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Induction of Intercellular Adhesion Molecule-1 on Human Brain Endothelial Cells by HIV-1 gp120: Role of CD4 and Chemokine Coreceptors

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SUMMARY: Central nervous system dysfunction is commonly observed in children with HIV-1 infection, but the mechanisms whereby HIV-1 causes encephalopathy are not completely understood. We have previously shown that human brain microvascular endothelial cells (HBMEC) from children are responsive to gp120 derived from X4 HIV-1 by increasing expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1. However, the mechanisms involved in gp120-mediated up-regulation of cell adhesion molecule expression is unclear. In the present study, we found that gp120 derived from both X4 and R5 HIV-1 induced increased expression of ICAM-1 on HBMEC, but the degree of this up-regulation differed among the various HBMEC isolates. The up-regulation of ICAM-1 was inhibited by anti-CD4 antibodies as well as by specific antibodies directed against chemokine receptors and small-molecule coreceptor inhibitors. Anti-CD4 antibodies inhibited the increase in ICAM-1 expression mediated by gp120 derived from X4 and R5 HIV-1, whereas antibodies against chemokine receptors displayed a differential inhibition depending on the source of gp120. Both X4 and R5 gp120-induced ICAM-1 expression was sensitive to pertussis toxin and involved the nuclear factor-kB pathway. These findings indicate a direct involvement of CD4 and a differential involvement of chemokine receptors in the activation of pediatric HBMEC by X4 and R5 gp120. The activation of brain endothelium of children by HIV-1 protein gp120 by way of CD4 and chemokine receptors may have implications for the pathogenesis of HIV-1 encephalopathy in the pediatric population. (*Lab Invest 2003, 83:1787–1798*).

HIV-1 enters the central nervous system (CNS) and causes encephalopathy in AIDS patients. HIV-1 may cross the blood-brain barrier (BBB) endothelium by way of several possible mechanisms. HIV-1 may enter the CNS using the Trojan Horse mechanism by way of transmigration of HIV–1-infected monocytes (Nottet et al, 1996; Persidsky et al, 1997), absorptive endocytosis (Banks et al, 1997), macropinocytosis (Liu et al, 2002), or by directly infecting brain endothelium (Edinger et al, 1997; Moses et al, 1993; Poland et al, 1995). In addition, the brain endothelium may be activated or injured by HIV proteins (eg, gp120) and cytokines and thereby allow increased penetration of cell-free HIV or HIV-infected monocytes into the CNS (Fiala et al, 1997; Stins et al, 2001).

Although it is generally accepted that perturbations of the BBB are common in HIV–1-infected patients, the underlying mechanisms for endothelial dysfunction are unclear. Under normal conditions, the BBB efficiently forms a tight barrier between the blood and the brain, strictly regulating the passage of substances across this barrier. However, during HIV-1 infection, the endothelium of the BBB is affected by many inflammatory substances, viral proteins, viral cofactors, and viral particles originating both from the blood and the brain side, and structural and functional perturbations of the BBB may occur. Evidence for alteration of BBB function in HIV-1 encephalitis (HIVE) is commonly featured after examination of postmortem tissues (Berger et al, 2000; Petito and Cash 1992; Power et al, 1993). A direct compromise of tight junctions (eg, focal alterations in junctional markers such as ZO-1/occludin) was demonstrated in postmortem brain specimens of HIV-1 patients, in a severe combined immunodeficiency disease mouse model for HIVE, and in in vitro cell culture experiments (Persidsky et al, 2000). Additional evidence for increased permeability of the BBB was shown by the leakage of serum proteins into the brain, alteration of vessel diameter, and thinning of basal lamina (Buttner et al, 1996; Power et al, 1993; Rhodes, 1991; Weis et al, 1996).

Alterations in protein expression (eg, decrease in general glycoproteins), a specific decrease in

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P-glycoprotein, and increased cell adhesion molecule expression on the endothelium were demonstrated in human brain specimens with HIVE and in a severe combined immunodeficiency disease mice model (Buttner et al, 1996; Persidsky et al, 2000). In simian models with simian immunodeficiency virus infection, changes in protein expression were found in brain endothelium (eg, increase in vascular cell adhesion molecule [VCAM]-1 expression) (Sasseville et al, 1992) and decrease in glucose transporter expression (Mankowski et al, 1999). Endothelial activation and damage is also reflected in an increase of endothelial cell products circulating in plasma, such as soluble adhesion proteins and procoagulant proteins (eg, von Willebrand factor), soluble thrombomodulin, tissue type plasminogen activator, plasminogen activator inhibitor, angiotensin converting enzyme, and endothelin-1 (Lafeuillade et al, 1992; Rolinski et al, 1994; Schved et al, 1992; Seigneur et al, 1997).

To study the role of the brain endothelium in the pathogenesis of HIV-1 encephalopathy, we developed an in vitro model of the human BBB by isolating and culturing human brain microvascular endothelial cells (HBMEC) (Stins et al, 2001). We were the first to show that gp120 can activate HBMEC from children in up-regulation of VCAM-1, intercellular adhesion molecule (ICAM)-1, and increased monocyte transmigration (Stins et al, 1997). Other investigators have also shown the gp120-mediated induction of ICAM-1 on endothelial cells derived from different origins: lung, skin, coronary artery, and umbilical vein (Ren et al, 2002). However, the underlying mechanisms involved in gp120-mediated activation of HBMEC are unclear (eg, roles of CD4, CCR3, CCR5, and CXCR4 receptors or other nonchemokine receptors).

