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Short Communication

Virus Detection Using Viro-Adembeads, a Rapid Capture System for Viruses, and Plaque Assay in Intentionally Virus-Contaminated Beverages

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SUMMARY: Intentional contamination of beverages with microbes is one type of bioterrorist threat. While bacteria and fungus can be easily collected by a centrifuge, viruses are difficult to collect from virus-contaminated beverages. In this study, we demonstrated that Viro-Adembeads, a rapid-capture system for viruses using anionic polymer-coated magnetic beads, collected viruses from beverages contaminated intentionally with vaccinia virus and human herpesvirus 8. Real-time PCR showed that the recovery rates of the contaminated viruses in green tea and orange juice were lower than those in milk and water. Plaque assay showed that green tea and orange juice cut the efficiency of vaccinia virus infection in CV-1 cells. These results suggest that the efficiency of virus detection depends on the kind of beverage being tested. Viro-Adembeads would be a useful tool for detecting viruses rapidly in virus-contaminated beverages used in a bioterrorist attack.

Intentional contamination of beverages with microbes is a relatively easy way for terrorists to transmit microbes to anonymous persons and to induce a public panic. Although the few incidents of intentional viral contamination of drinks in Japan to date have not been real bioterrorist attacks, a similar act as a form of terrorism could be devastating. Therefore, an efficient and rapid detection system to detect microbes in contaminated drinks should be developed as an anti-bioterrorism tool. While bacteria and fungi can be easily collected by a centrifuge, viruses are difficult to collect from virus-contaminated beverages. An ultracentrifuge is a useful tool for virus collection in liquid samples, but not every laboratory is equipped with an ultracentrifuge. In addition, virus concentration with an ultracentrifuge usually takes more than 3 h.

Viro-Adembeads (Ademtech, Pessac, France) are recently developed magnetic beads intended for capturing viruses in liquid samples. They are specifically designed to function in virus-containing cell culture media (1,2). An anionic polymer coating on the magnetic beads binds to the surface of virus particles electrically; the complex of virus and beads can then be collected using a magnet. In the present study, we examined the capacity of Viro-Adembeads to collect viruses in virus-contaminated beverages. We also investigated whether the kind of beverage tested affected the efficiency of virus detection in this manner.

To represent intentional contamination of beverages with viruses, we mixed a solution containing two viruses with beverages. Human herpesvirus 8 (HHV-8) and vaccinia virus (LC16m8) were collected as reported previously (3,4). Milk, green tea, water, orange juice, and barley tea were purchased from a convenience store in Tokyo. To create virus-contaminated beverages, we added 0.1 mL of virus solution containing 1×10^7 copies of HHV-8 or vaccinia virus into 0.9 mL of beverages in 1.5-mL tubes. Each virus-contaminated beverage was serially diluted $\times 10$ with the beverage at each stage, to $\times 10,000$. If a terrorist was to contaminate beverages with viruses in a food store, the period of incubation would probably be from 5 min to several days; for our sample incubation time, we incubated the contaminated beverages for 1 h. To collect viruses from virus-contaminated beverage samples, we then used Viro-Adembeads according to the manufacturer's instructions. Briefly, 25 μL of washed Viro-Adembeads solution was added to each 1-mL aliquot of a virus-contaminated beverage sample. After 20 min of agitation at room temperature, the Viro-Adembeads were collected with a magnet. The beads were then washed with PBS twice. DNA was directly extracted from the beads with a DNA extraction kit (DNeasy; Qiagen, Hilden, Germany). The DNA was dissolved in 100 μL of water. Virus copy numbers were measured with a TaqMan Real-Time PCR (Applied Biosystems, Foster City, Calif., USA) as previously described (5). To detect HHV-8, we amplified ORF-26 using a previously reported probe and primer set (6). To detect vaccinia virus, we used a consensus probe and primer set targeting the F2R region of orthopoxvirus as follows: forward primer 5′-gatctagttttcagcacggtggta-3′, reverse primer 5′-cagatatatgattggatgtagaacaccat-3′, and probe 5′-FAM-agaggtggaggaatttatagatgatggaagacaagtt-TAMRA-3′. The recovery rate was calculated by the retrieved virus copy number with Viro-Adembeads, divided by the input virus copy number in 1 mL of each sample. The results of real-time PCR showed that the amount of collected virus was proportionally reduced as the sample was diluted (Figure 1A and 1B). Copies of HHV-8 and vaccinia virus were reduced almost one-tenth per dilution. However, copies of HHV-8 in $\times 100$, $\times 1,000$, and $\times 10,000$ dilutions of green tea, and vaccinia virus in the $\times 10$ dilution of green tea were extremely reduced. Although 34% of input HHV-8 was detected in the water sample, other beverages such as milk, orange juice, and green tea demonstrated lower recovery rates (Figure 1C). The recovery rate of HHV-8 with Viro-Adembeads was 39% in culture media (RPMI 1640 medium supplemented with 10% fatal bovine serum) containing the same amount of HHV-8 to the $\times 10$ dilution, suggesting similar efficacy of virus collection with Viro-Adembeads between water and culture

