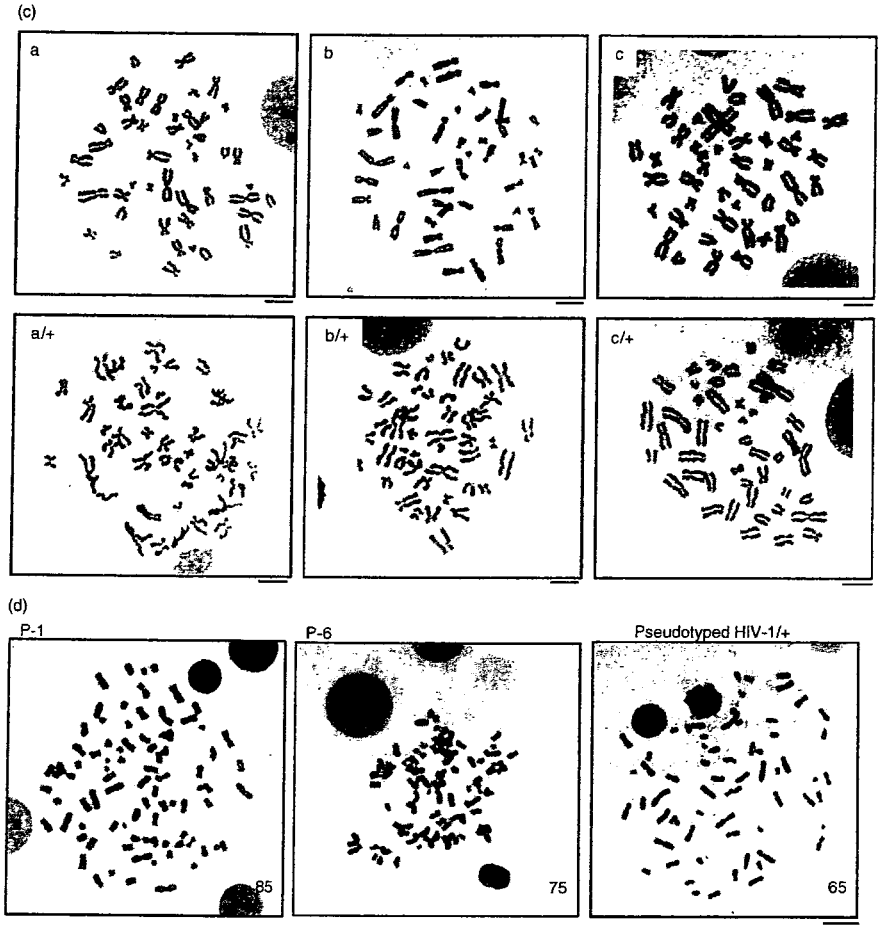

Fig. 1. *(continued)*

[12–14]. One of the major factors accelerating aneuploidy is thought to be abnormal chromatid separation [15–17]. At metaphase, paired sister chromatids are folded at the centric region until the onset of anaphase [18–22]. If the attachment of the sister chromatids is abolished before the onset of anaphase, premature sister chromatid separation (PCS) occurs. Subsequently, chromosome mis-segregation is induced, often resulting in aneuploidy [16,17]. PCS has been found in several clinical conditions, including aging, familial dominant inheritance [23–25], Roberts syndrome [26,27], cancer-prone syndrome mosaic variegated aneuploidy [28,29] and general tumours [30,31]. Note that all of these cases of PCS are associated with aneuploidy, indicating that a high PCS rate is a sign of chromosome instability. To investigate the cellular mechanism of HIV-1-related aneuploidy, we examined PCS in peripheral blood cells of HIV-1-infected individuals.

Peripheral blood was collected in sodium heparin (20 U/ml) from HIV-1-infected patients or healthy volunteers. We added 0.5 ml whole blood to 9.5 ml RPMI-1640 growth medium containing 10% fetal calf serum and 2% phytohemagglutinin M-form, and incubated it for 82 h at 37°C. Then colcemid (30 ng/ml) was treated for 2 h at 37°C. Recovered cells were resuspended in 75 mM potassium chloride and incubated for exactly 15 min at 37°C. To the cell suspension, freshly prepared Carnoy's solution (methanol : glacial acetic acid = 3 : 1) was added and mixed gently. After three changes of Carnoy's solution, a drop of the cell suspension was placed on a slide and air dried. Subsequently, the metaphase spread was stained with Giemsa.

Surprisingly, the HIV-1 patients examined showed PCS at high frequencies of 2.1 to 9.0% (mean ± standard deviation; 5.36 ± 2.92%; Fig. 1a, panels b–f and Fig. 1b). A high incidence of PCS was observed in HIV-1-infected individuals with high viral RNA copy numbers (Fig. 1b), in which total PCS was often observed (patient case no. 1 and no. 6; panels b and f). By contrast, peripheral blood mononuclear cells (PBMC) from healthy volunteers

showed normal attachments at the centromere (Fig. 1a, panel a), and PCS was detected in less than 2% ($1.22 \pm 0.48\%$).

We next clarified whether the PCS was attributable to HIV-1 infection. The PBMC (1.5×10^6) [32] were infected with vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1 [33] at the concentration of 2 ng/ml of p24 Gag antigen of pseudotyped virus (multiplicity of infection at 0.007). They were incubated for 82 h in the presence of 2% phytohemagglutinin M-form, and metaphase spread was analysed as described above. All of the specimens from three volunteers showed an increased incidence of PCS after HIV-1 infection (Fig. 1c, lower panels), whereas PCS was barely detectable without infection (Fig. 1c, upper panels). The frequencies of PCS after HIV-1 infection in the three samples were 8.40 ± 1.09, 5.28 ± 1.40, and 7.34 ± 1.67, whereas the frequencies without infection were 1.26 ± 0.40, 0.72 ± 0.22, and 1.68 ± 0.86, respectively. Our present data suggest that HIV-1 infection is a primary factor inducing PCS.

In the patients' case, the frequency of PCS was positively correlated with the reduction in total white blood cells (Pearson product–moment correlation coefficient $r = 0.837$, $P < 0.01$; Fig. 1b) rather than CD4 positive lymphocytes ($r = 0.011$, $P > 0.05$). Although VSV-G-pseudotyped HIV-1 was infected to PBMC at a multiplicity of infection of 0.007 (0.7%), the average incidence of PCS with HIV-1 infection exceeded 7%. Taken together with the information that pseudotyped HIV-1 induces a single round of infection, these data suggest that PCS occurs not only in response to the infection itself but also as a result of the effects of other virus products or cellular proteins stimulated by HIV-1 infection.

Simultaneously, we found aneuploidy in hyperploid cells of HIV-1-infected individuals who had high viral loads and high PCS frequency (Fig. 1b and Fig. 1d, left and middle panels). We also found aneuploidy in PBMC with HIV-1 infection *in vitro* (Fig. 1d, right panel). By contrast, aneuploidy was not found in control PBMC. Although it remains to be determined whether PCS is directly related to neoplasms in AIDS, we speculate that a high incidence of PCS and constitutive virus infection augment the susceptibility of the cells to aneuploidy and may play a critical role in the development of AIDS-related neoplasms. It will be important to track the epidemiological and biological features of the incidence of PCS in HIV-1 infection.

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[a]*Department of Intractable Diseases, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan;* [b]*Department of Pathology, National Institute of Infectious Disease, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan;* [c]*Center for Infectious Diseases, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan;* [d]*Department of Clinical Pathology, Research Institute, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan; and* [e]*Department of Internal Medicine, National Hospital Organization Chiba Medical Center, 4-1-2 Tsubakimori, Chuo-ku, Chiba 260-8606, Japan.*

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HIV-1 Vpr Induces DNA Double-Strand Breaks

Hiroaki Tachiwana,[1,2] Mari Shimura,[2] Chikako Nakai-Murakami,[2] Kenzo Tokunaga,[3] Yoshimasa Takizawa,[1,2] Tetsutaro Sata,[3] Hitoshi Kurumizaka,[1] and Yukihito Ishizaka[2]

[1]Graduate School of Science and Engineering, Waseda University; [2]Department of Intractable Diseases, International Medical Center of Japan; and [3]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan

Abstract

Recent observations imply that HIV-1 infection induces chromosomal DNA damage responses. However, the precise molecular mechanism and biological relevance are not fully understood. Here, we report that HIV-1 infection causes double-strand breaks in chromosomal DNA. We further found that Vpr, an accessory gene product of HIV-1, is a major factor responsible for HIV-1–induced double-strand breaks. The purified Vpr protein promotes double-strand breaks when incubated with isolated nuclei, although it does not exhibit endonuclease activity *in vitro*. A carboxyl-terminally truncated Vpr mutant that is defective in DNA-binding activity is less capable of Vpr-dependent double-strand break formation in isolated nuclei. The data suggest that double-strand breaks induced by Vpr depend on its DNA-binding activity and that Vpr may recruit unknown nuclear factor(s) with positive endonuclease activity to chromosomal DNA. This is the first direct evidence that Vpr induces double-strand breaks in HIV-1–infected cells. We discuss the possible roles of Vpr-induced DNA damage in HIV-1 infection and the involvement of Vpr in further acquired immunodeficiency syndrome–related tumor development. (Cancer Res 2006; 66(2): 627-31)

Introduction

A high incidence of malignant tumors, such as non-Hodgkin's lymphoma, Kaposi's sarcoma, and invasive cervical cancer [acquired immunodeficiency syndrome (AIDS)–defining cancers], is epidemiologically associated with HIV-1 infection (1, 2). These neoplasms are attributable mainly to diseases that accompany immunodeficiency, including coinfection with EBV, human herpes virus 8, and human papillomavirus (1, 2). In addition to these AIDS-defining cancers, several non-AIDS-defining cancers also occur with a higher incidence in HIV-infected individuals (3, 4). These reports lead to the assumption that HIV-1 has the potential to induce neoplasms before AIDS develops. Recently, DNA damage responses have been observed in precancerous lesion before inactivation of p53 (5, 6). Interestingly, it has been reported that HIV-1 infection induces DNA damage responses by activating Rad3-related or ataxia-telangiectasia mutated proteins and promoting phosphorylation of their downstream substrates (7, 8). The elucidation of the factor triggering the DNA damage responses to HIV-1 infection is essential to determine the as yet unknown mechanism causing AIDS-related neoplasms. In the present study, we found that HIV-1 infection induces double-strand breaks of chromosomal DNA, as detected using pulsed-field gel electrophoresis (PFGE). We further showed that *vpr*, an accessory gene of HIV-1 encoding a virion-associated nuclear protein, which induces cell cycle accumulation at G_2-M phase and increases ploidy (9), was a factor responsible for double-strand breaks. We discuss the potential ability of Vpr-induced double-strand breaks to develop into neoplasms in HIV-1 infection.