HIV-1 coat protein gp120 can activate a variety of cell types, such as secretion of TGF-β by astrocytes (non-CD4 dependent) (Da Cunha et al, 1995), IL-1β and TNF-α in the rat brain (Ilyin and Plata-Salaman, 1997), and arachidonic acid metabolites and IL-1 by monocytes (Wahl et al, 1989), and also increase the permeability of rat brain endothelium (Annunziata et al, 1998). Gp120 also induces apoptosis in T lymphocytes (Finkel et al, 1995) and neurons (Hesselgesser et al, 1998; Lannuzel et al, 1997). Gp120 may exhibit these phenotypes by way of CD4, chemokine coreceptors, or other non-CD4 and nonchemokine receptor mechanisms (eg, substance P) (Annunziata et al, 1998). Endothelial cells are heterogeneous and differ based on the source (eg, various organs, macro- versus microvessels, age, or sex) (Craig et al, 1998; Kumar et al, 1987; MacLean et al, 2001). Endothelium from the brain has been shown to display a differential panel of receptors for HIV-1 gp120, such as chemokine coreceptors (Andjelkovic and Pachter, 2000; Berger et al, 2000; Edinger et al, 1997; Mukhtar et al, 2002), sulfatide (Prasadarao et al, 1993), and CD4 (Stins et al, 2001). We showed that the gp120-mediated enhancement of monocyte transendothelial migration in pediatric HBMEC was blocked by anti-CD4 antibodies, indicating that CD4 in pediatric brain endothelial cells is functional and responsive to gp120. In contrast, HBMEC derived from adults is not responsive to gp120. We have previously shown the presence of CD4 in pediatric endothelial cells using four different methods (eg, RT-PCR, FACS analysis, and immunocytochemistry of isolated HBMEC and frozen brain sections) (Stins et al, 2001). However, the involvement of HIV-1 receptors such as CD4 and chemokine coreceptors in the gp120-induced ICAM-1 expression is unclear.

In this report, we examined the involvement of CD4 and chemokine coreceptors in gp120-induced ICAM-1 expression by using antibodies against CD4 and chemokine coreceptors as well as small-molecule coreceptor inhibitors (Tamamura et al, 2001) and examined the underlying signaling pathways using pertussis toxin (ptx), an inhibitor of Gi/o-protein coupled receptors such as chemokine coreceptors and Bay 11–7082, a nuclear factor (NF)-kB inhibitor.

Results

Gp120 Induces ICAM Expression on HBMEC

We previously showed that X4 gp120 can induce ICAM-1 expression on pediatric HBMEC. Here, we examined the effects of different gp120 concentrations and the kinetics of ICAM-1 expression in pediatric HBMEC. HBMEC were incubated with 0.01 to 1 μg/ml gp120 derived from X4 HIV-1 for up to 72 hours. Figure 1A shows that after 24 hours of treatment, gp120 (0.1 μg/ml) could increase ICAM-1 expression by 20%, and ICAM-1 up-regulation up to 170% was achieved with 0.5 μg/ml of gp120. No up-regulation of ICAM-1 expression was observed with gp120 concentrations at 0.01 μg/ml. TNF-α (10 ng/ml), which was used as a positive control, increased ICAM-1 expression by 60%. Figure 1B shows that a maximal expression of ICAM-1 was achieved after 24 hours of exposure to gp120, and ICAM-1 expression decreased after 48 to 72 hours of incubation. Inclusion of polymyxin B did not affect gp120-induced ICAM-1 expression, whereas preabsorption with anti-gp120 antibodies abrogated the gp120-mediated increase in ICAM-1 expression (not shown), indicating that this gp120 effect was not mediated by any contaminating endotoxin in the gp120 preparation and was specific to gp120.

We next examined whether gp120 derived from R5 HIV-1 would be equally effective as gp120 derived from X4 HIV-1. For this, we tested more than 10 different HBMEC isolates, and Figure 2, A to C, shows typical patterns of the increase in ICAM-1 expression induced by gp120 (0.1 μg/ml) derived from R5 and X4 HIV-1 in HBMEC derived from three different children. We found that X4 gp120 can consistently increase ICAM-1 expression, whereas the effects of M-tropic gp120 on ICAM-1 expression were variable. Of interest, one HBMEC isolate failed to exhibit ICAM-1 up-regulation in response to R5 gp120 (Fig. 2C). It is not clear why the responses of the HBMEC to R5 gp120 differ from those of X4 gp120.

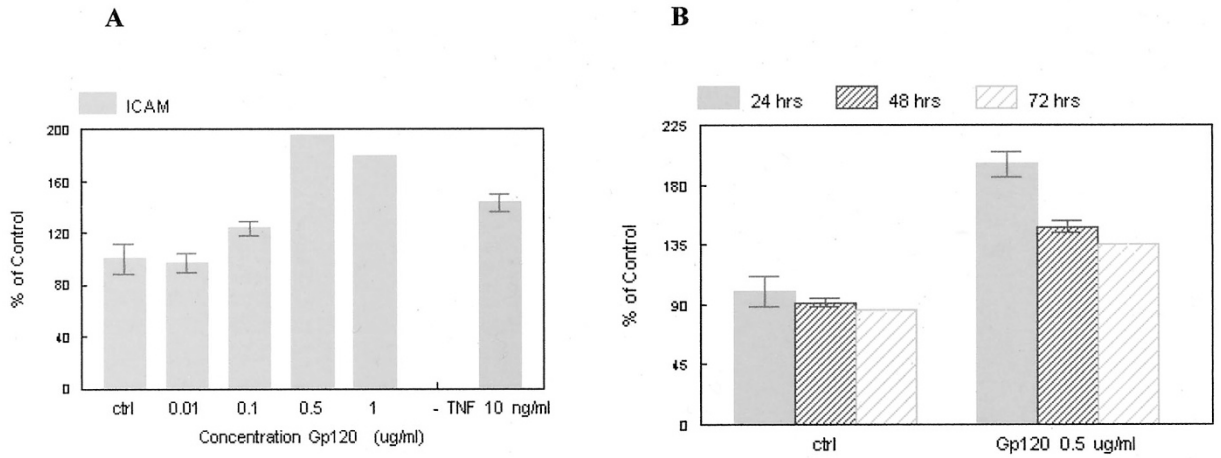

Figure 1.