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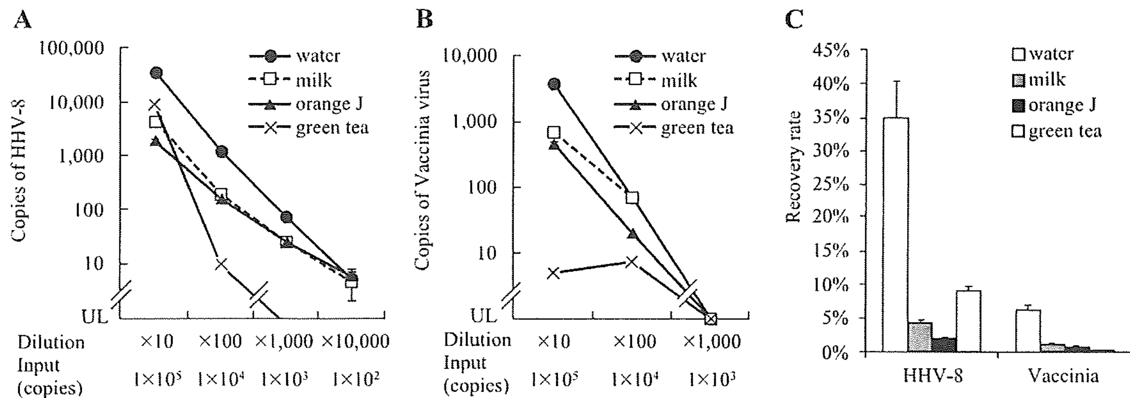

Fig. 1. HHV-8 and vaccinia virus detection using Viro-Adembeads. (A) Copies of HHV-8. (B) Copies of vaccinia virus. The y-axis indicates copy numbers of the virus in 1% of the extracted DNA. 'UL' indicates 'under limitation'. 'Dilution' and 'Input' under the x-axis indicate dilution factors and 1% of the virus copy numbers in 1 mL of each sample, respectively. (C) Recovery rates. Virus solutions were diluted ×10 with beverages; viruses were then recovered with Viro-Adembeads. Recovery rates were calculated based on the results of real-time PCR. Error bars indicate standard deviations.

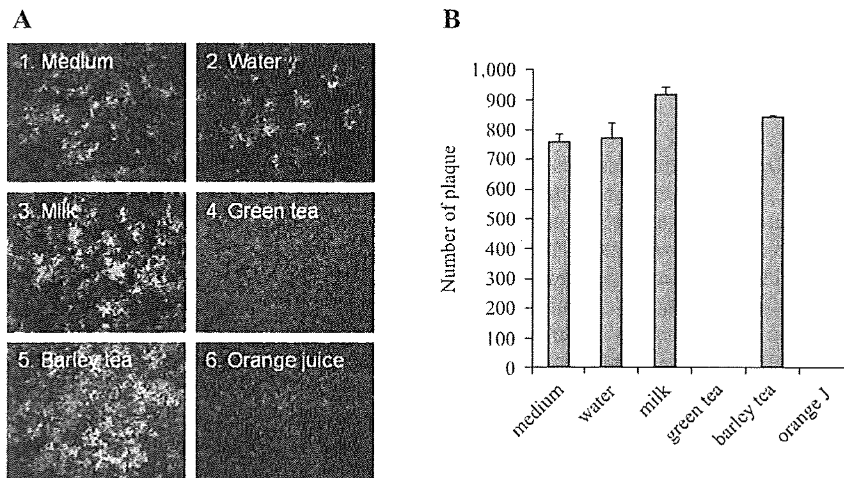

Fig. 2. Green tea and orange juice reduce the efficiency of vaccinia virus infection. (A) Microscopic view of plaques. (B) Numbers of plaque by vaccinia virus. Error bars indicate standard deviations.

media. Vaccinia virus was also recovered at low rates in all beverages. In both viruses, the recovery rates differed among beverages. For example, the recovery rates in milk, orange juice, and green tea were significantly lower than that in water. These results indicate that the efficiency of virus detection depends on the kind of beverage being tested.

To know whether the beverage type affects viral infectivity, we examined a plaque assay using vaccinia virus in the beverages, because it is difficult to perform a plaque assay in HHV-8 (7). Virus solution containing 10,000 plaque-forming units (pfu) was mixed with 1 mL of each beverage and incubated with rotation for 20 min. From these solutions, 100-μL samples were added to CV-1 cells with 1 mL of serum-free medium per well in a 6-well plate. The plaque assay showed that the number of plaques was dramatically reduced in green tea and orange juice (Figure 2). The plaque number in barley tea did not change from that of water and milk. These results suggested that green tea and orange juice reduced the infectivity of vaccinia virus. The pH conditions of the water, milk, orange juice, and green tea used in this experiment were pH 7.2, 6.8, 4.0, and 6.2, respectively, suggesting that acidity is not the only factor in reducing virus infectivity.

The results in the present study suggest that the type of beverage affects virus detection in virus-contaminated beverages and that Viro-Adembeads would be a useful tool for virus detection in virus-contaminated beverages. Our results clearly demonstrated that orange juice and green tea reduced virus infectivity of vaccinia virus. One of the reasons could be a low pH in orange juice, but other factors were unveiled. We checked pH conditions of several kinds of orange juice in a food store and found they ranged from pH 3.3 to pH 4.2, suggesting that the orange juice used in the present study was not special, at least with regard to its pH condition. Incubation of viruses with orange juice or green tea reduced not only the number of plaques in a plaque assay but also the copy numbers of the virus detected by a real-time PCR after virus collection, suggesting that orange juice and green tea affect the binding efficiency of viruses to Viro-Adembeads, in addition to reducing virus infectivity. No plaque was observed in any well of CV-1 cells with 10,000 pfu of vaccinia virus incubated with orange juice or green tea. On the other hand, we could detect the viruses from orange juice or green tea containing the same amount of viruses by Viro-adembeads and real-time PCR, although the recovery rate was low. These data indicated that Viro-adembeads and real-time PCR was more sensitive for detecting vaccinia virus in orange juice

and green tea than was the plaque assay.

