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Increased Serpin A5 levels in the cervicovaginal fluid of HIV-1 exposed seronegatives suggest that a subtle balance between serine proteases and their inhibitors may determine susceptibility to HIV-1 infection

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ABSTRACT

HIV-exposed seronegative individuals (HESNs) are persons who remain seronegative despite repeated exposure to HIV, suggesting an in vivo resistance mechanism to HIV. Elucidation of endogenous factors responsible for this phenomenon may aid in the development of new classes of microbicides and therapeutics. We compared cervicovaginal protein abundance profiles between high-risk HESN and two control groups: low-risk HESN and HIV-positives. Four iTRAQ-based quantitative experiments were performed using samples classified based on presence/absence of particular gynaecological conditions. After statistical analysis, two proteins were shown to be differentially abundant between high-risk HESNs and control groups. Serpin A5, a serine proteinase inhibitor and Myeloblastin, a serine protease, were up- and downregulated, respectively. Commercially available ELISA assays were used to confirm differential Serpin A5 levels. These results suggest that HIV resistance in CVF of HESNs is the result of a delicate balance between two complementary mechanisms: downregulation of serine proteinases and upregulation of their inhibitors.

Introduction

HIV is responsible for one of the most disastrous epidemics throughout human history, and approximately 34 million people are currently infected (UNAIDS, 2013). Unfortunately, the development of a prophylactic vaccine will likely not be available soon. Other strategies include topically applicable (e.g., rectal or vaginal) or oral antiretrovirals (ARV), which are chemical entities that can prevent or reduce HIV transmission. However, although these ARVs are a very promising strategy to reduce HIV spread, some issues remain, like the uptake and adherence of these compounds and the improvement of their efficiency (Cutler and Justman, 2008; Rohan and Sassi, 2009; Baeten and Grant, 2013).

HIV-exposed seronegative individuals

HIV-exposed seronegative individuals (HESNs) are frequently exposed to HIV but are not infected and are thus apparently HIV resistant in vivo. They comprise less than 5% of the general population and can be found among commercial sex workers, haemophiliacs receiving HIV contaminated blood, healthcare workers, children from HIV-infected mothers, intravenous drug users and seronegative partners in a discordant couple (Hirbod and Broliden, 2007; Horton et al., 2010; Broliden, 2010; Lederman et al., 2010; Shearer and Clerici, 2010; Miyazawa et al., 2009; Kulkarni et al., 2003; Shacklett, 2006). Elucidation of endogenous factors that inhibit HIV transmission and prevent the establishment of a productive infection are of high importance as

Abbreviations: CAN, acetonitrile; ARV, antiretroviral; BCA, bicinchoninic acid assay; BV, bacterial vaginosis; CI, confidence interval; CVF, cervicovaginal fluid; FA, formic acid; FC, fold change; HESNs, HIV-1 exposed seronegative individuals; HIV, human immunodeficiency virus; HR, high-risk; IHGT, infection of the higher genital tract; IL, interleukin; iTRAQ, isobaric tags for relative and absolute quantification; LR, low-risk; MALDI, matrix assisted laser desorption/ionisation; MCD, mucopurulent cervical discharge; MCP, monocyte chemotactic protein; NC, not calculated; NI, not infected; RP, reversed phase; SCX, strong cation exchange; TLR, toll-like receptor

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they can be used as a base for the development of new types of ARV and microbicides with higher efficiency. Therefore, many studies have been performed to identify physiological factors correlated with the HESN-status. Mutations of chemokine or Toll-like receptors (TLR), upregulation of chemokines due to genetic polymorphisms, specific human leukocyte antigen haplotypes, natural killer cell activity regulated by the killer Ig-like receptor (KIR)/HLA interaction, presence of autoantibodies and/or alloantibodies, cytotoxic and helper T lymphocyte responses against HIV epitopes, altered cytokine profiles and production of anti-HIV antibodies have all been linked to HIV resistance (Guerini et al., 2011; Ghadially et al., 2012; Arenzana-Seisdedos and Parmentier, 2006; Hirbod and Broliden, 2007; Kulkarni et al., 2003; Marmor et al., 2006; Shacklett, 2006; Lajoie et al., 2012; Choi et al., 2012; Sironi et al., 2012; Tomescu et al., 2011; Turk et al., 2013; Yao et al., 2013; Prodger et al., 2013). In addition, some studies hypothesize that HIV resistance by HESNs occurs at the viral entry gate before HIV interacts with dendritic or other target cells (Belec et al., 2001; Soderlund et al., 2007). Because the cervicovaginal mucosa is the most important entry point for HIV in women, resistance to sexually transmitted HIV infection in female HESNs may be the result of factors present at the lower female genital tract. Among these, the mucosal epithelium and especially proteins or peptides present in the cervicovaginal fluid (CVF) may play an important role (Iqbal et al., 2009; Shen and Smith, 2014).

