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Expression of CCR5, CXCR4 and DC-SIGN in Cervix of HIV-1 Heterosexually Infected Mexican Women

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Abstract: *Background*: A number of studies have demonstrated that receptor and co-receptor expression levels which may affect viral entry, promoting cervical HIV infection. The aim was to evaluate the expression levels of CCR5, CXCR4 and DC-SIGN mRNA in a sample of heterosexually HIV infected Mexican women.

Methods: We enrolled twenty-six HIV heterosexual infected women attending a local infectious diseases medical unit. RNA was isolated from the cervix and gene expression analysis was performed using real-time PCR.

Results: Expression rates for mRNA of CCR5 (median 1.82; range 0.003–2934) were higher than those observed for CXCR4 (0.79; 0.0061–3312) and DC-SIGN (0.33; 0.006–532) receptors ($p < 0.05$). A high correlation was found between the mRNA expression levels of these three receptors ($r_s = 0.52$ to 0.85, $p < 0.01$).

Conclusion: Levels of expression of the tested chemokine receptors in the cervix are different from each other and also vary from woman to woman, and seem to support the suggestion that chemokine receptor expression in genital tissues may be playing a role in the HIV transmission.

Keywords: AIDS, HIV-1, Mucosae, CCR5, CXCR4, DC-SIGN.

BACKGROUND

The Acquired Immunodeficiency Syndrome (AIDS) is a serious public health problem worldwide with 33.4 million people currently living with the Human Immunodeficiency Virus (HIV) according to UNAIDS and WHO [1]. In Mexico, the number of persons living with HIV is estimated to be 200,000 [2]. Although HIV infection initially affected homosexual men, the virus is now spreading to a growing number of heterosexual individuals. The male-to-female ratio among HIV-1-infected people in Mexico is estimated to be 4.9:1 [3].

A number of HIV-1 infected patients acquired the virus during heterosexual exposure where the virus binds to the CD4 receptor and other co-receptors present on immune cells of the genital mucosa [4, 5]. HIV infection is mediated by a complex interaction between HIV envelope proteins and receptors on target cells, particularly CD4 receptors; however, expression of this molecule alone is not enough to establish the infection. It is well known that CCR5 and CXCR4 co-receptors are necessary for the virus entrance [6]. Viral strains that utilize CCR5 are classified as R5-tropic (which replaced the designation M-tropic), whereas those that utilize CXCR4 are termed X4-tropic (replacing the designation T-tropic) [7]. R5 variants have been suggested to be the predominant strains following sexual transmission [7, 8]. Experimental evidence indicates that co-receptor expression levels have impact on the efficiency of viral entry and may affect the pathogenesis of the HIV-1 infection [7]. However, data from previous studies have shown differences in the chemokine receptor expression, which possibly indicate that the transmission of R5 variants may not be directly associated with the levels of CCR5 within the genital tract [9]. Another important factor in viral transmission is the C-type lectin DC-SIGN (Dendritic Cell – Specific ICAM-3 Grabbing Nonintegrin, CD-209), which plays a central role in the mucosal HIV-1 infection from

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Dendritic cells (DCs) to T cells. DC-SIGN binds the glycoprotein 120 (gp120) with a high affinity and independently of the other receptors and then transmits virions to T lymphocytes using the physiological functions of the dendritic cells to gain access to the lymphoid tissues [10-12].

Recently, the interest in HIV tropism has re-emerged due mainly to the generation of new promising antiretroviral drugs that target CCR5, CXCR4, and DC-SIGN. Unfortunately, information regarding the events immediately following the HIV-1 infection in humans is insufficient. Therefore, it is important to identify cellular and molecular factors of the host that may be contributing to HIV-1 transmission, not only to understand the subjacent immunopathogenesis but also to obtain information that can be useful to develop strategies to prevent the infection [13]. The aim of this study was to evaluate the mRNA expression of CCR5 and CXCR4 co-receptors and of the DC-SIGN receptor in cervical biopsies in a case series of HIV-1 heterosexually infected Mexican women.