The use of Viro-Adembeads is an easy method for collecting viruses from a virus-contaminated liquid. One of the merits of this method is its rapidity. In our experiment, it took about 30 min to collect viruses from virus-contaminated beverages, which is much quicker than the using the ultracentrifuge method. Another merit is that Viro-Adembeads can be used in liquid samples with some precipitation and turbidity, like milk and orange juice. Such precipitation and turbidity interfere with efficient isolation of viruses in any method using an ultracentrifuge. In addition, the use of Viro-Adembeads does not require any machine. Thus, although further studies are required to determine optimal techniques and conditions, Viro-Adembeads could be a useful tool for rapidly detecting viruses in virus-contaminated beverages.

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Genotypic and Clinicopathological Characterization of Kaposi's Sarcoma-Associated Herpesvirus Infection in Japan

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Kaposi's sarcoma-associated herpesvirus (KSHV) is related causally to Kaposi's sarcoma, primary effusion lymphoma, and a subset of cases of multicentric Castleman's disease. As the numbers of acquired immunodeficiency syndrome (AIDS) patients have increased, KSHV-associated diseases have also increased in Japan. Sporadic cases of classic Kaposi's sarcoma have also been reported in Japan. In the present study, the clinicopathological characteristics of 75 samples, comprising 68 cases of Kaposi's sarcoma, 5 cases of primary effusion lymphoma, and 5 cases of multicentric Castleman's disease were investigated. All of these cases were positive for KSHV by immunohistochemistry or PCR analysis. All fifty-two of the AIDS-associated Kaposi's sarcoma cases were males, whereas 7 of the 13 non-AIDS-associated Kaposi's sarcoma cases were females. The mean age of patients with AIDS-associated Kaposi's sarcoma or primary effusion lymphoma was 46 years, whereas the mean age of patients with non-AIDS-associated Kaposi's sarcoma or primary effusion lymphoma was 71.8 and 97.5, respectively. KSHV genotypes were determined based on the sequence of variable region 1 in the *K1* gene. Genotypes A and C of KSHV were detected in both AIDS- and non-AIDS-associated Kaposi's sarcoma. Genotype A was detected more frequently in AIDS-associated cases than non-AIDS-associated cases, suggesting that genotype C is broadly distributed in Japan, and genotype A spreads among AIDS patients. Genotype D was detected only in non-AIDS-associated Kaposi's sarcoma. These data confirmed the difference between AIDS- and non-AIDS-associated KSHV diseases with regard to age of onset, gender, and genotypes in Japan. ***J. Med. Virol. 82:400–406, 2010.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: Kaposi's sarcoma; Kaposi's sarcoma-associated herpesvirus; AIDS; genotype

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INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV, also known as human herpesvirus-8, HHV-8) was identified from a Kaposi's sarcoma (KS) specimen by representational difference analysis in 1994 [Chang et al., 1994]. KSHV has been detected in KS, primary effusion lymphoma (PEL) and a subset of multicentric Castleman's disease (MCD) cases [Moore and Chang, 2001]. KS was first described in 1872 by Moriz Kaposi, a Hungarian dermatologist, as an idiopathic, multi-pigmented sarcoma of the skin [Kaposi, 1872]. KS is classified into four types: classic, endemic, iatrogenic, and acquired immunodeficiency syndrome (AIDS)-associated KS (AIDS-KS) [Antman and Chang, 2000]. Classic KS affects typically elderly men in Mediterranean littoral, endemic KS affects typically people in Africa, iatrogenic KS affects most commonly organ-transplant recipients receiving immunosuppressive therapy and AIDS-KS is mainly associated with homosexual men infected with human immunodeficiency virus (HIV). In Japan, KS was a very rare condition prior to 1980 [Fujii et al., 1986]. A few patients with adult

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T-cell leukemia were described who had developed KS, from the Kyushu region and the southern island of Okinawa, but very few classic cases of KS were reported from other areas of Japan [Kamada et al., 1992]. After 1980, cases of AIDS-KS increased dramatically in Japan because of the rapid spread of AIDS. The introduction of highly active anti-retroviral therapy (HAART) reduced the number of KS cases in Western countries [Jones et al., 1999]. However, KS cases still increased in Japan after 2000 because of the dissemination of HIV infection within the homosexual male community. As well as AIDS-KS, other KSHV-associated diseases, such as AIDS-PEL and AIDS-MCD, also increased during the past 10 years in Japan [Katano et al., 1999a; Hasegawa et al., 2004; Abe et al., 2006]. Therefore, to prevent the spread of AIDS-KS, it is important to determine the clinicopathological features of KSHV-associated diseases in Japan. To date, reports describing the clinicopathological features of Japanese AIDS-KS have all involved only small sample sizes [Fujii et al., 1986; Kamada et al., 1992; Kondo et al., 2000; Yamada et al., 2000; Meng et al., 2001; Sato-Matsumura et al., 2001; Kamiyama et al., 2004; Minoda et al., 2006; Yoshii et al., 2006; Ueno et al., 2007].

The association of KSHV infection with KS pathogenesis has already been well investigated. A latency-associated nuclear antigen 1 (LANA-1) encoded by KSHV is detected in almost all KS cells, indicating that KS cells are infected with KSHV [Dupin et al., 1999; Katano et al., 1999b]. The genome of KSHV is a double-stranded linear DNA of about 170 kbp, flanked by GC-rich terminal repeats [Russo et al., 1996]. The *K1* gene in KSHV contains highly variable regions 1 and 2 (VR1, VR2) and phylogenetic analysis of the *K1* gene classified KSHV into genotypes A–F [Zong et al., 1997, 1999; Meng et al., 1999; Hayward and Zong, 2007]. These genotypes are differently distributed throughout the world: KSHV genotype A is predominant in North America, B in Africa, C in Eurasia and the Mediterranean, D in the Pacific islands, E in Brazilian Amerindians, and F in the Ugandan Bantu tribe [Zong et al., 1999; Biggar et al., 2000; Kajumbula et al., 2006]. Previous studies involving a small number of cases detected genotypes C and A in cases of KS, and genotype D in some cases of classic KS in Japan [Meng et al., 2001; Kamiyama et al., 2004]. However, it is unknown whether these genotypes are associated with any of the clinical features or pathogenesis of KS or other diseases.