Materials and Methods

Cell culture. MIT-23 and ΔVpr, a mock transfectant, were established from HT1080 (JCRB9113; the Health Science Research Resources Bank) as previously described (9). In MIT-23, Vpr expression is controlled by the rtet promoter on incubation with 3 μg/mL doxycycline (Sigma, St. Louis, MO) for 48 hours.

Virus infection. Vesicular stomatitis virus G protein (VSV-G)–pseudotyped HIV-1 was produced by cotransfection with a plasmid encoding VSV-G (pHIT/G) and the pNL-Luc-E$^-$R$^+$ or pNL-Luc-E$^-$R$^-$ proviral clone (10). (10). The preparation and titration of viruses are described elsewhere (11). Briefly, the concentration of p24 antigen in the culture supernatant was measured using a p24 Gag antigen capture ELISA kit (ZeptoMetrix, Buffalo, NY). The infectivity of the prepared viral stock was examined using MAGIC5 cells. HT1080 cells were infected for 48 hours with viruses that had 200 ng/mL of p24 Gag antigen, giving a multiplicity of infection (MOI) of 0.7.

Immunostaining. Immunostaining was carried out as described (9). A rabbit polyclonal Rad51 antibody raised against the bacterially expressed protein and a mouse monoclonal antibody raised against synthesized peptides of full-length of Vpr (mAb8D1) were used as the primary antibody. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR) and goat anti-mouse IgG conjugated with Cy3 (Zymed Laboratories, Inc., San Francisco, CA) were used as the secondary antibodies. Images were captured on a phase contrast microscope, BX50 (Olympus Corp., Tokyo Japan), or a Radiance 2100 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Overexpression and purification of Vpr and its mutant. The HIV-1 *vpr* gene was ligated into the *Nde*I and *Bam*HI sites of the pET15b vector (Novagen, Madison, WI). The Vpr protein and VprΔC12 mutant were produced in the *Escherichia coli* BL21 (DE3) Codon(+)RIL strain (Novagen) by induction with isopropyl-β-D-thiogalactopyranoside (IPTG; Nacalai Tesque, Inc., Kyoto, Japan) and were purified as described in Supplementary Method. The concentration of the purified Vpr protein was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as the standard.

Isolation of nuclei. Cells scraped from culture dishes were washed once with ice-cold PBS and resuspended in 3 mL of ice-cold 20 mmol/L Tris-HCl buffer (pH 7.6) containing 60 mmol/L KCl, 15 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 250 mmol/L sucrose, 0.6% NP40, and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Hitoshi Kurumizaka, Graduate School of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, 169-8555 Tokyo, Japan. Phone: 81-3-5286-8189; Fax: 81-3-5292-9211; E-mail: kurumizaka@waseda.jp and Yukihito Ishizaka, Department of Intractable Diseases, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, 162-8655 Tokyo, Japan. Phone: 81-3-5272-7527; E-mail: zakay@ri.imcj.go.jp.

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protease inhibitor mixture (Sigma). The cell suspension was incubated for 10 minutes on ice and the sucrose concentration was adjusted to 1.6 mol/L. Then, the sample was loaded onto a sucrose cushion of 2.3 mol/L sucrose solution and centrifuged at 35,000 × *g* for 30 minutes. The isolated nuclei were obtained in the 2.3 mol/L sucrose fraction. For immunostaining, isolated nuclei were cytocentrifuged to the MAS-coated slide glass (Matsunami Glass IND., LTD., Tokyo, Japan) for 6 minutes at 800 rpm (Thermo Shandon, Chadwick Road, United Kingdom).

PFGE assay. Isolated nuclei were incubated with 10 μmol/L of purified Vpr or VprΔC12 for 15 hours at 30°C. The cells (isolated nuclei) were embedded in agarose plugs at a density of 3 × 10^5 cells/100 μL. The plugs were treated with proteinase K solution [0.5 mol/L EDTA (pH 8.0), 1% sarcosyl, and 0.5 mg/mL proteinase K] for 38 hours at 50°C. After PFGE was done in a CHEFF Mapper (Bio-Rad Laboratories), the gels were stained with Vistra Green (Amersham Bioscience, Piscataway, NJ).

The DNA-binding assay. The Vpr protein was incubated with ϕX174 single-stranded DNA (ssDNA; 20 μmol/L) or ϕX174 superhelical dsDNA (10 μmol/L) in 10 μL of 8 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA. The reaction mixtures were incubated for 1 hour at 37°C and were analyzed by electrophoresis on a 0.8% agarose gel in 1× TAE buffer (40 mmol/L Tris acetate and 1 mmol/L EDTA) at 3.3 V/cm for 2 hours. The bands were visualized using ethidium bromide staining.

Nuclease activity. The Vpr protein (18.8 μmol/L) or DNaseI (Invitrogen Corporation, Carlsbad, CA; 0.02 unit/μL) were incubated with ϕX174 superhelical double-stranded DNA (dsDNA; 2.5 μmol/L) in 40 μL of 15 mmol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L DTT and 100 μg/mL BSA, in the presence of 5 mmol/L $MgCl_2$, $MnCl_2$, $ZnSO_4$, or $CaCl_2$. The reaction mixtures were incubated at 37°C for 30 minutes. After incubation, the samples were treated with proteinase K (0.3 mg/mL) in the presence of 0.1% SDS and the DNA was extracted using phenol-chloroform. The DNA was precipitated by ethanol and was analyzed by electrophoresis on a 0.8% agarose gel in 1 × TAE buffer at 6.6 V/cm for 30 minutes. The bands were visualized with ethidium bromide staining.

The Ni-NTA agarose pull-down assay. Isolated nuclei were disrupted in 20 mmol/L Tris-HCl buffer (pH 8.5) containing 200 mmol/L KCl, 2 mmol/L 2-mercaptoethanol, 0.25 mmol/L EDTA, and 10% glycerol. The extract was incubated with His_6-Vpr (53 μmol/L) for 15 hours at 30°C. After incubation, His_6-Vpr was precipitated with 4 μL of Ni-NTA agarose beads and the beads were washed thrice with 500 μL of 20 mmol/L Tris-HCl buffer (pH 7.6) containing 100 mmol/L NaCl, 5 mmol/L DTT, 10 mmol/L imidazole, 1 mmol/L EDTA, and 0.2% Tween 20. The proteins precipitated with the Ni-NTA beads were analyzed by 16% SDS-PAGE. The bands were visualized by silver staining.

Results

Vpr expression induces chromosomal double-strand breaks. To test whether HIV-1 infection causes double-strand breaks, we used PFGE, which was able to clearly detect the double-strand breaks induced by X-ray irradiation (Fig. 1*A*, *lane 2*; ref. 12). HT1080 cells were infected with HIV-1 that had 200 ng/mL of p24 Gag antigen, giving a MOI of 0.7, and the cellular DNA was fractionated using PFGE. Figure 1*A* (*lane 6*) shows that HIV-1 infection induced double-strand breaks. Interestingly, the amount of HIV-1-dependent double-strand breaks was reduced significantly (Fig. 1*A*, *lane 5*) when the *vpr* gene was deleted from the HIV-1 viral genome (HIV-1ΔVpr). To show that HIV-1-dependent double-strand breaks are attributable to Vpr expression, we examined double-strand break formation in Vpr stable transfectant, MIT-23 (9), in which Vpr expression is controlled by the rtet promoter by doxycycline, and, in ΔVpr, a mock transfectant. As shown in Fig. 1*B*, double-strand breaks were observed in the Vpr-expressing cells (*lane 5, arrow*) but not in the mock transfectants (*lane 4*). Furthermore, Rad51 foci, which are formed

