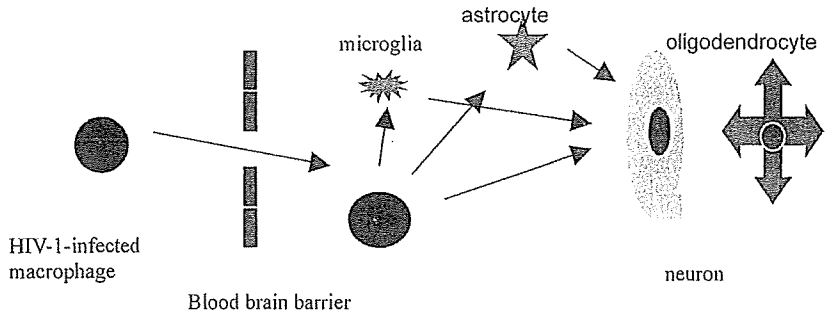

図 3 エイズ脳症の病態
エイズ脳症の病態の中心はHIV-1に感染したマクロファージが脳血液関門を越えて脳実質内に侵入することで始まると考えられている。このマクロファージから放出される因子により，マイクログリアやアストロサイトの活性化，神経細胞やオリゴデンドロサイトの障害を引き起こす。また，活性化されたミクログリアやアストロサイトからの因子も細胞障害に関わっており，複雑な病態である。

Persidsky らはヒト単球由来マクロファージを脳内移植した。その一方で，小柳らは1型糖尿病モデルマウスである nonobese diabetic（NOD）マウスとSCIDマウスを戻し勾配にてNOD-SCIDマウスを作製し，さらにヒト末梢血単核球細胞を腹腔内に移植して，hu-PBL-NOD SCIDマウスを報告し，このマウスの脳血管ではヒトT細胞が浸潤していることを報告した。さらに，その後グラム陰性桿菌の細胞表面生理活性物質のひとつであり，かつ単球系細胞に優位に活性化を引き起こすLipopolysaccharideの腹腔内接種を追加することにより脳解析系のモデルを開発した[5]。

また，一方で2002 Poluektova らはヒト単球由来マクロファージの脳内接種と，hu-PBL-NOD SCIDマウスを組み合わせたマウスを報告した。

これらマウスを用いた研究の特徴は，大型の実験動物に比べて，解析系として非常に有用であることがあげられる。また，特にHIVの場合は，マウス細胞や臓器には直接感染が起こらないため，感染実験系としての工夫が重要となってくる。そして特にヒト細胞を移植した実験系であるSCIDマウス研究などは，マウスとヒト細胞が混在することから，異種動物細胞間作用があることにも注意しなければならない。そして病態のすべてというよりもその一部を反映していると考えられる。

LPS-i.p.injected hu-PBMC-NOD SCIDマウス

我々が報告したマウスモデルであるLPS-i.p.injected hu-PBMC-NOD SCIDマウスでは，LPS投与によりヒト単球由来マクロファージのマウス脳内移行が促進され，細胞レベルでの障害，および組織学的変化が検出され，ヒトエイズ脳症の変化に類似していた。そして神経細胞死とTNF-related apoptosis-inducing ligand（TRAIL）発現マクロファージが検出され，中和実験より両者は密接な関係があると考えられた[5]。しかし一方で広範囲な組織障害は再現できず，T-CATなど行動解析では有意な異常が検出できなかった。

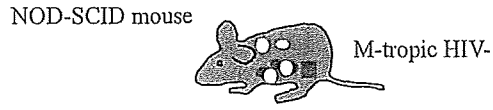

図 4 LPS-i.p.injected hu-PBMC-NOD SCIDマウスの作製法
1型糖尿病モデルマウスとして知られるNOD（Non-obese Diabetic）マウスと重症複合型免疫不全（SCID）マウスを兄妹勾配にてNOD SCIDマウスを作製する。このマウスの腹腔内にヒト末梢血単核球細胞（hu-PBMC），感染性マクロファージ指向性HIV-1，Lipopolysaccharide（LPS）を順に投与する。

マウス実験系結果の検証

次にマウスモデルを用いた解析結果をヒトエイズ脳症解剖脳，そしてマウス脳細胞培養系を用いて検証した。ヒトエイズ脳症剖検脳でも神経細胞のアポトーシス，活性型カスパーゼ陽性の神経細胞が検出された。また血管周囲のウイルス感染マクロファージではTRAILが発現していた。また，マウス脳神経細胞培養系に，ウイルス感染マクロファージやTRAIL発現マウス細胞株を共培養すると，優位に神経細胞死が増加することが証明された[6]。

今後の展望

エイズ脳症マウスモデルLPS-i.p.injected hu-PBMC-NOD

SCIDマウスは，病態の一部の反映であると考えられる。従って総合的病態の解明という観点では，マウスモデルの開発は未だ発展途上である。hu-MDM-i.c.transplanted hu-PBL-NOD SCIDやhu-CD34+HPC-i.p.transplanted NOD SCIDの開発，そしてTgマウスに対するLPS投与なども報告されてきており，これらの発展が期待される。そしてこれらのマウスモデルでの病態を，ヒトエイズ脳症剖検例や，in vitro培養系実験を合わせて総合的に検討してゆくことが重要である。

NeuroAIDSとサイトカイン：ヒト剖検例とサルエイズモデルをもちいた免疫組織学的検討

(鹿児島大学難治ウイルス研　分子病理　邢　惠琴，早川仁，出雲周二，国立感染研　森　一泰，ウィーン大学神経学研究所　Herbert Budka)

エイズ脳症の病理像として多核巨細胞を伴うHIV脳炎と大脳皮質の神経変性病態が知られているが，我々はサルエイズモデル[7,8]，ヒト剖検脳[9]を用いた解析をすすめ，この二つの病変はそれぞれ独立して生じうることを明らかにした。どちらの病変についても，ミクログリアやアストロサイトにより産生される炎症惹起性サイトカインIL-1β，TNF-αが神経傷害のエフェクターとなることが想定されているが，多くはin vitroの研究結果に基づいており，脳症病巣部で直接証明した報告は少ない。我々は大脳皮質変性病態ではアストロサイトによるグルタミン酸除去作用を担うEAAT-2の発現低下とミクログリアの瀰漫性活性化が生じており，それらは相関していることを定量的解析により明らかにした[9]。活性化ミクログリアでは炎症惹起性サイトカインIL-1β，TNF-αの発現はみられず，一方で，一部のミクログリアはEAAT-2を発現しており，アストロサイトの障害により生じたEAAT-2発現低下を補うためにミクログリアが活性化し，アストロサイトの神経保護作用を代償していることが推察された。これらの結果を踏まえ，もう一つの病態であるHIV脳炎病変について，炎症惹起性サイトカインIL-1β，TNF-αの炎症病変形成への関与を免疫組織染色にて解析した。

ヒト剖検脳とサルエイズ脳の検討

マクロファージ指向性ウイルスSIV239env/MERTを感染させたサル2頭と，非感染サル3頭をコントロールとした。ヒト剖検例はウィーン大学神経病学研究所剖検例について，1983年以後の剖検台帳を閲覧し，HIVに感染しエイズで死亡した429剖検例を抽出，日和見感染症・腫瘍・脳血管性病変が中枢神経の主病変として認められる症例を除外したHIV脳炎病変群11例を対象に，HIV脳炎病変部位について，CD3, CD20, CD68, IL-1β, TNF-α, SIVenvgp

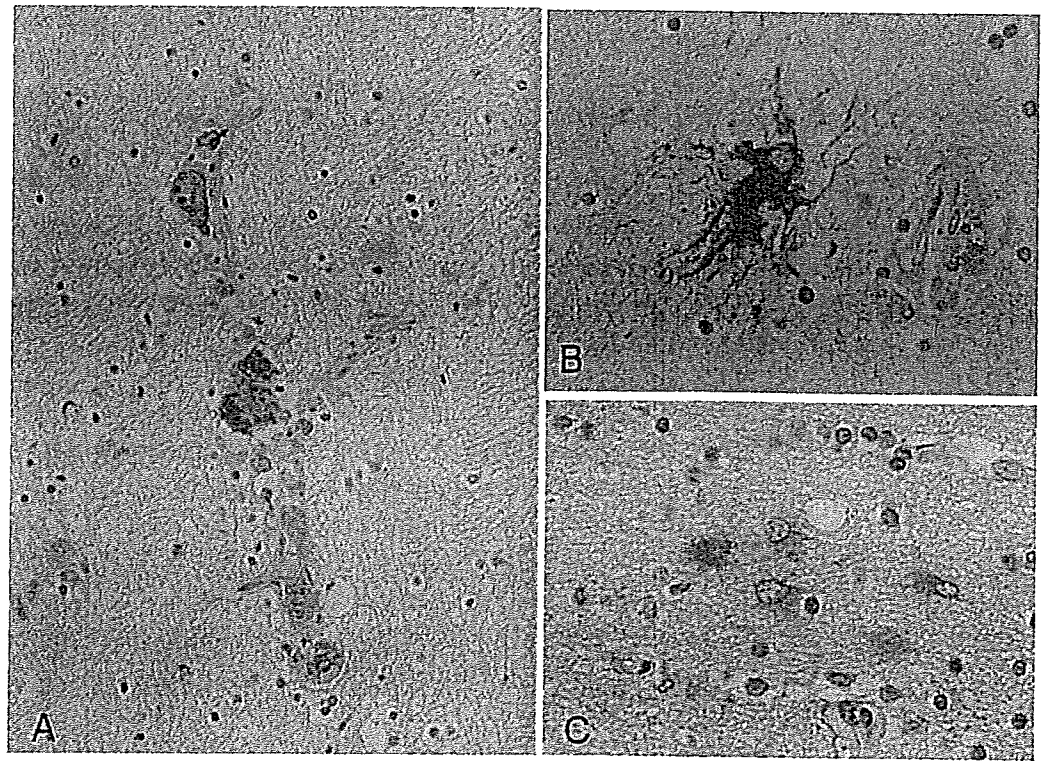

図5　IL-1βの免疫組織染色

160/gp32 に対する抗体を用いて免疫染色し，さらにミクログリア結節病変について IL-1β と SIVenvgp160/gp32 の二重免疫染色をおこない，光顕にて観察した。

