have shown the dual

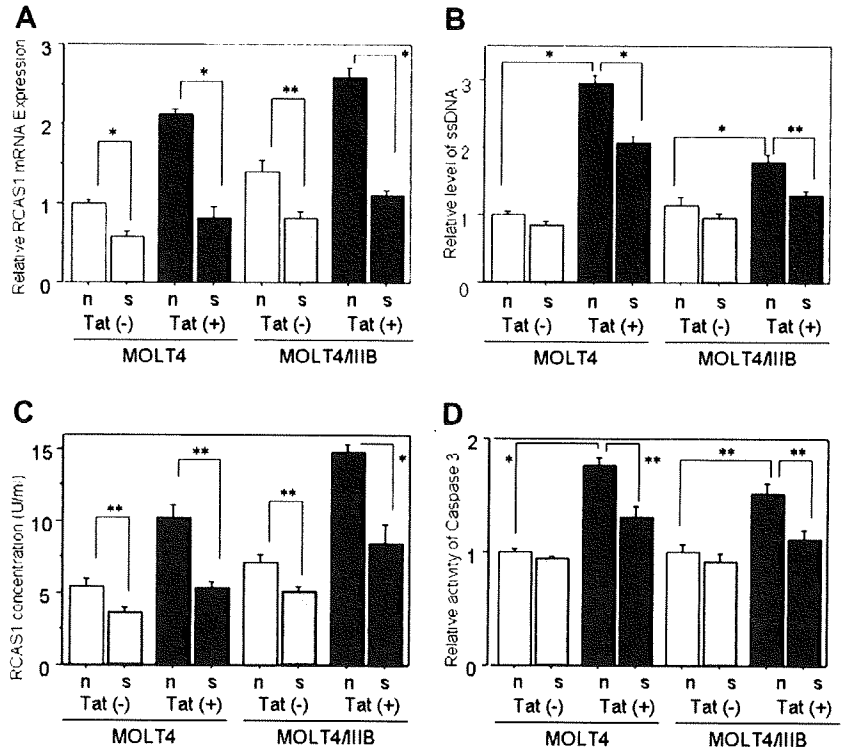

Fig. 4. Inhibition of RCAS1 expression by siRNA decreased Tat-induced apoptosis. (A) SiRNA-RCAS1 reduced the RCAS1 mRNA expression in MOLT4 cells and MOLT4/IIIB cells. Cells pre-treated with siRNA were incubated with or without Tat (0.01 μg/ml). After an 8 h culture, cells were isolated and subjected to RT-PCR. mRNA expression was measured with real-time PCR method. The results have been normalized to β-actin mRNA and to untreated MOLT cells. (B) ELISA of conditioned medium from MOLT4 cells, MOLT4/IIIB cells pre-treated with siRNA. Cells (1×10^5/ml) were incubated with or without Tat (0.01 μg/ml) for 48 h. The cells were collected by centrifugation, and an aliquot of the cell-free supernatant was analyzed using ELISA. (C) siRNA-RCAS1 inhibits Tat-induced apoptosis. Cells (1×10^4) pre-treated with siRNA were incubated with or without Tat (0.01 μg/ml) for 24 h. Apoptosis was measured by the detection of denatured DNA with monoclonal antibody to single-strand DNA. The results have been normalized to MOLT cells treated with negative control siRNA and without Tat. (D) siRNA-RCAS1 inhibits Tat-induced activation of caspase 3. Cells (2×10^6) pre-treated with siRNA were incubated with or without Tat (0.01 μg/ml) for 24 h. The caspase 3 activity was measured, using the Caspase-3 Colorimetric Activity Assay Kit. The results have been normalized to MOLT cells treated with negative control siRNA and without Tat. (A–D) The data are the mean values with SEs of three independent experiments. n, treated with negative control siRNA; s, treated with RCAS1 siRNA. $^*p < 0.01$, $^{**}p < 0.05$.

and inverse roles of HIV Tat in regulating apoptosis in T cells. Exogenous Tat transduces a signal that activates the death pathway, such as Fas/FasL system, TRAIL receptor/TRAIL system, and down-modulation of Bcl-2. On the other hand, endogenous Tat blocks the death pathway, such as by inducing c-FLIP [26], bcl-2/bcl-x, and IL-2, or by the down-regulation of caspase10 [27].