METHODS

Patients and sample collection. HIV-1 heterosexually infected women undergoing medical care at the "Dr. Juan I. Menchaca Unidad de Infectología" Hospital General Regional No. 45, Instituto Mexicano del Seguro Social in Guadalajara, Western Mexico, were eligible. Written informed consent was obtained from each patient, and the study was approved by the institutional research and ethics review board of the Hospital General Regional No. 45. Superficial cervical biopsies (probably containing transitional epithelium with some few endocervical glands) were taken from each patient and stored in 1 ml of RNA stabilization solution (RNAlater; Ambion, Austin, TX) at room temperature. Menstruating patients, those who engaged in sexual activity within 48 hours prior to sampling, those with symptoms of any known genital tract infection, and those with a history of intravenous drug use or blood transfusion were excluded. Women younger 48 years of age were classified as premenopausal, while those with 48 years old and over were considered postmenopausal [14].

RNA isolation and cDNA synthesis. Cervical tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and then processed following the manufacturer's instructions. RNA isolate was eluted in RNase-free water treated with diethylpyrocarbonate (DEPC). RNA concentration was determined by the absorbance at 260 nm. For synthesis of the single strand cDNA, SuperScript II Reverse Transcriptase (Invitrogen) and Oligo dT Primer (Invitrogen) were used. The reactions were adjusted to 5 μg of total RNA concentration, and were mixed with 1 μL of 10 mM dNTP's Mix (Amersham Biosciences, Piscataway, NJ). This was completed at a final volume of 12 μL with sterile Milli-Q water. Next, the mixture was heated at 65°C for 5 min and then immediately chilled in an ice bath. In the second step of the reaction, 4 μL of the reaction solution (5x First Strand Buffer, Invitrogen) and 2 μL of DTT 0.1 M were added. This was maintained at 42° C for 2 min. Finally, 0.5 μL of reverse transcriptase enzyme were added and the reaction was completed at a final volume of 20 μL with sterile Milli-Q water. The mix was incubated for 50 min at 42° C and then for 15 min at 70° C to inactivate the reaction.

Real-time PCR. Receptor mRNA relative expression was measured with real-time PCR using the $2^{-\Delta\Delta C}{}_T$ Livak method [15]. The relative expession of the target (HIV-infected), normalized to an endougenous reference (18S rRNA) and controls (15 non–HIV-infected women), was obtained by $2^{-\Delta\Delta C}{}_T$, whereas $\Delta\Delta C_T$ was defined as the difference of $\Delta C_{T\ (target)}$ and $\Delta C_{T\ (controls)}$, and the ΔC_T was defined as the difference in $C_{T\ (CCR5,\ CXCR4\ or\ DC\text{-}SIGN)}$ and the $C_{T\ (endogenous\ reference)}$ [16]. Serial dilutions from one of the samples were made to validate the efficiency of the quantitative PCR (qPCR) assay and were tested with the Taqman probes. The results were analyzed in a calibration curve considering that the regression value must be close to 1 and the slope close to -3.32 [17]. All reactions were adjusted to a concentration of 10 ng. Double distilled water was used as a negative control. Ten microliters of the TaqMan Universal PCR master mix (Applied Biosystems/Roche, Foster City, CA) were mixed with 1 μL of the corresponding TaqMan assay. Reactions were completed at 20 μL total volume by adding double distilled water and were performed using the iCycler thermocycler (Bio-Rad, Hercules, CA) in the required settings for TaqMan assays. Primers and probes were designed based on the mRNA sequence, and the sequences were 5'-TGT AGA AGG AGA CAG AGC TGG TT-3' for the forward primer, 5'-GCC GTC AAG GTT CTT CAT GAT CTA G-3' for the reverse primer and 6FAM-5'- CCT CCC CAT GTC TTC C-3'-TAMRA fluorogenic probe for the CCR5 co-receptor. 5'-GTT GTC TGA ACC CCA TCC TCT ATG-3' forward, 5'-CGT GCT GGG CAG AGG TTT TA-3' reverse and 6FAM-5'-AAT TTG GCT CCA AGG AAA G-3'-TAMRA probe for CXCR4; and for the DC-SIGN, 5'-GTC CCT CAG TGG AGC AAG TT-3' forward, 5'-TTC GTT TCT CCT TCT TCA GGG C-3' reverse and 6FAM-5'-CCT GCT GGC GTT TCT-3'- TAMRA probe. Each qPCR reaction was made in triplicate.

