Seminal plasma promotes the attraction of Langerhans cells via the secretion of CCL20 by vaginal epithelial cells: involvement in the sexual transmission of HIV

W.Berlier, M.Cremel, H.Hamzeh, R.Lévy, F.Lucht, T.Bourlet, B.Pozzetto and O.Délezay

GIMAP, Groupe Immunité des Muqueuses et Agents Pathogènes, Faculté de Médecine J. Lisfranc, Saint Etienne, France

1To whom correspondence should be addressed at: GIMAP, Faculté de Médecine Jacques Lisfranc, 42023 Saint-Etienne cedex 02, France. E-mail: delezay@univ-st-etienne.fr

BACKGROUND: Heterosexual human immunodeficiency virus (HIV) transmission implies the crossing of the vaginal mucosa by virions present in the semen, potentially using Langerhans cells as transporters. The recruitment of these cells in the mucosa is mediated by the chemokine macrophage inflammatory protein 3α (CCL20). The aim of this study was to evaluate the capacity of the semen to induce Langerhans cell recruitment via the production of CCL20 by vaginal epithelial cells. METHODS AND RESULTS: Using a vaginal epithelium model based on the SiHa cell line and human seminal plasma, we demonstrated that semen enhanced the production of CCL20. This secretion was regulated by the nuclear factor-κB intracellular signalling pathway. Fractionation of the seminal plasma indicated that the secretion of CCL20 was stimulated by high molecular weight compounds present in semen. Migration assays demonstrated that secreted CCL20 was able to promote the recruitment of Langerhans cell precursors (LCps), which remain permissive to X4 and R5 HIV infection. CONCLUSIONS: Our results demonstrate that epithelial cells respond to factors present in semen by secreting CCL20, leading to the enhancement of LCp recruitment. These data argue in favour of the implication of epithelial cells in the heterosexual transmission of HIV.

Key words: CCL20/HIV/Langerhans cell/semen/vaginal mucosa

Introduction

Heterosexual contact is the main mode of human immunodeficiency virus (HIV) transmission. This virus, present in the semen as free or cell-associated particles, crosses the vaginal epithelium mucosa and joins the blood circulation, leading to efficient contamination. In the absence of breaches in the vaginal mucosa, the mechanisms involved in the crossing of vaginal epithelium by HIV imply the recruitment of immune cells that possess migratory properties, including macrophages, lymphocytes or Langerhans cells (LCs), which can be considered as the first target cells for this virus (Lore and Larsson, 2003). Indeed, it has been demonstrated that LCs could interact with HIV particles (with or without efficient infection) via the CD4 molecule as well as CXCR4 and CCR5, the two HIV coreceptors leading to infection by X4 and R5 HIV strains, respectively (Tchou et al., 2001; Kawamura et al., 2003).

The regulation of the recruitment of LCs has been demonstrated to be controlled by the macrophage inflammatory protein 3α (MIP3α/CCL20). This chemokine, which attracts LC precursors (LCps) by interacting with the CCR6 receptor (Baba et al., 1997; Dieu-Nosjean et al., 1999), is secreted by epithelial cells from numerous tissues such as skin (Charbonnier et al., 1999; Dieu-Nosjean et al., 2000) and oral (Abiko et al., 2003) or vaginal epithelium (Cremel et al., 2005). As LCs capture the HIV particles, phenotypic changes promote LC maturation and emigration from the mucosa to the draining lymph nodes (Dieu et al., 1998), in which they transmit virions to lymphocytes for further dissemination to the whole organism (Wilflingseder et al., 2005).