In the present study, the clinicopathological features and genotypes of KSHV-associated diseases were investigated in 75 samples originating from all over Japan. The aim was to determine the characteristics of KSHV-associated diseases in Japan.

MATERIALS AND METHODS

Tissue Specimens

Studies using human tissue were performed with the approval of the Institutional Review Board of the National Institute of Infectious Diseases (Approval No. 158). Seventy-five cases of KSHV-associated disease were filed in the Department of Pathology, National Institute of Infectious Diseases, Japan, from 1995 to April 2009 as consultation cases. They include 68 KS cases, 5 PEL cases, and 5 MCD cases. Two MCD patients and a PEL patient had KS lesions. Frozen tissue samples were available for 21 of these cases. For some other cases, only formalin-fixed paraffin-embedded tissue sections were available. The samples were sent from all over Japan, from the northern island of Hokkaido to Okinawa, the southern island of Japan.

Histological Grading and Immunohistochemistry

Paraffin sections were hematoxylin and eosin stained and subjected to immunohistochemical staining to detect LANA-1, as described previously [Katano et al., 1999b]. Samples were categorized into three clinical stages of KS (patchy, plaque, or nodular stage) according to the clinical data and the histological findings.

Preparation of DNA

Total DNA was extracted from fresh-frozen clinical materials or formalin-fixed paraffin-embedded sections as described previously [Asahi-Ozaki et al., 2006]. For the isolation of DNA from formalin-fixed paraffin-embedded biopsies, three or four 5 μm-sections were placed into sterile eppendorf tubes, deparaffinized with xylene, digested with proteinase K, then extracted using the phenol/chloroform method. For fresh-frozen samples, DNA was extracted using the DNeasy Blood & Tissue kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany).

PCR Amplification and DNA Sequencing

A 160 bp fragment containing VR1 of the *K1* gene was amplified by PCR from DNA samples as described previously [Dilnur et al., 2001]. The primer set used was as follows: forward primer 5′-TTG CCA ATA TCC TGG TAT TGC-3′; reverse primer 5′-CAA GGT TTG TAA GAC AGG TTG-3′. PCR amplification was carried out at 94°C for 2 min (one cycle); 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min (35 cycles); and 72°C for 5 min (one cycle) using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). PCR products were purified using the QIAquick PCR purification kit (Qiagen), followed by direct sequencing with an ABI sequencer 3130 (Applied Biosystems) using a Big-Dye terminator ready reaction kit (Applied Biosystems) according to the manufacturer's instructions.

Phylogenetic Tree Analysis

Nucleotide sequences, excluding primer sequences, were multiple aligned with CLUSTAL W version 1.83 [Jeanmougin et al., 1998], and a phylogenetic tree was constructed using the neighbor-joining-plot method and Genetyx software (Genetyx, Tokyo, Japan). In addition

to our samples, 20 previously reported *K1* gene sequences were obtained from the GenBank database and used as reference sequences for comparison [Dilnur et al., 2001]. The genotypes of KSHV samples and the GenBank accession numbers of the reference strains are as follows: BCBL-R (genotype A, accession no. AF133038), BCBL-B (A, AF133039), 431KAP (B, AF133040), ASM72 (C, AF133041), BC2 (C, AF133042), TKS10 (D, AF133043), ZKS3 (D, AF133044), US3 (A, AF151688), Ug3 (A, AF151690), US6 (C, AF151686), Au1 (D, AF151687), Ug1 (B, AF151689), 78/48 (C, AF201851), 75/10T (A, AF201848), 80/56 (A, AF2-1853), KS-F (C, U93872), Tupi-1 (E, AF220292), Tupi-2 (AF220293), Wagu128 (E, AY940426), and BCBL-1 (A, U86667) [Meng et al., 1999; Zong et al., 1999; Lacoste et al., 2000; Kazanji et al., 2005]. BCBL-R was used as a consensus sequence.

GenBank Accession Numbers

GenBank accession numbers of Japanese KSHV sequences are AF278837 (J1), AF278842 (J2), AF278847–AF278849 (J3–J5), AF278850–AF278852 (J7–J9), AF278838 (J14), AF278839 (J16), AF278840 (J17), AF278841 (J19), AF278843 (J21), AF278844–AF278846 (J24–J26), and GQ848990–GQ849006 (J27–J43).

Statistical Analysis

Analysis of statistical significance was carried out using the Chi-squared test or Fisher's extract for bivariate tabular analysis and the Mann–Whitney test for comparison of two independent groups of sampled data.

