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Chemokine receptor mRNA expression at the *in vitro* blood–brain barrier during HIV infection

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VIRAL entry through the blood–brain barrier (BBB) has not been fully defined and identification of co-receptors that can facilitate this phenomenon is crucial in understanding disease progression. Using a RNAse protection assay to examine chemokine receptor families simultaneously, we analyzed the total RNA of *in vitro* BBB cultures treated with purified preparations of HIV gp120, gp41, TAT proteins and TNF-α. HIV tat protein affected CCR1 and CCR3 mRNA expression whereas the other viral by-products had no effect. Interestingly, TNF-α was able to induce CCR1, CCR3 as well as CXCR1, CXCR2, CXCR4 receptors and Burkitt's lymphoma receptor BLR2. These results suggest that HIV-induced molecules can manipulate the surface receptor expression of the BBB to allow for their preferential entry into brain. *NeuroReport* 10:53–56 © 1999 Lippincott Williams & Wilkins.

Key words: AIDS dementia complex; Blood–brain barrier; Chemokine receptors; HIV

Introduction

The neurobiology of HIV infection is poorly understood. Currently one-third of HIV-infected individuals have mild to severe forms of dementia, termed AIDS dementia complex (ADC), and, as survival rates of HIV individuals continue to increase, the likelihood of this neurodegenerative condition will also rise [1]. Current anti-retroviral drug combinations are able to control the systemic replication of HIV but are largely ineffective in crossing the blood–brain barrier (BBB). Therefore, characterization of the mechanism of viral entry will help in the development of therapeutic interventions that can halt viral progression and sequestration into normally immunoprotected areas, such as the brain. Although many central nervous system (CNS) cells are capable of being infected *in vitro*, it is thought that initial viral infiltration *in vivo* into the brain is due to a 'Trojan horse' scenario involving the recruitment of infected monocytes and macrophages [2]. Studies of HIV-infected brain show a lack of extensive viraemia seen at autopsy; thus, it is more likely that neuropathologic damage occurs via an inflammatory cascade. Once the infection is established, viral proliferation into surrounding cells and chemokine production can amplify the recruitment of other (possibly infected) inflammatory cells (T cells, neutrophils, monocytes).

Logistically speaking, there are relatively low numbers of circulating HIV infected cells, and increasing evidence supports the concept that preferential selection and acceleration of these cells occurs in the CNS. In the ADC brain, myelin pallor is extensive, suggesting that there is local damage of the cerebral microvasculature by HIV [1]. *In vivo*, the BBB may initially provide a first line of defense against blood-borne virus but, following initial infection, HIV-induced soluble factors (cytokines, chemokines and secreted HIV by-products) can modulate BBB permeability and allow the progression of HIV encephalitis [3]. One such modification is the expression of chemokine co-receptors which, in conjunction with primary receptors such as CD4, enable binding and entry of the virus. In this study, we examined the ability of TNF-α and HIV glycoproteins (molecules locally present during infection of the brain microvasculature) to induce chemokine receptor expression in an established model of the BBB. Such a controlled analysis of select HIV-induced factors affecting the integrity of the BBB is an appropriate study to elucidate the pathogenesis of neuro-AIDS and may aid in the development of new therapeutics to contravene this disease.

Materials and Methods

Preparation and treatment of cell cultures: To determine the effect of HIV glycoproteins on the

BBB, we established an artificial system using a co-culture of HUVEC 304, a human immortalized endothelial cell line and C6, a rat glial cell line as described by Hurst and Fritz [4]. In these experiments, HUVEC 304 cells are grown in M199 medium supplemented with 10% fetal bovine serum (Gemini), and 2 mM glutamine. Stock cultures of C6 astrocytes were grown in DMEM containing 10% fetal bovine serum and 2 mM glutamine; 1 week prior to experiments, the cells were gradually passaged into M199 medium. Co-cultures of ECV 304 and C6 cells were plated at a ratio of 2:1 into 10 mm dishes. The cells were grown for 3 days until they reached 75–80% confluency. The spent medium was removed, and the cells were placed in 5 ml fresh medium containing the following proteins for either 6 or 24 h: 10 ng/ml TNF-α (kindly provided by Dr Dennis Taub, Gerontology Research Center, IRP, NIA, NIH), 1 ng/ml recombinant HIV gp41 (Intracel Corporation), 100 ng/ml HIV TAT (obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH: HIV-1 TAT protein from Dr John Brady), 100 ng/ml HIV gp120 LAV (obtained through the AIDS Research and Reference Program, NIAID, NIH: HIV-1 gp120 LAV protein from MicroGeneSys, Inc.).