However, in addition to the involvement of LC, heterosexual transmission of HIV may also be influenced by sperm components that could interact either with HIV or with the vaginal epithelial cells. In this way, seminal plasma has been demonstrated to modulate HIV particles or vaginal epithelial cell properties (Gorodeski and Goldfarb, 1998; Bouhhal et al., 2002). It has also been shown that, after a sexual contact, sperm elicited a transient infiltration of the cervix mucosa by leukocytes (Pandya and Cohen, 1985; Thompson et al., 1992), suggesting a role for seminal plasma in the recruitment of immune cells. Because seminal plasma carries a huge repertoire of molecules, including chemokines and cytokines (Maegawa et al., 2002), it seems reasonable to investigate their possible role in the first steps of HIV transmission (Scofield, 1998). Indeed, very few studies relative to the interactions between seminal plasma, HIV and human vaginal epithelial cells have been performed, rendering the molecular
mechanisms implied in the early steps of sexual HIV transmission unclear.

This study was aimed to analyse the co-operation of seminal plasma, vaginal epithelial cells and LCs in the first steps of HIV heterosexual transmission, using a human reconstructed vaginal mucosal model (Cremel et al., 2005). We demonstrate that human semen, whatever the HIV status of the donor, promotes the secretion of CCL20 by the SiHa vaginal epithelial cell line through the nuclear factor-κB (NF-κB) intracellular signalling pathway, leading to an efficient recruitment of LCs. After migration, LCs were shown to be permissive to both X4 and R5 HIV strains.

Materials and methods

Seminal plasma samples

Semen samples were collected from HIV+ patients entering or undergoing a programme of highly active antiretroviral therapy (HAART) and from HIV+ patients attempting medically assisted conception, after they gave their fully informed written consent. HIV− and from HIV− going a programme of highly active antiretroviral therapy (HAART)

SiHa cells were incubated with HIV + seminal plasma samples 1:2 in phosphate-buffered saline (PBS), seminal plasma was separated from the cell fraction by centrifugation (800 g, 30 min) and stored at –80°C until use.

Cell culture

The SiHa cell line (ATCC® number HTB-35™, LGC-Promochrom, Teddington, UK) was routinely grown in Dulbecco’s minimal essential medium (DMEM)-F12 medium (Cambrex BioScience, Verviers, Belgium) supplemented with 10% of fetal bovine serum (FBS) and antibiotic-antimycotic solution (penicillin-streptomycin-amphotericin B, Sigma-Aldrich, St. Louis, MO, USA). For stimulation experiments, the cells were seeded onto 24-well culture plates (BD Falcon, Franklin Lakes, NJ, USA) at a density of 200 000 cells/well and cultured until confluence.

LCs were derived from CD34+ haematopoietic progenitor cells (umbilical cord blood origin). Firstly, cord blood mononuclear cells were separated from granulocytes and erythrocytes by centrifugation (900 g, 30 min) on a Ficoll density gradient (Histopaque®-1077, Sigma-Aldrich). Monocyte depletion was performed by differential cell adherence in 75 cm² culture flask in Roswell Park Memorial Institute (RPMI) 1640 medium (Cambrex BioScience) containing 10% FBS and an antibiotic-antimycotic mixture. CD34+ progenitor cells were isolated through a positive immunoselection procedure (CD34 MultiSort Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in the presence of stem cell factor (SCF, 10 U/ml, Peprotech, London, UK), granulocyte/macrophage-colony-stimulating factor (GM-CSF, 200 U/ml, Peprotech), tumour necrosis factor (TNFα, 50 U/ml, Peprotech) and 2.5% human AB+ serum in RPMI 1640 medium.

Cells were used at day 6 for chemotaxis experiments after being assayed for CCR6 expression by flow cytometry using phycoerythrin-labelled anti-CCR6 (BD Biosciences) prior to each experiment.

Measurement of CCL20 secretion by SiHa cells

SiHa cells were incubated with HIV− or HIV+ seminal plasmas (diluted 1:10 in culture medium) or recombinant human interleukin IL1-β (25 ng/ml, Peprotech), as positive control, with or without the NF-κB-signalling pathway inhibitor BAY 11-7085 (2.5 μg/ml, Biomol, Plymouth Meeting, PA, USA). The production of CCL20 in the supernatants of SiHa cells after 17 h (overnight) of treatment was quantified using ELISA detection kits (Quantikine, R&D systems, Abingdon, UK) according to the manufacturer’s instructions. Optical densities were measured at 450 nm. Each assay was performed in triplicate.