Gp120-induced ICAM-1 expression on human brain microvascular endothelial cells (HBMEC): concentration and time dependence. (A) HBMEC were incubated with X4 gp120 (0.01–1 μg/ml) for 24 hours. As a positive control, HBMEC were stimulated with tumor necrosis factor (TNF)-α (10 ng/ml) for 24 hours. (B) HBMEC were incubated with X4 Gp120 (0.5 μg/ml) for 24, 48, and 72 hours. ICAM expression is indicated as percent of control (nonstimulated HBMEC).

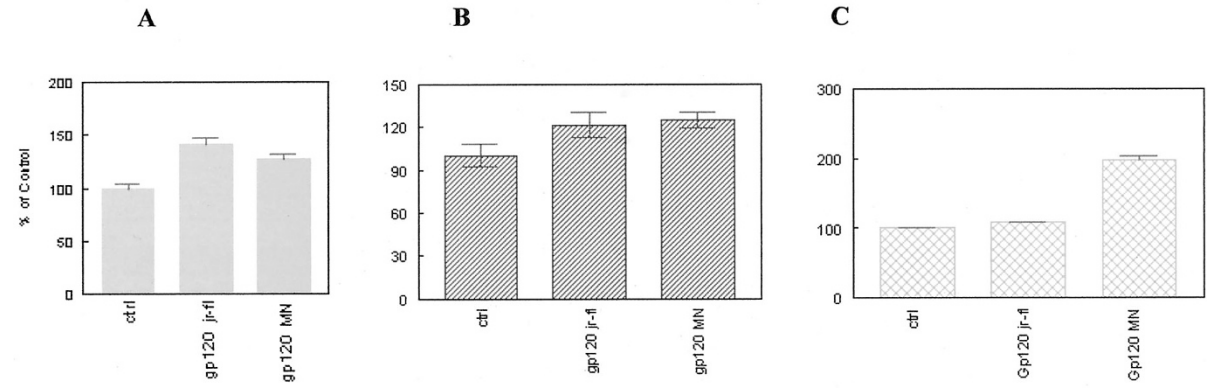

Figure 2.

ICAM-1 expression on HBMEC: R5 versus X4 gp120 on HBMEC derived from three different donors. Gp120 (0.1 μg/ml) was derived from R5 HIV-1 (JR-FL) or X4 HIV-1 (MN). ICAM-1 expression was expressed as percentage of control (nonstimulated HBMEC).

Real-Time qPCR Analysis for ICAM-1 in HBMECs

To determine whether the changes in ICAM-1 occurred at the transcriptional level, we performed real-time quantitative PCR analysis for ICAM-1. Three hours after addition of gp120-MN (0.5 μg/ml), the level of ICAM-1 mRNA expression doubled compared with the nontreated control (Fig. 3). Gp120 treatment persistently up-regulates the expression of ICAM-1 up until at least 6 hours, whereas it is reduced in the untreated sample (not shown). This indicates that gp120 up-regulates ICAM-1 expression by causing an increase at the level of mRNA expression.

Chemokine Receptor Involvement in gp120-Mediated Increase in ICAM-1 Expression

We showed that X4 and R5 gp120 increased ICAM-1 expression on pediatric HBMEC, but some disparities exist in responses to gp120 of different origin and among different HBMEC isolates. We did find that CD4 and chemokine receptor expressions vary among different HBMEC isolates (Stins et al, unpublished data), which could explain some of these disparities. We examined the involvement of CD4/chemokine co-receptors in the gp120-mediated increase in ICAM-1

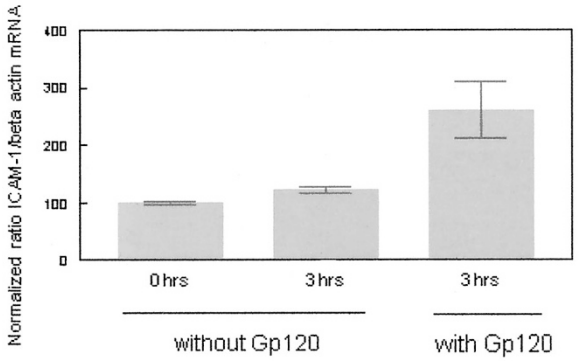

Figure 3.

Real-time quantitative polymerase chain reaction (PCR) analysis of ICAM-1 expression in HBMECs. Gp120-MN (0.5 μg/ml) was added to HBMEC and at 0, 3, and 6 hours. Real-time quantitative PCR was performed as indicated in Material and Methods. Data are expressed as ratio of ICAM-1 to beta actin mRNA.

expression by using nonactivating antibodies directed against CD4 and chemokine coreceptors CCR3 and CCR5.

Because all anti-CXCR4 Mab were activating antibodies and elicited an ICAM-1 up-regulation, we used the small-molecule inhibitors of CXCR4: T140, TN140, and TC140 to assess the contribution of CXCR4 in X4 gp120 mediated up-regulation of ICAM-1.

Figure 4A shows that Mab against CCR3 and CCR5 and the small-molecule inhibitors against CXCR4 and TN140 alone cannot inhibit X4 gp120-mediated increases in ICAM-1 expression. However, when used in combination, anti-CCR3 and anti-CCR5, anti-CCR5 and TN140, but not anti-CCR3 and TN140, could inhibit X4 gp120-induced ICAM-1 expression. Figure 4B shows that the R5 gp120-mediated response can be inhibited with antibodies against chemokine coreceptors CCR3 and CCR5. As a control, small-molecule CXCR4 inhibitor TN140 could not block R5 gp120-mediated ICAM-1 responses (not shown). Thus, anti-CCR3 and anti-CCR5 antibodies, as expected, could not block activation of HBMEC by X4 gp120 but blocked the response to R5 gp120. Surprisingly, combinations of anti-CCR3/CCR5 and anti-CCR5/TN140 but not CCR3/TN140 could inhibit X4 gp120 activation of HBMEC, suggesting that blockade of two receptors such as CCR3/CCR5 and CCR5/CXCR4 inhibits X4 gp120-mediated activation of HBMEC. It is, however, unclear why such an inhibition was not observed with the combination of CCR3/CXCR4.