HIV 脳炎群で IL-1β は脳炎のミクログリア結節を形成する HIV 感染多核巨細胞で発現しており，周囲の異型アストロサイトの一部でも発現していた（図 5，6）。また，HIV-P24 陰性の非感染細胞でも弱い発現がみられた。連続切片での検討では，IL-1β 陽性細胞の分布はほぼ HIV-p24 陽性細胞の分布と類似しており，二重染色による検討でも IL-1β は主として HIV-1 感染細胞に発現しているものと思われた（図 6）。TNF-α 強陽性細胞の分布は血管周囲に集族する Iba1 陽性小円形細胞と類似しており，浸潤マクロファージが主な TNF-α 発現細胞であると思われた（図 7）。一部の症例ではミクログリア結節周囲のアストロサイトでも発

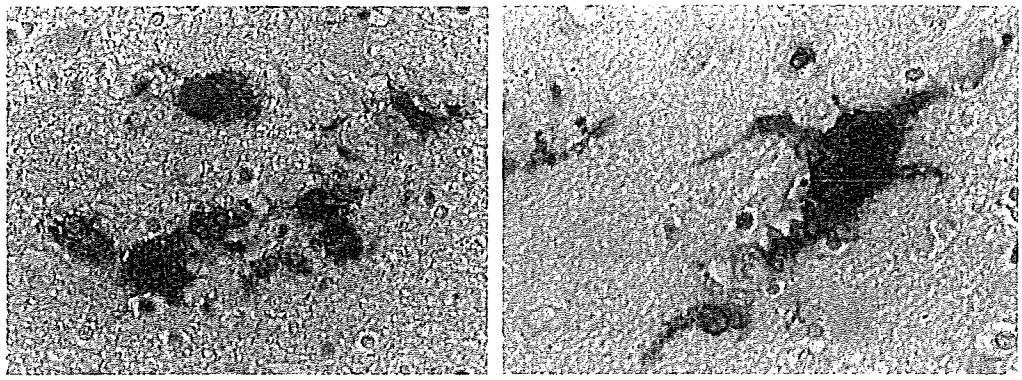

図 6　IL-1β と HIVp24 の二重免疫組織染色

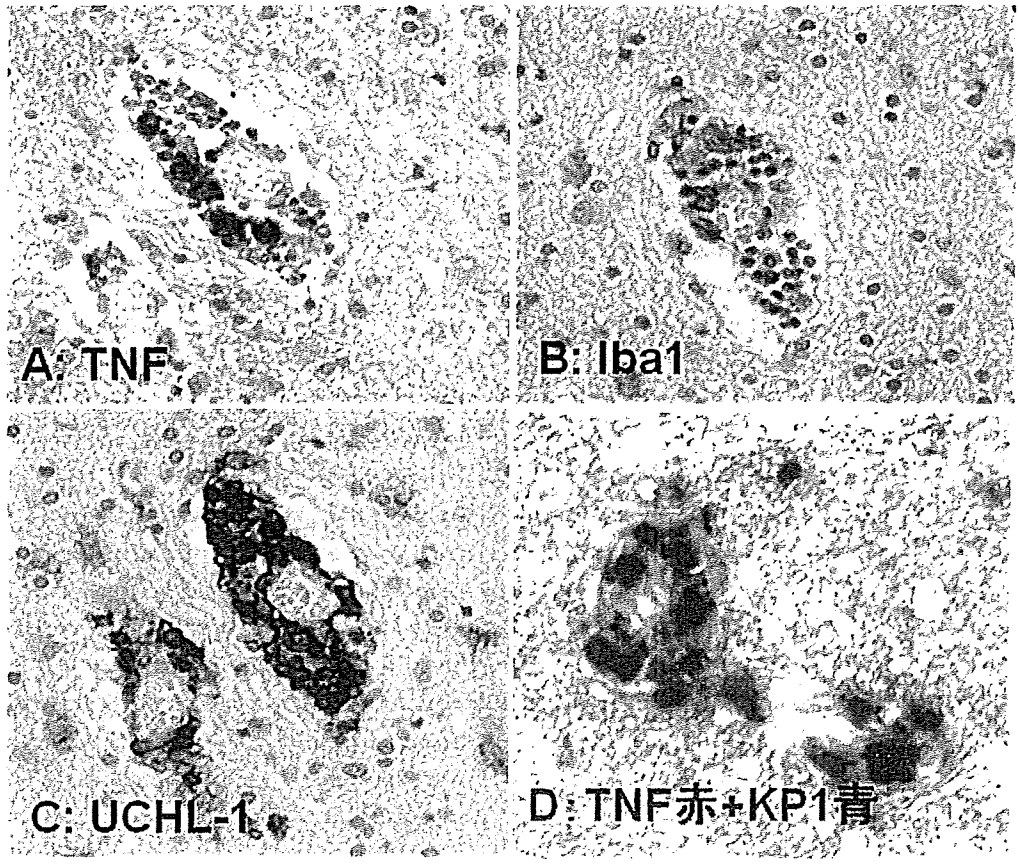

図 7　TNF-α の免疫組織化学染色

現していた。一方，炎症病変周囲の活性化ミクログリアはIL-1β，TNF-αの発現は認められなかった。サルモデルでもTNF-α，IL-1βは炎症性病変で観察されたが，ウイルス感染細胞とは一致しなかった[10,11]。

HIV脳症の発症病態

脳症の発症病態の一つとしてTNF-α，IL-1βなどの炎症惹起性サイトカインの関与が想定されており，その発現はHIV脳炎病変を形成する細胞であることが報告されている。今回のウィーン大学剖検例の検索でもHIV脳炎病巣にTNF-α，IL-1β発現細胞の存在が確認された。特にIL-1βは病変を形成するHIV-1感染細胞の，HIV脳炎に特徴的な多核巨細胞に一致して強く発現しており，HIV脳炎の炎症の持続，組織の変性に関与していることが想定される。一方TNF-αは主に浸潤マクロファージに発現しており，発現がみられる病巣周囲の変性は軽度にとどまっており，TNF-αは必ずしもHIV脳症を特徴付ける因子ではないように思われる。大脳皮質に瀰漫性に増勢するIba1陽性ミクログリアやHIV脳炎周囲の非感染ミクログリアにIL-1β，TNF-αの発現は認められなかった。大脳皮質の変性病変における活性化ミクログリアの役割について我々は神経保護作用を担っている可能性を想定しているが，HIV脳炎における活性化ミクログリアの役割についても検討が必要である。

最後に

世界的にはHIV感染者数・AIDS患者数ともに頭打ち傾向にある中で，わが国ではHIV感染者・AIDS患者数は年々増加している。HAARTによって欧米でもNeuroAIDSは減少したが，米国での調査[12]やヨーロッパのCASCADEでも[13]，抗ウイルス療法が奏功しても神経障害が持続すること，HAART治療後も軽症認知障害が発症すること，長期生存により認知機能障害のリスクが高まることが指摘されている。われわれがNeuroAIDS研究班で行った調査でも，神経症状がないHIV感染者でも比較的初期より脳血流が低下しており，今後，神経内科医，感染症科医，臨床心理士，神経病理医などとの学際的な協力のもとHIV感染者を感染早期より長期間フォローアップする体制が必要である。

なお，本研究は，厚生労働科学研究費補助金（エイズ研究事業）「NeuroAIDSの発症病態と治療法の開発を目指した長期フォローアップ体制の構築に関する研究（H18—エイズ—一般—009）」の助成を受けて行われた。

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Human Herpesvirus 8 DNA Load in the Leukocytes Correlates with the Platelet Counts in HIV Type 1-Infected Individuals

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Abstract

Human herpes virus 8 (HHV-8) is known to be reactivated in immunocompromised situations and it is associated with Kaposi's sarcoma (KS) and some hematological diseases. The aim of this study was to analyze the effect of HHV-8 on HIV-1 infection, especially on thrombocytopenia complicated with HIV infection. The HHV-8 DNA load was determined by a quantitative real-time PCR, using leukocytes from 125 HIV-1-infected individuals. HHV-8 DNA was detected in 37 individuals. The increased HIV-1 load and reduced percentage of CD4-positive T cells were significantly associated with the presence of HHV-8. The prevalence and load for HHV-8 are higher in patients with KS than in patients without KS, but the difference is not significant. The increased HHV-8 DNA load was significantly correlated with thrombocytopenia, and platelet counts were significantly lower in individuals with HHV-8 than in individuals without HHV-8. We also obtained the negative correlations between changes in platelet counts and changes in HHV-8 DNA loads. The association between thrombocytopenia and HHV-8 has never been reported previously, apart from some case reports of Castleman's disease and KS. Various cytokines or chemokines are produced by HHV-8-infected cells, some of which have been reported to inhibit hematopoiesis. This may be one of the mechanisms by which HHV-8 infection induces thrombocytopenia. These results indicate that HHV-8 DNA in leukocytes may provide useful information for the assessment of the clinical appearance of HIV-1 infection.

Introduction

HHV-8, A NEW MEMBER OF THE GAMMAHERPESVIRINAE, was identified as the etiologic agent of Kaposi's sarcoma (KS). The main transmission routes of human herpes virus 8 (HHV-8) seem to be sexual contact,[1] but transmission by saliva,[2] blood products, and organ graft[3,4] has also been proposed. The distribution of HHV-8 is related to a combination of geographic and behavioral risk factors. Serological studies have shown that HHV-8 seroprevalence is high in Africa and the Middle East and low in Europe and the United States. In Japan, it is reported that HHV-8 seroprevalence among healthy controls is 0.2–1.4% and HHV-8 seroprevalence among HIV-1-positive homosexual men is 11.6–63.6%.[5,6]

HHV-8 can infect circulating B cells, monocytes, macrophages, T cells, and KS-like spindle cell progenitors,[7–9] and usually persists in a latent state in these cells. The reactivation of this latent HHV-8 infection can be induced by a number of conditions, including superinfection by other viruses, stress, chronic illnesses, malignancies, and immunosuppressive disorders, such as HIV infection. HHV-8 contains more than 80 open reading frames, including several homologues of oncogenes, cytokine, and cytokine response genes. During latent and lytic infection, some viral genes are expressed and play a causative role in the genesis of some diseases, such as AIDS and non-AIDS-related KS, multicentric Castleman's disease, body cavity-based lymphoma, and some lymphoproliferative diseases. Our previous study reported a case of Castleman's disease with HIV-1 infection in which repeated episodes of thrombocytopenia were correlated with an increase in HHV-8 DNA loads in leukocytes.[10] The aim of this study was to determine the prevalence and loads of HHV-8 DNA in peripheral blood leukocytes in HIV-1-positive individuals and to investigate the correlation with the clinical appearance of HIV-1 infection, especially with thrombocytopenia.

