Functional Correlation of P-Glycoprotein Expression and Genotype with Expression of the Human Immunodeficiency Virus Type 1 Coreceptor CXCR4

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The aim of this study was to investigate the relationship between lymphocyte P-glycoprotein (P-gp) expression and genotype in vivo and the expression of lymphocyte receptors critical in the life cycle of human immunodeficiency virus type 1 (HIV-1), i.e., CD4, CCR5, and CXCR4. Using flow cytometry to quantify each membrane receptor/transporter, we demonstrate a highly significant correlation between P-gp protein expression and the expression of CXCR4 (rho = 0.874; P < 0.0001). Furthermore, confocal microscopy showed colocalized expression of CXCR4 and P-gp in the lymphocyte membrane. This significant relationship was also apparent at the mRNA level by use of reverse transcriptase-PCR (rho = 0.61; P < 0.005) and was present in both phytohemagglutinin-stimulated and unstimulated peripheral blood mononuclear cells. Genotypic analysis of the C345T single-nucleotide polymorphism of P-gp confirmed significantly higher levels of P-gp in C (range, 2.45 to 11.00 relative fluorescence units [RFU]) than in T (range, 0.25 to 5.00 RFU)-homozygous individuals (P = 0.0088; 95% confidence interval [95% CI], 0.7 to 6.3 RFU). An equivalent association between CXCR4 levels and C (range, 12.7 to 44.1 RFU) versus T (range, 3 to 18.9 RFU) genotype was also demonstrated (P = 0.0019; 95% CI, 5.4 to 23.7). Functionally, although these correlates had no impact on HIV-1 production from either X4- or R5-tropic virus, expression correlated significantly with the activity of the HIV-1 protease inhibitor (PI) saquinavir for both P-gp (rho = 0.75; P = 0.0019) and CXCR4 (rho = 0.71; P = 0.0041). This study defines an association between P-gp (expression and genotype) and CXCR4 that may have implications for the selection of viral tropism and the access of drugs to protease for specific tropic types. The interplay between these two proteins may also influence the viral genotypes which escape effective chemotherapy and which therefore have the opportunity to evolve resistance to PIs.

A number of G protein-coupled CC and CXC chemokine receptors have been shown to act as human immunodeficiency virus (HIV-1) coreceptors in vitro (47, 48). CCR5 and CXCR4 are the major HIV-1 coreceptors in vivo (46). The selective use of the CCR5 and/or CXCR4 coreceptor is the predominant determinant of cellular tropism observed for different HIV-1 isolates (3, 7). CCR5 is the principal coreceptor for primary and early infection (R5 isolates). The appearance of variants that use CXCR4 or both coreceptors (X4 and R5X4 isolates) results in accelerated CD4+ T-cell loss and disease progression (6, 36), and evidence suggests that patients with higher expression of CXCR4 in lymphocytes acquire X4-tropic strains of virus more rapidly (24).

The introduction of protease inhibitors (PIs) has dramatically improved the prognosis for HIV infections. However, PIs such as saquinavir (SQV) have a variable and frequently low bioavailability (29). High dosages are often required; this has clinically improved the prognosis for HIV infections. However, PIs which therefore have the opportunity to evolve resistance to PIs.

P-gp is a member of the largest class of membrane transport proteins, designated the ATP-binding cassette (ABC) superfamily (19). A number of recent studies have also implicated P-gp in the infectivity of HIV (22, 32, 37). P-gp overexpression blocks insertion of the influenza virus fusion protein (hemagglutinin-2) into the plasma membrane (32), and this inhibits membrane fusion and infectivity. Furthermore, HIV-1 infectivity is lower in CD4+ T-cell lines, which overexpress P-gp (22). The authors concluded that P-gp expression inhibited HIV-mediated membrane fusion, as well as a subsequent step(s) in the HIV-1 life cycle. Recently, Speck et al. reported similar data for drug-selected (P-gp-overexpressing) CEM cells; the effect was reversible by verapamil (a known P-gp inhibitor), and the authors speculated that overexpression of P-gp and its localization to lipid rafts may disrupt critical protein-protein interactions because of the physical size and abundance of P-gp (37). Indeed, evidence suggests that CD4 and CXCR4 form clusters within lipid rafts that are necessary for efficient HIV infection (23). However, this does not explain the sensitivity to verapamil, and subsequently, a significant difference in the expression of CD4 and CXCR4 between CEM and drug-selected CEM cells grown in our lab was observed (27, 33).