Furthermore, Fig. 4C shows that siRNA-RCAS1 significantly inhibited the apoptosis in the MOLT4 cells and MOLT4/IIIB cells induced by Tat. These results suggest that RCAS1 plays an important role in apoptosis mechanisms mediated by Tat, as well as Fas/Fas ligand, and TRAIL/TRAIL receptor mechanisms.

We also examined the activity of caspase 3, which plays a critical role in the apoptotic pathways. As shown in Fig. 4D, similar results were obtained with the data of detecting ssDNA. These findings confirm that RCAS1 thus plays a critical role in the apoptosis induced by Tat.

Immune activation as a response to HIV-1 infection occurs in uninfected bystander cells as well as in HIV-1 infected $CD4^+$ T cells [10,28]. The treatment of primary $CD4^+$ T cells with soluble HIV-Tat protein has been reported to mimic many of the properties of HIV infection in these cells [29]. These observations suggest that RCAS1 might also regulate the apoptosis of bystander cells, and that the RCAS1 induced by Tat might be one of the important mechanisms for $CD4^+$ T cell depletion in patients with HIV infection, namely a disease progression of HIV infection.

As described previously, RCAS1 is expressed on the surface and in the cytoplasm of various kinds of cancer cells and it induces apoptosis of immune cells with RCAS1 receptor. Such evidence indicates that RCAS1 on tumor cells may act as a defense mechanism of tumor cells against the cells in the immune system. In patients with invasive cancers, the serum levels of sRCAS1 are higher than those in healthy controls and the elevation of RCAS1 is correlated with a poor prognosis [30]. In addition, in HIV infection, as we reported previously, elevated serum levels of RCAS1 have been reported to be associated with a poor immunological prognosis in HIV-1-infected patients [31].

This study focused on $CD4^+$ T cells, because $CD4^+$ T cell apoptosis has been proposed to be an important mechanism in the pathogenesis of HIV-1 infection. However, it will be interesting in future studies to investigate whether the RCAS1 secreted by monocytes plays a critical role in the apoptosis in HIV-1 infected $CD4^+$ T cells or uninfected bystander cells.

In summary, our findings are the first to demonstrate the induction and secretion of RCAS1 in HIV-Tat-stimulated $CD4^+$ T cells and monocytes, and these findings suggest that RCAS1 might be involved in the $CD4^+$ T cell apoptosis observed in HIV-1 infection, together with FasL and TRAIL. Further functional studies are therefore necessary to clarify the precise mechanisms of $CD4^+$ T cell apoptosis induced by RCAS1 in HIV-1 infection.

Acknowledgments

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Original Research Report

Elevated Serum Levels of RCAS 1 Are Associated with a Poor Recovery of the CD4+T Cell Count after ART in HIV-1-infected Patients

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Objective : RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) is an apoptosis-associated protein that induces apoptosis in activated T-cells. The aim of this study is to investigate the role of RCAS1 in HIV-1 infection.

Methods : We examined the serum levels of RCAS1 in HIV-1-infected patients at different clinical and immunological stages.

Results : Although the RCAS1 levels did not correlate with the clinical stage, they did correlate significantly with the CD8+T cell numbers. Furthermore, the RCAS1 levels were also significantly higher in patients whose CD4+T cell counts did not respond to antiretroviral therapy (ART) than in those who responded to ART.

Conclusions : The present findings therefore suggest that the RCAS1 level affects the CD 4 + T cell counts in HIV-1-infected patients with ART.

Key words : RCAS1, HIV-1 infection, immunological prognosis

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Introduction

RCAS1 has been demonstrated to be a type II membrane protein expressed on human tumor cells. RCAS 1 can also be secreted, and both the secreted and transmembrane forms act as a ligand thereby inducing apoptosis in receptor-positive cells including T cells, B cells and NK cells[1]. These results suggest that RCAS 1 may assist tumor cells in their survival or escape from immunosurveilance[2]. Several reports have shown the serum RCAS1 level to be a significant prognostic factor in patients with certain malignancies[3-5]. In addition, monocytes/macrophages have also been shown to express RCAS1, and soluble RCAS1 molecules have been detected in culture supernatants of lipopolysaccharide-stimulated macrophages[6]. Furthermore, an association between RCAS1 and Epstein-Barr virus infection has also been reported[7]. In the present study, to elucidate whether RCAS1 plays a role in HIV-1 infection, we examined the serum levels of RCAS1 in patients during various clinical and immunological stages of HIV-1 infection.