Materials and Methods

Patients and samples

All consecutive HIV-1-infected patients who attended Kyushu Medical center between April 2005 and August 2006

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were eligible for this study. The exclusion criteria were HCV infection, active hepatitis, and treatment for antiherpes therapy at the time of sampling. Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of antiretroviral therapy (ART). For controls, 12 HIV-1-seronegative patients with autoimmune thrombocytopenia and 17 HIV-1-seronegative healthy volunteers were sampled for baseline comparisons. Informed consent for blood sampling was obtained from all participants. The study was conducted according to the ethical guidelines of the hospital and was approved by an authorized representative of the hospital. EDTA-treated blood was taken from the subjects, and leukocytes were collected after removing red blood cells with hemolysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Cell pellets were stored at –20°C until use.

Real-time quantitative PCR

DNA was extracted from the cell pellets using a QIAamp Blood Mini kit (QIAGEN Inc., Tokyo, Japan). Real-time PCR was conducted with the LineGene33 (BioFlux, Tokyo, Japan) using Premix Ex Taq (TAKARA, Shiga, Japan). As an internal control measurement, to normalize for input DNA, copy numbers of ß$_2$-microglobulin (ß$_2$M) were determined in every sample tested. The primers used for amplification were as follows: HHV-8 forward, 5′-CCTCTGGTCCCCATTCATTG-3′, and reverse, 5′-CGTTTCCGTCGTGGATGAG-3′, and probe 5′-FAM-CCGGCGTCAGACATTCTCACAACC-TAMRA-3′;[11] Epstein–Barr virus (EBV) forward, 5′-CGGAAGCCCTCTGGA CTTC-3′, and reverse, 5′-CCCTGTTTATCCGATGGAATG – 3′, and probe 5′-FAM-TGTACACGCACGAGAAATGCGCC-TAMRA-3′;[12] ß $_2$M forward, 5′-CAGCAAGGACTGGTCTTT CTATCTCT-3′, and reverse, 5′-ACCCCACTTAACTATCTT GG-3′, and probe 5′-FAM-CACTGAAAAAGATGAGTATG CCTGCCGTGT-TAMRA-3′.[13] Standards were obtained by amplification of a control sample in a polymerase chain reaction (PCR) reaction using the same primers. The data were normalized as copies/10^6 cells by measuring copy numbers of the ß$_2$M gene, since two ß$_2$M copy numbers correspond to one cell. The lower limit of detection was defined as 1 copy/10^6 cells.

Statistical analyses

Frequency analysis was performed using Fisher's exact test for 2×2 tables. The viral DNA copy numbers of HHV-8, EBV, and HIV-1 were $\log_{10}$ transformed and compared between different groups by means of the Mann–Whitney *U* test. The associations between them were determined using an analysis of covariance (ANCOVA), with EBV-DNA and HIV-RNA as covariates, where HHV-8 DNA was adjusted on the bases of EBV-DNA and HIV-RNA. Spearman's rank correlation coefficient was used to compare changes in platelet counts and changes in HHV-8 DNA loads. A partial correlation was used to assess this relationship, while controlling for any changes in HIV-RNA and EBV-DNA.

Results

Subjects characteristics

A total of 125 patients fulfilled the inclusion criteria. Demographic and clinical characteristics of the subjects included in this study are shown in Table 1. There were 8 women and 117 men, 112 of whom were men who have sex with men; the others were heterosexual. These patients were ranged from 20 to 69 years of age (mean age, 37.8 years). Of these, 58 received ART and 67 remained untreated. The duration of ART was 3 months to 9.5 years (mean 3.25 years). Nine HIV-1-positive individuals were analyzed longitudinally before and after the initiation of ART. Among 125 HIV-1-positive subjects included in our study, we evaluated 97 subjects for the prevalence of splenomegaly by means of an abdominal ultrasound examination; 23 subjects were thus found to have splenomegaly.

HHV-8 qualitative and quantitative DNA analysis

The presence and load of HHV-8 DNA were investigated in 125 HIV-1-positive subjects and 17 HIV-1-negative healthy controls (Fig. 1). HHV-8 DNA was detected in 37 of 125 (29.6%) leukocyte samples from HIV-1-positive subjects, with values ranging from 2 to 91,171 copies/10^6 leukocytes. HHV-8 DNA was not detected in any healthy controls.

Relationship between HHV-8 DNA and KS

HHV-8 DNA was detected in three of five (60%) HIV-infected subjects with KS, whereas they were found in 34 of 120 (28.3%) subjects without KS ($p = 0.15$). Figure 2 shows that the HHV-8 DNA load of subjects with KS to be higher than that of the subjects without KS, but not significantly so.

The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters

The HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that of HHV-8 DNA-negative subjects (Fig. 3a). In addition, the correlation between HHV-8 DNA and hypergammaglobulinemia (the percentage of gammaglobulin is more than normal range) was examined, which is often observed in HIV-1-positive subjects. The HHV-8 DNA load in leukocytes in subjects with

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF 125 HIV-POSITIVE SUBJECTS[a]

	Total	*Pretreatment of ART*	*Treatment of ART*	p
N (male/female)	125 (117/8)	67 (63/4)	58 (54/4)	
Age (years)	37.8 ± 10.4	34.3 ± 8.2	42.1 ± 11.2	<0.001
CD4 (cells/ml)	396 ± 218	387 ± 226	407 ± 211	0.616
Viral load (copies/ml) ($\log_{10}$)		4.36 ± 0.86	Under detection limit	
Duration of ART (years)		0	3.25 ± 2.68	

[a]The mean values ± SE are shown. Pretreatment of ART versus treatment of ART.

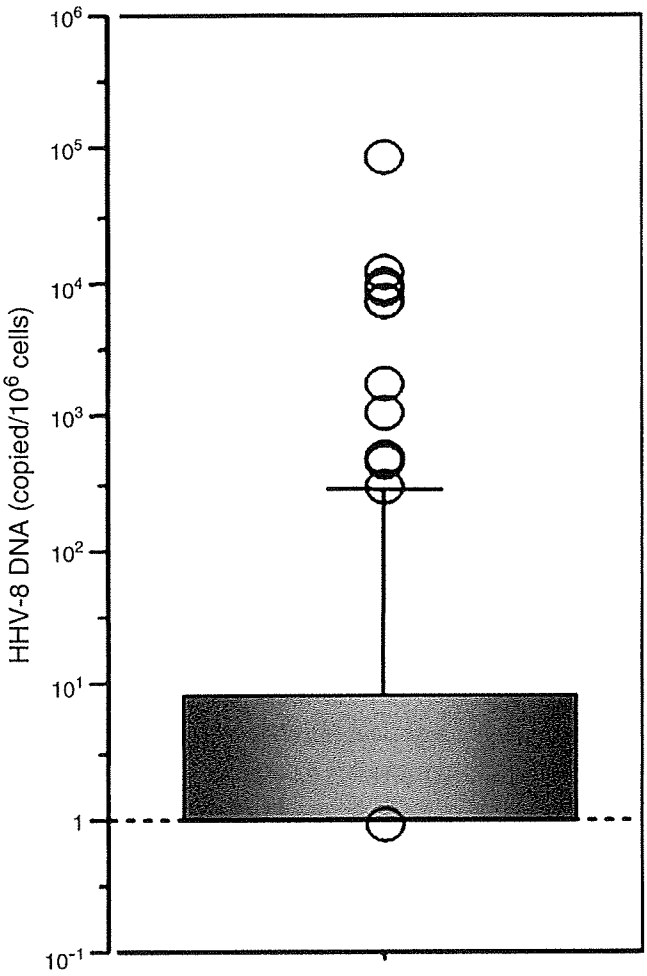

FIG. 1. Quantification of HHV-8 DNA by real-time PCR. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. The dotted line shows the detection limit of the assay.

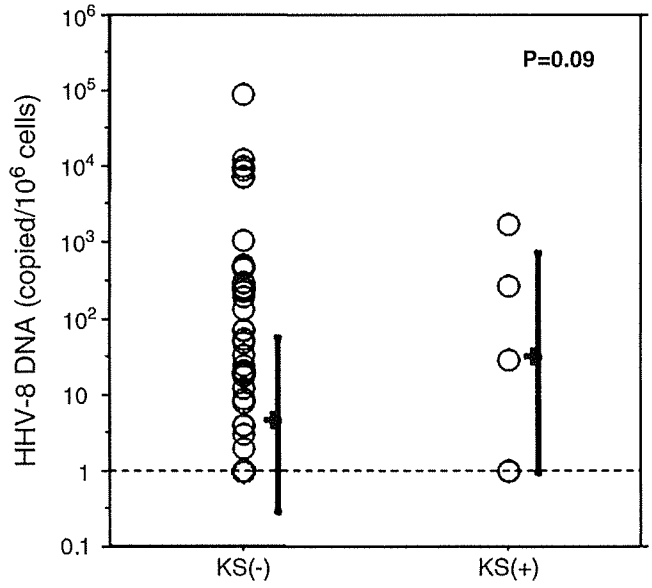

FIG. 2. HHV-8 DNA loads among the HIV-1-positive subjects with or without KS. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. p-values were estimated by the Mann–Whitney U test. The dotted line shows the detection limit of the assay.

hypergammaglobulinemia was significantly higher than in subjects without hypergammaglobulinemia (Fig. 3b). HHV-8 DNA was detected in 24 of 44 (54.5%) HIV-infected subjects with hypergammaglobulinemia, whereas it was found in 13 of 81 (16.0%) subjects without hypergammaglobulinemia ($p < 0.0001$). We could not find any significant correlation between HHV-8 DNA loads and CD4-positive T cell counts.

The effect of ART on HHV-8 DNA

HHV-8 DNA load in leukocytes in subjects with ART was significantly lower than in subjects without ART (Fig. 4a). HHV-8 DNA was detected in 12 of 58 (20.7%) HIV-infected subjects with ART, whereas it was found in 25 of 67 (37.3%) subjects without ART ($p = 0.042$). There was no difference in the duration of ART between HHV-8 DNA-negative and HHV-8 DNA-positive subjects with ART (3.161 ± 2.764 vs. 3.575 ± 2.439 years, $p = 0.624$). HHV-8 DNA loads (log_{10}) per 10^6 leukocytes in subjects with a short duration of ART (<6 months) were higher than in subjects with a long duration of ART (≥6 months), but the difference was not statistically significant (0.758 ± 0.447 vs. 0.323 ± 0.111, $p = 0.309$).

Longitudinal analysis of HHV-8 DNA loads after initiation of ART

The longitudinal profiles of HHV-8 DNA loads were examined in nine subjects after initiation of ART (Fig. 4b). Within 1 month after starting ART, HHV-8 DNA transiently increased in three patients (Fig. 4b, black straight lines); in two of the three patients, KS had progressed transiently. These findings show the reactivation of HHV-8 by initiation of ART. Three months after the initiation of ART, all nine patients had achieved and maintained the decreased levels of HHV-8 DNA.