RNAse protection assay: Total RNA (100 μg) was extracted and prepared from treated co-cultures using a RNA-STAT kit (Tel-test). Each set of RNA samples was prepared according to the instructions in the Riboquant multi-probe RNAse protection assay system (Pharmingen). Comparative analyses of different RNA species in each sample of total RNA were examined using a multi-probe template against human chemokine receptor family CCR mRNA (hCR-5 template) or a multi-probe template against human chemokine receptor family CXCR mRNA (hCR-6 template), which additionally recognizes Burkitt's lymphoma receptor (BLR2) [5,6].

Results

As shown in Figure 1A, a 6 h treatment of cell cultures resulted in the peak induction of CCR chemokine receptor mRNA. The 24 h incubation period did not result in any positive expression. TNF-α was able to induce the mRNA expression of two CCR chemokine receptors, CCR1 and CCR3. Induction of the CCR family receptors by this cytokine was positively identified after readjustment to GADPH and L32 internal controls. Interestingly, HIV Tat protein was also able to induce expression

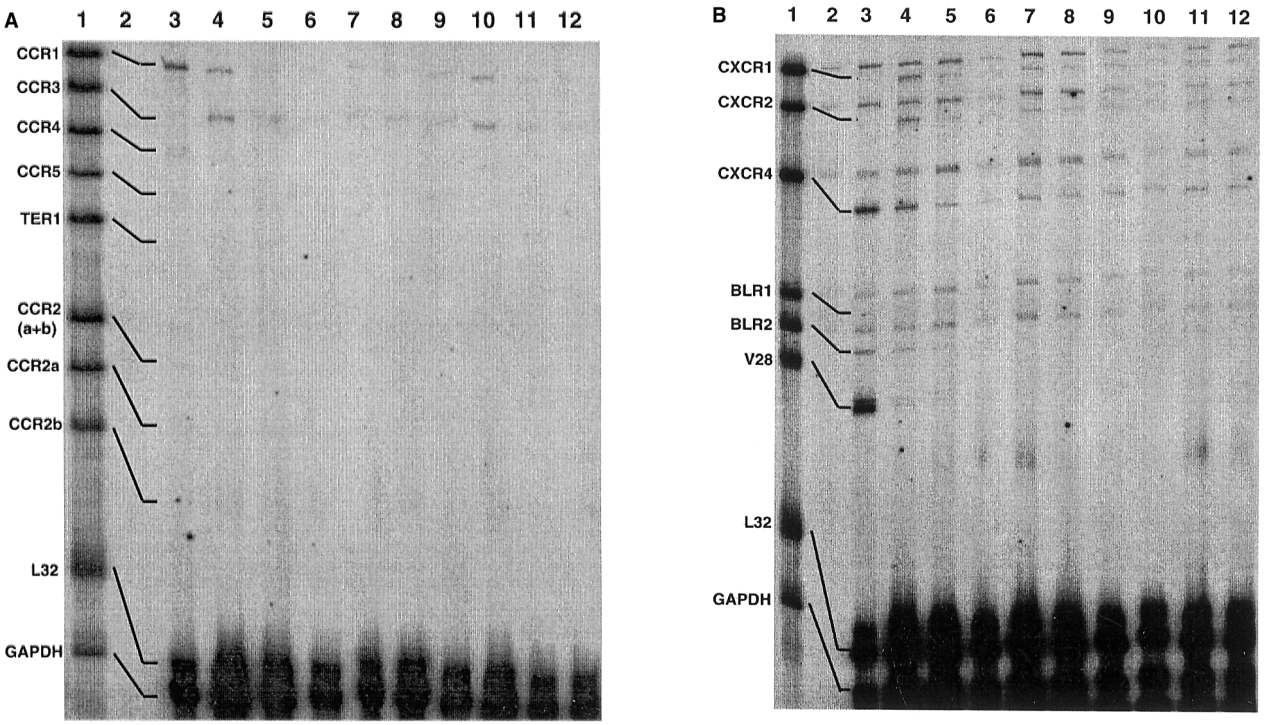

FIG. 1. RNAse protection assay of endothelial/glia co-cultures treated with TNF-α and HIV glycoproteins. Total RNA of treated HUVEC 304 and C6 glioma co-cultures were analyzed by RNAse protection assay using a template probe of the CCR chemokine receptor family (**A**) or a template probe of the CXCR chemokine receptor family (**B**). For both A and B, lane 1 shows the probe set not treated with RNAse, lane 2 represents the RNAse protected probes hybridized with yeast tRNA (lane 2) and lane 3 shows the probe hybridized with a provided standard control, RNA-2 (a pool of Raji, U937, Molt2, Daudi, NC37 and THP1 stimulated with TPA/ionomycin). The CCR or CXCR receptor family RNAse protected probes were hybridized with RNA from co-cultures stimulated with (a) 10 ng/ml TNF-α for 6 and 24 h (lanes 4, 5); (b) 100 ng/ml HIV gp120 for 6 and 24 h (lanes 6, 7); (c) 1 ng/ml HIV gp41 for 6 and 24 h (lanes 8, 9); (d) 100 ng/ml HIV Tat for 6 and 24 h (lanes 10, 11); (e) untreated for 24 h (lane 12).