The contribution of CD4 was further investigated by using soluble CD4 and a nonactivating anti-CD4 Mab. Premixing of soluble CD4 with gp120 before their addition to HBMEC prevented ICAM-1 expression in response to X4 and R5 gp120 (Fig. 4C). Similarly, the addition of anti-CD4 Mab to HBMEC before gp120 decreased ICAM-1 expression induced by X4 and R5 gp120. These findings illustrate the important contri-

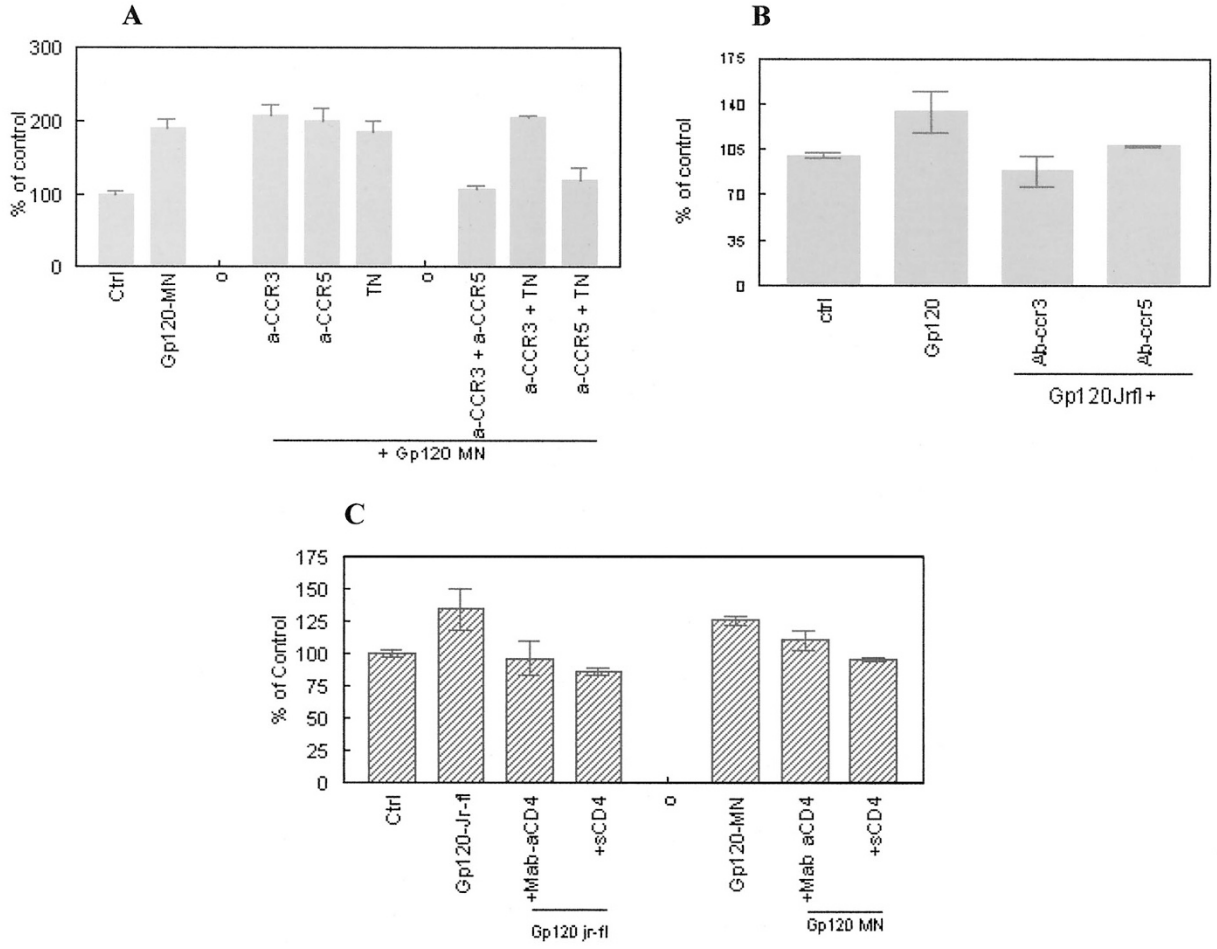

Figure 4.

Role of chemokine receptors and CD4 in gp120-induced ICAM-1 expression. A, Gp120–MN-induced ICAM-1 expression HBMEC were exposed to gp120 (0.1 μg/ml) derived from X4 HIV-1 in the absence or presence of "inhibiting" antichemokine coreceptor antibodies (aCCR3 Mab RD155, aCCR5 Mab 5C7) or small molecule CXCR4 antagonist TN and the ICAM-1 expression on HBMEC determined by enzyme-linked immunoadsorbent assay (ELISA). B, Gp120–JR-FL induced ICAM-1 expression HBMEC were exposed to gp120 (0.5 μg/ml) derived from R5 HIV-1 in the absence or presence of "inhibiting" antichemokine coreceptor antibodies (aCCR3 Mab RD155, aCCR5 Mab 5C7). Subsequently, ICAM-1 expression on HBMEC was determined by ELISA. C, Role of CD4 in gp120-mediated increase in ICAM-1 expression. HBMEC were incubated with gp120 (0.1 μg/ml) in the absence or presence of inhibiting anti-CD4 antibodies (SIM4, DAKO) or soluble CD4, and ICAM-1 expression was determined. Shown are results obtained with X4 gp120 and R5 gp120.

bution of CD4 to gp120-mediated activation on HBMEC.