In addition to these biochemical analyses, information on the relationship between HIV and P-gp has emerged by anal-
Expression of CD4, CXCR4, CCR5, and P-gp on peripheral blood mononuclear cells isolated from healthy volunteers.
TABLE 1. Correlations between proteins and mRNA species analyzed in this study and expression in different MDR1 genotypes

<table>
<thead>
<tr>
<th>Species</th>
<th>Correlation with the following protein</th>
<th>Expression in the following MDR1 genotype:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXCR4</td>
<td>CCR5</td>
</tr>
<tr>
<td>P-gp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$r = 0.87$</td>
<td>$r = -0.22$</td>
</tr>
<tr>
<td>mRNA</td>
<td>$r = 0.61$</td>
<td>ND$^f$</td>
</tr>
<tr>
<td>CXCR4</td>
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<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$r = -0.1$</td>
<td>$r = -0.28$</td>
</tr>
<tr>
<td>mRNA</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CCR5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>$r = 0.47$</td>
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<td>mRNA</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD4</td>
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<tr>
<td>mRNA</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$In median RFU for protein and arbitrary units relative to GAPDH expression for mRNA.

$^b$P < 0.0001.

$^c$P > 0.05.

$^d$P < 0.05.

$^e$P < 0.01.

$^f$P < 0.005.

$^g$ND, not determined.

$^h$P > 0.5.

$^i$P < 0.5.

Data on correlations between CD4, CXCR4, CCR5, and P-gp on PBMC from healthy donors are summarized in Table 1. P-gp expression on healthy donor PBMC (n = 21) was 2.75 RFU (range, 0.25 to 11.00 RFU). CXCR4 expression was 11.2 RFU (range, 3.00 to 44.10 RFU), and CCR5 expression was 0.23 RFU (range, 0.010 to 0.48 RFU). CD4 expression on PBMC of healthy volunteers was 11.2 RFU (range, 3.00 to 44.10 RFU), and CCR5 expression was 23.49 RFU (range, 3.63 to 71.96 RFU). CXCR4 expression was 0.12 RFU (range, 0.25 to 5.00) RFU, respectively (Fig. 3a). P-gp expression was significantly higher in CC individuals than in CT (P = 0.0088; 95% CI, 0.7 to 6.3) individuals. When CXCR4 expression on PBMC was assessed in the same genotype groups, values were 19.1 RFU (range, 12.7 to 44.1 RFU) in CC individuals, 9.1 RFU (range, 7.15 to 18.15 RFU) in CT individuals, and 5.7 RFU (range, 3.0 to 18.9 RFU) in TT individuals (Fig. 3b). Expression was again significantly higher in CC individuals than in CT (P = 0.0045; 95% CI, 3.5 to 21.1) and TT (P = 0.0019; 95% CI, 5.4 to 23.7) individuals.

No significant differences in expression of CCR5 or CD4 were observed between C3435T genotypes (data not shown).
Localization of P-gp and CXCR4 by confocal laser scanning microscopy. Confocal laser scanning microscopy revealed P-gp and CXCR4 to be expressed on the same cells within the lymphocyte population (Fig. 4b and c). Furthermore, expression of these proteins was colocalized within individual cells (Fig. 4d). This was not the case for P-gp and CD11a (Fig. 4e to h).

Relative expression of CXCR4 and MDR1 mRNAs in peripheral blood mononuclear cells isolated from healthy volunteers. Data on the expression of CXCR4 and MDR1 mRNA in PBMC, expressed relative to GAPDH expression in arbitrary units, are summarized in Table 1. In PBMC isolated from healthy donors (n = 19), MDR1 expression was 1.0 arbitrary unit (range, 0.16 to 2.95) and CXCR4 expression was 1.16 arbitrary units (range, 0.43 to 2.41). A significant positive correlation was observed between the MDR1/GAPDH ratio and the CXCR4/GAPDH ratio (rho = 0.61; P = 0.005) (Fig. 5).