of these identical receptors. Chemokine receptor mRNA expression was not detected in the total RNA samples of untreated controls nor in HIV gp120- or HIV gp41-treated cultures. The RNAse protection assay using the CXCR chemokine family template probe showed constitutive expression of CXCR1, CXCR2 and CXCR4 mRNA from untreated and treated cultures (Figure 1B). TNF-α treatment was able to induce the increased expression of CXCR4 mRNA as well as that of a novel receptor, BLR2. Treatment of the cells with the three HIV glycoproteins did not show distinct increases in mRNA expression and after readjustment of each sample with both GADPH and L32 internal controls, no significant mRNA expression of CXCR family of chemokine receptor were identified in these samples.

Discussion

The mechanism of HIV entry through the BBB has not been fully characterized. The classical pathway of HIV infection involves viral penetration into target cells through fusion of HIV gp120 glycoprotein with CD4 receptors. Recent studies [7] have shown that select chemokine receptors, which normally function in lymphocyte circulation and homing, are also used as co-receptors to facilitate entry; therefore, factors that modulate the BBB to express these receptors would promote efficient infection of the CNS by HIV. Analysis of brain tissue described by Sanders *et al.* [8] show that brain sections of HIV demented patients have increased chemokine receptor expression when compared with their non-demented counterparts. In particular, their findings show a rise in CCR1 and CCR3 expression primarily in macrophages and endothelial cells and increased CXCR4 expression in macrophages, neurons and astrocytes. Our study is supported by these observations as we are also able to detect in our co-cultures, increased mRNA expression of identical receptors along with other chemokine receptor family members, (CXCR1, CXCR2 and BLR2). Chemokine receptor CCR3 has been shown to facilitate entry of M trophic strains of HIV whereas CXCR4 enables T trophic strain entry of HIV into target cells [7]. As yet, there is no report describing the involvement of chemokine receptors CCR1, CXCR1 and CXCR2 in HIV infection and, therefore, their role remains largely undefined. Interestingly, this study is the first to report the mRNA expression of BLR2 receptor in an endothelial/glia culture. Initially reported by Burgstahler *et al.* [5] BLR2 is strongly up-regulated in activated peripheral blood lymphocytes [6], and its ligand, MIP-3, has been shown to be chemotactic for T and B cells [9]. The co-expression of BLR2, along with CXCR4 receptors detected in our endothelial/glia cultures suggests that it may synergistically promote the entry of lymphocyte trophic HIV strains.