By using "nonactivating" anti–β-chemokine coreceptor antibodies and small-molecule CXCR4 inhibitors, the roles of chemokine coreceptors in gp120-mediated activation of HBMEC were found to differ between R5 and X4 gp120. For example, antibodies to β-chemokine coreceptors (CCR3, CCR5) alone inhibited R5 gp120 response. In contrast, the small-molecule CXCR4 inhibitor could not inhibit T-tropic gp120-mediated ICAM-1 expression. However, an unexpected finding was the involvement of β-chemokine receptors (anti-CCR3, CCR5 antibodies) in the inhibition of X4 gp120-mediated increase of ICAM-1 expression of HBMEC.

Signaling Pathways Involved in Gp120-Mediated ICAM-1 Expression

ICAM-1 signaling pathways have been shown to involve NF-kB (Collins et al, 1995). We showed that R5 and X4 Gp120 induced translocation of NF-kB by gelshift assay (Fig. 5A). Addition of an inhibitor of NF-kB (Bay 117082) prevented X4 gp120-mediated increase in ICAM-1 expression (Fig. 5B). Chemokine coreceptors are shown to be G–protein-coupled receptors (Feng et al, 1996). Next, we used ptx, an inhibitor of Gi/o-protein coupled receptors. Preincubation of HBMEC with ptx (100 ng/ml) for 1 to 24 hours before addition of gp120 partially prevented the increase in ICAM-1 expression by X4 gp120 (from 177% to 132% of the control) (Fig. 5C). Similar results were found for R5 gp120. These findings indicate that G–protein-coupled receptors, such as chemokine coreceptors, are involved in R5 and X4 gp120-mediated ICAM-1 increase, but additional non-$G_{i/o}$-protein coupled receptors may be involved.

Discussion

HIV–1-associated CNS disorders are severe complications of HIV-1 infection. It is generally assumed that HIV-1 enters the CNS by way of HIV–1-infected monocytes in an early stage of the disease. Monocytes and macrophages do not show a great propensity to transmigrate across the brain endothelial barrier unless a stimulus that attracts macrophages is present or the endothelium is activated. HIV-1 infection of macrophages by itself does not lead to an increased transmigration (Persidsky et al, 1997). Therefore, activation of brain endothelium must play an important

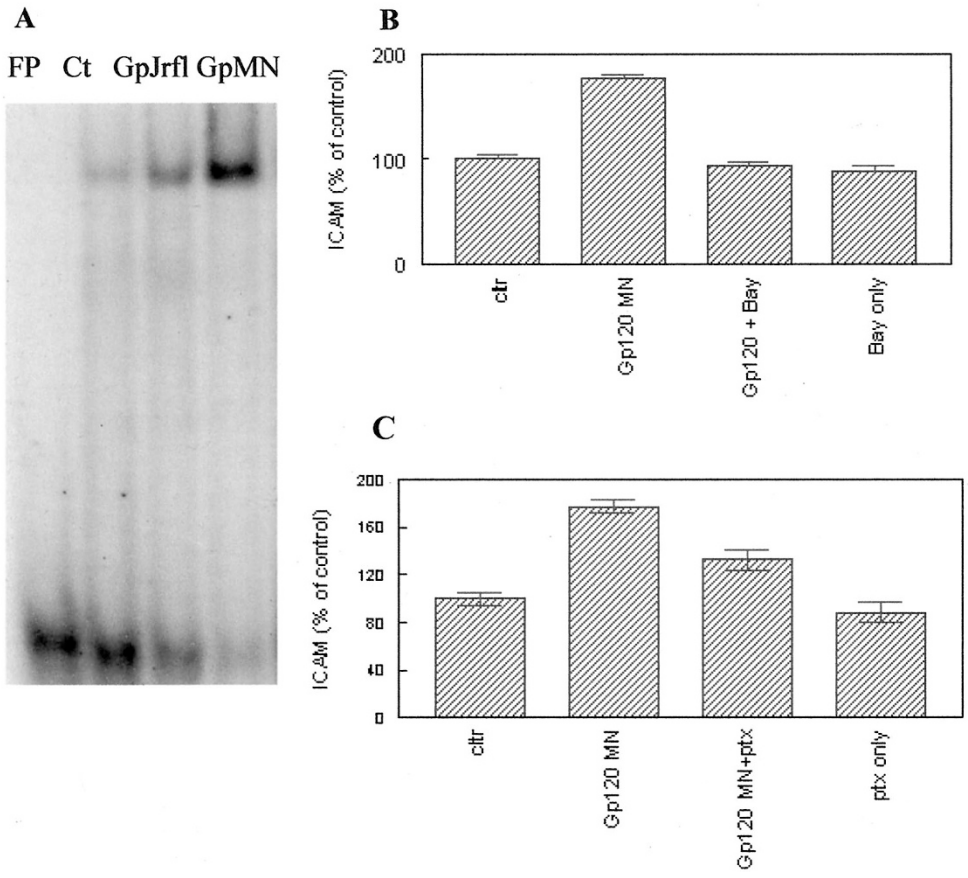

Figure 5.

Signaling pathways involved in gp120-induced ICAM-1 expression on HBMEC. HBMEC were preincubated with the indicated inhibitor for 1 hour with Bay (10 μM) or pertussis toxin (ptx) (100 ng/ml) before addition of R5 or X4 gp120 (0.1 μg/ml MN or JRFL). A, Electrophoretic mobility shift for nuclear factor (NF)-kB indicates translocation induced by gp120 (FP, free probe; Ct, control). B, NF-kB inhibitor Bay 11–7082 inhibits gp120-induced ICAM-1 expression, indicating involvement of NF-kB signaling pathway. C, ptx inhibits gp120-induced ICAM-1 expression in HBMEC, indicating involvement of G-protein receptors.

role in transmigration of monocytes. Factors that may activate brain endothelium in HIV-1 infection include cytokines, chemokines, and HIV-1 proteins (eg, gp120 shed by HIV-1 and HIV–1-infected cells). We have previously shown that gp120 can activate human brain endothelium derived from children in increasing ICAM-1/VCAM-1 expression and monocyte transmigration (Stins et al, 2001). ICAM-1 expression in response to gp120 was later reported for other endothelial cell types derived from the skin (dermal), lungs, and coronary artery but not for VCAM-1 (Ren et al, 2002). In accordance with our findings, these authors found that human umbilical vein endothelial cells did not respond to gp120. Gp120-mediated induction of ICAM-1 has also been demonstrated in glial cells and astrocytes (Seilhean et al, 1997; Shrikant et al, 1996). Because the underlying mechanisms responsible for ICAM-1 induction by gp120 were unclear, we set out to study them in our HBMECs.