MDR1 expression for CC individuals (n = 6), CT individuals (n = 7), and TT individuals (n = 6) was compared. Expression of MDR1 mRNA from individuals with CC, CT, and TT genotypes was 1.45 (range, 0.97 to 2.43), 0.97 (range, 0.51 to 2.95), and 0.90 (range, 0.16 to 2.41) arbitrary units, respectively. CXCR4 mRNA expression was 1.69 (range, 1.02 to 2.41) arbitrary units in PBMC isolated from CC individuals, 1.23 (range, 0.57 to 1.94) arbitrary units for CT individuals, and 0.70 (range, 0.43 to 1.38) arbitrary units for TT individuals. Expression of CXCR4 mRNA in CC individuals was significantly higher than that in TT individuals (P = 0.0027; 95% CI, 0.3 to 1.5).

FIG. 2. Representative scatter plots obtained from dual-color flow cytometry of PBMC stained with (a) isotypically matched negative-control antibodies and with (b) CXCR4-specific, (c) P-gp-specific, and (d) P-gp- and CXCR4-specific antibodies. Values in each quartile indicate number of events within the region.

FIG. 3. Expression of (a) P-gp and (b) CXCR4 in relation to C3435T genotype in healthy volunteers (n = 6 CC, 7 CT, 6 TT). Expression was determined by flow cytometry and is expressed in relative fluorescence units for each protein and genotype by TaqMan allelic discrimination. Statistical analysis was carried out by one-way analysis of variance (*, P < 0.05; ***, P < 0.01).
Relationship between P-gp or chemokine receptor expression and viral replication in activated peripheral blood mononuclear cells. To determine the influence of membrane proteins on HIV replication, activated PBMC were infected with either HIV-IIIB (X4-tropic) or JRCSF (R5-tropic), and p24 recovery was measured after 7 days. Median HIV-IIIB (n = 24) and HIV JRCSF (n = 24) p24 recoveries were 61,210 (range, 20,040 to 263,400) and 27,704 (range, 3,727 to 2,335,000) pg·ml⁻¹, respectively. No significant correlation was observed between HIV-IIIB p24 recovery and expression of CXCR4 (rho = 0.22; P = 0.30; n = 23) or P-gp (rho = 0.39; P = 0.06; n = 23). Results were similar for HIV-JRCSF p24 recovery compared to expression of CXCR4 (rho = 0.40; P = 0.06; n = 23) and P-gp (rho = 0.29; P = 0.17; n = 23).

When P-gp activity was blocked by addition of XR9576, there was no significant difference between p24 recovery for HIV-IIIB (median, 129,045 [range, 15,782 to 1,351,481] pg·ml⁻¹; P = 0.19) or HIV-JRCSF (median, 32,382 [range, 4,894 to 2,169,319] pg·ml⁻¹; P = 0.24) and p24 recovery in the absence of XR9576.

Relationship between membrane-bound protein expression and the SQV IC₅₀ and IC₉₀ against viral replication in activated peripheral blood mononuclear cells. The IC₅₀ and IC₉₀ of SQV against HIV-IIIB in activated PBMC were 8.05 nM (range, 1.3 to 25.2 nM; n = 16) and 11.97 nM (range, 1.3 to 69.18 nM; n = 16), respectively. The SQV IC₅₀ and IC₉₀ correlated significantly with both P-gp expression (for the IC₅₀, rho = 0.83, P = 0.0002, and n = 15; for the IC₉₀, rho = 0.75, P = 0.0019, and n = 15) (Fig. 6a) and CXCR4 expression (for the IC₅₀, rho = 0.57, P = 0.028, and n = 15; for the IC₉₀, rho = 0.71, P = 0.0041, and n = 15) (Fig. 6b) on PBMC.

FIG. 4. Combined fluorescence and bright-field images of peripheral blood mononuclear cells. (a and c) Bright-field images of collections of cells. (b and d) Localization of fluorescence of anti-CXCR4 and anti-P-gp antibodies, respectively, in the cells shown in panel a. (d) Superimposition of the three images in panels a to c, with areas of colocalization in yellow. (f and g) Localization of fluorescence of anti-CD11a and anti-P-gp antibodies, respectively, in the cells shown in panel e. (h) Superimposition of the three images in panels e to g, with areas of colocalization in yellow.