The influence of EBV on HHV-8 infection

Like HHV-8, EBV is lymphotropic and is a member of the gammaherpesvirinae; it is reactivated by HIV-1 infection. Therefore, the influence of EBV on HHV-8 infection was investigated. EBV-DNA loads (log_{10}) per 10^6 leukocytes were significantly higher in HHV-8 DNA-positive subjects (0.69 ± 0.11 vs. 1.46 ± 0.21, $p = 0.0007$) and EBV DNA was detected in 32 of 88 (36.4%) HHV-8-negative subjects, whereas they were found in 25 of 37 (67.5%) HHV-8-positive subjects ($p = 0.002$).

Relationship between HHV-8 DNA and thrombocytopenia

Figure 5 shows a case of Castleman's disease in which the platelet counts changed in inverse association with HHV-8 DNA in the leukocytes. Therefore, the association between platelet counts and HHV-8 DNA in leukocytes was examined. First, the changes in platelet counts and HHV-8 DNA were analyzed in the nine subjects before and after the initiation of ART (Fig. 6). Both the decrease in HHV-8 DNA and the

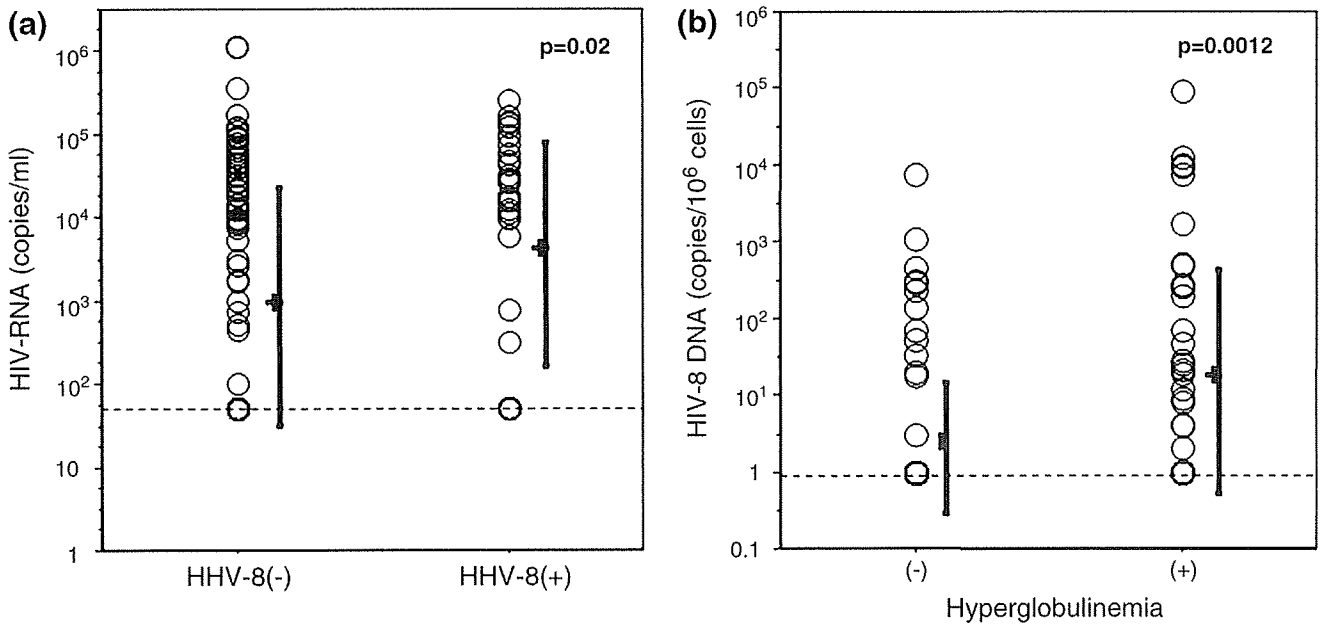

FIG. 3. The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters. (a) HIV-RNA loads among the HIV-1-positive subjects with or without HHV-8 DNA. Log_{10}-transformed HIV-1 copy numbers/ml of serum are shown. The bar shows the mean and standard deviation. *p*-values were estimated by the Mann–Whitney *U* test. (b) HHV-8 DNA loads among the HIV-1-positive subjects with or without hypergammaglobulinemia. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. *p*-values were estimated by the Mann–Whitney *U* test. The dotted line shows the detection limit of the assay.

increase in platelet counts were significant. Furthermore, the correlation between changes in platelet counts and changes in HHV-8 DNA was obtained (Spearman; $r = -0.783$, $p = 0.0267$). Patients with HIV infection sometimes develop thrombocytopenia, and EBV is also reported to cause mild thrombocytopenia. Therefore, partial correlations were also used to assess this relationship while controlling for EBV-DNA and HIV-RNA, and the correlation between the change in HHV-8 DNA and platelet counts was obtained (partial correlation coefficient; $r = -0.733$, $p = 0.0219$).

Next, all 125 subjects with HIV infection were analyzed. There was no significant difference between the group with

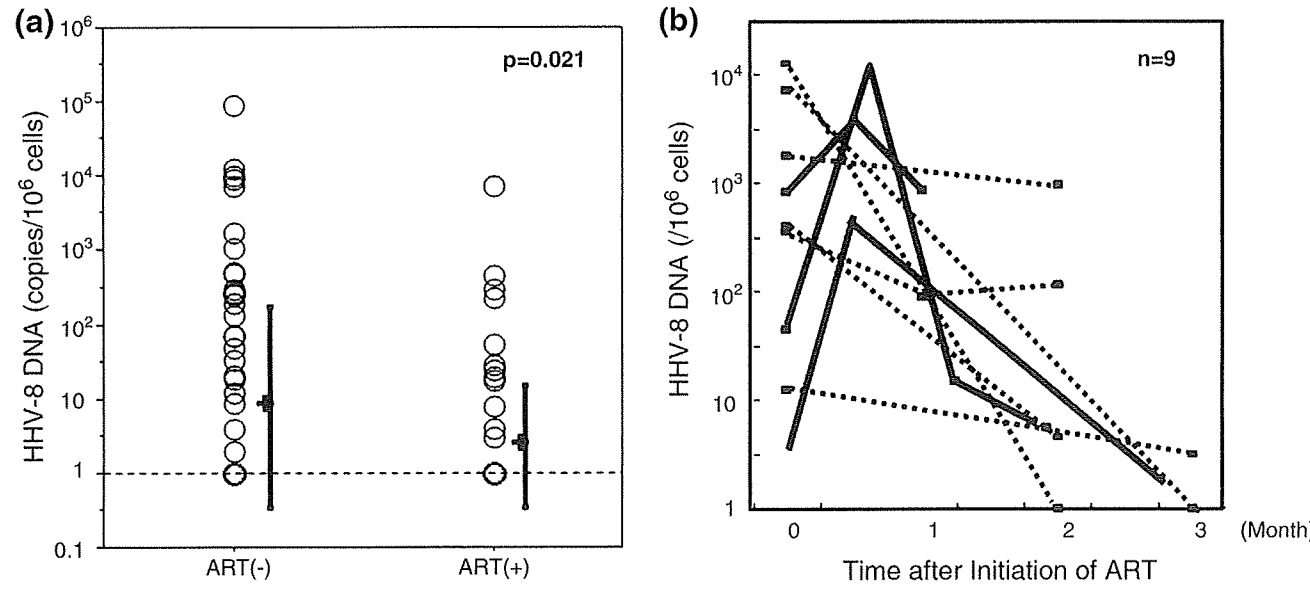

FIG. 4. The effect of ART on HHV-8 DNA. (a) HHV-8 DNA load in PBMCs in subjects with or without ART. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. *p*-values were estimated by the Mann–Whitney *U* test. (b) HHV-8 DNA load after initiation of ART. HHV-8 DNA loads of the nine subjects were measured at the indicated time points. The dotted line shows the detection limit of the assay.

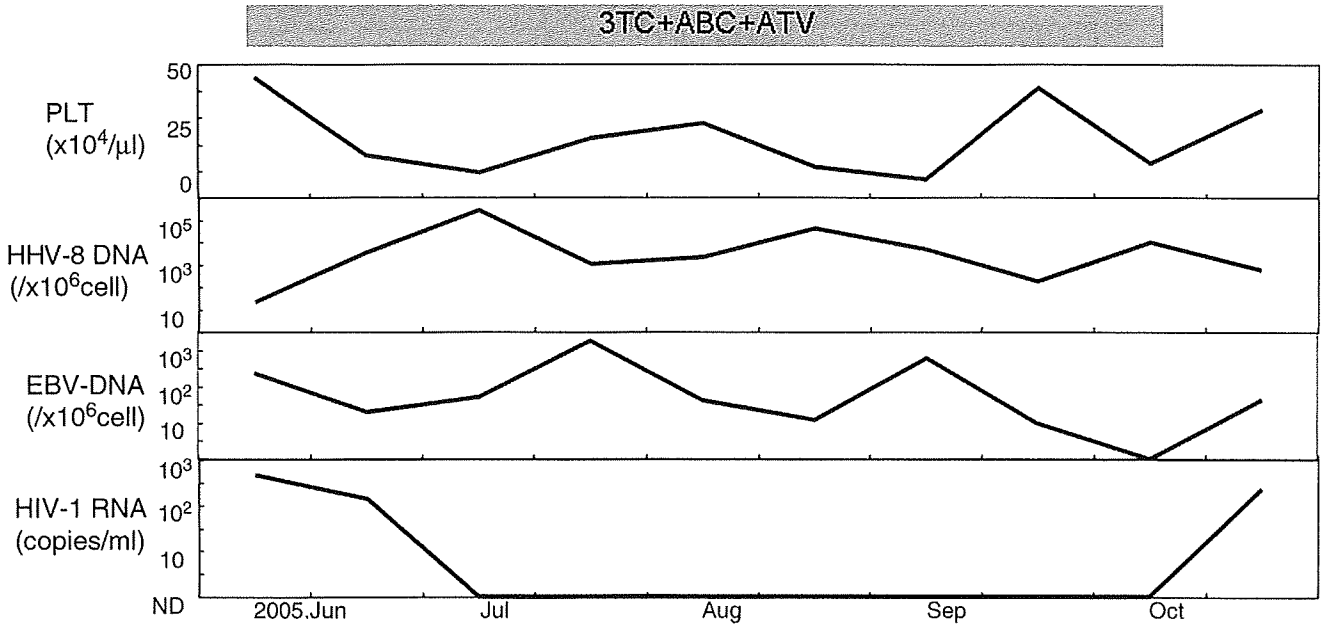

FIG. 5. Time course of the case of Castleman's disease with HIV-1 infection. Platelet counts, $\log_{10}$-transformed HHV-8 and EBV copy numbers per 10^6 leukocytes, and $\log_{10}$-transformed HIV-1 copy numbers/ml of serum are shown. The data were excerpted from Minami and Yamamoto.[10] PLT, platelet; HHV-8, human herpes virus 8; EBV, Epstein–Barr virus; 3TC, lamivudine; ABC, abacavir; ATV, atazanavir.