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Methods

The subjects consisted of 82 HIV-1-infected patients and 12 HIV-1-seronegative healthy controls. The characteristics of these patients and controls are shown in Table 1. None of the HIV-1-positive patients exhibited any signs of acute HIV-1 infection. Informed consent for blood sampling was obtained. The study was conducted according to the ethical guidelines of our hospital, and was approved by an authorized representative of the hospital. We first measured the levels of sRCAS 1, sFasL and sTRAIL in the 82 HIV-infected patients at two different clinical stages (AIDS vs. non-AIDS) and at two different immunological statuses (>800 CD8+ T cells/μl vs. <800 CD8+T cells/μl). The serum levels of RCAS1, FasL and sTRAIL were measured using specific ELISA kits (RCAS1, Medical and Biological Laboratories Co. Nagoya, Japan ; sFasL and

Table 1 Serum levels of RCAS1 of the subjects

	No. of cases	Age	sRCAS1
Healthy Control	12	32.5 ± 7.0	6.81 ± 1.87
HIV infected Pt.	82	38.6 ± 11.1	6.23 ± 3.31
ART naïve	41	37.0 ± 10.6	5.91 ± 3.11
CD4-responder	26	36.6 ± 10.8	5.34 ± 2.16
CD4-non-responder	15	41.8 ± 11.1	8.72 ± 4.29*

*$p<0.01$ for the difference between CD4-responder and CD4-non-responder

Table 2 Characteristics of patient with ART

	CD4-responder	Cd4-non-responder	*p*-value
Age	37.4±10.4	42.9±10.1	0.1106
CD4+T cell counts before ART (/μl)	189.3±132.6	154.2±125.5	0.4209
duration of ART (years)	4.88±2.38	4.58±4.52	0.7844

sTRAIL, DIACLONE Research Co. Besancon, France) according to the manufacturer's recommendations. We used Student's *t* test for comparisons between the two groups.

Results

The levels of sRCAS1were not significantly different between the AIDS group and the non-AIDS group (AIDS vs. non-AIDS ; 6.42±3.18 vs. 5.72±3.65). Levels of sRCAS1 were significantly higher in the patients with >800/μl CD8+T cells than in the patients with< 800/μl CD8+T cells (CD8< 800/μl vs. CD8≧ 800/μl ; 7.33±3.77 vs. 4.96±2.06). There were no significant correlations between the serum levels of sFasL, sTRAIL and sRCAS1 (data not shown). We next measured the levels of sRCAS1 in 41 ART-naive HIV-1-infected patients. There was no significant difference in the levels of RCAS1 between ART-naive HIV-1-infected patients and the controls (Table 1). We next examined 41 HIV-infected patients who had undetectable levels of plasma HIV-1 RNA after they were treated with ART. To evaluate the effects of ART on the recovery of the CD4+T cell count in the peripheral blood, we divided these 41 patients into two groups according to their CD4+T cell counts : CD4 responders (n=26), CD4+T cells increased by more than 300/μl after the initiation of ART ; CD4 non-responders (n=15), CD4+T cells increased by less than 300/μl after the initiation ART. There were no statistically significant differences in age, the CD4+T cell counts before ART, or the duration of ART between the responders and non-responders (Table 2). As shown in Table 1, the serum levels of RCAS1 were significantly higher in the CD4 non-responders.

Discussion

In HIV-1 infection, infected CD4+T cells undergo apoptotic cell death. In addition, a significant number of uninfected CD4+T cells in HIV-1-infected patients undergo apoptosis, induced either by immunological activation, by the effects of HIV-1 proteins, or by elevated levels of death-inducing ligands. These apoptotic mechanisms contribute to an impairment of the immune system in HIV-1-infected patients. It has recently been shown that FasL and TRAIL contribute to the apoptosis of HIV-1-uninfected CD4+T cells, and that the serum levels of sFas and sTRAIL correlate with the clinical, immunological and virological status[8,9]. The present results are thus consistent with these previous findings.