FIG. 5. Relationship between expression of CXCR4 mRNA in PBMC and the IC₅₀ and IC₉₀ of SQV against viral replication in activated peripheral blood mononuclear cells. The IC₅₀ and IC₉₀ of SQV against HIV-IIIB in activated PBMC were 8.05 nM (range, 1.3 to 25.2 nM; n = 16) and 11.97 nM (range, 1.3 to 69.18 nM; n = 16), respectively. The SQV IC₅₀ and IC₉₀ correlated significantly with both P-gp expression (for the IC₅₀, rho = 0.83, P = 0.0002, and n = 15; for the IC₉₀, rho = 0.75, P = 0.0019, and n = 15) (Fig. 6a) and CXCR4 expression (for the IC₅₀, rho = 0.57, P = 0.028, and n = 15; for the IC₉₀, rho = 0.71, P = 0.0041, and n = 15) (Fig. 6b) on PBMC.

DISCUSSION

In this study we have utilized healthy donor PBMC as a model to investigate the effects of P-gp, receptor, and coreceptor expression on HIV susceptibility. In contrast to CEM and CEMVBL cells (27, 33), a strong positive correlation of P-gp and CXCR4 expression on the surfaces of PBMC was observed, with 80% of P-gp-expressing PBMC also expressing CXCR4 (Fig. 2), suggesting limitations to the cell line model. The two proteins were further shown to colocalize on the surfaces of individual cells. This relationship was also observed at the mRNA level, and expression of both P-gp protein and
P-gp mRNA was related to the C3435T genotype in the following order (from highest to lowest): CC, CT, TT. This relationship between P-gp expression and genotype is in agreement with the findings of previous studies (9, 14). However, a novel finding was that the coexpression of P-gp and CXCR4 was also reflected in relation to C3435T genotype, with CXCR4 protein and mRNA also expressed in the order (from highest to lowest) of CC, CT, and TT.

The mechanism by which P-gp expression and CXCR4 expression are linked is unclear, although correlation at the level of mRNA suggests that the mechanism may be in part transcriptional. Examination of a 1-kb sequence upstream of the CXCR4 gene indicates putative recognition sites for a number of transcription factors, including AP-1, NF-kB, SP-1, C/EBPβ, and NF-Y. Indeed, NF-kB has recently been shown to regulate CXCR4 via these sequences (13), and all of these proteins have been shown to influence P-gp expression via sequences in the MDR1 promoter. It is possible that individual variability in the activity of one or more of these nuclear proteins may account for differential regulation of the two genes under baseline conditions. However, this is speculative, and given the apparent lack of biological plausibility, it is now imperative that the relationship between CXCR4 expression and MDR1 genotype be examined in larger cohorts.

CD4 expression in PBMC reflects the pattern observed in CEM and CEMγvrl cells (27, 33), with increased P-gp expression correlating with decreased CD4 expression. However, although this relationship in PBMC was statistically significant, it was not as striking as that observed between P-gp and CXCR4; it appears to be the result of a few outliers expressing extremely high levels of CD4. Indeed, CD4 expression was independent of the C3435T genotype. This finding does, however, suggest that the relationship observed between P-gp and CXCR4 expression is specific to these proteins and is not a reflection of general coordination of membrane surface proteins.

We failed to show a correlation between CCR5 and P-gp expression in the cell membrane. However, as CCR5 expression is known to be low on PBMC (the majority of CCR5 is expressed on macrophages), it cannot be ruled out that in many individuals CCR5 protein expression is below the limit of detection by the assay employed in this study. For this reason, CCR5 expression was not investigated at the mRNA level. We are currently investigating whether a relationship between CCR5 and P-gp exists in monocyte-derived macrophages.

In order to further investigate the implications of membrane-bound protein expression on PBMC for cell-virus interactions, activated PBMC were assessed for expression of P-gp, CXCR4, and CCR5 and were infected with either an X4- or an R5-tropic virus. The protein expression relationships observed in nonactivated PBMC were maintained following activation: a strong correlation was observed between P-gp and CXCR4 expression, and CCR5 expression did not correlate with that of P-gp. These findings again suggest a common regulatory mechanism for P-gp and CXCR4, requiring further investigation.

Following 7 days of infection with HIV-IIIB (X4-tropic) or HIV-JRCSF (R5-tropic), there was large intraindividual variation in the amount of virus produced by PBMC, with a ca. 10-fold difference between the highest and lowest values obtained for HIV-IIIB and a 1,000-fold difference for HIV-JRCSF.