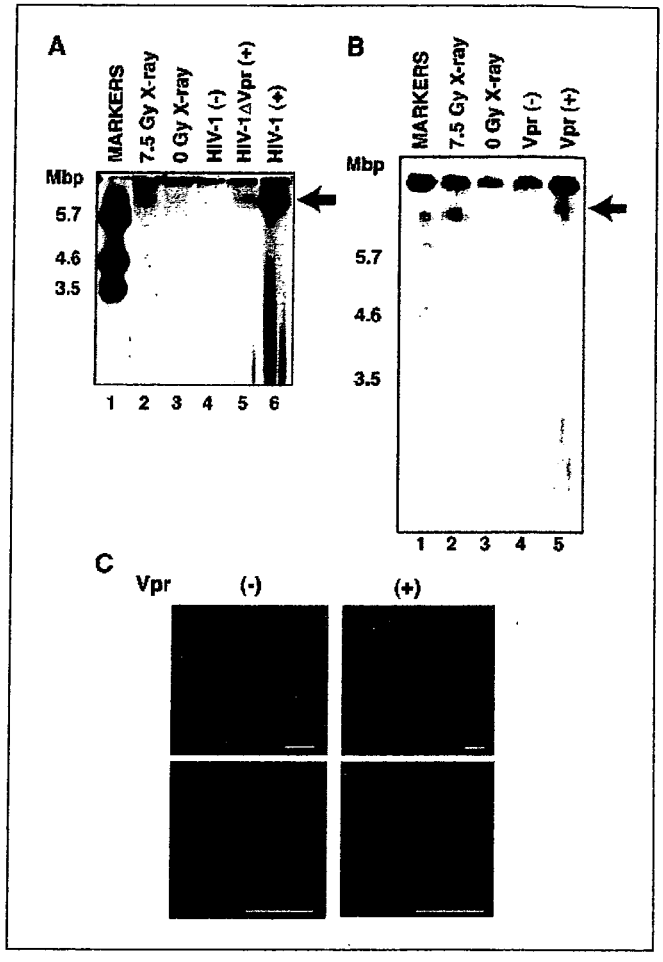

Figure 1. Vpr induces double-strand breaks *in vivo*. *A*, PFGE analysis of double-strand breaks after HIV-1 infection. HT1080 cells were infected with the same amount of HIV-1 or HIV-1ΔVpr (MOI = 0.7) and subjected to PFGE. As a positive control, uninfected cells were analyzed immediately after 7.5 Gy of X-ray irradiation. Molecular mass markers (*lane 1*), control cells (*lanes 3* and *4*), cells subjected to X-ray irradiation (*lane 2*), and cells infected with HIV-1ΔVpr (*lane 5*) or HIV-1 (*lane 6*) are shown. *Arrow*, position corresponding to the double-strand breaks. *B*, PFGE analysis in Vpr-expressing cells. Molecular mass markers (*lane 1*), cells irradiated with 7.5 Gy (*lane 2*), control cells (*lane 3*), mock transfectants (*lane 4*), and cells with Vpr expression (*lane 5*) are shown. *Arrow*, double-strand breaks. *C*, Rad51 focus formation with Vpr expression. An immunohistochemical analysis was used to detect Rad51 in cells with (*right*) or without (*left*) Vpr expression. Bar, 10 μm.

at double-strand break sites (13), were observed with Vpr expression (Fig. 1*C*). These results indicate that Vpr is responsible for double-strand break formation. The double-strand breaks shown in Fig. 1*B* were not the result of an apoptotic process as the DNA ladder typically observed in apoptotic cells (14) was not detected (data not shown).

Vpr has no endonuclease activity. Next, we studied whether Vpr directly induces double-strand breaks. The recombinant Vpr protein was purified to near homogeneity (Fig. 2*A*) and the DNA-binding activity of Vpr was examined. As shown in Fig. 2*B*, purified Vpr bound both ssDNA (*lanes 2-6*) and dsDNA (*lanes 8-12*) in an ATP- and Mg^{2+}-independent manner (15). Then, we examined whether Vpr has nuclease activity. Superhelical dsDNA containing small amounts of nicked circular dsDNA was incubated with Vpr in the presence of various divalent cations. After the incubation, the proteins were removed and the DNA was examined by

electrophoresis. If Vpr induces a double-strand break or nick, the superhelical dsDNA would give rise to linear or nicked circular forms, producing a different electrophoretic pattern. However, the DNA incubated with Vpr in the absence (*lane 2*) or presence of any divalent cation examined (*lanes 4, 6, 8,* and *10*) showed the same migration pattern with control (*lane 1*), indicating that Vpr does not cleave DNA (Fig. 2*C*). Positive control experiments showed that the DNA was digested by DNaseI with $MgCl_2$, $MnCl_2$, or $CaCl_2$ (*lanes 5, 7,* and *11*) but not with $ZnSO_4$ (*lane 9*; Fig. 2*C*). Therefore, these results indicate that Vpr lacks endonuclease or nicking activity.

Vpr induces double-strand breaks *in vitro*. In a second approach, we tested whether purified Vpr induces double-strand breaks in nuclei isolated from HT1080 cells (Fig. 3*A*). First, we confirmed by a laser confocal microscopy that Vpr localizes in nuclei after incubation *in vitro* (Fig. 3*B*). The nuclear DNA was then analyzed for double-strand breaks by using PFGE (Fig. 3*C*). Interestingly, purified Vpr induced double-strand breaks in the DNA of the isolated nuclei (Fig. 3*C*, *lane 5, arrow*). By contrast, few double-strand breaks were detected without Vpr (Fig. 3*C*, *lane 4*). Because Vpr alone did not show endonuclease activity (Fig. 2*C*), these results suggest that Vpr interacts with intrinsic nuclear protein(s), which required for double-strand break formation. To identify candidates for the Vpr-interacting nuclear proteins, we did the Ni-NTA pull-down assay. In this assay, recombinant His_6-tagged Vpr was incubated with the extract from isolated nuclei and Ni-NTA beads precipitated proteins bound to His_6-tagged Vpr (Fig. 3*D*). As shown in Fig. 3*D*, His_6-tagged Vpr associated with numerous proteins that were not detected in the control precipitates (*lane 2, asterisks*).

The DNA-binding activity of Vpr is correlated with double-strand break formation. The COOH-terminal region of Vpr is arginine rich and is thought to be an important site for DNA binding to Vpr (15). Nuclear magnetic resonance analysis shows that Vpr has three α-helixes (amino acids 17-33, 38-50, and 56-77) in solution, whereas the COOH-terminal region from amino acid residues 84 to 96 is disordered (16). This suggests that the deletion of the COOH-terminal 12 amino acid residues does not affect the tertiary structure of Vpr. We purified a Vpr mutant protein lacking the COOH-terminal 12-amino-acid residues (VprΔC12; Fig. 4*A*), and examined its DNA-binding activity. Purified VprΔC12 was significantly defective in both ssDNA- and dsDNA-binding activity compared with wild-type Vpr (Fig. 4*B*). Interestingly, VprΔC12 induced double-strand breaks in isolated nuclei but its efficiency was reduced significantly (Fig. 4*C*, *lane 6*). These results indicate that the DNA-binding ability of Vpr is important for the induction of double-strand breaks by Vpr.

Discussion

Here, we present evidence that HIV-1 Vpr induces double-strand breaks. Our data are consistent with previous observations in Vpr-expressing cells: the up-regulation of gene amplification events that are believed to be introduced by broken DNA strands (17) and the activation of activating Rad3-related/ataxia-telangiectasia mutated, followed by the phosphorylation of their downstream substrate, a histone H2A variant, H2AX, and γ-H2AX and BRCA1 focus formation (8). Biochemical analyses using purified Vpr indicated that Vpr alone has no endonuclease activity (Fig. 2*C*), suggesting that a cellular factor(s), possibly with endonuclease activity, is required for Vpr-dependent double-strand breaks. The factor(s) required for double-strand breaks must preexist in nuclei because double-strand breaks were observed upon incubating a mixture of isolated nuclei and purified Vpr *in vitro* (Fig. 3*C*). As one possible mechanism, Vpr may recruit a nuclease factor to chromosomal DNA, given that the Vpr-dependent double-strand breaks were correlated with the DNA-binding activity (Figs. 4*B* and *C*). Alternatively, Vpr itself may acquire endonuclease activity after modification in the nucleus. Further analyses are necessary to clarify this point.

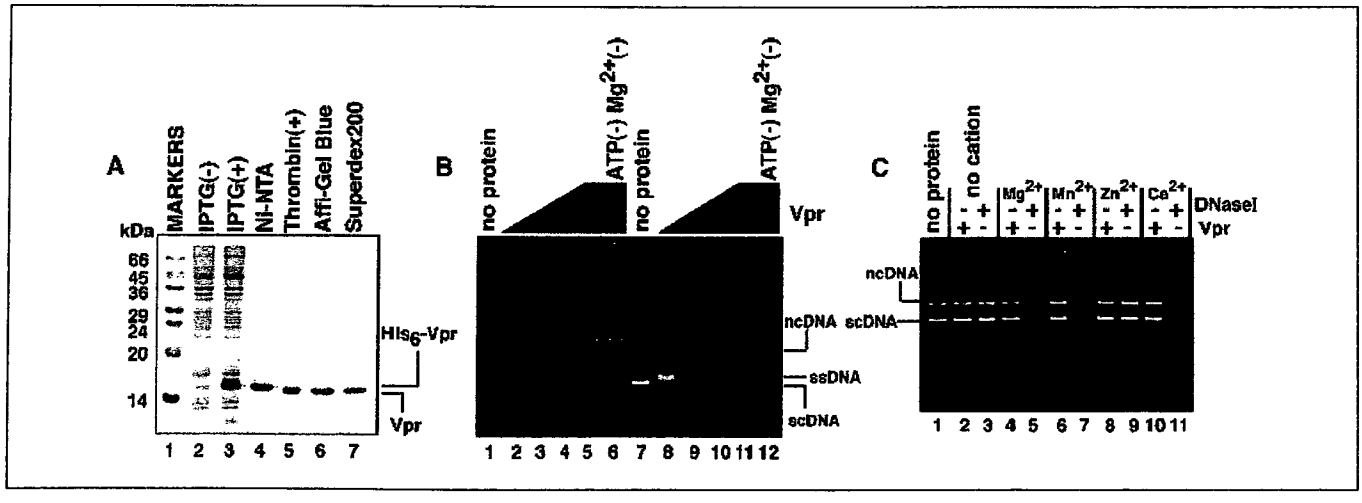

Figure 2. The Vpr-DNA interaction *in vitro*. *A*, purification of recombinant Vpr. Proteins from each purification step were analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. Molecular mass markers (*lane 1*), whole-cell lysates before (*lane 2*) and after (*lane 3*) induction with IPTG, samples from the Ni-NTA fraction (*lane 4*), the fraction after removing the hexahistidine tag (*lane 5*), the Affi-Gel Blue fraction (*lane 6*), and the Superdex 200 fraction (*lane 7*) are shown. *B*, the DNA-binding activity of Vpr. ϕX174 circular ssDNA (20 μmol/L; *lanes 2-6*) and ϕX174 superhelical dsDNA (*scDNA*; 10 μmol/L; *lanes 8-12*) containing a small amount of nicked circular DNA (*ncDNA*) were incubated with Vpr in the presence of 1 mmol/L ATP and 1 mmol/L $MgCl_2$. Control experiments without ATP and $MgCl_2$ (*lanes 6* and *12*) are included. The Vpr concentrations were 1.25 μmol/L (*lanes 2* and *8*), 2.5 μmol/L (*lanes 3* and *9*), 5 μmol/L (*lanes 4* and *10*), and 10 μmol/L (*lanes 5, 6, 11,* and *12*). *Lanes 1* and *7*, negative controls without protein. *C*, nuclease activity. ϕX174 scDNA (2.5 μmol/L) was incubated with Vpr (18.8 μmol/L; *lanes 2, 4, 6, 8,* and *10*) or DNaseI (*lanes 3, 5, 7, 9,* and *11*) in the absence of divalent cation (*lanes 2* and *3*) or in the presence of 5 mmol/L $MgCl_2$ (*lanes 4* and *5*), 5 mmol/L $MnCl_2$ (*lanes 6* and *7*), 5 mmol/L $ZnSO_4$ (*lanes 8* and *9*), or 5 mmol/L $CaCl_2$ (*lanes 10* and *11*). *Lane 1*, negative control without protein.