Cervicalvaginal fluid

The use of CVF as clinical samples has gained interest in recent years because analysis of the CVF proteome can be used for several purposes. Knowledge of the CVF proteome may: (1) yield information about the aetiology of specific gynaecological pathologies, (2) lead to the identification of biomarkers for disease diagnosis and progression or (3) provide insight into physiological phenomena such as HIV resistance. Using antibody-based techniques (e.g., ELISA and Western blotting), a plethora of potential biomarkers for preterm birth, preterm premature rupture of membranes, bacterial vaginosis and cervical cancer have been discovered (Zegels et al., 2010). In addition, several studies on HIV resistance have used CVF from HESNs for the identification of correlates of HIV protection using antibody-based techniques. Anti-HIV IgA and IgG antibodies were detected in CVF obtained from heterosexual HESN women (Archibald et al., 1992; Belec et al., 1994b, 1994a; Beyrer et al., 1999; Devito et al., 2000; Ghys et al., 2000; Mazzoli et al., 1997; Choi et al., 2012). Additionally, the levels of the HIV-suppressive β-chemokine RANTES were found significantly different in CVF from HESNs (Belec et al., 2001; Iqbal et al., 2005; Yao et al., 2013). These results indicate that CVF is an important factor for the establishment of HIV resistance in HESNs. However, the use of antibody-based techniques limits the research to the analysis of only a few selected proteins. Therefore, comprehensive studies on CVF, which take all proteins under consideration, may yield more HIV resistance factors (Zegels et al., 2010).

HIV resistance factors

Information from qualitative comprehensive proteomics studies on CVF showed that this biological fluid contains proteins/peptides with intrinsic anti-HIV activity such as defensins, lactoferrin, lysozyme, cathelicidin and SLPI (Cole and Cole, 2008; Hirbod and Broliden, 2007; Kazmi et al., 2006; Zegels et al., 2009). In addition, (Venkataraman et al., 2005) demonstrated that the cationic fraction of CVF has inherent anti-HIV activity and hypothesized that this activity is the result of a complex synergism between different proteins in CVF (Levinson et al., 2012). Later, a study of Levinson et al. (2012) confirmed these findings and pointed to HNP1-3 and LL-37 as possible mediators. In addition, Ghosh et al. (2010) showed that this anti-HIV activity correlated significantly with CVF levels of MIP-3α, HBD-2 and anti-gp160 IgG antibodies. Such factors may contribute to the highly inefficient sexual transmission of HIV, as most unprotected exposures to HIV ($>99.5\%$) do not result in infection (Gray et al., 2001).

Three quantitative proteomics studies for the isolation of HIV resistance biomarkers have been published. Burgener et al. (2008) employed resp. 2D-DIGE and LC-LTQ-FT (Burgener et al., 2011) and compared protein abundance profiles from HESN persons with those from healthy controls. The authors identified resp. 16 and 41 differentially expressed proteins with diverse biological functionalities, including several serine proteinase inhibitors. Iqbal et al. (2009) used surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry for comparison of protein abundance profiles from HESNs with those from control groups and found that the serine proteinase inhibitor elafin/trappin-2 is significantly upregulated in HESNs. In addition, elafin/trappin-2 has recently been identified as a new anti-HIV factor of the innate immune system of the lower female genital tract (Ghosh et al., 2009; Drannik et al., 2012b). However, the use of other proteomics techniques and test populations may help to characterize other CVF proteins correlated with in vivo HIV resistance. To improve the reliability of potential resistance factors that inhibit HIV transmission, it is important to confirm and to validate such factors in different independent HESN cohorts, whether or not with additional (genital) infections. Therefore, we analyzed the CVF from an HESN population of female sex workers from Abidjan, Côte d'Ivoire using iTRAQ-based quantitative proteomics to further unravel in vivo HIV resistance.

Results

Sample population

Three different subgroups were selected from a female commercial sex worker population from Abidjan, Côte d'Ivoire: HR, LR and HIV. We chose the HR group as the test group because these persons remained seronegative despite frequent HIV exposure for a long period of time. Therefore, this group is very likely to be enriched for HESN individuals. Two different control groups were included in the experiments. The individuals from the LR group were not (or extremely exceptionally) exposed to HIV due to the protective measures they have taken and the relatively low number of clients per week. This group is expected to be representative for the general population that includes only a small fraction of HESNs ($<5\%$ (Hirbod and Broliden, 2007; Kulkarni et al., 2003; Shacklett, 2006)). Therefore, these LR individuals act as a low-risk control group. Comparison between HR and LR may lead to the identification of proteins specifically correlated with HIV resistance. However, non-protective HIV-specific immune reactions occur in seropositive persons and HESNs (Biasin et al., 2000). Therefore, it is possible that adaptive immunity-related proteins can be significantly different in the HR and LR group, but not in comparison to HIV-positive persons. This type of result would indicate that the protein is derived from an HIV-specific immune reaction that does not contribute to the observed HIV resistance. Therefore, we incorporated HIV-infected persons as a non-resistant control group.

The large amount of potential biomarkers resulting from one experiment is one of the major downsides of MS-based quantitative methods such as iTRAQ. We conducted four different experiments so that the number of potential biomarkers could be reduced by statistics. As recently suggested in order to prevent confounding and bias, the HESN samples must be well documented, and pathological conditions need to be taken into consideration (Kaul et al., 2011). Indeed, because gynaecological pathologies can induce significant alterations of the CVF proteome (Zegels et