RESULTS

Clinical and Pathological Characteristics of KS in Japan

Table I provides a summary of the clinical data. All of the cases were positive for LANA-1 by immunohistochemistry. The 68 pathological samples of KS were taken from various anatomical sites: the skin (84%), the gastrointestinal tract (7%), the lymph node (4%), the lungs (1%), the oral cavity (1%), and the conjunctive (1%) (Fig. 1A). Non-AIDS-KS cases were all presented in the skin. Among the 68 KS cases, 52 were AIDS-KS (76.4%) and 13 were non-AIDS-KS (19.1%). HIV-1 seropositive data were not available for three KS cases (4.4%). AIDS-KS cases were all from male patients with a mean age of 45.8 years (range: 23–82). By contrast, only six non-AIDS-KS cases were male (46%) and the proportion of female in non-AIDS-KS cases was high (54%). The mean age of non-AIDS-KS cases was 71.8 years (range: 52–87), which was statistically higher than that of AIDS-KS cases (Mann–Whitney test, $P < 0.01$). In addition, the mean age of non-AIDS-associated PEL cases was 97.5 years (range: 94–101), indicating occurrence of PEL in predominantly very elderly patients. Among the non-AIDS-KS cases, nine cases were regarded as classic KS and four cases were iatrogenic KS in immunosuppressed patients. Seven out of 13 non-AIDS-KS cases were in females, including 4 cases of iatrogenic KS. Histologically, the skin lesions of KS were categorized into stages: patchy (27%), plaque (36%), and nodular (34%) (Fig. 1B,C). Among the 13 non-AIDS-KS lesions, 6 lesions (46.2%) were at the plaque stage. However, AIDS-KS lesions represented all three stages, patchy, plaque, and nodular, almost equally. No histological difference was found between AIDS-KS and non-AIDS-KS.

Phylogenetic Tree Analysis of VR1 of the *K1* Gene From KSHV Genotypes

KSHV genotypes were determined in 33 cases based on the sequence of VR1 in the *K1* gene [Meng et al., 1999; Zong et al., 1999]. Thirty strains were obtained from KS samples, three from each of the PEL and MCD samples (Fig. 2A). Sixteen strains (J1–J5, J7–J9, J14, J16, J17, J19, J21, and J24–J26) have been described previously (14 KS, one PEL and one MCD case) [Meng et al., 2001]. Construction of a phylogenetic tree revealed that the Japanese cases were categorized into genotypes A, C, and D (Fig. 2B). Genotypes A and C were observed in the AIDS-KS subjects, whereas genotypes A, C, and D were observed in non-AIDS-KS subjects (Fig. 3A). Thus, genotype D was observed only in non-AIDS-KS subjects. All three cases of PEL, including one case of non-AIDS-PEL, were genotype C. Two genotype C and one genotype A sequences were detected in three cases of AIDS-MCD. Genotype A was detected more frequently

TABLE I. Summary of the Clinical Data of All Disease Cases Used in This Study

	n	Mean age	Age range	No. of males (%)	HIV(+)
KS	**68**	**50.7**	**23–87**	**61 (89.7%)**	**52/65 (80.0%)**
AIDS-KS	52	45.8	23–82	52 (100%)	—
Non-AIDS-KS	13	71.8	52–87	6 (46.1%)	—
Unknown	3	46.0	33–53	3 (100%)	—
PEL	**5***	**64.0**	**42–101**	**5* (100%)**	**3*/5 (60%)**
AIDS-PEL	3*	45.5	42–49	3* (100%)	—
Non-AIDS-PEL	2	97.5	94–101	2 (100%)	—
AIDS-MCD	**5****	**38.8**	**27–56**	**5** (100%)**	**5**/5 (100%)**
All	75	51.2	23–101	68 (90.7%)	57/72 (79.2%)

*Including one case having KS. **Including two cases having KS. Bold indicates large categories.

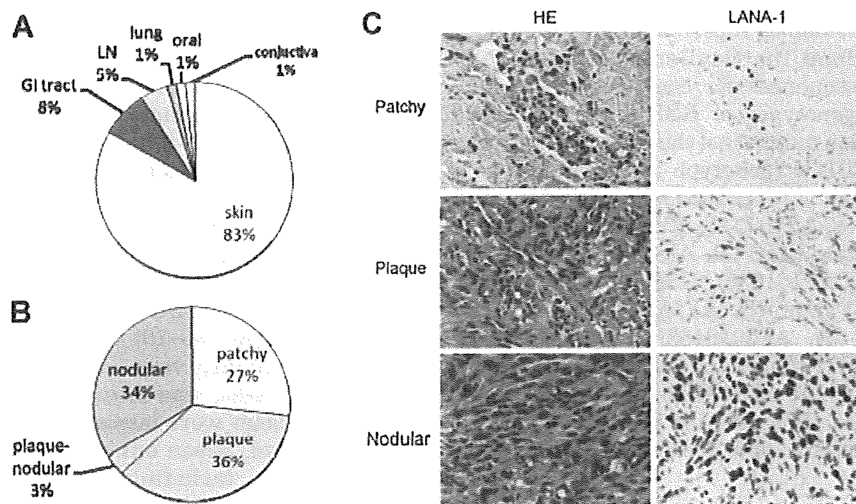

Fig. 1. Site and histology of Japanese Kaposi's sarcoma (KS) cases. Pie charts indicating (**A**) the site of KS and (**B**) the histological stage of KS in the skin, in the cases studied. GI: gastrointestinal, LN: lymph node. **C**: Hematoxylin and eosin (HE) staining (left) and latency-associated nuclear antigen 1 (LANA-1) immunohistochemistry (right) of patchy stage (upper), plaque (middle), and nodular stage (lower) of KS.

in AIDS-associated cases than non-AIDS-associated cases, but the difference was not statistically significant ($P = 0.28$, Chi-square test with Yate's correction) (Fig. 3B). Genotype C was common in both groups. The mean ages associated with genotypes A, C, and D were 48, 56, and 77, respectively. Genotype D was detected in more elderly patients than genotypes A and C ($P < 0.05$, Mann–Whitney test). These data indicated that genotype D was associated with non-AIDS-associated cases, not with AIDS-associated cases. The findings also suggest that genotype C is broadly distributed in Japan, and genotype A spreads among AIDS patients. There was no detectable histological difference among genotypes.