RCAS1 was originally discovered as a tumor-associated antigen, which induces apoptosis in RCAS1 receptor-positive immune-regulating cells, such as activated T cells, thereby helping such tumors escape immune surveillance.

Although we could not examine the molecular mechanisms of RCAS1 and the expression of RCAS1 receptor on HIV-1 infected CD4+T cells because of the unavailability of RCAS1, the expression of RCAS1 receptor has already been reported to increase when T cells are activated[1]. Given the fact that an HIV-1 infection is characterized by a state of chronic T cell activation[10], it is therefore very possible that the expression of RCAS1 receptor increases in HIV-1 infected CD

4+T cells.

Furthermore, the patients with high levels of RCAS1 showed a low CD4+T cell recovery in response to ART. This result shows that RCAS1 induces apoptosis in CD4+T cells, which are either HIV-1-infected cells or uninfected cells, even after ART has sufficiently suppressed HIV-1 production. Although the mechanisms of regulating RCAS1 and RCAS1 receptors in HIV-1 infection also remain unclear, the findings of a lack of any correlation between the serum levels of sFasL, sTRAIL and RCAS1 suggest the expression of RCAS1 to be regulated by a mechanism that is different from the FasL and TRAIL systems. Although our findings were not able to elucidate whether FasL, TRAIL, or RCAS1 plays a more important role in HIV-1 infection, RCAS1 was suggested to play a role as one of the mechanisms not only inducing the apoptosis of CD4+T cells in HIV-1-infected patients, but also causing a progression of the disease in HIV-1 infection.

In future studies, we plan to examine the precise mechanisms that regulate the RCAS1/RCAS1 receptor system in HIV-1.

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症　例

高熱，リンパ節腫脹を繰り返したのち発症したHIV-1陽性HHV-8関連Castleman病の1例

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（平成18年1月11日受付）
（平成18年2月24日受理）

Key words: Castleman disease, human immunodeficiency virus, human herpesvirus 8

序　文

Castleman病はリンパ節に形質細胞の増生を認め，腫脹リンパ節から産生されるIL-6により発熱，リンパ節腫脹等の臨床症状を呈する慢性炎症性疾患である．近年，human herpesvirus 8（HHV-8）はカポジ肉腫のみでなくCastleman病との関連が指摘されているが，本邦でのhuman immunodeficiency virus-1（HIV-1）陽性HHV-8関連Castleman病の報告例は少ない．今回，頻回に高熱を繰り返した後に発症したHHV-8関連Castleman病を経験したので報告する．

症　例

47歳男性．20歳時A型肝炎の既往あり．1999年にHIV-1感染症と診断．HIV-viral load（VL）10^4コピー/mL，CD4陽性Tリンパ球数（CD4）>500/μLであったため抗HIV療法を施行せずに経過観察していた．2000年9月以降，年に数回，血小板低下，CRP上昇を伴う発熱を認めたが，解熱剤使用にて数日で改善を認めていた．2003年6月，高熱，全身リンパ節腫脹出現，末梢血中EBV-DNA陽性でありEbstein-Barr virus（EBV）感染症を疑い，γグロブリン製剤使用したところ数日で改善した．以後，同様の高熱が頻回に認められた．無菌性髄膜炎の診断にてganciclovirを使用し改善を認めたこともあった．2005年3月，発熱，全身リンパ節腫脹，胸水，肝障害，脾腫出現したため，精査加療目的にて入院となった．入院時，体温37℃，皮疹は認めず，カポジ肉腫も認めなかった．両顎下部，両頸部，両鎖骨上窩部，両腋窩部，両鼠径部に1cm大のリンパ節腫大を複数認めた．肝は触知せず，脾を1横指触知した．神経学的異常所見は認めなかった．血液検査では血小板7.4万/μLと低下，CRP 18.69mg/dL，IgG 2,920mg/dLと増加を認めた．またIL-6 66pg/mL，可溶性IL-2レセプター11,900U/mLと上昇を認めた（Table 1）．血中のHHV-8 DNA，EBV DNAともにPCR法にて測定したところ陽性であった．骨髄像ではmegakaryocyteの増加を認めた．3系統の造血細胞に形態異常は認めず血球貪食像，異形細胞の浸潤は認めなかった．胸腹部CTにて肺門部，縦隔に1～2cm大，腸間膜に1cm大，傍大動脈領域，脾門部に2～3cm大のリンパ腫脹を複数