Increased expression of CXCR4 and CCR5 has recently been shown to enhance infection by X4- and R5-tropic strains of HIV (40). Similarly, a weak correlation was observed between either CXCR4 or P-gp expression and HIV-IIIB p24 recovery from PBMC in our study. However, when P-gp function was blocked by addition of XR9576, a potent and specific P-gp inhibitor, no effect was observed on HIV-IIIB p24 recovery, suggesting either that CXCR4 is the determinant of HIV-IIIB recovery and P-gp is merely a "bystander" as a result of coregulation of expression or that P-gp does not act directly on HIV infectivity. This is in contrast to the findings of Speck et al., who showed a reversal of infectivity by use of the P-gp inhibitor verapamil (37). This discrepancy may be explained by the observation that verapamil has numerous effects on cellular processes in lymphocytes. These include effects on Ca^2+ signaling, other transporters (2, 11), immunologically important proteins (41, 45), and membrane proteins (43, 44). However, a similar effect was observed by Lee et al. using both quinidine and PSC 833 as P-gp inhibitors (22). JRCSF production also correlated weakly with expression of P-gp and CXCR4, and addition of XR9576 to inhibit P-gp activity had no effect on p24 recovery.

Finally, there was a significant correlation between the expression of P-gp or CXCR4 and the concentration of SQV required to inhibit viral replication in PBMC. Because chemokine receptors are not thought to be involved in drug transport, it would seem likely that the increased IC_{50} of SQV in cells expressing high levels of membrane-bound proteins was due to decreased intracellular drug concentrations as a result of active efflux by P-gp. In vivo studies have not demonstrated a correlation between P-gp expression on lymphocytes and response to antiviral therapy including a PI (1, 26), although Fellay et al. noted increased immune reconstitution in HIV patients with the TT genotype (and therefore lower P-gp expression) at position 3435 in the MDR1 gene (9). This finding is also in contrast to previous studies reporting no significant differences in the IC_{50} of either SQV, ritonavir, indinavir, or nelfinavir between P-gp-overexpressing cells and their parental cell line (39). The authors suggested that intracellular drug concentrations would be affected by P-gp efflux only at high extracellular concentrations in excess of those required to inhibit viral replication. Our findings with PBMC suggest this is not the case, but which of these methods more accurately predicts the in vivo scenario is unclear at this time.

Our findings have a number of potential clinical implications. First, the correlation between P-gp expression and the IC_{50} of saquinavir suggests an important role for this transporter in HIV therapy. Second, since P-gp expression and CXCR4 expression correlate positively, it is possible that the relatively rapid emergence of more pathogenic X4-tropic strains of HIV that has been observed in patients with high lymphocyte CXCR4 expression (24) may be exacerbated by the increased IC_{50} of SQV against viruses in these cells. That is, in patients on therapy, higher CXCR4 expression may lead to increased CXCR4-dependent infection and concomitant higher P-gp expression, rendering the viruses within these cells less sensitive to drugs. This may facilitate the replication, and thereby speed the emergence, of X4-tropic viruses, a phenomenon that is related to accelerated disease progression.

The role of X4-tropic viruses in hastening disease progression has been the subject of debate but is supported by the
clinical observation that X4-tropic strains of virus are temporarily associated with a decline in CD4+ T-cell numbers and with AIDS (6, 20, 33, 38, 42). Also, X4 and X4/R5 strains (experimental strains as well as primary isolates) deplete CD4+ cells in vitro (activated PBMC, ex vivo lymphoid tissue, and noninflammatory human spleen tissue), whereas the effects of R5 strains on the CD4/CD8 ratio are much less marked despite similar rates of replication (12, 21, 28, 34). Furthermore, this CD4-selective toxicity is eliminated in these models by compounds that block CXCR4 (34). Finally, in support of our hypothesis, clinical data suggesting that antiretroviral treatment may create an environment for the emergence of CXCR4 tropism are now beginning to emerge (18, 30).

In summary, a correlation between P-glycoprotein and CXCR4 expression that influences virus production and the IC50 of saquinavir exists in PBMC. This is likely to be important for established antiretroviral drugs that are known to be substrates for P-gp as well as for new-generation compounds that target these coreceptors as well as fusion. The simultaneous higher levels of P-gp and CXCR4 may therefore have implications for the efficacy of both protease inhibitors and fusion inhibitors when given in combination. Further work to fully characterize this relationship with respect to HIV is now necessary, and studies are currently under way to investigate whether a similar relationship is observed on the surfaces of various cellular subsets and on PBMC isolated from HIV-positive individuals.

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