In the HIV-1 life cycle, DNA breakage and repair are thought to be essential steps for integrating the double-stranded viral cDNA into the host genome. In this study, we found that Vpr is one molecule responsible for the double-strand breaks that occur upon HIV-1 infection. However, it is also noteworthy that some double-strand breaks were induced in the cells with HIV-1ΔVpr (Fig. 1*A*, *lane 5*), suggesting that other viral factors are also involved. It has been shown that integrase activates the ataxia-telangiectasia mutated–dependent pathway (7) and, thus, the double-strand breaks observed with HIV-1ΔVpr infection are probably owing to integrase. For viral integration to occur, the amount of double-strand breaks induced by HIV-1ΔVpr (Fig. 1*A*, *lane 5*) may be sufficient, because viral production in peripheral blood mononuclear cells was not alleviated by infection with

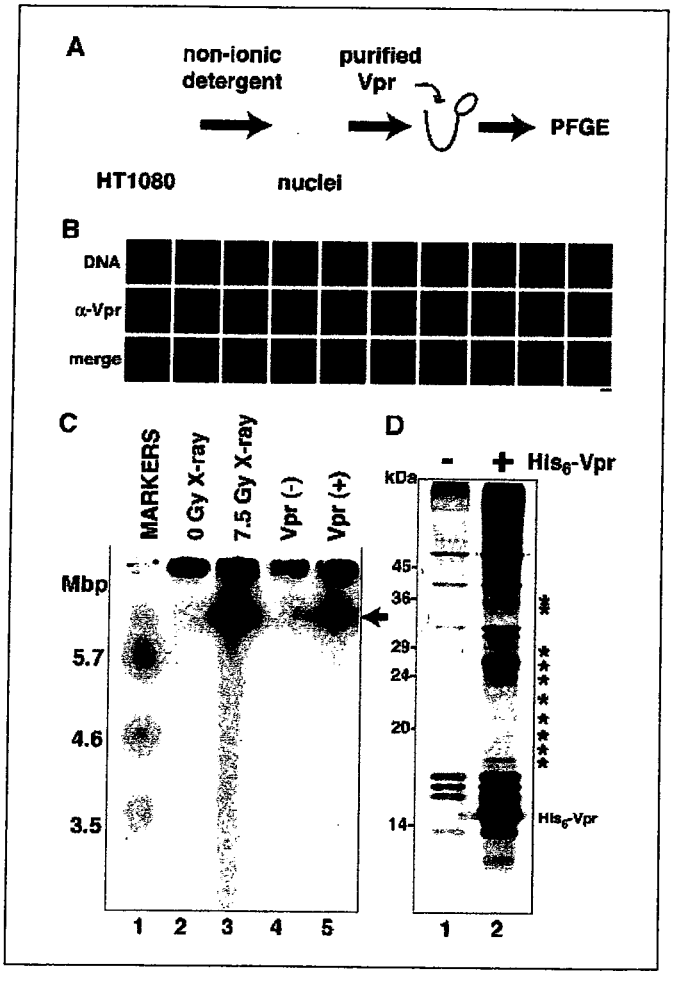

Figure 3. Purified Vpr induces double-strand breaks *in vitro*. *A*, a scheme of the protocol used to detect Vpr-induced double-strand breaks in isolated nuclei. *B*, Vpr localization in isolated nuclei. Isolated nuclei from HT1080 after incubation with Vpr were immunostained by α-Vpr (mAb8D1) and the images were captured by a laser confocal microscopy. The Z-series of optical sections collected at 1 μm steps of the cells were presented. Vpr (*red; middle*), DNA staining by Hoechst (*blue; top*) and their merged images (*bottom*) are shown. Without Vpr incubation, any signals by α-Vpr immunostaining were not detected in isolated nuclei (data not shown). Bar, 10 μm. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr. Molecular mass markers (*lane 1*), control cells (*lane 2*), cells subjected to X-ray irradiation (*lane 3*), and isolated nuclei without (*lane 4*) or with 10 μmol/L Vpr (*lane 5*). *Arrow*, double-strand breaks. *D*, Ni-NTA pull-down assay with His_6-tagged Vpr on isolated nuclei. Precipitated proteins bound to His_6-tagged Vpr (*lane 2*) and the control precipitates (*lane 1*) are indicated. *, His_6-Vpr–specific bands.

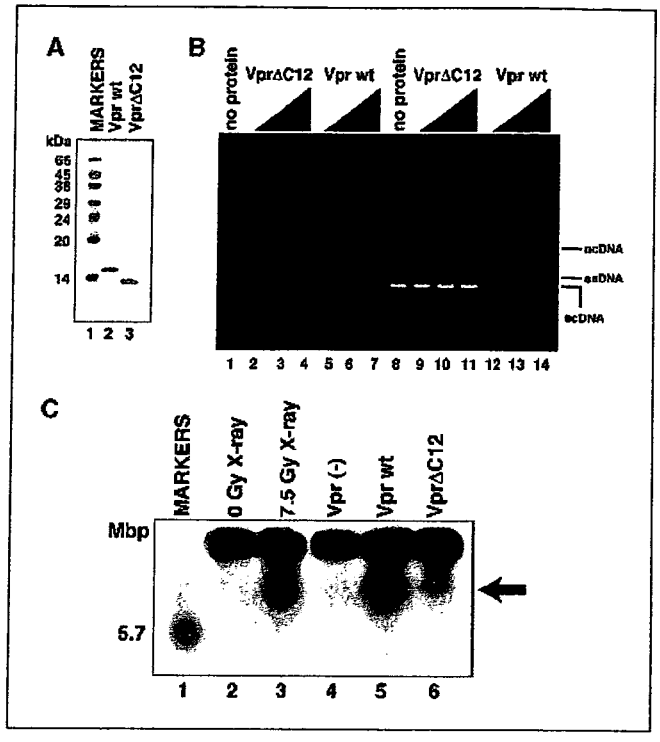

Figure 4. DNA-binding and double-strand break formation by Vpr. *A*, purification of VprΔC12. Purified VprΔC12 was analyzed using 16% SDS-PAGE with Coomassie brilliant blue staining. *Lane 1*, molecular mass markers. *Lanes 2* and *3*, purified wild-type Vpr and VprΔC12 protein, respectively. *B*, the DNA-binding activity of VprΔC12. The DNA-binding experiments were done using the protocol used to obtain Fig. 2*B*. The concentrations of VprΔC12 were 2.5 μmol/L (*lanes 2* and *9*), 5 μmol/L (*lanes 3* and *10*), and 10 μmol/L (*lanes 4* and *11*), and those of the wild-type Vpr were 2.5 μmol/L (*lanes 5* and *12*), 5 μmol/L (*lanes 6* and *13*), and 10 μmol/L (*lanes 7* and *14*). Negative controls without protein (*lanes 1* and *8*) are included. *C*, PFGE analysis of double-strand breaks in isolated nuclei treated with Vpr or VprΔC12. Molecular mass marker (*lane 1*), cells without (*lane 2*) or with (*lane 3*) 7.5 Gy of X-ray irradiation, control nuclei (*lane 4*), nuclei with Vpr (*lane 5*), and nuclei with VprΔC12 (*lane 6*). Vpr was used at 10 μmol/L. *Arrow*, double-strand breaks.

Vpr-deleted HIV-1 (18).[4] Vpr-induced double-strand breaks may be surplus to those required for viral integration (Fig. 1*A*, *lane 6*). The resultant DNA damage may reduce the integrity of the host genome.

Recently, DNA damage signaling was observed at an early stage of tumor development, suggesting that the DNA damage response is a mechanism to prevent the progression of pre-neoplasic lesions (5). If DNA repair is not accomplished correctly or is skipped because of unregulated checkpoint controls, the genomic structure would be altered severely (19). The progression of malignant tumors in AIDS-defining cancers is well documented in oncovirus infections (1, 2). If DNA damage increases the probability of neoplasia, Vpr-induced double-strand breaks with oncovirus infection may accelerate tumor progression during the clinical course of AIDS. In addition to AIDS-defining cancers, non-AIDS-defining cancers also occur at a higher incidence and the factor responsible for such oncogenesis is now a critical issue (3, 4). Vpr-induced DNA damage may result in

[4] M. Shimura, unpublished data.

these AIDS-related malignancies. It is essential to explore the molecular mechanism of Vpr-induced double-strand breaks to clarify their role in HIV-1 infection and their effect on the stability of the host cell genome.