CCL20 mRNA detection

A RT-PCR assay targeting the open reading frame of the CCL20 gene was performed after 4 h of treatment on 2 × 10⁶ cells as already described (Cremel et al., 2005) (forward primer: 5’-TTGCTCTGG CTGCTTTG; reverse primer: 5’-ACCCCTCATGATGTGCAAG). As control, the human gene of β-actin was amplified by RT-PCR for each sample.

LCp chemotaxis assay

Migration of CCR6+ LCps was assayed by measuring their capacity to migrate across Matrigel™-coated transwell inserts (BD BioCoat™ Matrigel™ Invasion Chamber, 8 μm pore size). Briefly, 500 000 LCps were added to the apical compartment of the filter (upper compartment), the basal compartment (lower compartment) containing the medium to be tested. After 17 h (overnight), the number of migrated LCps found in the basal medium was determined by microscope observation and counting. Inhibition of CCL20 chemotaxis was performed using neutralizing anti-CCL20 polyclonal antibodies (10 μg/ml, R&D systems) that were added to the cell supernatants 2 h before the chemotaxis assay. CCL20-unrelated neutralizing polyclonal antibodies (anti- leptin, 10 μg/ml, R&D systems) were used as negative controls.

Gel exclusion chromatography

Seminal plasmas diluted 1:2 in PBS were applied onto a gel filtration column (Superose 6, Amersham Biosciences, Freiburg, Germany), equilibrated at room temperature with PBS containing 1 mM CaCl₂ and 0.1 mM MgCl₂. Following application of the sample, the column was washed with the equilibration buffer at a flow rate of 0.2 ml/min. The different fractions were tested for their ability to induce CCL20 secretion by SiHa cells as described above.

Infection of LCps by HIV-1

After chemotaxis assay, migrated LCps found in the basal medium were incubated with 500 TCID₅₀ of HIV-1_R5 (X4-tropic) or HIV-1_R5 (R5-tropic) for 48 h. DNA was extracted using the Flexigene™ DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The integration of HIV proviral DNA in LCps was evaluated by a specific HIV PCR (LTR) using a quantitative protocol adapted from O’Doherty et al. (2002) on a Lightcycler® real-time PCR apparatus (Roche Diagnostics, Basel, Switzerland). Serial dilutions of 8 × 10⁵ cells containing one copy of HIV DNA per cell were used as quantification standard. The threshold of detection was estimated between 5 and 50 copies of HIV DNA. PCR directed against the human gene of β-actin was performed for each sample as control.

Statistical analysis

Values were analysed by Student’s t-test using the STATISTICA™ data analysis software system (StatSoft Inc., Tulsa, OK, USA).

Results

Seminal plasma promotes CCL20 production by human vaginal epithelial cells

First, experiments were carried out to study the secretion of the chemokine CCL20 by the human vaginal epithelial cell line SiHa after treatment with seminal plasma. The supernatants of
SiHa cells, incubated with DMEM-F12 medium alone (negative control) or with IL1-β (25 ng/ml, positive control) or with human seminal plasma (diluted 1:10 in culture medium), were analysed for CCL20 content by ELISA after 17 h of treatment. As shown in Figure 1A, a weak constitutive secretion of CCL20 (120 ± 8 pg/ml) was noticed in the supernatant of untreated SiHa cells. This production was strongly enhanced by IL1-β treatment (1908 ± 106 pg/ml, *P < 0.001) or by incubation of the cells with a pool of human seminal plasmas from HIV− healthy subjects (587 ± 26 pg/ml, *P < 0.001). No significant cell death was observed in these conditions and the absence of CCL20 in the pool of seminal plasmas confirmed the epithelial origin of the chemokine (data not shown). To evidence a potential difference between semen from HIV+ and semen from HIV− subjects on the secretion of CCL20 by epithelial cells, the seminal plasma of 15 HIV+ and 19 HIV− subjects was tested individually. Despite a slight difference in favour of samples from HIV+ subjects, no statistical difference was observed between the two groups (*P = 0.187) (Figure 1B).