Table 1　Laboratory Data on Admission

【Urinalysis】		【Immunology】	
Prot	(1+)	CRP	18.69 mg/dL
OB	(+/−)	IgG	2,556 mg/dL
【Hematology】		IgA	251 mg/dL
RBC	402×10^4 /μL	IgM	122 mg/dL
Hb	11.5 g/dL	$CD4^+$T cells	347 /μL
Ht	33.6 %	$CD8^+$T cells	1,017 /μL
WBC	5,100 /μL	ANA	×40
Neu	50 %	PAIgG	193.3 ng/10^7cells
Ly	36.8 %	anti-CL IgG Ab	9 U/mL
Mono	12.1 %	anti-CL-β 2GPI Ab	6.2 U/mL
Eo	0.3 %	IL-6	66 pg/mL
Baso	0.8 %	sIL-2 R	11,900 U/mL
PLT	7.4×10^4 /μL	【Infection】	
【Biochemistry】		β-D glucan	(−)
TP	7.1 g/dL	endotoxin	(−)
Alb	2.8 g/dL	HIV-RNA	82,000 copies/mL
T-bil	0.4 mg/dL	CMV-C7HRP	(−)
LDH	243 IU/L	HHV-8 DNA	6,200 copies/mL
AST	24 IU/L	EBV DNA	1,300 copies/mL
ALT	26 IU/L	EBV Ab	×320
BUN	14 mg/dL	VCA-IgM	< ×10
Cr	0.7 mg/dL	EBNA	×40
Amylase	96 IU/L	EADR-IgG	×40
CK	16 IU/L	EADR-IgA	×20
		VCA-IgG	×320

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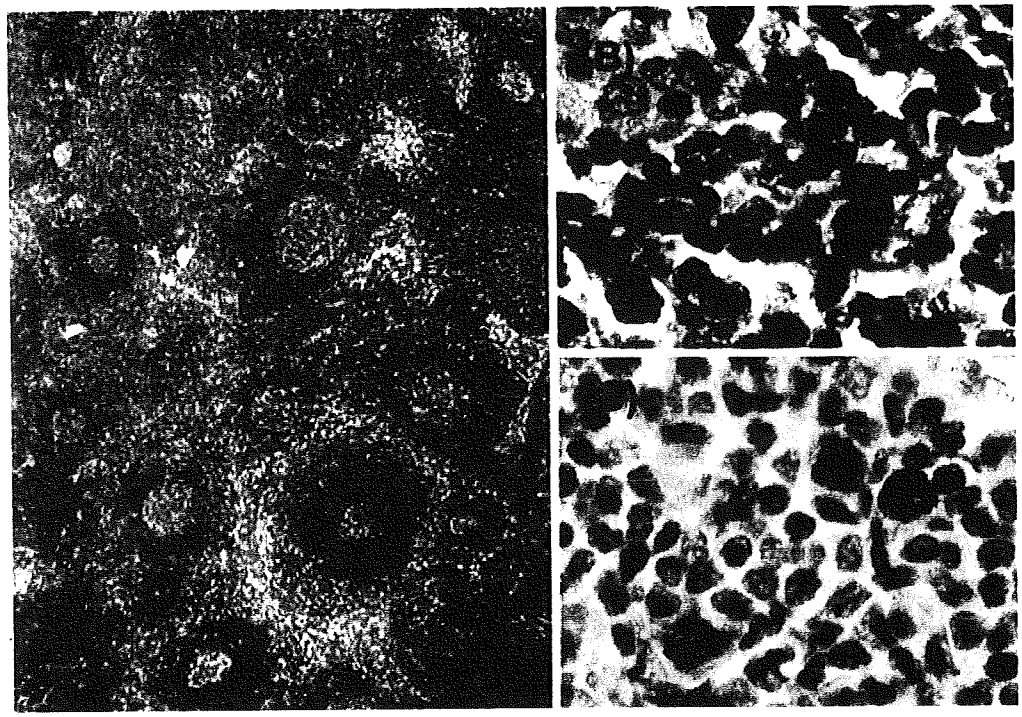
平成18年7月20日