Statistical Analysis. Medians and ranges were used to describe the main results. The Kolmogorov–Smirnov test was used to evaluate the normality of variables. The non-parametric Wilcoxon's signed rank test and the Friedman's analysis of variance (ANOVA) for repeated measures were used to compare expression levels between the evaluated co-receptors. To compare expression of these co-receptors according to the HAART status of the patients the Mann–Whitney U test and the Kruskal–Wallis' ANOVA were used. The Spearman's test was used to evaluate the correlation of the co-receptor expression levels with the VL and T CD4+ lymphocytes counts. Analysis was carried out using the SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

RESULTS

Patient sample. Cervical biopsies from 26 HIV-1 heterosexually infected women, 22 to 69 year old, were examined. All patients were asymptomatic and had been diagnosed with HIV-1 infection 1 to 13 years prior to enrollment. At the time of sampling, fourteen patients were on Highly Active Antiretroviral Therapy (HAART) and nine had a history of antiretroviral treatment. Most of these

Table 1. Clinical Characteristics of 26 HIV-1 Heterosexually Infected Mexican Women Included in the Study

Patient No.	Age	Time of Infection[a]	Sexual Partners[b]	Current CD4+ Cell Count[c]	Current Viral Load[d]	HAART Treatment Status	Contraceptive Use
1	69	6	1	560	<50	Currently	Abstention
2	67	3	1	260	<50	Previously	None
3	41	4	1	200	1870	Currently	Intrauterine device
4	32	3	1	357	5600	Naïve	Abstention
5	36	11	1	421	<50	Previously	Hormonal oral
6	47	2	1	350	<50	Naïve	None
7	38	13	1	1200	<400	Currently	Condom
8	34	1	1	201	<50	Previously	Hormonal oral
9	38	8	1	258	<50	Previously	Abstention
10	26	10	1	445	<50	Currently	Abstention
11	40	2	2	360	<50	Currently	Bilateral tubal occlusion
12	48	9	1	560	<50	Currently	None
13	39	1	2	450	<50	Previously	Abstention
14	48	1	1	800	<50	Currently	Abstention
15	51	2	1	430	<50	Previously	Condom
16	48	6	1	208	1800	Previously	Abstention
17	51	3	1	798	<50	Currently	None
18	41	2	1	546	<50	Previously	None
19	46	1	1	452	<400	Currently	Hormonal oral
20	35	4	1	246	<50	Currently	None
21	27	1	1	450	<50	Previously	None
22	22	6	2	310	400	Currently	Condom
23	46	1	1	969	400	Previously	Abstention
24	29	3	1	300	1200	Naïve	Abstention
25	38	9	1	609	<50	Currently	Abstention
26	62	3	1	550	<400	Currently	Hormonal oral

[a]From the date of the HIV diagnosis in years.
[b]Personal information obtained through direct questioning.
[c]As the number of cells/mm^3 of blood.
[d]As the number of RNA copies/ml of plasma using the COBAS Amplicor HIV-1 Monitor (Roche Diagnostics, Branchburg, NJ, USA).

patients had previously received or were receiving the combination of one protease inhibitor boosted with ritonavir plus two Nucleoside Reverse Transcriptase Inhibitors (NRTI). The other three patients had never received antiretroviral therapy. The viral load (VL) of most of the patients was under the detection level (<50 RNA copies/ml) with the exception of four patients whose VL was between 1200 and 5600 copies/ml. Absolute CD4+ cell counts of all patients were above 200 /µl (Table **1**).

Expression of CCR5, CXCR4 and DC-SIGN mRNA. Different expression levels were found for the three co-receptor mRNAs evaluated in the cervical biopsies ($p<0.0001$). Expression of CCR5 ($Ratio_{[test/calibrator]}$ median 1.82; range 0.003–2934) was higher than that observed for CXCR4 (0.79; 0.0061–3312) ($p=0.025$), and the expression of the former was in turn higher than that of the DC-SIGN receptor (0.33; 0.006–532) ($p=0.046$) (Fig. **1**). A high positive correlation was observed between the expression levels of the three receptor-mRNAs evaluated (r_s ranging from 0.52 to 0.85, $p<0.01$). No correlation was found between the expression levels of the three co-receptors evaluated with the CD4+ cell counts in blood (r_s ranging from −0.104 to −0.155, p=NS), but we found a moderate correlation of the levels of plasma viral load with the expression of CCR5 ($r_s=0.41$, $p=0.04$) and DC-SIGN ($r_s=0.40$, $p=0.04$). On the other hand, we did not find any difference when expression levels of the three evaluated co-receptor mRNAs were compared according to the HAART status. Thus, median and ranges of CCR5 levels were 1.76 [0.02–759.42], 1.36 [0.00–2934.20] and 31.31 [0.04–98.27] for patients currently on HAART, previously on HAART and naïve, respectively ($p=0.91$). The expression levels of CXCR4 were 0.81 [0.01–696.39], 0.27 [0.01–3312.30] and 6.69 [0.06–146.40] for the same groups of patients ($p=0.87$),