thrombocytopenia and the group without thrombocytopenia in terms of the number of subjects with ART, the duration of ART, and the prevalence of subjects with splenomegaly (with thrombocytopenia vs. without thrombocytopenia; 30.8% vs. 22.6%, $p = 0.520$). Similarly, no significant difference was observed between the group with HHV-8-DNA and the group without HHV-8-DNA in terms of the duration of ART and the prevalence of subjects with splenomegaly (with HHV-8 vs. without HHV-8; 26.7% vs. 22.4%, $p = 0.647$). The effects of HHV-8 DNA in leukocytes on platelet counts were examined using ANCOVA, with EBV-DNA as a covariate. ANCOVA revealed a significant interaction between EBV-DNA and HHV-8 DNA but no significant interaction between thrombocytopenia and EBV-DNA. Post hoc tests revealed that HHV-8 DNA was significantly higher in subjects with platelet counts <150,000 platelets/μl than in subjects with platelet counts ≥150,000 platelets/μl (Fig. 7a). HHV-8 DNA was detected in 11 of 14 (78.6%) subjects with low platelet counts,

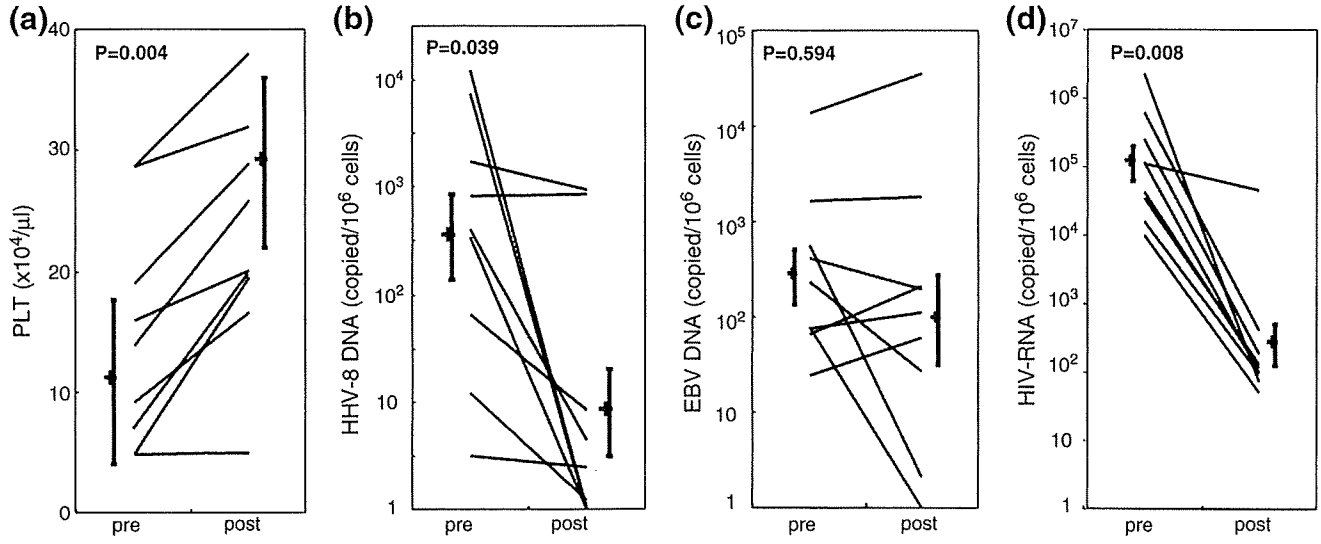

FIG. 6. Changes in the platelet counts, HHV-8 DNA loads, EBV-DNA loads, and HIV-RNA of pre- and post-ART. Platelet counts (a), HHV-8-DNA (b), EBV-DNA (c), and HIV-RNA (d) of the nine subjects were analyzed before (pre) and after 2 or 3 months of initiation of ART (post). The viral loads were $\log_{10}$ transformed. Bar shows the mean and standard deviation. p-values for the difference between the two time points were estimated by the Wilcoxon signed rank test.

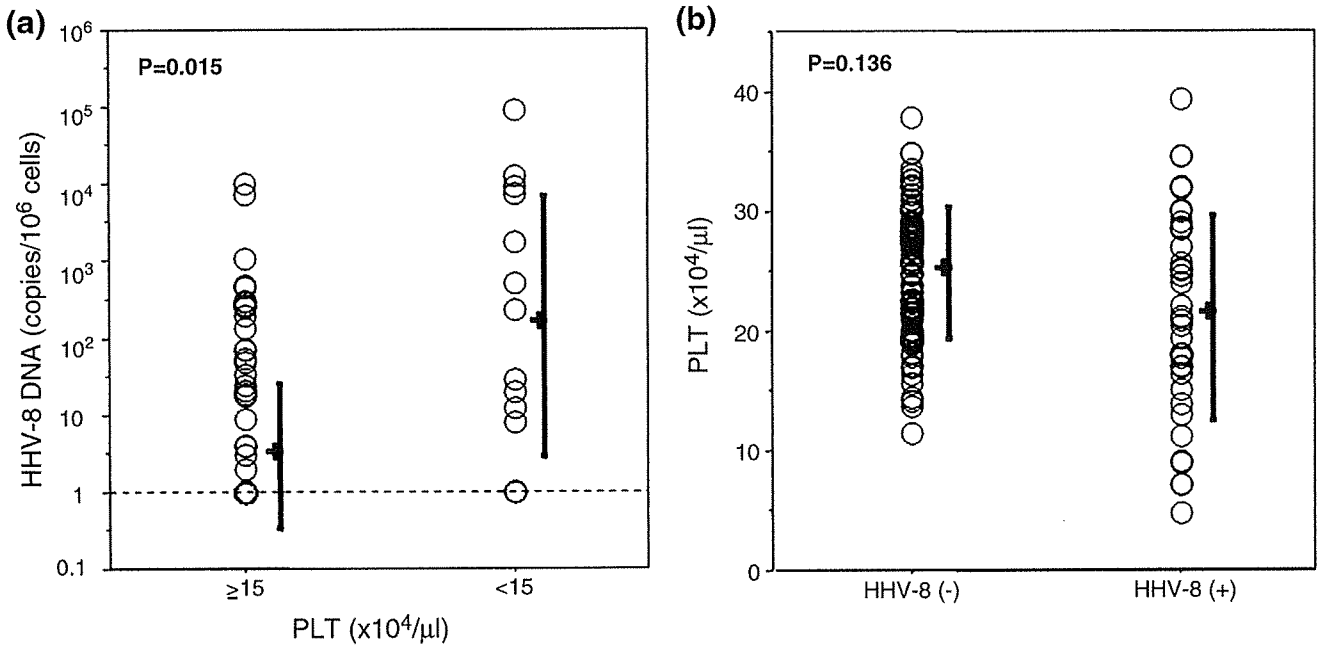

FIG. 7. Relationship between HHV-8 DNA and mild thrombocytopenia.(a) HHV-8 DNA load in leukocytes in subjects with platelet counts of <150,000 platelets/mm^3 or with platelet counts of ≥150,000 platelets/mm^3. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. The p-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. (b) Platelet counts among the HIV-1-positive subjects with or without HHV-8 DNA. The bar shows the mean and standard deviation. The p-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. The dotted line shows the detection limit of the assay.

whereas it was found in 26 of 111 (23.4%) of subjects with normal platelet counts ($p = 0.0059$: estimated by post hoc test after ANCOVA with the prevalence of EBV-DNA as a covariate). The platelet counts were lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects, *but not significanlyt* (Fig. 7b). In subjects without ART, HIV-RNA might be a confounding factor, and thus ANCOVA was conducted with HIV-RNA and EBV-DNA as covariates.

In subjects without ART, HHV-8 DNA was also significantly higher in subjects with mild thrombocytopenia with an adjustment for EBV-DNA and HIV-RNA ($p = 0.013$, data not shown). HHV-8 DNA was not detected in the 12 subjects with autoimmune thrombocytopenia. We also examined the correlation between HHV-8 DNA loads and platelet counts in subjects without KS, because the subject with KS might have occult Castleman's disease, which can cause thrombocytopenia. HHV-8 DNA was significantly higher in subjects with platelet counts of <150,000 platelets/μl than in subjects with platelet counts of ≥150,000 platelets/μl [$\log_{10}$ (HHV-8) = 2.103 ± 1.797 vs. 0.438 ± 0.924; $p = 0.0285$]. HHV-8 DNA was detected in 9 of 12 (75.0%) subjects with low platelet counts, whereas it was found in 25 of 108 (23.1%) subjects with normal platelet counts ($p = 0.0002$). The platelet counts were significantly lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects ($21.5 \pm 8.61/\mu$l vs. $25.0 \pm 5.56/\mu$l; $p = 0.1319$).

Discussion

Some previous studies have examined the prevalence of HHV-8 infection in HIV-1-infected subjects. A unique aspect of this study is that the effect of HHV-8 DNA in the peripheral blood leukocytes on HIV-1 infection was examined; a significant association between HHV-8 DNA and the clinical appearance of HIV-1 infection, especially thrombocytopenia complicated with HIV-1, was found.

Some reports have shown that the presence of KS was associated with the prevalence of HHV-8 DNA in leukocytes[14] and the severity was associated with HHV-8 DNA loads in leukocytes.[15] The current data also showed a certain association with the presence of KS and HHV-8 DNA load, but in this study there were only five subjects with KS, because the prevalence of KS in HIV-1-infected patients in Japan is low. As a result, it was impossible to determine whether the HHV-8 DNA load predicted the onset and severity of KS. To draw any conclusions concerning the clinical and prognostic utility on KS, more subjects with KS need to be analyzed.