Fig. 1 Histopathological image of cervical lymph node biopsy showing Castleman disease, plasma cell type pattern.
(A) H.E. staining of cervical lymph node. The section shows enlarged lymph nodes with hyperplastic lymphoid tissue and prominent lymphoid follicles with marked germinal centers. (×10) (B) H.E. staining of cervical lymph node. Abundant plasma cells with no definite atypia are frequently found in the cortex. (×400) (C) Immunohistchemical staining of cervical lymph node. Lymph node was stained with anti-HHV-8 antibody (NCL-HHV8-LNA ; Novocastra). Some HHV-8 positive cells are noticed. (×400)



認めた．可溶性IL-2レセプターの著高より悪性リンパ腫も疑い頸部リンパ節生検を施行したところ，リンパ濾胞の過形成および濾胞間組織のplasma cellの増生を認め，Castleman病plasma cell typeと診断された．免疫染色ではHHV-8およびEBERs陽性リンパ球が濾胞間に散見された（Fig. 1）．末梢血中およびリンパ節中のHHV-8陽性所見よりHHV-8関連のCastleman病と考えられた．Retrospectiveに保存血清中のHHV-8 DNA量（リアルタイムPCR法にて測定）およびIL-6を測定したところ有熱期間では双方とも増加しており解熱とともに低下していた．2005年4月よりHHV-8感染症に対する免疫能回復を目的にlamivudine（3TC），abacavir（ABC），atazanavir（ATV）にて抗HIV療法（Highly Active Antiretroviral Therapy；HAART）を開始した．開始後，一時期発熱の見られない時期があったが，再度，CRP上昇，血小板低下，HHV-8 DNA量の増加を伴う発熱を認めるようになった（Fig. 2）．EBV DNA量（リアルタイムPCR法にて測定）の増加は認められなかった．有熱時のリンパ節生検を再度行ったところ前回と同様，Castleman病の所見が得られた．Castleman病にHHV-8が関与していること，また本症例でもHHV-8 DNA量の増加を認めたことより抗HHV-8療法としてvalganciclovir 1,800mg/日の投与を開始した．その結果，血中HHV-8 DNA量は低下し有熱期間も短くなったが，内服を中止すると再度HHV-8 DNA量は増加し発熱もみられた．EBV DNA量とHHV-8 DNA量には関連は見られなかった．HAARTとvalganciclovirの併用は，患者の倦怠感が強く，継続が困難と思われたため現在，HAARTを中止しvalganciclovir 900mg/日のみで経過をみているが，末梢血中のHHV-8 DNA量は検出感度以下になっており，症状の再燃もみられていない．