The expression of CCL20 mRNA by SiHa cells was further tested (Figure 1C). A weak specific signal was detected in the absence of any treatment. The incubation of the SiHa cells with

---

**Figure 1.** CCL20 production by vaginal epithelial SiHa cell line. Cells were treated overnight with interleukin (IL1-β) or seminal plasmas (SP, diluted 1:10). Supernatants were assayed for CCL20 content by ELISA. (A) Histogram representative of 10 experiments using different seminal plasmas or IL1-β treatment (25 ng/ml, positive control). Asterisks represent statistically significant differences compared with untreated cells (*P < 0.001) (B) CCL20 production induced by HIV− or HIV+ seminal plasmas. (C) CCL20 mRNA expression by SiHa cells. Cells were treated for 4 h with diluted seminal plasmas from HIV+ (lane 3) or HIV− (lane 4) donors. Untreated and IL1-β-treated SiHa cells served as negative (lane 1) and positive controls (lane 2), respectively. Results are representative of three independent experiments.
IL1-β (25 ng/ml) or with pools of seminal plasmas from HIV+ or HIV− donors resulted in an increase of the specific signal.

**CCL20 production is mediated by the NF-κB intracellular signalling pathway**

The effect of BAY 11-7085, an inhibitor of the NF-κB pathway, was tested to study the regulation process involved in the stimulation of CCL20 production by epithelial cells in the presence of seminal plasma. As shown in Figure 2, BAY 11-7085 strongly inhibited the secretion of CCL20 by SiHa cells, from 1048 ± 36 pg/ml to 267 ± 1 pg/ml (P = 0.002) after IL1-β stimulation and from 848 ± 27 pg/ml to 181 ± 2 pg/ml (P = 0.002) after treatment with a pool of seminal plasmas. BAY 11-7085 also diminished the basal secretion of CCL20, suggesting that CCL20 production in the absence of any stimulation was also dependent on the same intracellular mechanisms. The decrease of CCL20 secretion was not associated with cell death as evaluated by an XTT-based cytotoxicity assay (data not shown). Moreover, the regulation of mRNA expression under these different conditions was in agreement with the above results, because the amount of CCL20 mRNA in SiHa cells was down-modulated by BAY 11-7085 treatment, after stimulation by either IL1-β or seminal plasma (Figure 2).

**CCL20 secreted by SiHa cells induces migration of LCps**

Different supernatants of SiHa cells were tested for their ability to specifically attract CCR6+ LCps through an 8-μm pore Matrigel™-coated filter. LCps, expressing the CCR6 receptor in more than 90% of the cells in flow cytometry, were able to migrate across an 8-μm pore filter when rhCCL20 was deposed in the basal compartment (Figure 3A). Similarly, the supernatant of SiHa cells treated with IL1-β induced attraction of LCps compared with the supernatant of untreated SiHa cell (52 000 ± 1400 and 8000 ± 100 cells, respectively, P = 0.002). The supernatant of SiHa cells stimulated by a pool of seminal plasmas was able to promote the attraction of LCps, whatever the HIV status of the semen donors (35 000 ± 4200 cells and 45 500 ± 7800 cells, P = 0.012 and P = 0.02, for samples from HIV− and HIV+ subjects, respectively) (Figure 3A).

To verify the specific involvement of CCL20 in CCR6+ LCp migration, the supernatant of SiHa cells was pre-incubated with anti-CCL20 neutralizing polyclonal antibodies before to be tested for their capacity to attract LCps. As shown in Figure 3B, this treatment abolished the LCp migration induced by the supernatant of IL1-β-treated SiHa cells (more than 95% of inhibition compared with the migration of LCps obtained with medium alone, P = 0.007). A similar effect was observed on the supernatant of SiHa cells treated with a pool of seminal plasmas from HIV− donors but with lower efficiency (61% of inhibition as compared with untreated SiHa cells, P = 0.022), which suggests that CCL20-unrelated factors present in the supernatant of SiHa cells treated with seminal plasma could also participate to the attraction of LCps. Pre-incubation of SiHa supernatants with CCL20-unrelated antibodies did not result in significant decrease of LCp migration (data not shown).