HHV-8 encodes homologues of cytokine and cytokine response genes, such as viral interleukin-6 (vIL-6)[16] and viral interferon regulatory factor (vIRF). vIL-6 can bind the gp130 receptor to activate IL-6 response genes and promote B cell activation, which is the mechanism of hypergammaglobulinemia induced by HHV-8 infection. It was also found that HHV-8 plays an important role in hypergammaglobulinemia, which is often found in subjects with HIV-1 infection.

The current data showed that HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that found in the serum of HHV-8 DNA-negative subjects. HHV-8 replication is activated by HIV-Tat,[17] and it has been reported that HHV-8 reactivation is associated with HIV-1 acute infection.[18] Furthermore, an open reading frame (ORF), the major transactivator of the HHV-8 lytic cycle, can also induce increased levels of HIV replication. In conclusion, these findings show that HHV-8 coinfection is associated with

immunological characteristics and disease severity of HIV infection.

ART reduced HHV-8 DNA loads as well as HIV-RNA (Fig. 4). The inhibition of HIV-1 replication with ART probably leads to the reconstitution of the immune system and regenerates effective immune responses against HHV-8. Moreover, protease inhibitors block the production of inflammatory cytokines,[19] which in turn may result in the downregulation of HHV-8 replication. In this longitudinal study during ART, HHV-8 DNA loads transiently increased in three subjects soon after the initiation of ART. This might be due to the disorder of cytokine balance, which occurs transiently in the process of the reconstitution of the immune system, and this might be one of the explanations for the immune reconstitution syndrome of KS. Some inflammatory cytokines are induced in immune reconstitution syndromes. It has recently been reported that some cytokines, which are associated with the development of KS, were elevated in other reconstitution syndromes.[20] It is possible that reactivation of HHV-8 by ART might activate the inflammation of the other reconstitution syndromes by the increase in vIL-6 or other cytokines.

The difference between HHV-8 DNA in the subjects with platelet counts of <150,000 platelets/μl and in the subjects with platelet counts of ≥150,000 platelets/μl was significant, but the difference between the platelet counts in the HHV-8 DNA-positive subjects and the platelet counts in the HHV-8 DNA-negative subjects was not significant. This is because HHV-8 DNA correlates with "mild" thrombocytopenia.

This is the first report that analyzed the association between HHV-8 DNA and thrombocytopenia in HIV-1-infected subjects, though there are a few studies that showed the relationship between HHV-8 infection and hematological disorders[21] or posttransplantation bone marrow failure in HIV-1-negative subjects.[22] Some case reports have described thrombocytopenia complicated with HHV-8-induced Castleman's disease, hemophagocytic syndrome, or other inflammatory syndromes. The HIV-1-positive subjects sometimes develop thrombocytopenia, which has been thought to be caused by an autoimmune mechanism. Recently, antibody cross-reactivity between a known epitope region of HIV-1 proteins and platelet GPIIIa49-66 has been reported to be responsible for HIV-1-associated thrombocytopenia.[23] However, the present study showed that HHV-8 also contributes independently to the thrombocytopenia complicated in HIV-1 infection.

The molecular mechanism of thrombocytopenia induced by HHV-8 remains to be established, but it is possible that some cytokines or chemokines induced by HHV-8 might be involved in thrombocytopenia. For example, interleukin-8 (IL-8), which is induced by HHV-8 infection,[24] binds the receptors on megakaryocytes and platelets and inhibits megakaryocytopoiesis.[25] Furthermore, HHV-8 is reported to infect CD34-positive hematopoietic progenitor cells[26,27]; HHV-8 harboring CD34-positive cells might also contribute to hematological disorders including thrombocytopenia.[28] Another possible explanation for thrombocytopenia is an autoimmune mechanism such as immune thrombocytopenia (ITP). Chronic stimulation of the B cell clones induced by HHV-8 could favor the production of autoantibodies, some of which might react against platelet. In fact, Evan's syndrome complicating multicentric Castleman's disease[29] and ITP complicating KS have been reported.[30]

In Italy, the area of subendemicity of HHV-8, the presence of HHV-8 DNA in HIV-1-negative elderly people has been reported to be associated with mild thrombocytopenia.[31] In Japan, HHV-8 infection in HIV-1-negative subjects is rare; as a result, when the 12 subjects with autoimmune thrombocytopenia were examined, the association between HHV-8 and autoimmune thrombocytopenia in HIV-1-negative subjects was not distinct. However, the fact that HHV-8 DNA was not detected in the HIV-1-negative subjects with autoimmune thrombocytopenia suggests that HHV-8 is not a main causative agent of autoimmune thrombocytopenia.

In conclusion, a significant association was observed between HHV-8 infection and the clinical appearance of HIV-1 infection, especially in regard to mild thrombocytopenia, hypergammaglobulinemia, and disease severity. It is possible that some of these effects of HHV-8 infection are related to other independent etiological cofactor or some other viruses. However, it may be useful to monitor HHV-8 DNA in leukocytes of HIV-1-positive subjects to assess and predict disease severity and to select the optimal treatment modalities.

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Disclosure Statement

No competing financial interests exist.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

High Molecular Weight Form of Adiponectin in Antiretroviral Drug-induced Dyslipidemia in HIV-Infected Japanese Individuals Based on *in vivo* and *in vitro* Analyses

Rumi Minami, Masahiro Yamamoto, Soichiro Takahama, Hitoshi Ando, Tomoya Miyamura and Eiichi Suematsu

Abstract

Objective High molecular weight (HMW)-adiponectin has been found to be a better negative regulator of insulin resistance than total adiponectin. The aim of this study was to investigate the influence of HMW-adiponectin on antiretroviral therapy (ART)-induced dyslipidemia in Japanese human immunodeficiency virus (HIV)-infected individuals. We also examined the effect of some antiretroviral drugs (ARVs) on adipocytes *in vitro*.

Patients and Methods Fifty-seven HIV-infected patients were enrolled in four clinical groups; (I) patients who started ART containing efavirenz (EFV); (II) patients who started ART containing a protease inhibitor without atazanavir (ATV); (III) patients who started ART containing ATV; (IV) patients who switched from ART without ATV into ART containing ATV. We measured the serum HMW-adiponectin before and one year after starting or changing ART, using an enzyme-linked immunoSorbent assay (ELISA). Furthermore, we treated the mouse adipocytes (3T3-L1) with some ARVs. The lipid content was assessed using Oil Red O staining. The expression of adiponectin was measured by quantitative real-time PCR.

Results The serum HMW-adiponectin decreased significantly in groups (I) and (II) after starting ART, and increased significantly in group (IV) after changing from ART without ATV to ART with ART. EFV, ritonavir (RTV) and nelfinavir (NFV) inhibited the expression of adiponectin mRNA in mature 3T3-L1 and to a greater extent in pre-mature 3T3-L1. This phenomenon was reversible when ARV was changed to ATV.

Conclusion Effects of the ARVs on adiponectin may vary depending on the administration of different drugs. These data suggest that the distinct metabolic effects of ARV could therefore be a consequence of their differential effects on the production of adiponectin.

Key words: HMV-adiponectin, antiretroviral therapy, dyslipidemia

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Introduction

Adiponectin is an adipokine secreted exclusively by adipocytes: this protein plays an important role in the control of systematic lipid metabolism and insulin sensitivity (1). Adiponectin forms multimers and is present in the serum as a trimer, hexamer, or as a high molecular weight (HMW) form (2). The HMW isoform most avidly binds to its receptors and stimulates AMP-activated protein kinase, one of the key molecules mediating the metabolic actions of adiponectin.

Antiretroviral therapy (ART) has prolonged survival in human immunodeficiency virus (HIV)-infected individuals, but most individuals receiving ART develop metabolic abnormalities, which include dyslipidemia (elevated plasma triglycerides and cholesterol), increased visceral and dorsocervical adipose tissue and peripheral lipoatrophy (3). Although

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HIV infection itself (4) and nucleoside reverse transcriptase inhibitors (NRTI) (5-7) have been associated with metabolic abnormalities, there are increasing clinical and epidemiological data that suggest a central role for HIV protease inhibitors (PIs) in the causation of metabolic complications (8) and atazanavir (ATV) use has been associated with a decrease in hyperlipidemia, less insulin resistance and reversal of lipodystrophy (9).

Several recent clinical studies suggest that hypoadiponectinemia might play an important role in the causation of metabolic abnormalities associated with HIV infected individuals treated with PI-based ART, especially for those with lipodystrophy (8, 10, 11), but to date, limited data have been published whether this hypoadiponectimia is due to a disturbance of adipocyte differentiation, or to the decrease in the number of adipose cells number, or a dysfunction of adipocytes. In addition, there are still no data regarding the influence of antiretroviral drugs (ARVs) on serum HMW-adiponectin levels in Japanese, about 40% of whom have a genetic variation in the adiponectin gene associated with a reduced adiponectin level (12).

This study compared the effect on HMW-adiponectin level between ATV, PIs (except for ATV), ATV replacement therapy and non-nucleoside reverse transcriptase inhibitors (NNRTI), efavirenz (EFV) in HIV-infected Japanese subjects. In addition, the influence of each of the ARVs on adipocyte development and the expression of adiponectin were evaluated, using in vitro models.

Materials and Methods

Study population

The subjects evaluated in this study consisted of 57 HIV-positive patients admitted to National Hospital Organization Kyushu Medical Center, who started ART or were changing the ART combination. The present study was conducted in accordance with the regulations of the institutional ethics committee. These individuals were enrolled into four clinical groups; (I) patients who started ART containing EFV; (II) patients who started ART containing PIs without ATV; (III) patients who started ART containing ATV; (IV) patients who replaced the ART without ATV into ART containing ATV. The Body Mass Index (BMI), serum triglyceride, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, HMW adiponectin were measured before and one year after starting or changing ART. HMW-adiponectin was measured by enzyme-linked immunosorbent Assay (ELISA), using the Human Adiponectin ELISA kit for Total and Multimers (Daiichi Pure Chemicals Co., Tokyo, Japan). Lipodystrophy was determined by a standardized, lipodystrophy-specific physical examination which recorded lipoatrophy and/or diffuse fat accumulation in the face, neck, dorsocervical spine, arms, breasts, abdomen, buttocks and legs.

Cells

The 3T3-L1 cells were purchased from the Japanese Collection of Research Bioresources. The cells were maintained in DMEM supplemented with 10% fetal bovine serum. For differentiation, post confluent cells were induced by incubation with 0.5 mM 3-isobutyl-methylxanthine and 1 μM dexamethasone for 2 days. This is followed by incubation with 10 μg/mL insulin for 2days. The cells were then maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for another 2 days. To investigate the effects of ARVs on adipogenesis, and to investigate the difference between the effect of ARVs on preadipocytes and the effect on mature adipocytes, several ARVs were added to the medium before and after differentiation of 3T3-L1 cells.