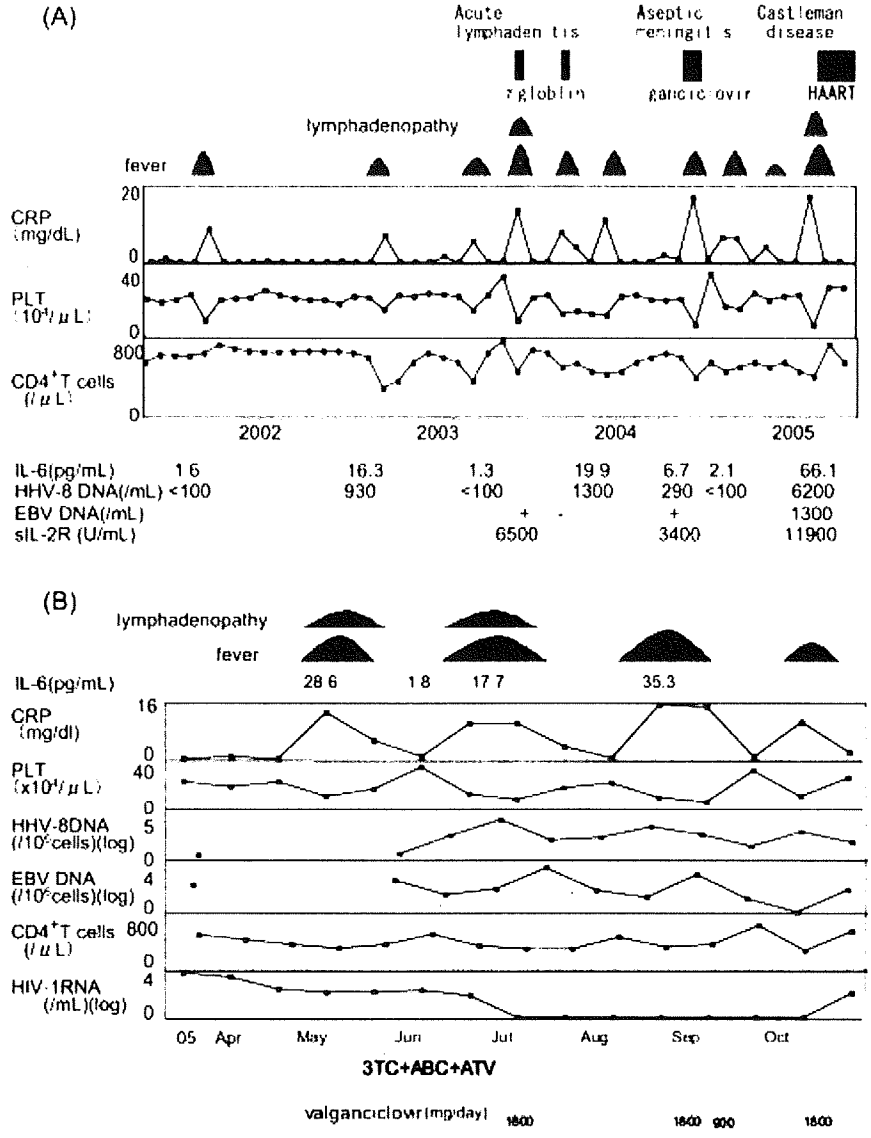
考 察

Castleman病は1956年にCastlemanらにより報告された良性のリンパ節腫脹を呈する慢性炎症性疾患である．多くは臨床的に無症状のhyaline-vascular typeであるが，本症例のように多クローン性高γグロブリン血症，易疲労感，肝脾腫，発熱などの多彩な症状を呈するplasma cell typeもある．このように全身症状をともなう場合，multicentric Castleman disease（MCD）とよばれている．本症の成因は不明であるが，近年HHV-8感染と密接な関係がある症例，あるいはHHV-8感染はないが血清中にIL-6や血管内皮細

Fug. 2 Clinical course

(A) Clinical course before starting HAART. Quantitative analysis of EBV DNA from the plasma was performed by PCR assay. Quantitative analysis of HHV-8 DNA and EBV DNA from the plasma was performed by real-time PCR assay. (B) After starting HAART. Quantitative analysis of HHV-8 DNA and EBV DNA from the peripheral blood mononuclear cells were measured by real-time PCR assay.

PLT : platelet, IL-6 : interleukin-6, HHV-8 : human herpes virus 8, EBV : Ebstein-Barr virus, sIL-2 R : soluble IL-2 receptor, 3TC : Lamivudine, ABC : Abacavir, ATV : Atazanavir



胞増殖因子が上昇している症例，これらのサイトカイン異常が腫瘍随伴症候群として現れている症例などがある．本症例は，発熱を繰り返したのち，リンパ節腫脹が認められるようになり Castleman 病と診断されたが，以前より発熱時には，HHV-8 DNA 量および IL-6 の増加を認めており，以前の症状も Castleman 病によるものであったと考えられる．HHV-8 は多くの感染細胞において潜伏感染しておりほとんどウイルスを産生していない．感染細胞が活性化されると溶解感染へ移行しウイルスを産生するとともにウイルス蛋白の産生も増加する．HHV-8 内にはヒト IL-6 と 25% の相同性をもつ viral IL-6（vIL-6）遺伝子が組み込まれており，IL-6 依存性の細胞を増殖させる．本症例においても感染等を契機にリンパ球が活性化し HHV-8 DNA 量および vIL-6 が増加したと考えられる．また，vIL-6 が発熱などの炎症反応，plasma 細胞の増生，ヒト IL-6 の増加，高γグロブリン血症に関与していたと考えられる．HHV-8 と EBV の重複感染による Cas-