**High molecular weight compounds of the seminal plasma are involved in the secretion of CCL20 by epithelial vaginal cells**

To further characterize the molecules present in human seminal plasma that were involved in the stimulation of CCL20 secretion...
by SiHa cells, we fractionated pools of seminal plasmas from HIV− subjects on a Superose 6 exclusion column, and the different fractions were tested for their ability to stimulate CCL20 production by SiHa cells. Four experiments using different pools of seminal plasmas led to similar results. As shown in Figure 4, the stimulatory effect was restricted to a well-individualized peak corresponding to fractions with molecular masses ranging from 41 to 69 kDa.

**HIV infection of LCps after migration**

The sensitivity of LCps to HIV after their migration through the filter was then evaluated by incubating them with R5-tropic (BAL) or X4-tropic (LAI) HIV-1 strains for 48 h. The migration assays were performed using the supernatant of untreated or seminal plasma-treated SiHa cells and HIV DNA integration was evaluated by quantitative PCR. Two independent experiments were performed. LCps could be infected by R5 as well...
as by X4 HIV strains whatever the conditions of their migration (data not shown).

Discussion

Heterosexual intercourse is the main route of transmission for numerous pathogens. It is well assumed that sexual contact currently represent the major mode of HIV transmission. It has already been described that HIV, present in the ejaculate as free or cell-associated particles, could cross the vaginal epithelial barrier by interacting with immune cells and particularly LCs. These cells are able to transport HIV particles from vaginal lumen to lymph nodes, because they bear receptors able to interact with the virus (Turville et al., 2002). Moreover, they are sensitive to both R5 and X4 HIV strains (Tchou et al., 2001; Kawamura et al., 2003). Despite the fact that it is well admitted that such cells are the main target for HIV in the vaginal mucosa and that they represent the ‘Trojan horses’ involved in this contamination (Piguet and Blauvelt, 2002), the first mechanisms allowing the recruitment of these cells at the epithelial/sperm contact site have not been described. In addition, the mechanisms involved in the selective transmission of R5 HIV particles are still unknown. To investigate these different points, we used an in vitro model of human reconstructed vaginal mucosa to study the interactions between seminal plasma, vaginal epithelial cells and LCps.

First, our results demonstrate that human seminal plasma is able to stimulate the secretion of CCL20 by human vaginal epithelial cells. This secretion was not associated to the HIV status of the donors of seminal plasma, suggesting a non-specific response of the vaginal epithelial cells to seminal plasma components following a sexual contact. The ability of seminal plasma to increase the expression of CCL20 mRNA was shown to be regulated at the transcriptional level through the NF-κB pathway. This result is in accordance with previous studies showing that this pathway is involved in the control of the secretion of numerous cytokines, including CCL20 (Kopp and Ghosh, 1995; Naumann, 2000; Sugita et al., 2002). We previously showed that SiHa cells, which constitute a relevant model of vaginal epithelium, were able to produce significant amounts of CCL20 in response to the pro-inflammatory cytokine IL1-β (Cremel et al., 2005). In the present study, we demonstrate for the first time that seminal plasma from normal donors produced a similar effect on these cells, suggesting that semen contains a compound able to generate a non-specific warning signal, constituted by the secretion of CCL20, which leads to the recruitment of LCs in the female genital tract. It is interesting to notice that, despite large amounts of the anti-inflammatory cytokine transforming growth factor-β in seminal plasma (Nocera and Chu, 1993), vaginal epithelial cells were able to secrete CCL20 after contact with sperm fluid as already described for other tissues in the course of inflammatory diseases (Nakayama et al., 2001; Kaser et al., 2004). The production of numerous factors by the female genital tract after contact with seminal fluid has already been described both in vitro and in vivo (Denison et al., 1999; Gutsche et al., 2003;
O’Leary et al., 2004). Nevertheless, focusing on the CCL20 secretion at the genital level seems of particular relevance, because this molecule is the main chemoattractant of LCps (Dieu-Nosjean et al., 2000). Up to now, we failed to identify the factor(s) involved in the stimulation of CCL20 secretion by seminal plasma; the results presented above indicate that it exhibits a molecular masses ranging from 41 to 69 kDa, which could correspond to numerous molecules present in semen (Fung et al., 2004), including lighter proteins forming complexes with other components such as prostasomes (Utelig et al., 2003). Further work is in progress to identify this (these) component(s).