while DC-SIGN levels were 0.32 [0.01–327.50], 0.27 [0.01–532.36] and 8.61 [0.03–12.18] (p=0.94). No significant differences were found in the expression of the three receptors when we compared pre- and post-menopausal HIV infected patients; however, in pre-menopausal patients all of them were higher. Thus, median and ranges of CCR5 levels were 4.34 [0.00–2934.20] and 0.30[0.02–759.42] in pre-menopausal and post-menopausal women, respectively (p=0.16). The expression levels of CXCR4 were 2.49 [0.01–3312.60] and 0.26 [0.01–693.39] for the same groups of women (p=0.17) and for DC-SIGN 4.74 [0.01–532.36], and 0.09 [0.01–327.70] (p=0.19).

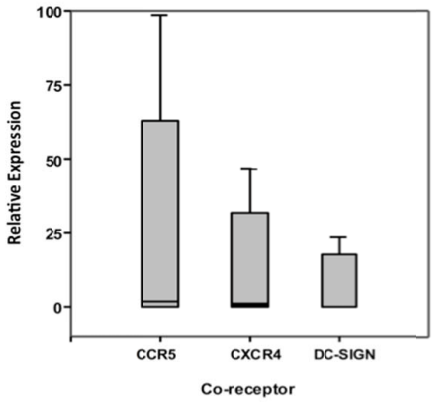

Fig. (1). Box and whisker plot of CCR5, CXCR4 and DC-SIGN receptor expression relative to receptor expression in uninfected women in cervical biopsies of 26 HIV-1 heterosexually infected Mexican women determined by the real-time PCR relative quantification scheme. Extreme values are hidden.

DISCUSSION

The global spread of the HIV-1 infection has been achieved by sexual transmission through the genital tract and women seem to be at a higher risk than men are [18]. Infection of cells with HIV-1 in sexual contact sites requires an additional chemokine receptor and it has been suggested that CCR5 is the first receptor utilized, followed by CXCR4 during advanced stages of the disease. Some studies have suggested that the higher expression of CCR5 may be the primary explanation for preferential transmission of CCR5-tropic (R5) isolates within the genital tract; however, this concern is still controversial [9]. In this study, the expression levels of CCR5, CXCR4 and DC-SIGN co-receptor mRNA in cervical biopsies of HIV-1 infected Mexican women were evaluated.

In the present study, the expression of CCR5 mRNA was higher than that observed for CXCR4 or DC-SIGN. Patterson *et al*. [8] also found that the expression levels of CCR5 mRNA were significantly higher than the CXCR4 levels in cervical biopsies obtained from five HIV infected patients. However, McClure *et al*. [9] evaluated cervical scrape samples obtained from a cohort of healthy women and reported that the expression of CCR5 was lower than that of CXCR4. This inconsistency could be due to differences in the evaluated patients and tissue sampling. In contrast to the McClure study, we did not evaluate healthy women. Our findings also differ from those reported by Zhang *et al*. [19] who, by immunohistochemical staining, found a high expression of CXCR4 in vaginal and cervical samples obtained from both women and macaques. Although in the present study immunohistochemical techniques were not used as Zhang *et al*. did, we used a more sensitive and well-accepted method to quantitatively evaluate mRNA expression levels of these co-receptors [20, 21].

All patients included in the present study acquired HIV infection through sexual intercourse, the most common way of transmission. Sheffield *et al*. [22] showed increased numbers of CD4+ cells expressing CCR5 in biopsies obtained from genital ulcers and it is well known that this type of visible lesions on the cervical mucosa increases the risk of HIV transmission in women [22, 23]. We may exclude such lesions as an explanation for the observed levels of expression of the receptors evaluated because patients with genital ulcers were excluded from our study, however, we did not evaluate the expression levels of mRNA of these receptors on CD4+ cells alone, but on all cells of the endocervix.