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【HIV-1感染に伴う染色体異常】

Chromosomal abnormalities in HIV-1 infection

星野　重樹・志村　まり・石坂　幸人
Hoshino　Shigeki　Shimura　Mari　Ishizaka　Yukihito

Key words
HIV-1 vpr, NADCs; Non-AIDS defining cancers, Chromosomal abnormality

要　約

エイズ症例では悪性腫瘍を高頻度に認める。Highly active anti-retrovirus therapy（以下HAART）の導入によりHIV-1感染症による日和見感染症のコントロールが可能になり，カポジ肉腫など癌の発症率は激減した。しかし近年，non-AIDS defining cancers（NADCs）と呼ばれる一群の悪性腫瘍が，免疫不全を示さないHIV-1感染者で報告されて以来，HIV-1感染による癌化機序が問われている。NADCsとして，皮膚がん・肛門周囲腫瘍・ホジキンリンパ腫や前立腺癌などが10万人に対して980人の頻度で発症する。なぜか？本編ではHIV-1感染に伴って誘導される染色体異常に関する知見を紹介するとともに，私達が解析してきたVprによるゲノム不安定性についてを紹介する。

はじめに

Highly active anti-retrovirus therapy（以下HAART）の導入により，HIV-1感染症の予後決定因子であった日和見感染症は激減した。しかし近年，HIV-1感染者に必ずしも免疫不全を伴わないnon-AIDS defining cancers（以下NADCs）が認められることが分かるとともに，NADCsがHIV-1感染者の予後を決定する重要な病態として注目されている。私達は，1997年の解析当初からHIV-1を「癌ウイルス」と想定し，ウイルス感染そのものによる悪性腫瘍誘発の可能性を検証してきた。

本編ではNADCsに関する現状を紹介し，ウイルス感染に伴うゲノム不安定性の様子とその機序について私達の知見を供覧する。

1.　増え続けるHIV-1感染者とウイルス感染に伴うゲノム不安定性

厚生労働省「エイズ動向委員会」の報告によると，2004年の我が国の新規感染者数はほぼ一千人で，特に10-20代の若者を中心としながら依然増加傾向にある。複数の薬剤を組み合わせたHAARTにより，患者予後は飛躍的に改善されてはいるものの，現行HAARTでは体からウイルスを完全には駆逐することができない。即ち，HIV-1感染者のフォローは生涯必要であり，長期的な予後を考える上で，悪性腫瘍が最も重要な予後決定因子となる。

エイズ症例において高頻度に癌が発症することは以前から指摘されており，HIV-1感染者の発癌リスクは健常人よりも60倍以上高いことが提唱されてきた[1]。しかし，このような悪性腫瘍は一般的には，エイズ病態下での免疫担当細胞の機能不全により誘発されるものと考えられ，事実，カポジ肉腫に見るように免疫不全に伴ってヘルペスウイルスが感染し，これが肉腫発症機序の一因となっている場合も

国立国際医療センター研究所 難治性疾患研究部：Department of Intractable Diseases, International Medical Center of Japan
〒162-8655　東京都新宿区戸山1-21-1　FAX：03-3202-7364　E-mail：zakay@ri.imcj.go.jp

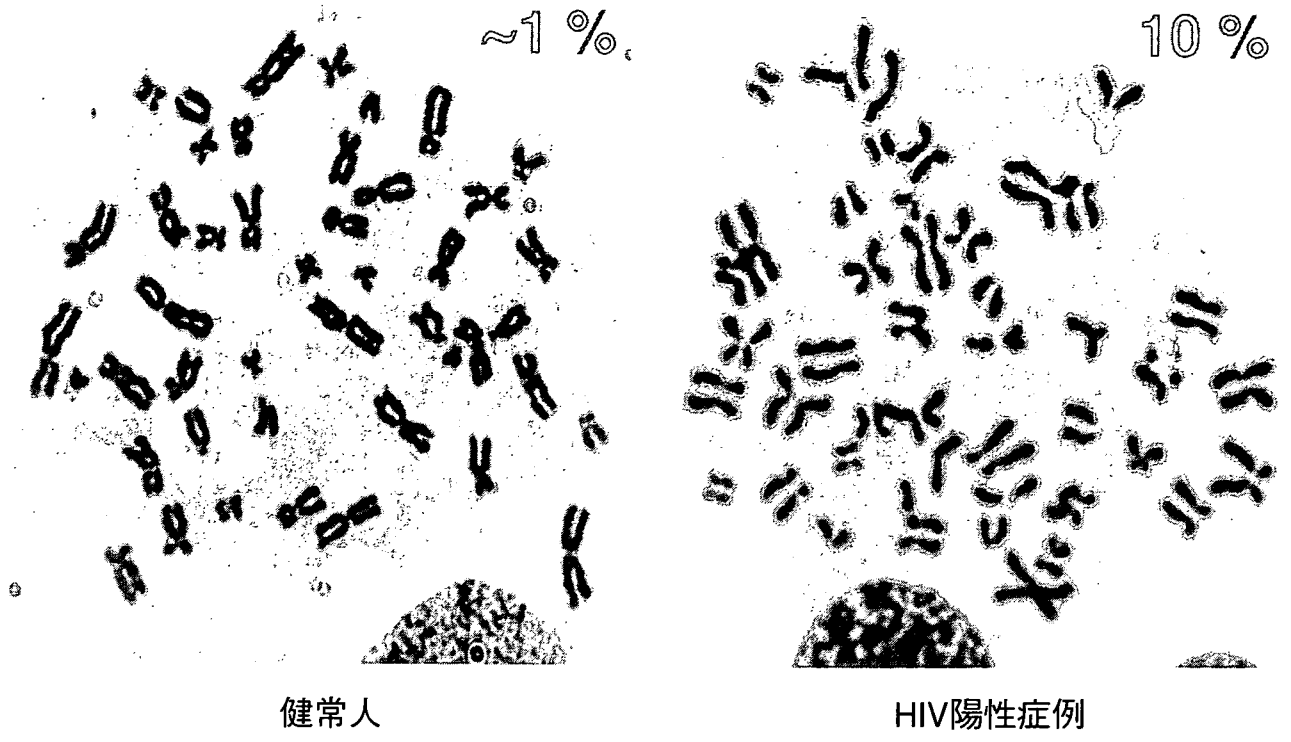


図1　HIV-1感染に伴う姉妹染色分体の早期分離。
健常人由来末梢血単核球細胞に試験管内で，ウイルスを感染させた後，分裂期染色体のギムザ染色による観察を行った。健常人由来末梢血単核球細胞の姉妹染色分体はセントロメア領域で束ねられているX字型を呈している（左図）が，感染後の細胞では，姉妹染色分体は離れて存在している（早期分離，右図）。その頻度は，健常人由来では１%未満，感染後の細胞では10%と高頻度であった。

ある。HAART導入後，著しい免疫不全病態は回避できるようになり，カポジ肉腫の頻度は激減した。しかし，近年行われたコホート研究により，免疫不全を示さないHIV-1感染者でも高頻度に癌の発症が認められることが指摘された[2]。NADCsの頻度は10万人当たり約980人と試算され[2]，皮膚がん，肛門周囲腫瘍，ホジキンリンパ腫や前立腺癌などが認められる。また，悪性腫瘍を初期症状として外来に訪れるHIV-1症例もあることから，今後，臨床の現場でも無視できない疾病概念となることが予測される。

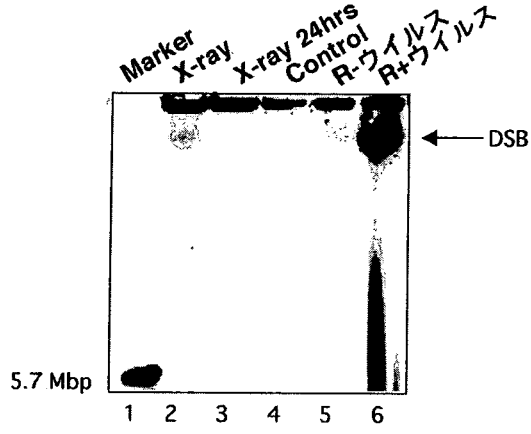
HIV-1陽性患者では，染色体転座，遺伝子再構成，p53の遺伝子変異など，様々なゲノム不安定性が検出され，悪性腫瘍発症との積極的な関連が指摘されている[3),4)]。また，ゲノム不安定性の要因である染色体異常がHIV-1陽性者の末梢血細胞中でも検出されている[3),4)]。私達はHIV-1陽性者の末梢血細胞をPHA存在下で培養すると早期姉妹染色分体分離（PCS; premature sister chromatid separation）が誘発され（図1，右図，姉妹染色分体が解離している），染色体の異数倍体化も生じることを見出した[5)]。また，健常人由来末梢血細胞に試験管内でHIV-1ウイルスを感染させた場合でも，同様にPCSが誘導されたことから，HIV-1自身に癌ウイルス様の機能があることが示唆された。この発見は，試験管内で感染させた不死化細胞がマウスに対して造腫瘍性を示した実験結果と良く合致する[6)]。

2.　HIV-1遺伝子Vprによるゲノム不安定性

当研究部は，HIV-1遺伝子の一つであるVpr（Viral protein R）に早期から着目し，その解析を行

2a.

2b.



2c.