Antiretroviral drugs

Efavirenz (EFV), ritonavir (RTV), nelfinavir (NFV) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada). ATV was provided by Bristol-Myers Squibb Company (NY, USA). Drug stocks in dimethyl sulfoxide (DMSO) were stored at -20°C and diluted into culture media. Vehicle control incubations received the same final DMSO concentration as all drug-treated incubations (0.1%). The IC50 /IC95 and the Cmax of each drug are as follows; EFV (IC95=1.7-25nM, Cmax= 14.2-28.8 μM), RTV (IC50= 65-289nM, Cmax= 0.84-21.9 μM), NFV (IC50=30-60nM, Cmax= 5.0-8.6 μM), and ATV (IC50=2-5nM, Cmax=4.96-8.38 μM). The cells were treated with 20 μM of EFV, RTV and ATV and 10 μM of NFV.

Quantitative real time RT-PCR

Total cellular RNA was isolated from 3T3-L1 cells, using QIAamp RNA Blood Mini (QIAGEN, Tokyo, Japan), including treatment with DNase. cDNA was generated from the RNA using TAKARA RNA PCR kit (TAKARA BIO, Shiga, Japan). Real time PCR was conducted with the LineGene33 (BioFlux, Tokyo, Japan) using SYBR Green Realtime PCR Master Mix (TOYOBO Co, Osaka, Japan). As an internal control measurement, to normalize for input DNA, copy numbers of β-actin were determined in every sample tested. The ratio of the normalized mean value for drug-treated samples was calculated and is given in the graphs.

Measurement of adiponectin levels in culture medium

The secretion of adiponectin from 3T3-L1 cells were determined by measuring the adiponectin concentration of culture medium, using the Mouse Adiponectin/Acrp30 Immunoassay (R&D systems, Minneapolis, USA).

Oil Red O staining

The cellular lipid content was assessed by lipid staining with Oil Red O. Staining was quantified at 520 nm after

Table 1. Characteristics of 57 HIV-infected Individuals and the Changes of Metabolic Markers

	group I	group II	group III	group IV (pre)	group IV (post)
Number of cases (M/F)	15 (13/2)	14 (13/1)	14 (13/1)	14 (12/2)	
Mean age (SD) (years)	37.7 (10.8)	42.1 (14.2)	38.3 (9.1)	46.1 (12.6)	
NRTI combination (number)(frequency %)					
Abacavir/ lamivudine	0 (0)	0 (0)	1 (7.1)	0 (0)	2 (14.3)
Zidovudine/ lamivudine	4 (26.7)	0 (0)	4 (28.6)	5 (35.7)	4 (28.6)
Stavudine/ lamivudine	7(46.7)	7 (50)	5 (35.7)	6 (42.9)	4 (28.6)
Didanosine/ lamivudine	0 (0)	0 (0)	0 (0)	2 (14.3)	2 (14.3)
Didanosine/ zidovudine	0 (0)	2 (14.3)	0 (0)	0 (0)	0 (0)
Tenofovir/ emtricitabine	4 (26.7)	5 (35.7)	4(28.6)	1 (7.1)	2 (14.3)
the ratio of after to bebore staring or changing ART [mean (SE)]					
Body Mass Index	1.00 (0.01)	0.99 (0.01)	1.02 (0.01)	0.98 (0.04)	
Tryglyceride	1.63 (0.19)*	1.72 (0.24)*	1.22 (0.17)	1.00 (0.304)	
HDL-cholesterol	1.29 (0.12)*	1.17 (0.16)	1.14 (0.11)	1.01 (0.07)	
LDL-cholesterol	1.17 (0.12)	1.27 (0.12)*	1.10 (0.05)	0.85 (0.06)*	
HMW-adiponectin	0.61 (0.24)*	0.65 (0.09)*	1.19 (0.20)	1.61 (0.11)*	

P values are evaluated by one sample sign test. * indicates significant difference (* $p<0.001$, ** $p<0.05$)
NRTI, nucleotide reverse transcriptase inhibitor; ART, antiretroviral therapy
"pre" indicates before changing ART, and "post"indicates after changing ART.

solubilization, using an Adipogenesis Assay Kit (Chemicon International Temecula, CA, USA)

Statistical analysis

To evaluate the changes of BMI, serum triglyceride, LDL-cholesterol, HDL-cholesterol, HMW-adiponectin after starting or changing ART, the one sample sign test was applied. The Mann-Whitney test was used to compare the serum HMW-adiponectin according to lipodystrophy. In vitro experiments were reproduced in at least three independent experiments. The results are presented as the mean ± SD. Significance was determined as described in the figure legends.

Results

The effect of ARV on the lipid profile and HMW adiponectin

The demographic and clinical characteristics for the 57 patients included in the study are shown in Table 1. There were no significant differences in the age and the NRTI combinations used as the backbone of PIs or EFV among the four groups (χ-square test). The serum triglycerides and LDL-cholesterol increased significantly in individuals in groups I and II and LDL-cholesterol decreased significantly in individuals with group IV. The serum HMW-adiponectin decreased significantly in individuals in groups I and II and increased significantly in individuals with group IV. On the other hand, there was no change in the triglyceride, LDL-cholesterol, and HMW-adiponectin levels in individuals in group III (Table 1). These results show that serum HMW-adiponectin level decreased in individuals with ART including EFV or PIs except ATV, but the decrease was reversible and it was recovered by changing the ART into that including ATV.

HMW-adiponectin and lipodystrophy

The development of lipodystrophy was observed in 14 of the 43 patients who newly initiated ART (the patients of groups I, II, and III). Lipodystrophy was more prevalent in group I (53.3%) and group II (28.6%) than in group III (14.3%). The differences in lipodystrophy were not driven by the overall weight gain. There was no significant difference in the serum HMW-adiponectin before ART between individuals with and without lipodystrophy followed by ART, but the serum HMW-adiponectin after starting ART and the ratio of HMW-adiponectin after to before starting ART decreased significantly in individuals with lipodystrophy (Fig. 1).

The effect of ARV on adiponectin mRNA levels in differentiating 3T3-L1 cells

As shown in Table 1, the effect of ART on dyslipidemia and lipodystrophy differed among the ART menus, but it is nearly impossible to fully separate the effects of the drug classes in the clinical data, because the patients received a combination of several classes of ART. As a result, *in vitro* models were used to examine the precise influence of these drugs on adipocyte development or metabolism, using well-characterized preadipocyte 3T3-LI cells. The concentrations of ARVs used in this assay were within the range (RTV, EFV) or a little higher (ATV, NFV) than what is generally observed in plasma from individuals receiving therapeutic doses of ARV. Considering that some ARVs can accumulate in fat tissue, it is possible that the effects of ARVs on 3T3-L 1 cell lines observed *in vitro* may also occur *in vivo*. When pre-adipocytes were treated with ARV, lipid accumulation was severely reduced by EFV and NFV (Fig. 2A). The adiponectin mRNA level was reduced by RTV, EFV, NFV, and was not affected by ATV (Fig. 2B). Similar results were obtained concerning the secretion of adiponectin into the culture medium (Fig. 2C). Since it is possible that the effect of ARV on the adipocyte metabolism is known to differ between preadipocytes and mature adipocytes, the mature adipocytes were also treated with ARVs. Mature adipocytes were less sensitive to ARVs' effects on lipid accumulation and adiponectin mRNA level than premature adipocytes. Lipid accumulation was reduced by EFV, RTV and NFV (Fig. 2A). The adiponectin mRNA level was reduced by RTV, EFV, and NFV, whereas ATV did not affect the adi-

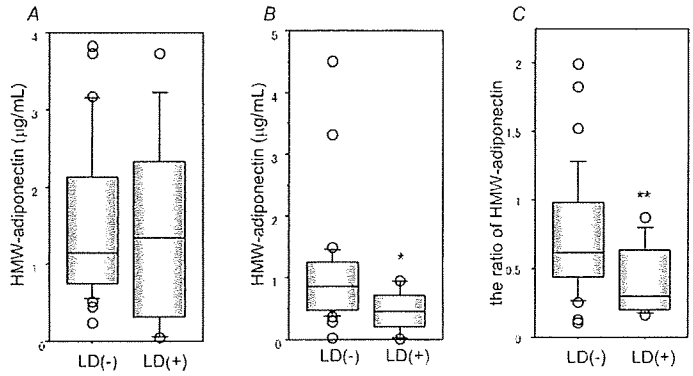

Figure 1. The levels of serum HMW-adiponectin in HIV-infected individuals with/without lipodystrophy. (A) HMW-adiponectin before starting ART, (B) HMW-adiponectin after starting ART, (C) the ratio of HMW-adiponectin after starting ART to that before starting ART. The medians are indicated with horizontal bars. The vertical bars indicate the range between 10% and 90% and the horizontal boundaries of the boxes represent the interquartile range. P values are evaluated by the Mann-Whitney U-test. * $p<0.01$, ** $p<0.05$ versus individuals without lipodystrophy. LD: lipodystrophy

ponectin mRNA level (Fig. 2B). Similar results were obtained concerning the secretion of adiponectin into the culture medium (Fig. 2C).

The effect of changing PI/EFV to ATV on adiponectin mRNA levels in 3T3-L1 cells

In the clinical study, the decreased adiponectin induced by PI/EFV was restored by switching the drugs to ATV. In addition, the precise effect of ATV on decreased adiponectin mRNA induced by PI/EFV was examined in vitro, using 3T3L1 cells. Adiponectin mRNA recovered significantly in mature adipocytes after switching drugs from EFV and NFV to ATV, but it did not recover on EFV- and NFV-treated pre-adipocytes and RTV-treated pre- and mature adipocytes (Fig. 3).

Discussion

Several studies have reported that metabolic syndrome is more common in subjects with HIV infection than in HIV-negative individuals. Although uncontrolled HIV replication can cause an adverse modification of the lipid metabolism, these modifications can be mainly induced by ART. The present study described the lipid abnormalities and lipodystrophy associated with ART. Hypertriglyceridemia was more common than abnormalities of HDL cholesterol and LDL cholesterol. LDL cholesterol increases were only observed in the population of group II (PI without ATV). These abnormalities were recovered by changing ART to the ATV containing regimen. In HIV-negative populations, the most common features associated with metabolic syndrome are obesity and hypertension. On the other hand, in HIV-positive populations, hypertriglyceridemia and hypertension are reported to be common components and the most frequent abnormalities that lead to metabolic syndrome (3). That is why HIV-positive patients might need to have their ART regimen tailored to their lipid abnormalities.