In addition, the functional activity of CCL20 secreted after stimulation by seminal plasmas was demonstrated by its capacity to attract CCR6+ LCps. Once again, no difference was observed in LCp migration when SiHa cells were treated with semen from HIV− or HIV+ donors. When pre-incubated with neutralizing anti-CCL20 antibodies, the chemoattraction properties of the SiHa cell supernatants on LCps were significantly reduced, but not totally abolished, in contrast to the results obtained with supernatants of IL1-β-treated cells. In addition to the chemoattraction mediated by CCL20, these results suggest the presence of other chemoattractive factors either present in semen or secreted by SiHa cells after seminal plasma contact. In this way, seminal plasma contains a huge repertoire of molecules including potential chemoattractive factors such as defensins able to interact with the CCR6 receptor, resulting in cell attraction (Yang et al., 1999); β-defensins have also been demonstrated to be secreted by epithelial cells (Seo et al., 2001; King et al., 2003). The respective role of CCL20 and other cytokines such as β-defensins in the chemoattraction of CCR6+ LCps deserves further investigation. Whatever the precise mechanism involved in the process, our data highlight that, either directly or via the epithelial vaginal cells, seminal plasma from normal donors is able to recruit LCps at the vaginal mucosal surface. This phenomenon could play an important role in the initial non-specific immune intake of infectious agents possibly present in semen.

To evaluate the relevance of these findings in the course of HIV infection, LCps were incubated with R5 or X4 HIV strains following the migration induced by supernatants of SiHa cells treated with seminal plasma. Quantitative RT-PCR experiments evidenced that the cells were still sensitive to X4 and R5 HIV infection. Indeed, during the initial events of HIV heterosexual transmission, a preferential contamination by R5-tropic HIV isolates has been described despite the presence of both X4 and R5 strains in the semen (Zhu et al., 1996). The involvement of the CCR5 coreceptor in HIV sexual transmission was also evidenced by the relative resistance to HIV infection of individuals bearing homozygous CCR5 Δ32 mutation (Liu et al., 1996; Samson et al., 1996). The present results indicate that these cells are not involved in this selection process of R5 strains. As recently published (Berlier et al., 2005), we suggest that epithelial cells could participate in the HIV tropism selection by sequestering HIV X4 particles, leading to the depletion of X4 strains and then increasing the probability of infection of LCs by R5 strains. A possible scenario of the initial steps of HIV infection could be as follows: first, seminal plasma bearing X4 and R5 viruses interacts with epithelial vaginal cells, leading to the sequestration of X4 viruses and to the attraction of CCR6+ LCps via, at least in part, the production of CCL20 by epithelial cells; then, LCs capture free R5 virions and migrate to lymph nodes, in which they transmit HIV particles to lymphocytes.

The involvement of epithelial cells and semen in the first steps of heterosexual HIV transmission should be considered for a better understanding of the mechanisms leading to HIV contamination. Such findings could have interesting implications for the development of new antiviral approaches at the mucosal level.

Acknowledgements

Willy Berlier is a recipient of an ANRS fellowship, and Magali Cremel is a recipient of a MENRT grant. We also thank Philip Lawrence for English revision of the paper.

References


Submitted on October 24, 2005; resubmitted on December 7, 2005; accepted on December 20, 2005