DISCUSSION

In this study, the clinicopathological features and genotypes of Japanese cases of KSHV-associated diseases were demonstrated. These data confirmed that non-AIDS-KSHV-associated diseases occurred predominantly in elderly patients. Genotype analyses suggested the broad distribution of genotype C, association of genotype D with non-AIDS-KS and spread of genotype A among AIDS patients in Japan.

There were few reports describing KS in Japan before 1986, and only 14 cases of classic KS were reported between 1917 and 1982 [Fujii et al., 1986]. A group in Okinawa reported six KS cases, including one adult T-cell leukemia-associated and two AIDS-associated cases in 1992 [Kamada et al., 1992]. After the discovery of KSHV in 1994, the association of KSHV infection in Japanese KS cases was proposed [Tachikawa et al., 1996]. Serological assays revealed that the seroprevalence of KSHV was 1.4% among the general population in Japan [Katano et al., 2000]. Almost all patients with AIDS-KS and non-AIDS-KS had serum antibody to KSHV, and 64% of Japanese AIDS patients, infected with HIV via sexual transmission were positive for anti-KSHV antibody [Katano et al., 2000]. KSHV was detected in all KS cases in Japan with positive immunohistochemical results for LANA-1 [Katano et al., 1999b]. Thus, the correlation between KSHV infection and KS pathogenesis has already been demonstrated in many Japanese cases. However, to date clinical information on Japanese KS cases was rarely reported [Fujii et al., 1986; Kamada et al., 1992; Kondo et al., 2000; Yamada et al., 2000; Kamiyama et al., 2004; Minoda et al., 2006; Yoshii et al., 2006; Ueno et al., 2007]. The difference in the mean age of patients affected by AIDS-KS and non-AIDS-KS was demonstrated in the present study. These results may reflect the population of origin for these patients. Several case studies reported that non-AIDS-KS in Japan is associated with immunosuppression, old age, or iatrogenic factors [Kondo et al., 2000; Yamada et al., 2000; Sato-Matsumura et al., 2001; Yoshii et al., 2006]. Regarding AIDS-KS, an epidemiological survey revealed that 70% of newly-HIV-infected individuals were infected via homosexual behavior (AIDS Surveillance Committee Japan, 2008). HAART decreased the incidence of KS in HIV-infected patients, but the increase of HIV-infection in homosexual men resulted in an increase of AIDS-KS cases in Japan. Although it is suggested that KSHV may be spread among homosexual men in Japan, further epidemiological studies on HIV-infected and uninfected males would be required to clarify the association of KSHV infection with the increase of KS in Japan.

KSHV genotypes are determined based on the sequence of VR1 in the *K1* gene sequence of KSHV [Meng et al., 1999; Zong et al., 1999]. Several variable regions were identified in the KSHV genome [Poole