In the present study, we found that gp120 increased expression of ICAM-1 in a time- and dose-dependent manner. A maximal increase in ICAM-1 expression was found after 24 hours, which parallels the findings of cytokine-induced increase in ICAM-1 expression (Wong and Dorovini-Zis, 1992). Gp120-induced expression of ICAM-1 was not caused by contaminating endotoxin in the gp120 preparation because polymyxin B did not inhibit the response. Moreover, antibodies against gp120 blocked gp120-induced responses in ICAM-1 expression (not shown), which is consistent with the results of Ren et al (2002) and our previous findings on gp120-induced monocyte migration (Stins et al, 2001). The gp120-mediated increases in ICAM-1 expression are mediated at the mRNA level, which is in accordance with the findings of Ren et al (2002). Gp120 derived from X4 HIV-1 showed a consistent up-regulation of ICAM-1 expression, whereas the response to gp120 derived from R5 HIV-1 varied among HBMEC derived from different children. These differences could be caused by differences in the gp120-receptor(s) profile on HBMEC or the presence of the mutant CCR5-delta-32 receptor.

It is well known that gp120 uses CD4 or the chemokine coreceptors CCR3, CCR5, and CXCR4 (Choe et al, 1996; Deng et al, 1996; Doranz et al, 1997a; Dragic et al, 1996), but it is unclear how gp120 uses these receptors in up-regulation of ICAM-1 on HBMEC. Endothelium from the brain has been shown to display a differential panel of receptors for HIV-1 gp120, such as CD4 (Stins et al, 2001) and chemokine coreceptors CCR3, CCR5, and CXCR4 (Andjelkovic and Pachter, 2000; Berger et al, 1987; Edinger et al, 1997; Lavi et al, 1997; Mukhtar et al, 2002). A heterogeneity in receptor profile or the presence of mutant receptors in brain endothelial cells of different patients and regions may explain differences in responses to gp120 among HBMEC isolates derived from different patients. Our preliminary investigations revealed that receptor profiles differed among HBMEC derived from different patients, and some HBMEC were heterozygous for the delta 32-CCR5 receptor (not shown), which could explain the different responses to R5 versus X4 gp120.

We examined the involvement of CD4 or chemokine coreceptors in the gp120-mediated ICAM-1 expression by using several anti-CD4, CCR3, CCR5, and CXCR4 antibodies. Chemokine coreceptors are G–protein-coupled seven-transmembrane receptors (Bleul et al, 1996; Feng et al, 1996; Oberlin et al, 1996), and antibodies raised against these coreceptors are commercially available. To demonstrate the (co-) receptor involvement in gp120-mediated ICAM expression, we used nonactivating anti-CCR3 and CCR5 antibodies and small-molecule inhibitors (TN) for CXCR4. This approach demonstrated that X4 gp120 response could not be inhibited by anti-CCR3 and CCR5 antibodies or TN alone, but, surprisingly, a combination of anti-CCR3 and CCR5 antibodies or anti-CCR5 antibody and TN abrogated the response. As expected, both anti-CCR3 and CCR5 antibodies inhibited the increase in ICAM-1 expression in response to R5 gp120. Soluble CD4 and antibodies to CD4 inhibited ICAM-1 expression in response to R5 and X5 gp120. These findings indicate that, for R5 gp120, the individual chemokine receptors CCR3 and CCR5 or CD4 could be sufficient to elicit a response in HBMEC. For X4 gp120, soluble CD4 completely inhibited the ICAM-1 increase, whereas antibodies against CD4 were only partially inhibitory. The responses of HBMEC to X4 gp120 seem to be more complicated than those to R5 gp120 and were regulated by a collaboration of two chemokine receptors of CCR3 and CCR5 or CCR5 and CXCR4. In contrast, the combination of CCR3 and CXCR4 failed to inhibit X4 gp120-mediated ICAM-1 expression, and the reasons for this discrepancy between CCR5/CXCR4 and CCR3/CXCR4 are not clear. Of interest, X4 gp120-mediated tissue factor production in smooth muscle cells was also shown to be dependent on two coreceptors, CCR5 and CXCR4, and also dependent on the presence of functional CD4 (Schecter et al, 2001). As far as the involvement of chemokine receptors, gp120 induced other cellular responses; for human umbilical vein endothelial cells, mainly CXCR4 but also CCR5 were shown to be involved in apoptosis induction by X4 gp120 (Huang et al, 2001; Ullrich et al, 2000), and, in neuroblastoma cells, cell death involved both CXCR4 and CCR5 (Catani et al, 2000). The involvement of both alpha and beta chemokine coreceptors (CXCR4 and CCR3/CCR5, respectively) in a X4 gp120 response appears unusual, and this may be because of possible promiscuity of gp120 for different coreceptors.