We examined the effects of the inflammatory cytokine TNF-α and three HIV glycoproteins, all of which have known neurotoxic effects in the brain and are likely to be present in substantial or potent concentrations that are capable of modulating changes in the surrounding environment. It was surprising to observe that the intensively investigated HIV glycoproteins, gp41 and gp120, did not induce chemokine receptor mRNA expression in our endothelial/glia cultures. The involvement of gp41 in ADC is supported by Adamson *et al.* [10], who reported a direct association between gp41 levels and HIV dementia. In these studies, gp41 expression correlated with increased production of nitric oxide, a potent neurotoxic agent that directly induces neuronal cell death. We can postulate that gp41, either directly or through its actions, via nitric oxide, will eventually cause a loss of BBB integrity; but gp41, in our study, was unable to modulate BBB permeability through the up-regulation of chemokine receptor expression.

CNS damage by HIV gp120 is well documented. The addition of this neurotoxic molecule to neuronal cultures results in the disruption of intracellular calcium levels and membrane integrity [11]. The extent of damage due to HIV gp 120 is emphasized in transgenic mouse models which show that neuronal injury correlates with gp 120 expression and, more importantly, the pathology of CNS damage in these animals is similar to that observed in HIV infected human brains [12]. The ability of gp120 to influence BBB function was reported by Bragardo *et al.* [13]. HIV gp120 is able to increase adhesion molecule expression on lymphocytes, and this action up-regulates lymphocyte homing and interaction with endothelial cells. Banks *et al.* [14] have only recently examined the direct effects of gp120 on the endothelium. In their study, gp120 induced adsorptive endocytosis but required the presence of a chemokine co-receptor to complete viral fusion into endothelial cells. It was therefore of surprising interest that we could not demonstrate any induction of chemokine receptor expression in our co-cultures. Thus, despite the potency of this glycoprotein to regulate many aspects of HIV progression, we demonstrate that gp120 has limited action to modulate chemokine receptor expression at the BBB.

In our hands, HIV Tat protein was the only viral product that induced chemokine receptor expres-

sion. HIV Tat protein, a transactivator of HIV replication [15], is released by infected lymphoid and glia cells, and it can provide extacellular regulation of cells in the local environment [16]. Similar to gp120, Tat protein, can bind to neurons with high affinity and cause depolarization of calcium and membrane damage [17]. This glycoprotein has been demonstrated to alter BBB permeability by stimulating the expression of adhesion molecules on HUVEC cells and subsequently recruiting inflammatory cells to the region [18]. Tat protein is also a potent stimulator of IL-6, a cytokine that is accredited with the ability to increase endothelial permeability [19]. In our study, the induction of mRNA of CCR1 and CCR3 receptors in the endothelial/glia provides evidence for a new route by which this molecule can regulate the HIV infection of the CNS. The action of TNF-α on mediating HIV entry into the CNS has been extensively studied. Fiala et al. [20] have shown that TNF-α can enhance the paracellular passage of HIV-1 and, in addition, that TNF-α in association with cocaine can also act to increase the brain microvascular permeability to inulin [21]. In ADC brain, microglia activation is particularly elevated, and TNF-α is one of several inflammatory proteins produced by these cells. Interestingly, Griffin [22] reported that brains from HIV-infected demented and non-demented individuals showed a significant difference in their concentration of TNF-α. As described above, Tat protein is able to up-regulate HIV entry into the brain through several mechanisms, but when combined with TNF-α the actions are augmented [18]. In our study, TNF-α had the most potent effect on chemokine receptor mRNA expression. In particular, the up-regulation of CXCR4 mRNA substantiates the findings of Gupta *et al*. [23], who reported a biphasic effect on the receptor expression by TNF-α. The co-expression of several members of both chemokine receptor families indicates that TNF-α is a non-partisan stimulator and the upregulation of these receptors to the BBB surface will probably recruit both M and T trophic strains of HIV into the CNS.

Conclusion

The toxic effects of HIV glycoproteins can cause neuronal damage that underlies the cognitive dysfunction seen in ADC patients. During the course of infection, these glycoproteins can also modulate the BBB to recruit infected cells through an increased expression of chemokine receptors. We have demonstrated that HIV Tat protein and TNF-α both induce several members of the CCR and CXCR family of receptors. These results suggest that viral entry into the brain is not a random opportunistic event but an organized one. In identifying these modulating factors, we can provide a clearer understanding that will enable the design of new therapies that will help to stem the neuropathological progression of this disease.

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