A number of studies have shown that the menstrual cycle does not significantly affect the immune status of the genital tract [24-26], nevertheless, Howell *et al*. found that receptor and co-receptor expressions in the genital tract vary as a function of menstrual cycle stages [27]. Therefore, we excluded menstruating patients and those with recent vaginal intercourse to reduce the possibility of mucosal changes related to these events. Prakash *et al*. [28] demonstrated that the use of oral contraceptives is associated with an increased expression of CCR5 on CD4+ T endocervical lymphocytes, which may increase the susceptibility of these cells to HIV-1 infection [29]. Although the microenvironment of the female genital tract may be altered by the use of oral contraceptives, it is highly unlikely that our findings were secondary to their use owing to a low percentage of our patients using them. Moreover, we did not find significant differences between the mRNA expression levels of the three co-receptors, but they tended to be higher in pre-menopausal women when compared with those of post-menopausal women. This suggests that the pre-menopausal women are at a higher risk of re-infection by sexual intercourse with HIV-1 infected partners, but further studies are needed to elucidate the role of the sex hormone status in modifying HIV receptors' and co-receptors' expression and changing the susceptibility of target cells to the HIV infection.

We found no significant differences in the expression levels of mRNA of the three receptors tested according to the antiretroviral treatment status. Previous studies have demonstrated an upregulation of CCR5 expression on several immune cells occurring early in the HIV infection that seems to provide a selective advantage to the virus but that is downregulated toward a normal expression by the antiretroviral therapy [30, 31]. The lack of differences we found might be attributed to the wide variability in the levels of expression observed which in turn might be due to the small number of patients evaluated. In the study by McClure *et al*. [9], a wide range of CXCR4 expression in the female ecto and endocervix was found but not for CCR5.

It has been demonstrated that in addition to the conventional binding of HIV to CD4 and the chemokine coreceptors (CCR5, CXCR4), HIV can interact with

adhesion molecules as DC-SIGN, complement receptors, Fc receptors, and heparan sulfate proteoglycans [32]. We observed a low expression of DC-SIGN in comparison with the other evaluated receptors in the genital tract of HIV-1 heterosexually infected Mexican women. Approximately 1 to 5% of the cells in the rectal mucosa express DC-SIGN [31], which may be related to the low expression levels of this receptor found in the cervix. Jameson *et al*. [13] detected cells expressing DC-SIGN receptors in the subepithelial lamina propia of the vagina and cervix, with a small proportion of these cells co-expressing CCR5 in the vagina but not in the cervix. This finding appears to be different from our results. Additionally, Gurney *et al*. [33] demonstrated that differences in the expression levels of DC-SIGN, CCR5 and CXCR4 chemokine receptors might be relevant in the transfer of the virus from the peripheral to the secondary lymphoid organs or in supporting the local viral infection and replication. However, since the mechanisms involved in the mucosal HIV infection remain unresolved, new insights into the dynamics of these events are needed.

The contributing factors in the emergence of X4 variants in later stages of the HIV infection are unknown. Montfort *et al*. [34] reported that dendritic cells in mucosal sites do not easily transfer R5 viruses. The dendritic cells preferentially transfer X4 viruses to the CD4+ T lymphocytes in trans, an effect observed in cells expressing high levels of DC-SIGN receptors [34]. In this study, low levels of DC-SIGN expression in cervical biopsies of HIV heterosexually infected women were found, which might be related to the preferential transmission of R5 variants upon sexual contact.

In conclusion, our findings show that levels of expression in cervix of the evaluated chemokine receptors differ from one receptor to another and vary from woman to woman. In the cervical biopsies obtained from heterosexually HIV-1 infected Mexican women, the expression of the CCR5 co-receptor was higher than that of the CXCR4 and DC-SIGN. This finding seems to support the suggestion that the chemokine receptor expression may influence the transmission of R5 isolates. However, further studies evaluating cervical samples obtained from larger series of patients and, above all, cohorts of recently HIV-1 infected women followed over longer periods are needed before definitive conclusions about the actual role of these co-receptors may be drawn.

AUTHORS CONTRIBUTIONS

LGRM and PLG designed the study, carried out experimental and analysis work, and prepared the manuscript. JMVG and GCPS assisted in designing the study, analysis work and drafting the manuscript. AGRT, ARP and PIAG assisted in the interpretation of the data, and in writing and revision of this report. CRP supervised the study and provided substantial intellectual content to the manuscript. All authors critically reviewed and approved the final manuscript.

COMPETING INTEREST

The authors declare that they have no competing interests.

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