In this study, we also investigated the effect of ARVs on serum HMW-adiponectin, which has been reported to be a useful marker for evaluating insulin resistance and metabolic syndrome. This is the first study to investigate the influence of ARVs on the serum HMW-adiponectin levels in Japanese HIV-infected individuals. In general, the serum adiponectin levels are known to be inversely related to the adipose tissue mass (13). The serum adiponectin levels have been reported to rise when obese persons lose weight (14). We showed that adiponectin levels are relatively low in HIV-infected individuals with lipodystrophy who have a low fat mass, and this is consistent with previous reports (7, 10). These results suggest that the normal relationships between adiponectin concentration and adipose droplets appear to be lost or reversed in HIV-infected individuals. Since adiponectin expression is higher in subcutaneous fat than in visceral fat in humans (15), visceral fat accumulation and subcutaneous fat loss may thus lead to decreased adiponectin production both in lipoatrophic and lipohypertrophic patients. Therefore, fat redistribution may actually be responsible for the decreased adiponectin levels in HIV patients with lipodystrophy. As shown in Fig. 1(C), it is certain that some of the patients without lipodystrophy had a decrease in serum HMW-adiponectin levels, but these patients had dyslipidemia more frequently than the patients with either normal or high serum HMW-adiponectin without lipodystrophy (data not shown). Considering the fact that clinical HIV lipodystrophy has been reported to be associated with dyslipidemia, these patients might thus have the potential to be complicated by lipodystrophy. Therefore, the low levels of serum HMW-adiponectin are correlated with lipodystrophy and/or dyslipidemia induced by PIs and NNRTI (EFV).

The present study also showed the direct effects of ARVs

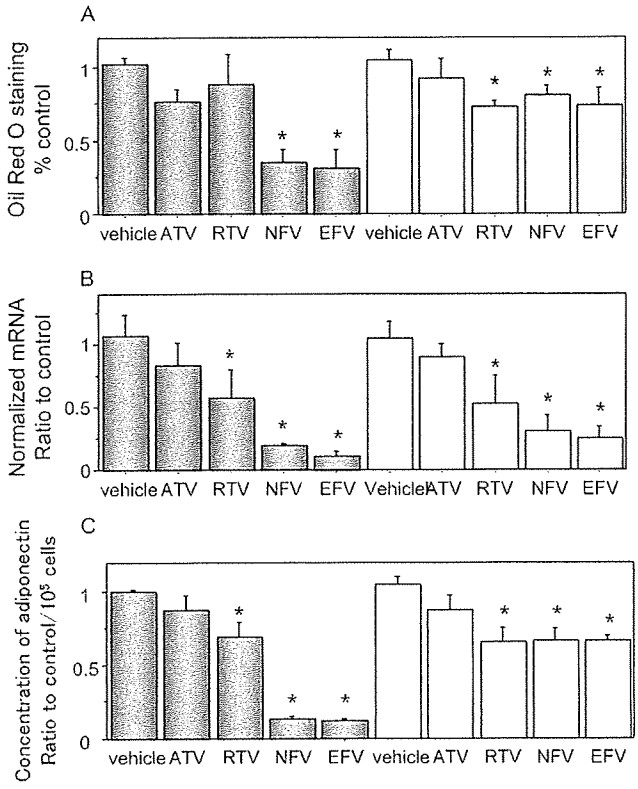

Figure 2. The effect of ARV in pre-adipocytes and mature adipocytes. From confluence (day 0), 3T3-L1 cells were treated with differentiation medium in the absence (control) or in the presence of vehicle or various ARVs. To compare the effects of ARVs on preadipocytes with mature adipocytes, ARVs were added to the medium on day 0, when 3T3-L1 cells are still preadipocytes (gray bar) or on day 6, when 3T3-L1 cells differentiate to mature adipocytes (white bar). (A) The effects of ARVs on triglyceride accumulation during 3T3-L1 adipose conversion. On day 7, the cells were stained with Oil red O. Staining was quantified at 520 nm after solubilization and expressed as %±SE of the control on day 7. (B) The effect of ARV on adiponectin mRNA levels in 3T3-L1 cells. On day 7, total RNA was prepared and mRNA levels were determined by real time RT-PCR. The results shown are after correction for the levels of β actin mRNA and normalized to the controls and represent the mean±SE. (C) The effect of ARV on adiponectin secretion in 3T3-L1 cells. At day 7, each supernatant was collected. Then, concentrations of the adiponectin were determined using an ELISA. Results shown are normalized to the controls and represent the mean±SE. Significance of difference between vehicles and other ARVs was evaluated by using the Dunnett test. EFV: efavirenz, RTV: ritonavir, ATV: atazanavir, NFV: nelfinavire

on the expression of adiponectin mRNA. In 3T3-L1 cells, the expression of adiponectin mRNA was decreased by RTV, NFV and EFV, but not by ATV. These data are consistent with our *in vivo* data. The pre-mature adipocytes were more sensitive to the effect of NFV and EFV on adiponectin mRNA and lipid accumulation than mature adipocytes. Since adiponectin is mainly produced from mature adipocytes, some factors, such as the regulatory mechanisms for the differentiation of adipocytes, might be involved in the expression of adiponectin. In fact, in subcutaneous fat from individuals from HIV-associated lipodystrophy, decreased expression of some differentiation-associated gene, such as sterol regulatory element binding protein 1, CAAT enhancer binding protein α, and peroxisome proliferators-activated receptor-γ have been described (16). On the other hand, the effects of RTV and ATV on the expression of adiponectin mRNA and lipid accumulation were not significantly different between pre-mature adipocytes and mature adipocytes. Further, the effects of RTV, NFV, and EFV on adiponectin expression were observed also in mature adipocytes, though to a lesser extent than in NFV- and EFV-treated pre-mature adipocytes. These results showed some mechanisms other than differentiation-associated gene might be involved in the expression of adiponectin. Adipose cells are highly sensitive to oxidative stress, and it has been reported that oxidative stress is one of the mechanisms that regulates adiponectin expression. Using a reporter construct containing the adiponectin promoter, reactive oxygen species (ROS) have been shown to reduce the transcriptional activity of the adiponectin gene in 3T3-L1 adipocytes (17). From the current data, it is certain that various mechanisms are involved in the regulation of adipokine expression and that the effects of ARVs on adipogenesis and adiponectin expression may vary among different drugs. The distinct metabolic effect of ARVs could therefore be a consequence of their differential effects on both the production of adiponectin and the adipocyte physiology.

We have shown that ATV, in comparison to RTV, NFV and EFV, causes less inhibition of adiponectin secretion and lipid accumulation. Furthermore, the replacement of RTV, NFV, and EFV to ATV did not decrease the serum HMW adiponectin level and ATV replacing therapy has been associated with a decrease in hyperlipidemia and an increase in serum HMW adiponectin in HIV-infected patients. In the same way, ATV leads to a reversal in the impairment of adiponectin secretion or other metabolic abnormalities in 3T3-L1 cells. These properties could underlie the favorable metabolic side effect profile of ATV observed in its clinical use.

This study showed the direct effect of ARV on the lipid metabolism, but it is possible that such abnormalities in adiponectin and lipid metabolism in HIV-infected individuals are the result of either the consequence of HIV infection itself or of cytokine/chemokine released from infiltrating macrophages, or several other factors.

This study provides important new information for clinicians and patients regarding the relative risk and benefits of available antiretroviral regimens for the initial therapy of HIV-1 infection. EFV and some PIs except for ATV containing ART decreased serum HMW-adiponectin, which is associated with dyslipidemia and lipodystrophy. Some ARVs, with the exception for ATV decreased the expression of adiponectin in adipocytes *in vitro* and the phenomenon seems

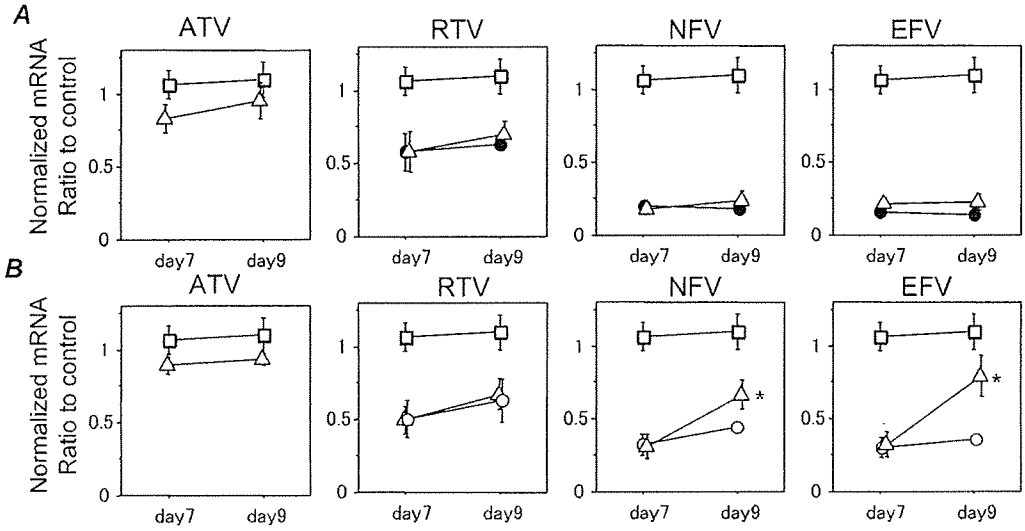

Figure 3. The effect of replacing ARVs with ATV on adiponectin mRNA levels in 3T3-L1 cells. From confluence (day 0), 3T3-L1 cells were treated with differentiation medium in the absence (control) or in the presence of vehicle (□) or various ARVs, which were added to the medium at day 0, when 3T3-L1 cells are still preadipocytes, (●)(A), or on day 6, when 3T3-L1 cells differentiate to mature adipocytes (○) (B). On day 7, the cells were washed and then treated with the medium containing ATV(△) or with the medium with the same ARV as used until day 7 (●, ○). On days 7 and 9, total RNA was prepared and mRNA levels were determined by real time RT-PCR. Results shown are after correction for the levels of β actin mRNA and normalized to the control and represent the mean ± SE. P values are evaluated by Student's t-test. *$p<0.01$ versus the same ARV as used until day 7. EFV: efavirenz, RTV: ritonavir, ATV: atazanavir, NFV: nelfinavir

to be caused by several different mechanisms. A greater understanding of the mechanisms underlying the development of this metabolic effect could lead to safer ARVs, and at the same time lead to the most appropriate treatment for these metabolic side effects of ARVs.

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