The degree whereby gp120 can activate human cells may not only be dependent on the viral origin but also on the expression level or efficiency of the interaction of the coreceptors with CD4 or the gp120–CD4 complex (Dimitrov et al, 1999; Mondor et al, 1998). It has been shown that this interaction may differ for different cells types (eg, CD4–CXCR4 interaction is more efficient in lymphocytes and monocytes than in macrophages) (Dimitrov et al, 1999). Efficiency of gp120 binding and subsequent signaling by corecep-

tors may depend on receptor mutations and differences in posttranslational modifications (eg, glycosylations, sulfations). In addition, gp120 viral origin may display differences in binding to one or more regions of the receptor. For example, CXCR4 displays a considerable heterogeneity, SDF-1 can bind to the N-terminal of CXCR4 without conferring signaling, and binding to the second extracellular loop triggers signaling, whereas mutations in the third extracellular loop affects transduction of G-protein signaling (Baribaud et al, 2001; Brelot et al, 2000; Lu et al, 1997; Wang et al, 1998). For CCR5 specifically, posttranslational sulfations were shown to be of importance for CCR5 binding to gp120–CD4 complexes (Farzan et al, 1999). In addition, other factors exist that may affect presentation or the availability of receptors on the membranes, such as membrane composition (cholesterol-lipid or sphingoglycolipids raft formation), and heparan sulfate proteoglycans may modulate the responsiveness of CXCR4, CCR3, CCR5, or CD4 to gp120 (Fantini et al, 2000; Liao et al, 2001; Mbemba et al, 1999; Mondor et al, 1998; Patel et al, 1993). Taken together, subtle differences in gp120 binding affinities, or gp120/CD4 complexes to chemokine coreceptors of different HBMEC, which may possess different mutations or posttranslational modifications in a variable membrane environment, may lead to the observed discrepancies in ICAM-1 responses of HBMEC derived from different patients.

ICAM-1 is a cytokine-inducible cell adhesion molecule, the physiologic ligand for leukocyte function associated antigen-1 and membrane attack complex-1, and is involved in extravasation of leukocytes (for review, see van de and van der Sag, 1996). Migration of HIV–1-infected monocytes across the BBB seems to be a major pathway for HIV-1 entry into the CNS. Thus, up-regulation of ICAM-1 expression on the brain endothelium could lead to increased adhesion and transmigration of (HIV–1-infected) monocytes/macrophages, thereby perpetuating HIV-1 pathogenesis. We have previously shown, using our in vitro model of the human BBB, that gp120 can enhance transmigration of monocytic cells (Stins et al, 2001). In vivo, it was shown that ICAM-1/VCAM-1 are significantly up-regulated in the brain of AIDS patients (Seilhean et al, 1997), and endothelium of gp120-transgenic mice show a significant up-regulation of ICAM-1 expression (Toneatto et al, 1999). ICAM-1 present on endothelium can be cleaved of the membrane and subsequently released into the serum or cerebrospinal fluid. Levels of soluble ICAM-1 are indeed found to be increased in plasma and cerebral spinal fluid of pediatric and adult AIDS patients (Gaddi et al, 2000; Most et al, 1993; Rieckmann et al, 1993) and have been indicated as a marker for disease progression. Moreover, it was shown that ICAM-1/LFA-1 may play a role in HIV–1-dependent cell fusion, cytopathicity (Hildreth and Orentas, 1989), and endothelial infection by HIV-1 (Scheglovitova et al, 1995). Therefore, it is of importance to study the mechanisms whereby HIV-1 proteins up-regulate ICAM-1 expression on the endothelium of the BBB. This may lead to development of specific inhibitors of either adhesion and extravasation of HIV–1-infected monocytes into the brain or of the inhibition of ICAM–1-dependent HIV-1 infection or endothelial activation.

In summary, in this article, we showed that gp120 increases ICAM-1 expression by way of its interaction with CD4 and chemokine coreceptors. Chemokine receptors are G–protein-coupled seven-transmembrane receptors (Bleul et al, 1996; Feng et al, 1996; Oberlin et al, 1996) and sensitive to ptx (Davis et al, 1997). We confirmed the involvement of the ptx-sensitive coreceptor pathway in gp120-mediated increase in ICAM-1 expression in our HBMEC. It is also known that the promotor for ICAM-1 expression contains NF-kB, which is involved in cytokine up-regulation of ICAM-1 (Collins et al, 1995), and, as shown here, the translocation of NF-kB by gelshift assay and the use of NF-kB inhibitor Bay indicated that gp120 uses the NF-kB signaling pathways in a manner analogous to the cytokine-mediated pathways. Gp120 can signal through other pathways (eg, signal transducer and activator of transcription-1α and Janus kinase) (Shrikant et al, 1996) or indirectly by way of TNF-α, IFN-γ, or IL-10 (Capobianchi, 1996). However, we were not able to find a gp120-mediated increase in TNF-α, IFN-γ, or IL-10 secretion by gp120 in HBMEC (not shown), and it is therefore unlikely that these "indirect" mechanisms are involved in the gp120-mediated increase in ICAM-1 expression in HBMEC. It appears that gp120 signaling pathways resulting in different phenotypes are likely to differ between HBMEC and other cell types such as T cells, neurons, and glial cells and that gp120 has a choice of triggering alternate signaling pathways in various cell types, including HBMEC.

Materials and Methods

Isolation and Culture of Human Brain Microvessel Endothelial Cells

Human brain microvessels were isolated from children's brain specimens derived from surgeries for seizure disorders and cultured as described previously (Stins et al, 1997). Human brain microvessels were plated on rat tail collagen and cultured in RPMI 1640 based medium with 10% fetal bovine serum, 10% NuSerum, endothelial cell growth supplement (30 μg/ml) (Collaborative Biomedical Products, B&D, Bedford, Massachusetts), heparin (5 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), minimal essential medium vitamins, minimal essential medium nonessential amino acids, penicillin, and streptomycin (100 U/ml) (Irvine Scientific, Irvine, California). Endothelial cell cultures were incubated at 37° C in a humid atmosphere of 5% CO_2 and characterized for the presence of Factor VIII-Rag and AcLDL uptake. HBMEC with a purity greater than 99% endothelial cell population were obtained by FACS as previously described (Stins et al, 1997) and used in our experiments.