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tleman病は特に免疫不全状態での発症が報告されており[1]，多くは全身症状を伴うMCDの病態を呈すると言われている．本症例でも，重複感染がありリンパ節内でも双方の感染が認められた．末梢血中のEBV DNA量はHHV-8 DNA量および病勢と関連は認められなかったがHHV-8とともにB細胞の活性化に関与している可能性がある．なおCastleman病はB細胞が慢性的に活性化した状態であることからEvans症候群[2]，橋本病[3]などの自己免疫疾患の合併も報告されている．本症例においても特に発熱時にPAIgGの増加を認め，血小板減少には自己免疫機序が関与していると考えられる．

Castleman病は，悪性リンパ腫，形質細胞腫への移行が報告されている．特にHIV陽性HHV-8陽性のCastleman病はしばしばより悪性度の高い形質芽球性リンパ腫へ進行するといわれており[1]，本症例のように全身症状を伴う場合は治療が必要となる．外科的切除やステロイド治療のほかに，近年，経口Etoposid，抗IL-6レセプター抗体療法[5]，幹細胞移植[6]，抗CD20抗体であるRituximab[2]による治療なども行われている．HIV感染合併の場合には，HAARTによる治療効果が期待できると言われている．本症例でもHAARTを開始したが，効果は得られず，逆に再燃時の症状は増悪し，HHV-8 DNA量の著増も認められた．HAARTによりHHV-8が再活性化した可能性もある．最近抗ウイルス剤としてHHV-8に対するvalganciclovirの効果が報告されている[7][8]ため，本症例でもvalganciclovirを使用した．その結果，valganciclovirを使用しなかったときに比べ有熱期間，CRP正常化までの期間が短くなった．しかし，投与中止により再燃を認めた．HAARTとvalganciclovirの併用は本人の倦怠感の訴えが強かったためHAARTを中止し，現在valganciclovir 900mgの持続投与のみで経過を見ているが，再燃は認めていない．HHV-8関連Castleman病に対してfoscarnet,cidofovir[9]を使用した報告があるがいずれも不成功であった．valganciclovir使用例の報告は検索範囲内では我々の報告を含めて3例のみであるがいずれも寛解状態になっている．本症例においても，寛解状態が持続するか経過を見ていく必要がある．また，valganciclovirの投与量，投与期間等についても，検討していく必要がある．

以上，HIV-1感染症に併発したHHV-8関連Castleman病の一例を報告した．Castleman病は比較的稀な疾患であるが，HIV-1感染者に再発性の炎症所見，リンパ節腫脹，炎症所見が認められた場合には，本疾患も考慮に入れる必要がある．なお，治療法については，今後も検討が必要である．

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A Case of HIV-1 and HHV-8-Associated Castleman Disease with a Relapsing High Fever and Lymphoadenopathy

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A 48-year-old man infected with an HIV-1 experienced intermittent bouts of fever, lymphadenopathy, elevated CRP level, and thrombocytopenia, each lasting about 2 weeks, and recurring at 2－3 month intervals. His CD4 count was about 500/μL, and he had never received antiretroviral therapy(ART). In March 2005, he experienced the same symptoms, accompanied by liver damage, splenomegaly, pleural fluid, and a high serum soluble IL-2 receptor level. Examination of a cervical lymph node specimen resulted in a diagnosis of Castleman disease, plasma cell type. Immunohistochemical studies confirmed the presence of HHV-8 and Ebstein-Barr virus(EBV). Since the plasma HHV-8 DNA and serum IL-6 were elevated during the flare-up, were negative between episodes, he was treated with ART to control the Castleman disease. He remained asymptomatic for 3 months, but, similar symptoms recurred with a high level of HHV-8 DNA in his PBMCs. Oral valganciclovir was them started at 1,800mg twice daily, and his symptoms immediately improved. The HHV-8 DNA level in the PBMCs decreased markedly over the course of 4 weeks, and valganciclovir was discontinued. One week later, he experienced another flare-up, and was successfully treated with 10 days of valganciclovir 1,800mg, followed by maintenance with valganciclovir 900mg. ART was discontinued, because the valganciclovir plus ART caused severe fatigue. No subsequent flare-ups have been observed, and, no HHV-8 DNA has been detected in his PBMCs. Castleman disease is an unusual complication in patients with HIV-1 and HHV-8 infection, but it should be included in the differential diagnosis of patients who exhibit a relapsing systemic inflammatory syndrome and lymphoadenopathy. Further study is needed to determine the appropriate usage and timing of the anti-HHV-8 and HIV-1 medication.

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