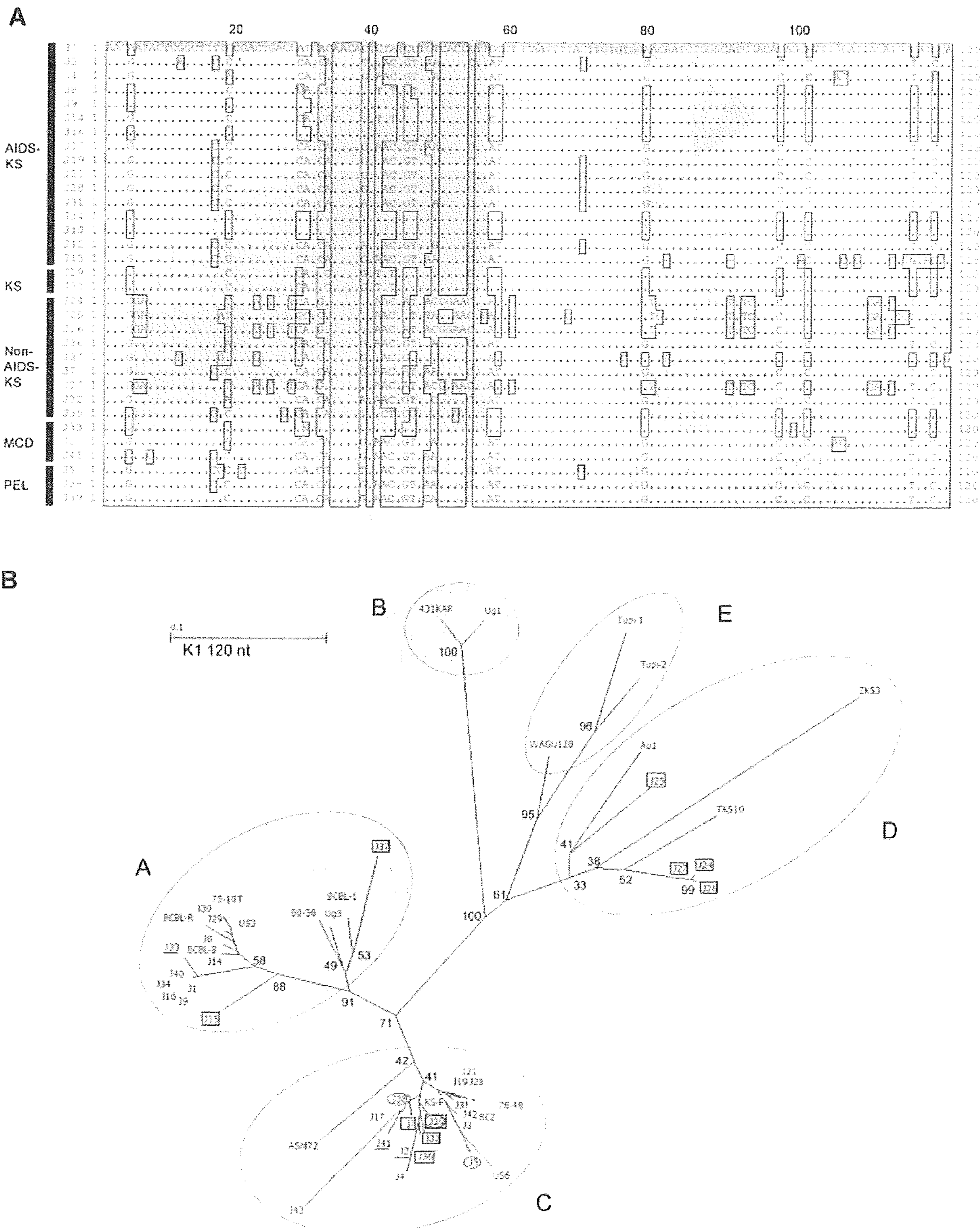

Fig. 2. K1 gene sequences in Japanese cases. **A**: Alignment of K1 gene sequences. One hundred twenty basepair fragments containing VR1 of the *K1* gene are shown. Case J33 had not only MCD, but also KS. HIV-1 seropositive data were not available for J29 and J30 cases. **B**: Radial unrooted phylogenetic tree generated by the NJ method on 120 bp segments of the *K1* gene. The numbers at some nodes (boot strap values) indicate frequencies of occurrence for 100 trees. Scale bar represents 0.1 substitutions per site. Genotypes A–E are indicated by circles. Japanese cases are indicated by J-numbers. J-numbers with boxes are non-AIDS cases of KS or PEL. J5 and J38 are AIDS-PEL cases (circled). J39 is a non-AIDS-PEL case (circled and boxed). J2, J33, and J41 are AIDS-MCD cases (underlined). All other sequences are included for reference. See text.

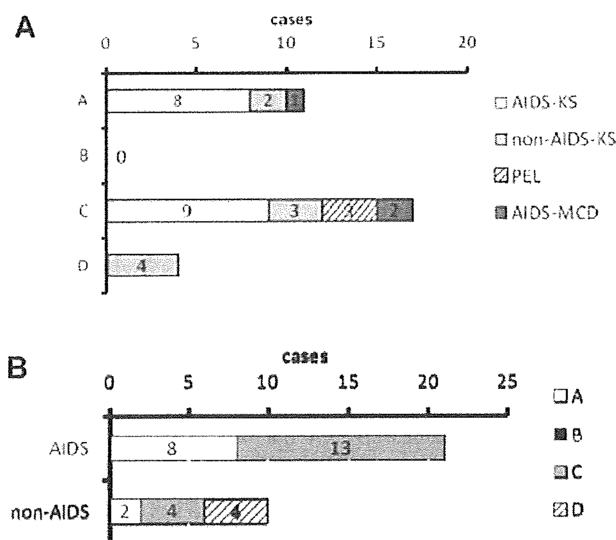

Fig. 3. Graphs indicating the association between: **A**: Kaposi's sarcoma-associated herpesvirus (KSHV) genotypes and diseases (each bar indicates the number of cases). **B**: Genotypes in AIDS-associated and non-AIDS-associated cases.

et al., 1999]. However, since frequent variations were detected in the *K1* gene among strains, the *K1* gene was well investigated and used as a standard for genotype determination [Meng et al., 1999; Zong et al., 1999; Hayward and Zong, 2007]. Genotypes A and C of KSHV are broadly distributed throughout the world. A previous study had already shown that genotype C was predominant not only in Japan, but also in Asian countries, such as Taiwan, Korea, and China [Zong et al., 2002]. Genotype C was detected in Uygur people in Xinjiang, west of China, that was located at the middle point of the Silk Road from Rome to Xian, China [Dilnur et al., 2001]. The virus may therefore have been transmitted via the migration of people from Europe, and the genotype C virus spread in Asian countries. Genotype D, found in the Oceania region, had already been detected in three cases of non-AIDS-KS in Japan in a previous study [Meng et al., 2001]. One additional case of genotype D was found in a non-AIDS-KS case in the present study, supporting the association of genotype D with non-AIDS-KS. Genotype A was detected in both AIDS-KS and non-AIDS-KS cases in the present study. To date, there has been no report of genotype A in non-AIDS-KS cases in Japan. Genotype A was more frequently found in AIDS-KS cases, suggesting that genotype A came from the USA via homosexual activity. However, detection of genotype A in non-AIDS-KS cases at a low rate suggests that genotype A is also a common virus in the general population in Japan, along with genotype C.

PEL and MCD are very rare diseases associated with KSHV infection. A previous study demonstrated that only AIDS-MCD is associated with KSHV infection, not non-AIDS-MCD in Japan [Suda et al., 2001]. All three cases of PEL investigated in this study were genotype C virus, while two genotype C and one genotype A were detected in three cases of MCD. There was no correlation between KSHV genotype and disease, suggesting that any genotype in Japan may induce any type of KSHV-associated disease. Considering 1.4% of KSHV seroprevalence in the general population in Japan, there may be many KSHV-infected individuals without symptoms [Katano et al., 2000]. Although genotype analysis suggests transmission routes of the virus from other countries, further studies using a large number of KSHV-infected patients are needed to clarify the route of KSHV infection among individuals.