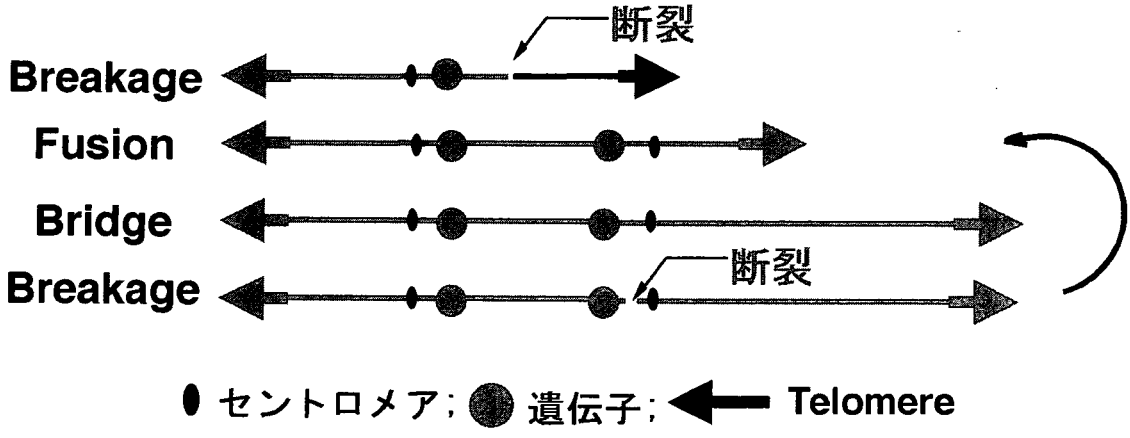


図2　VprによるDNA二重鎖切断

2a. Vprによる遺伝子増幅. コントロール（左），Vpr発現後（中央及び右図）のFISH解析結果を示す。ドットが増幅した遺伝子である。単一の染色体上に幾つもの遺伝子シグナルが検出される（中央図）。また，二動原体染色体を有する細胞も検出される（右図）。

2b. Vprプラスウイルス（図中R+ウイルス，レーン6）を培養細胞に感染させ，パルスフィールド電気泳動法にて解析した。陽性コントロールとしてX線照射後の細胞も解析した（レーン2）。矢印で示す位置に，高分子DNAよりも早い泳動度を示すDNAの塊が検出された。

2c. BFBサイクルの模式図. DSBが生じると染色体の融合が起こり，二動原体染色体が形成される。分裂期に動原体が娘細胞の両極に牽引され，物理的に切断されることでDSBが生じる。この部位を起点として再度このサイクルが開始し，何回も繰り返される事で遺伝子増幅が生じる。

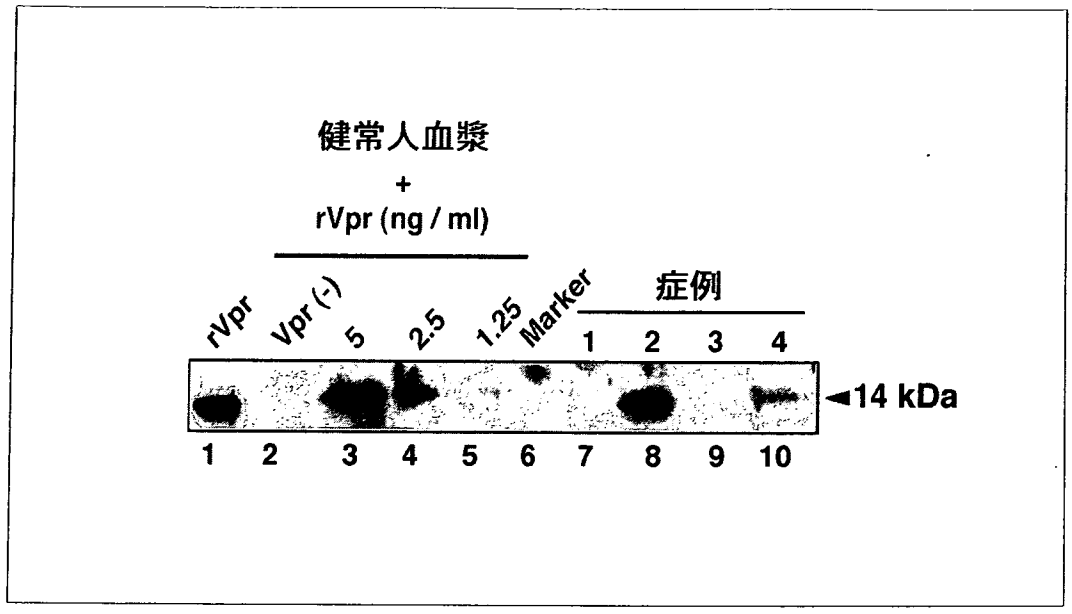


図3　HIV-1陽性者血中Vprの検出.
Vprに対する2種類の単クローン抗体を用いて免疫沈降-ウエスタン解析法を行った。52例中20例に分子量14 kDaのVpr を検出した。代表的な症例を示す(レーン7-10)。その濃度は最大10 ng/ml(0.7 nM)であった。種々の量の精製rVpr標品を健常人由来血漿に添加し，同様の解析を行い，得られたシグナルの強度を比較することで患者血漿中Vpr量を半定量した(レーン2-5)。

って来た。Vprは96個のアミノ酸からなる約14 kDaの核蛋白質で，究めて多くの宿主側蛋白質と結合する。当初，Vprは細胞周期のG2期における細胞増殖抑制を示すことが知られていた。しかし，Vprの機能を詳細に解析した結果，G2期の異常に加えて，M期の異常も誘導することを見いだした。さらにVpr発現を誘導した細胞中には，染色体の数の異常や小核の形成，遺伝子増幅(図2a，中央図)，中心体の数の異常等，様々なゲノム不安定性を認めた[7]。さらにここ1-2年の解析で，VprがゲノムDNA二重鎖切断(以下DSB: double strand breaks)を誘発することを証明した[8),9]。図2bは，DSBの様子を示した実験結果で，Vprプラスのウイルスを培養細胞に感染させると（レーン6），X線照射後（レーン2）に検出されるのと同じように早い泳動度を示すDNAの塊が生成された（図2b,矢印)。

遺伝子増幅とDSBは一見，関連性の無い現象のように見える。しかし，染色体上の広い領域にわたって生じる遺伝子増幅にはDSBが重要な役割を担っており，その機序を理解するためのモデルとしてbreakage-fusion-bridge（BFB）サイクルが提唱されている。

BFBサイクルは1942年，Barbara McClintockにより提唱されたダイナミックな染色体の動きで，トウモロコシの分裂の様子を詳細に観察することで発見された[10]。図2cに簡単な模式図を示す。染色体上に損傷DNAが生じると二動原体染色体が形成される(BreakageとFusion)。そしてそれぞれの動原体は，次の細胞分裂の際に娘細胞側に牽引され，対極に移動する。その結果，娘細胞間で一旦ブリッジが形成されるが（Bridge)，分裂が進む過程でこのブリッジに断裂が生じ（Breakage)，再び二動原体染色体が形成される。

このサイクルが繰り返されることで染色体上の広い領域に亘って再構成が進む。例えば薬剤耐性遺伝子のように増殖を支持する遺伝子が染色体上に局在すれば，結果として「遺伝子増幅」となる。BFBサイクルが遺伝子増幅に関与していることを示唆する重要な所見は，二動原体染色体の存在であるが，Vpr発現により得られた遺伝子増幅陽性細胞中に二

動原体染色体を認めている(図2a，右図)。余談だが，このようにゲノムがダイナミックに動く事を発見したMcClintockは，1983年「可動遺伝子の発見」でノーベル生理学・医学賞を受賞した。

3. 患者血中Vpr濃度の把握とリコンビナントVpr蛋白質(rVpr)によるDSB誘発

このような恐ろしい機能を有するVprが，HIV-1感染者の血液や脳脊髄液などの体液中に存在することが報告されてきた。しかし，その濃度については不明であった。そこで，52例の患者血漿について2種類の単クローン抗体による免疫沈降-ウエスタン解析を行った。その結果，20症例にVprを検出し，その濃度は数ng/ml（最大濃度1 nM）であった(図3)[11]。さらに，大腸菌で発現・精製したrVprを数nMの濃度で健常人末梢白血球細胞の培養系に添加すると，DSBの指標であるATM (ataxia telangiectasia mutated）のリン酸化型蛋白質のフォーカス形成が誘導された[9]，(星野: 未発表データ)。

HIV-1感染者で認められる悪性腫瘍の主たる病型はB細胞性リンパ腫であるが，B細胞はCD4を発現しないためウイルスは感染しない。また，EBウイルス感染を示す頻度は悪性リンパ腫症例の60%で，EBウイルス非感染者の中には免疫不全を示さない症例も見いだされている[3]。即ち，B細胞性リンパ腫の発症にはEBウイルス以外の因子も関与していることが予測される。

このような背景の中で，私達はVprによる染色体異常とDSBに特に注目している。血液中に存在するVprは体中の細胞に対してDSBを誘導し得る。さらに，Vprはマクロファージからある種のサイトカインを産生させることも分かっており，このサイトカインが前癌細胞の増殖を支持する可能性も考えられる。現在，HIV感染患者における悪性腫瘍発症のモデルとして，VprによるB細胞性リンパ腫の誘発能を解析中である。HIV感染に関連するB細胞性リンパ腫発症では，c-myc遺伝子と免疫グロブリン遺伝子間の遺伝子組み替えを認める[4]。このような現象はX線照射後に形成される悪性リンパ腫の発症過程でも生じる異常な遺伝子再編である。VprがX線照射のようにDSBを誘導することから，B細胞に同様な異常な染色体転座を誘発する可能性は充分に考えられる。

おわりに

「有効な薬剤が開発された現在ではHIV-1感染は怖くない」という誤った考え方をする若者がもしいるとしたら，その方々には是非，「HIV-1感染と一見関連性のないように考えられてきた悪性腫瘍の発症が，現在では重要な問題となりつつある」ということを理解して頂きたいと願う。私達が本職とするエイズ病態の研究も大切であるが，何よりも大切なことはHIV-1感染予防であることをご理解頂けたら有り難い。

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Socioeconomic and Reproductive Factors Associated with Condom Use Within and Outside of Marriage Among Urban Pregnant Women in Zambia

Chipepo Kankasa, MD, PhD[1], Margaret Siwale, MD, PhD[1], Francis Kasolo, MD, PhD[2], Ayako Nishiyama, MD, MHSc[3], Hiroshi Terunuma, MD, PhD[4] and Naomi Wakasugi, MD, PhD[3]

Abstract

A cross-sectional questionnaire survey was conducted on 470 pregnant women in Lusaka, Zambia. Multivariate analysis revealed school attendance and child deaths as independently significant variables positively associated with HIV seropositivity. Among women with fidelity, HIV prevalence was not significantly lower, and condom use was much lower than among women who were having extramarital affairs. Factors significantly associated with condom use within and outside of marriage differed–age and number of live births within, and sexual transmission knowledge outside of marriage. School attendance was not effective for gaining knowledge on sexual transmission or condom use. Regular own earning was significantly effective for condom use in both groups, irrespective of school attendance. The following should be implemented intensively: effective education on HIV and sex in and out of school before early sexual debut, further implementation of family planning with emphasis on condom use, and empowering women by assisting with their economic independence. (*Afr J Reprod Health* 2005; 9[3]:128-136)