Antibodies and Inhibitors Used

Antibodies against chemokine coreceptors CD4 and soluble CD4 were obtained from the AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH, (Bethesda, Maryland) as follows: antibodies against CCR3, #MAB155, from R&D systems (Minneapolis, Minnesota); CCR5 antibodies 12D1 from Dr. Stephen Peiper (Doranz et al, 1997b), and 5C7 from LeukoSite, Incorporated (Cambridge, Massachusetts) (Wu et al, 1997). CD4 antibodies were obtained as follows: MAb SIM2 from Dr. James Hildreth (McCallus et al, 1992) and soluble CD4 from Dr. Sweet, (GlaxoSmithKline, Welwyn Garden City, Hertfordshire, UK) (Deen et al, 1988). Anti-gp120 antibodies (No 902, F105, and AD3) were from Dr. B Cheseboro, Dr. M. Posner (Posner et al, 1993) Dr. K. Ugen, and Dr. D. Weiner (Ugen, 1993). Antibody against CD4 (clone MT310) was purchased from DAKO (Carpinteria, California). Small-molecule chemokine coreceptor inhibitor T140, TN, or TC were kindly donated by Dr. Fujii of Kyoto University, Japan (Tamamura et al, 2001).

Activation of HBMEC with gp120

HBMEC derived from different patients were seeded in 96-well plates, grown to confluence, and subsequently treated with gp120 (0.1 up to 1 μg/ml) in RPMI with 5% fetal bovine serum for 6, 24, or 72 hours. Gp120 from X4 HIV-1 (MN-CM) and from R5 HIV-1 (JR-FL) were obtained through the Reference Reagent Program, Division of AIDS, NIAID, NIH, and supplied by the following companies: MicroGeneSys (Meriden, Connecticut), Immunodiagnostics (Bedford, Massachusetts), and Progenics Pharmaceuticals (Tarrytown, New York) (McCutchan et al, 1992),

Expression of ICAM-1 was assessed by ELISA using anti–ICAM-1 antibodies from Immunotech/Coulter (Westbrook, Maine) or biotinylated anti–ICAM-1 antibody from Chemicon (Temecula, California) using the ABC-alkaline phosphatase method as described previously (Stins et al, 1997). The results were expressed as percentage of untreated control cells.

For inhibition experiments, Bay 11–7082 (NF-kB inhibitor) or ptx (Gi/o-coupled protein inhibitor) (Sigma, St Louis, Missouri), antichemokine receptor antibodies, soluble CD4, or small-molecule chemokine coreceptor inhibitors (T, TN) were included in the medium during exposure of HBMEC to gp120. The effects of the indicated reagents on the gp120 mediated ICAM-1 expression on HBMEC were assessed.

Viability of the HBMEC were monitored by visual inspection using a microscope, live-dead stain (Molecular Probes, Eugene, Oregon), and by lactate dehydrogenase release in the supernatant using cytotoxicity detection kit (Roche, Mannheim, Germany). After 24 hours at 37°C treatment with gp120 (up to 1 μg/ml), we did not find any evidence of cell death by any of the above methods (not shown).

Real-Time Quantitative PCR Analysis of ICAM-1 Expression in HBMECs

HBMEC where grown to confluence on collagen-coated 24-wells plates and treated with gp120 (0.5 μg/ml) for up to 24 hours as previously indicated. Samples for RNA extraction were collected at time 0, 3 hours, and 6 hours. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, California). The amount and the quality of the RNA was verified by measuring the absorbance at 260 and 280 nm.

Oligo (dT) primed Reverse Transcription of RNA was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California) using 50 ng of RNA for each reaction; reactions without reverse transcriptase were concurrently prepared for each RNA sample to verify the absence of DNA contamination.

Real-time PCR analysis of ICAM-1 expression was performed using specific primers 431f (5'-AGCCAGTGGGCAAGAACC) and 1036r (5'-GGTCCCTTCTGAGACCTC), and β-actin amplification using commercial primers (Quantum*RNA* β-actin Internal Standards; Ambion, Austin, Texas) was used as reference.

Serial dilutions of cDNA were PCR amplified using a LightCycler System (Roche Applied Science, Indianapolis, Indiana) in 1x LightCycler-FastStart DNA Master SYBR Green I reaction mix with 3 mM Mg^{2+}. The PCR program consisted of one preincubation at 95° C for 10 minutes and 40 cycles at 95° C for 10 seconds, 55° C for 5 seconds and 72° C extension, followed by the melting curve analysis program described by the manufacturer; extension time was 20 seconds for ICAM-1 amplification and 12 seconds for β-actin amplification.

Gelshift Assay

HBMEC were seeded in 35 mm tissue culture dishes and stimulated with gp120 (0.1–0.5 μg/ml) for 30 minutes and 1 hour. Subsequently, nuclear extracts were prepared according to Schreiber et al (1989) with the modifications of Molitor (1990). The nuclear extracts were snap frozen and stored at −80° C. Probes for NF-kB were obtained from Santa Cruz Biochemicals (Santa Cruz, California) and labeled with 32P using DNA endlabeling kit (GIBCO-BRL, Gaithersburg, Maryland) according to the manufacturers instructions. ^{32}P-labeled NF-kB probe was purified with a Stratagene (La Jolla, California) push column, incubated with the nuclear extracts, separated with PAGE, and the bands were subsequently visualized with autoradiography.

Conclusion

We showed that chemokine co-receptors and CD4 receptors are involved in the response of human brain endothelium to R5 and X4 gp120 by increasing ICAM-1 expression in ptx and NF–kB-dependent pathways. The degree of ICAM-1 up-regulation differs for R5 versus X4 gp120 and also among the various

HBMEC isolates. Endothelial cells are important regulators of immune function and inflammatory reactions. Thus, endothelial activation may lead to compromised function of the BBB. Because ICAM-1 is an adhesion molecule shown to be involved in extravasation of monocytic cells, increased ICAM-1 expression may lead to increased influx of (HIV-1) infected monocytes into the CNS. This may explain our previous demonstration that gp120 increased transmigration of monocytes across the human BBB model. In addition, the presence of CD4 on the brain endothelium of children may explain an increased incidence of HIV-1 CNS involvement in pediatric AIDS patients.

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