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雑誌

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南　留美、高濱宗一郎、安藤　仁、山本政弘	治療後ウエスタンブロット法にて抗HIV抗体が陰性化し持続しているHIV-1感染症の１例	感染症学雑誌	83	251-255	2009

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, Mimmeapolis, 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

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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-

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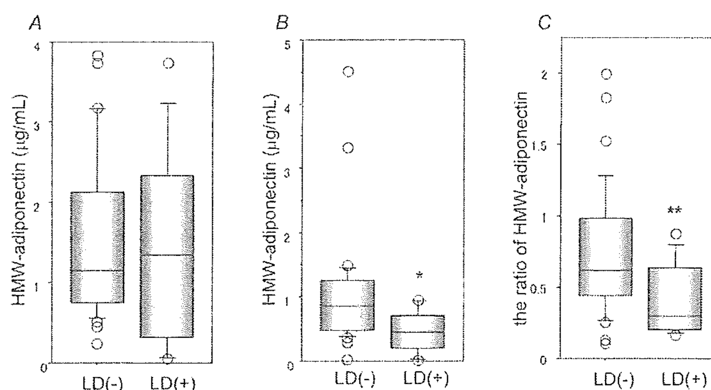

Figure 1. The levels of serum HMW-adiponectin in HIV-infected individuals with/without lipodystrophy. (A) HMW-adiponectin before starting ART, (B) HMW-adiponectin after starting ART, (C) the ratio of HMW-adiponectin after starting ART to that before starting ART. The medians are indicated with horizontal 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 preadipocytes 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

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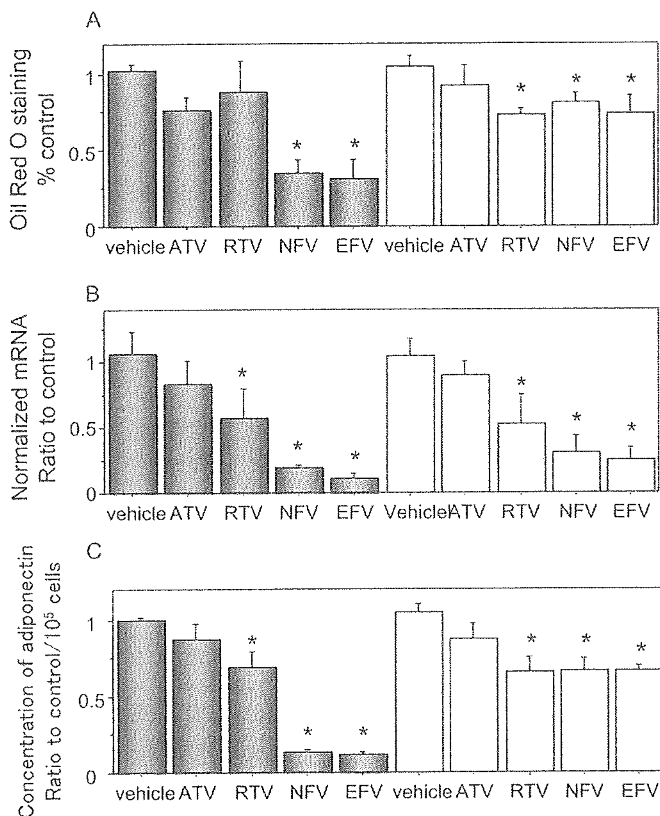

Figure 2. The effect of ARV in pre-adipocytes and mature adipocytes. From confluence (day 0), 3T3-L1 cells were treated with differentiation medium in the absence (control) or in the presence of vehicle or various ARVs. To compare the effects of ARVs on preadipocytes with mature adipocytes, ARVs were 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-L 1 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

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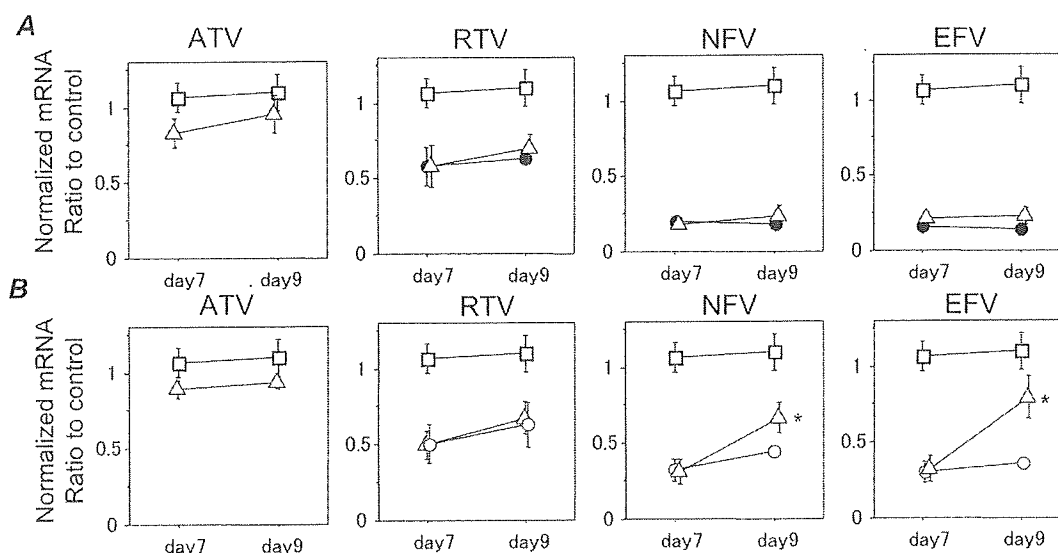

Figure 3. The effect of replacing ARVs with ATV on adiponectin mRNA levels in 3T3-L1 cells. From confluence (day 0), 3T3-L1 cells were treated with differentiation medium in the absence (control) or in the presence of vehicle (□) or various ARVs, which were added to the medium at day 0, when 3T3-L1 cells 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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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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