Résumé

Facteurs de reproduction et socio-économiques liés à l'usage du condom dans le mariage et hors de mariage chez les femmes urbaines enceintes en Zambie. Une enquête transversale basée sur un questionnaire a été menée auprès de 470 femmes enceints à Lusaka en Zambie. Les analyses multivariables ont montré que la fréquentation à l'école et la mortalité infantile sont des variables importants liés à la séropositivité du VIH. Parmi les femmes fidèles, le niveau de fréquence n'était pas inferieur de manière significative et le taux de l'usage du condon était moins élevé chez elles que chez les femmes qui trompaient leurs maris. Il y avait une différence à l'egard de l'âge et nombre de naissance vivantes dans le mariage et la connaissance de la transmission sexuelle hors de mariage ou l'usage du condom. La fréquentation à l'école n'était pas effective quant à l'acquisition de la connaissance sur la transmission sexuelle ou l'usage du condom. Un salaire personnel et régulier bien efficace pour l'utilisation du condom pour les deux groupes, que l'on fréquente l'école ou non. Il faut une mise en oeuvre rigoureuse des propositions suivantes: une éducation efficace du VIH et de la sexuelité dans l'école et hors de l'école avant le commencement précose de la vie sexuelle, une réalisation davantage de la planification familiale en mettant l'accent sur l'utilisation du condom et la capabilisation de la femme en l'aidant à accéder à l'independance économique. (*Rev Afr Santé Reprod* 2005; 9[3]:128-136)

Key Words: *Zambia, HIV, Condoms, Extramarital relationships, Income*

[1]*Department of Pediatrics, University Teaching Hospital, Lusaka, Zambia.* [2]*Virology & Immunology Laboratory, University Teaching Hospital, Lusaka, Zambia.* [3]*Graduate School of Political Science, Waseda University, 1-6 Nishiwaseda, Shinjuku-ku.* [4]*Department of Microbiology, Yamanashi University School of Medicine, Yamanashi, Japan*

CORRESPONDENCE: Naomi Wakasugi, MD, PhD, Professor, Graduate School of Political Science, Waseda University, 1-6 Nishiwaseda, Shinjuku-ku, Tokyo, 169-8050, Japan. E-mail: nwakasu@ri.imcj.go.jp Tel: 81-3-3202-7181,ext.2857; Fax: 81-3-3202-7364;

Introduction

The global burden of HIV infection is increasingly shifting to women and children.[1] This trend is a real and serious threat, particularly in Africa where infected females outnumber infected males[1,2], and the fertility rate is very high[3]. Zambia is no exception, since the estimated HIV prevalence rates among young people (15–24 years of age) are 16.8% – 25.2% for women and 6.5% – 9.7% for men[4]. Furthermore, 20% – 27% of the approximately 400,000 women who deliver every year in Zambia[5] are estimated to be HIV-positive. Consequently, around 30,000 to 40,000 Zambian infants are thought to contract HIV by vertical transmission every year.

Among the foremost reasons cited for the increased vulnerability of women to HIV infection is the higher efficiency of male-to-female HIV transmission than the reverse that has been shown in developed countries[6,7]. In addition to this universal reason, many other factors seem to play a role in African countries. The high rate of undiagnosed and untreated STI (sexually transmitted infection)[8] and the high frequency of transfusion of contaminated blood during high-risk deliveries under the insufficient safe blood policy[9] are increasing the biologic risk of HIV infection among African women.

The socioeconomic and cultural conditions faced by women, such as poverty, economic dependence, socio-sexual subordination, and power inequity, are also considered to contribute to their increased vulnerability to HIV infection[10,11]. Male resistance to condom use and women's inability to negotiate safer sex[12] are considered to be major obstacles to the reduction of non-protective sex. The promotion of condom use within marriage might be the most difficult challenge in this society, where child bearing is of great value. However, it should be more targeted, in order to reduce the advancing deadly threat of a generalized HIV epidemic in women and children.

Confronted with a very high prevalence of HIV infection among pregnant women in Zambia, we decided to investigate the factors related to women that facilitate condom use to help women protect themselves against contracting HIV infection.

Methods

The present study was performed at a primary-level health clinic offering antenatal services, located in an urban area of Lusaka city. The clinic covered a population of 120,000 people living in a compound named Chipata, with an estimated 6,400 annual pregnancies. The clinic is one of six antenatal clinics at which the national PMTCT (Prevention of Mother to Child Transmission) programme was conducted between 2000 and 2002, with the support of UNICEF/UNAIDS.

The pregnant women on their first attendance at antenatal care were invited to individual pre-test counselling after a brief explanation of the VCT Voluntary Counselling and Testing and PMTCT programme. Our cross-sectional questionnaire survey was given before the start of individual pre-test counselling. Zambian female staff trained for this study interviewed the women individually, using a structured questionnaire. After counselling, the consent of each woman for serologic testing of HIV was sought. Each woman was assigned a serial number in addition to the number on her clinical record sheet. HIV status was checked thereafter using the serial number, and the confidentiality of each individual was strictly respected.

The questionnaire included items aimed at defining the women's socioeconomic characteristics (age, recognition of own and husband's HIV sero-status before this VCT, years of school attendance, own earning, condom use) and reproductive profile (number of pregnancies, live births, abortions, and child deaths). The women were also asked which body fluid could transmit HIV among blood, breast milk, semen, and vaginal secretion. As for condom use, respond-

ents were asked how often their husbands and/or their partners used condoms in the past. The term 'partner' in this study was defined as sexual partner excluding husband. Each answer was classified into three categories, respectively: never use, sometimes use, and always use. Regarding sexual relationship, we classified the respondents into three groups according to their answers to two questions on condom use: 'only husband' for those who answered only the question for condom use with husband, 'husband and partner' for those who answered both questions for condom use with husband and with partner, and 'only partner' for those who answered only the question for condom use with partner. The group 'only husband' indicates, in its broad sense, women with fidelity, and the other group 'husband and partner' indicates women who were having extramarital affairs.

The sampling was conducted consecutively between November 2000 and May 2001. The HIV sero-status was determined at the laboratory attached to the clinic using an HIV-1&2 rapid test kit (ABBOTT), and confirmed using another test kit (BIONOR or GenieII). The results were announced to the women two days after, and then they received post-test counselling. Ethical approval for the study was obtained from The Zambian Government/University of Zambia Research Ethic Committee.

Data analysis

Associations between HIV sero-status and socio-economic/reproductive characteristics were examined by the chi-square test and unpaired Student's t-test. School attendance was divided into seven categories and subjected to the following analyses. The risk factors significantly associated with HIV seropositivity were identified by univariate analyses. Multivariate logistic regression analysis adjusted for those factors was then performed to identify variables that had a significant independent association. The multivariate logistic regression analyses for condom use were performed to two outcomes, one with the husband, and the other with partners. Since no subject answered that she always used condoms with her husband, we dichotomised the remaining two answers, never use and sometimes use. As for condom use with partners, the answers sometimes use and always use were combined together and used for further analysis. Statistical analyses were conducted using SPSS software, version-10.

Results

The general characteristics of the surveyed population, together with HIV seropositivity and its risk factors, are summarized in *Table 1*. A total of 490 pregnant women agreed to be tested for HIV. The rate of HIV test acceptance was 49.1%. Twenty cases were eliminated due to lack of serologic data (i.e., missing blood sample, one; rapid test declined, two; data not recorded, 17). Consequently, 470 pregnant women aged 15 to 44 were enrolled for analyses. The overall HIV prevalence was 24.5% (95% confidence interval [CI]=20.6–28.4). Only 6.2% were aware of their own HIV status before undergoing HIV testing, including two women who knew of their HIV infection and 27 who knew that they were HIV negative. Similarly, 5.7% of women were aware of their husband's HIV status (data not shown). A feature of age-specific prevalence of HIV infection was a slightly higher rate among women aged 15–19 years than that recorded in the sentinel surveillance study conducted in the same Lusaka city in 1998 (14.8% in 1998, 16.2% in the present study)[13]. The age of women was associated significantly with HIV seropositivity by univariate, but not by multivariate analysis. The prevalence of HIV infection increased significantly with increase in the years of school attendance by both univariate and multivariate analyses. The rate of correct answers on the routes of HIV transmission were 97.2% for blood, 80.8% for breast milk, 65.2% for semen, and 62.6% for vaginal secretion (data not shown). Thus, a gap

Table 1. **Characteristic of pregnant woemn and analyses of risk factors for HIV seropositivity**

Characteristics	N	%HIV +ve	Univariate analysis OR (95%CI)	P-value	Multivariate analysis AOR (95%CI)	P-value
Age (n=464)						
15-19	105	16.2	1.04 (1.00-1.08)	<0.05	1.05 (1.00-1.11)	0.09
20-24	185	22.7				
25-29	108	31.5				
30-39	60	30.0				
40+	6	16.7				
School attendance[a] (n=467)						
0 yr	38	18.4	1.17 (1.01-136)	<0.05	1.22 (1.05-143)	<0.05
1-2 yr	14	14.3				
3-4 yr	67	22.4				
5-6 yr	86	23.3				
7-8 yr	163	23.3				
9-10 yr	80	31.3				
11 yr+	19	42.1				
Sexual transmission knowledge[b] (n=468)						
wrong	163	25.2	1.00			
right	305	23.9	0.94(0.60-1.46)	0.8		
Own earning (n=454)						
regularly	88	23.9	1.00			
irregularly	60	26.7	1.16 (0.55-2.47)	0.7		
none	306	24.2	1.02 (0.58-1.77)	1		
Live births (n=468)						
none	128	18.0	1.00	1.00		
1	127	29.9	1.95 (1.08-3.52)	<0.05	1.52 (0.81-2.88)	0.2
2+	213	25.4	1.55 (0.90-2.68)	0.1	0.93 (0.43-2.00)	0.8
Abortions (n=470)						
never	422	24.6	1.00			
experienced	48	22.9	0.91 (0.45-1.85)	0.8		
Child deaths (n=470)						
never	351	22.2	1.00	1.00		
experienced	119	31.1	1.58 (1.00-2.51)	0.05	1.69 (1.01-2.83)	<0.05
Condom use with husband (n=449)						
never	344	24.1	1.00			
sometimes	105	22.9	0.93 (0.56-1.56)	0.8		
always	0	-	-			
Condom use with partner (n=167)						
never	118	29.7	1.00			
sometimes	43	23.3	0.72 (0.32-1.62)	0.4		
always	6	0.0	-	-		

[a]7 categories: 0=0yr. 1=1-2yr. 2=3-4yr. 3=5-6yr. 4=7-8yr. 5=9-10yr. 6=11yr+ P<0.05: unpaired t- test

[b]Sexual transmission knowledge means whether women know that semen can transmit HIV.

OR: Odds ratio AOR: Adjusted odds ratio CI: confidence interval

Table 2. **Characteristics of women by sexual relationship and those associated with HIV seropositivity**

		Condom use with husband			Condom use with partner			HIV seropositivity		
Sexual relationship	N	Non use (%)	Use[a]	P-vaule	Non use (%)	Use[b] (%)	P-value	%HIV+ve	OR (95%CI)	P-vaule
Only husband	299	242 (80.9)	57 (19.1)	<0.01	-	-	0.2	22.4	1.00	
Husband and partner	150	102 (68.0)	48 (32.0)		103 (68.7)	47 (13.3)		26.7	1.26 (0.80-1.98)	0.3
Only partner	17	-	-		15 (8.2)	2 (11.8)		29.4	1.44 (0.49-424)	0.5

OR: Odds ratio, CI: confidence interval

[a] *Use indicates sometimes use of condom*

[b] *Use indicates sometimes or always use of condom*

Table 3. **Multivariate analyses for the association of women's characteristics and condom use either with husband or with partner.**

	Condom use with husband (n=423)			Condom use with partner (n=156)		
	N	OR (95%CI)	P-vaule	N	OR (95%CI)	P-value
Age	423	0.92 (0.86-0.98)	<0.05	156	0.98 (0.88-1.09)	0.7
School attendance[a]	423	1.11 (0.94-1.32)	0.2	156	1.02 (0.80-1.31)	0.9
Sexual transmission knowledge[b]						
wrong	143	1.00		59	1.00	
right	280	0.70 (0.43-1.14)	0.2	97	2.11 (0.97-4.61)	0.06
Own earning						
regularly	84	1.00		35	1.00	
irregularly	54	0.39 (0.16-0.96)	<0.05	15	0.11 (0.01-0.97)	<0.05
none	285	0.74 (0.41-1.32)	0.3	106	0.83 (0.34-2.04)	0.7
Live births						
none	107	1.00		53	1.00	
1	116	2.49 (1.22-5.05)	<0.05	37	0.94 (0.32-2.79)	0.9
2+	200	4.13 (1.78-9.58)	<0.01	66	1.34 (0.36-4.92)	0.7
Abortions						
never	381	1.00		140	1.00	
experienced	42	1.94 (0.96-3.94)	0.07	16	2.93 (0.91-9.49)	0.07
Child deaths						
never	313	1.00		119	1.00	
experienced	110	0.73 (0.41-1.30)	0.3	37	0.68 (0.26-1.78)	0.4
HIV serotatus						
negative	323	1.00		114	1.00	
positive	100	0.93 (0.53-1.63)	0.8	42	0.58 (0.24-1.39)	0.2

[a] *7 categories: 0=0yr.1=1-2yr. 2=3-4yr. 3=5-6yr. 4=7-8yr. 5=9-10yr. 6=11yr+*

[b] *Sexual transmission knowledge means whether women know that semen can transmit HIV.*

OR =odds ratio adjusted for each variable listed above CI = 95% confidence interval

existed in the extent of knowledge between the non-sexual (blood or breast milk) and the sexual (semen or vaginal secretion) routes of HIV transmission ($P<0.01$, chi-square test). Those who answered correctly on the non-sexual routes had a trend toward longer school attendance (blood, $P<0.05$; breast milk, $P=0.09$; unpaired t-test). However, no association was found regarding

the sexual routes. Furthermore, knowledge of the routes of sexual transmission did not correlate significantly with HIV seronegativity in the present study. One-third of the women had their own earnings, whether or not the earning was regular. As for the factors comprising the reproductive profile, child deaths were associated significantly with HIV seropositivity in the multivariate analysis. None of the women answered that they always use condoms, and only one-fourth reported sometimes use of condoms with their husbands. Condom use both with husband and partner tended to decrease HIV prevalence, but a significant association was not revealed.

Table 2 shows the distribution of women by sexual relationship, condom use, and HIV seropositivity in each group. Of the 466 pregnant women, 32% had extramarital sexual relationships. The HIV prevalence among women with fidelity was not significantly lower than that among women without fidelity. With respect to condom use with the husband, it was much lower among the fidelity group than among extramarital affair group ($P<0.01$, chi-square test). Women involved in extramarital sexual relationships seemed to have increased condom use either with the husband or with the partner.

Table 3 shows the results of the multivariate analyses for the association of women's characteristics with condom use either with husband or with partner. Condom use with husband showed a significant association with age and number of live births (for age, odds ratio [OR]=0.92, 95% CI=0.86–0.98); for the number of live births, 1, OR=2.49, 95% CI=1.22–5.05; 2+, OR=4.13, 95% CI=1.78–9.58). On the other hand, condom use with partner was not associated with these factors. Sexual transmission knowledge tended to associate with condom use with the partner but not with the husband. Women with irregular earnings had significantly lower condom use with both husband and partner than those with regular earnings (with husband, OR=0.39, 95% CI=0.16–0.96; with partner, OR=0.11, 95% CI=0.01–0.97).

Discussion

The present study indicates the tendency of and the factors associated with the HIV epidemic among urban pregnant women in Zambia in 2001. The overall prevalence of HIV infection in the study population was slightly lower than that in the report of sentinel surveillance performed in 1998 (median 27.3%, min. 25.9%, max. 29.1% in antenatal care clinics-major urban areas)[13,14]. However, it may be noted that the prevalence in women 15–19 years of age was slightly higher than in that report.

We observed a contradictory effect of the length of education on the prevention of women contracting HIV infection. This result was not unexpected, since previous reports have found that a woman's educational attainment was linked positively with a high prevalence of HIV infection[15,16]. School attendance did not, in fact, increase condom use with husband or extramarital sexual partner. Furthermore, school attendance was not effective for these women to gain knowledge on sexual transmission of HIV, although it was effective for knowledge of non-sexual transmission routes, such as blood and breast milk. This suggests to us a lack of effective and consistent education regarding HIV transmission and sexual behaviour, at least within the school system. Actually, we had several cases of infected teenagers who had a correct knowledge of the sexual transmission routes. It may be possible that the knowledge was not gained before a risky event or the knowledge was the result of a risky event that led them to become infected with HIV. This again indicates insufficient or lacking education on HIV and sex both in and out of school, and that educational efforts should be implemented before the very early age of sexual debut among Zambian women[17,18]. Another reason for non-linkage between sexual transmission knowledge and HIV protection might be the power inequity

between men and women, which makes it exceedingly difficult for women even with sexual transmission knowledge to negotiate safer sex with their sexual partners[19,20].

Concerning whether a woman's fidelity protects her from HIV infection, our result was not affirmative. Among women with fidelity (the "only husband" group), the prevalence of HIV infection was not significantly lower, and condom use was much lower than among women who were having extramarital affairs (the "husband and partner" group). This finding suggests to us that the woman's fidelity only cannot prevent HIV transmission within marriage, given together with the high rate of polygamy in Zambia[21]. On the other hand, significantly higher use of condoms both with the husband and with other partners among women having extramarital relations, prompts us to consider that the meaning of condom use as an HIV protector is becoming accepted and is put into practice to some extent in Zambia.

Interestingly, the factors significantly associated with condom use within and outside of marriage were very different from each other. Reproductive factors, such as the number of live births, promoted condom use with the husband, which is consistent with the previous report[22]. It would appear that the motivation for condom use comes after a sufficient number of childbirths. In other words, condom use within marriage can be promoted when family planning is more accepted and practiced. On the other hand, for condom use with the extramarital sexual partner, not the reproductive factors but the knowledge of the sexual transmission route of HIV was barely associated, which indicates that sexual transmission knowledge can push condom use as HIV protection in the context of which childbearing is not expected.

Besides this, it is noteworthy, in comparison to the inefficacy of school attendance, that regular earning by a woman was significantly associated with condom use with both husband and partner. The possibility of sex work as the source of earnings was thought to be very low, since the percentage of women with fidelity among women with regular earnings was 59% compared with 37% for women without fidelity, and this ratio was similar in both the irregular earnings and no earnings groups (data not shown). Empowering women, or correcting the power imbalance between partners, has received much attention as critical to gaining the male partners' cooperation and acceptance of condom use[1,19,23]. However, it has not been clearly demonstrated what constitutes a woman's empowerment and how it can be acquired. Our results suggest that even relative but stable economic independence by their own earning, irrespective of their school attendance, might be indispensable, even though education has been believed to be a prerequisite for economic empowerment. It would appear that a woman's economic independence should receive proper attention to combat the spread of HIV infection through condom use.

In conclusion, the following should be implemented intensively in the HIV/AIDS prevention programme in Zambia: effective HIV and sex education for young girls less than 15 years of age in and out of school, further implementation of family planning with emphasis on condom use, and empowering women by helping them to become economically independent.

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