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HIV-1 envelope protein gp120 up regulates CCL5 production in astrocytes which can be circumvented by gp120-specific siRNA and inhibitors of NF-kappa B pathway

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Abstract

HIV associated neurological disorders (HAND) is a common neurological complication in patients infected with HIV. The proinflammatory cytokines and chemokines produced by astrocytes play a pivotal role in neuroinflammatory processes in the brain and viral envelope gp120 has been implicated in this process. In view of increased levels of CCL5 observed in the CSF of HIV-1 infected patients, we studied the effects of gp120 on CCL5 expression in astrocytes and the possible mechanisms responsible for those effects. Transfection of the SVGA astrocyte cell line with a plasmid encoding gp120 resulted in a time-dependent increase in expression levels of CCL5 in terms of mRNA and protein by 24.6 ± 2.67 fold and 35.2 ± 6.1 fold, respectively. The fluorescent images showed localization of CCL5 in the processes of the astrocytes. The gp120-specific siRNA abrogated the gp120-mediated increase in CCL5 expression. We also explored a possible mechanism for the effects of gp120 on CCL5 expression. Using a specific inhibitor for the NF- κ B pathway, we demonstrated that levels of gp120 induction of CCL5 expression can be abrogated by 44.6 ± 4.2 % at the level of mRNA and 51.8 ± 5.0 % at the protein level. This was further confirmed by knocking down NF- κ B through the use of siRNA.

Introduction

CCL5, also known as RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) is a member of β chemokine family. It binds to several members of the G protein-coupled receptor family including the HIV co-receptor CCR5 [1]. Elevated levels of CCL5 (RANTES) have been correlated with inflammatory responses [2]. CCL5 has also been shown to be involved in monocyte and lymphocyte migration and adherence to endothelial cells [3] as well as neuroinflammation associated with Alzheimer's disease [4], Parkinson's disease [5] and cerebral ischemia [6]. CCL5 along with MIP-1 α and MIP-1 β was shown to increase the replication of T-tropic strain of HIV-1 in peripheral blood lymphocytes [7]. High levels of CCL5 were also observed in inflammatory brain lesions [8] and CSF [9] of HIV+ patients, which may explain its potential role in AIDS dementia complex.. Although the incidence of HIV-associated dementia has been reduced significantly in the post-HAART era [10], HAND, minor cognitive motor dysfunction (MCMD) and other neuro-

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complications remain important problems [11]. Along with other mechanisms, alteration of innate immunity and consequential alteration of inflammatory molecules is thought to play a pivotal role in CNS disorders associated with HIV-1 infection such as HAND, MCMD, and HAD [12].

Astrocytes comprise 25–50% of the volume of the brain [13] and their activation results in increased oxidative stress, expression of cytokines and chemokines, and neuronal damage. Astrocytes been implicated in pathogenesis of neurological disorders including HIV-associated dementia (HAD), Alzheimer's disease and multiple sclerosis [14]. Li et al showed that intact HIV-1 virus can infect human fetal astrocytes and increase the expression of cytokines and chemokines [15]. Thus, studying the effect of HIV-1 on astrocytes can provide essential understanding about the mechanisms involved in neuro AIDS.

The envelope glycoprotein gp120 is a 120 KDa structural protein that facilitates entry of the virus in a variety of host cells through its interactions with either the CXCR4 or CCR5 chemokine receptors [16,17]. Among various mechanisms for neurotoxicity [18], gp120 is thought to increase oxidative stress [19], activate macrophage/microglia [16,17] and alter expression of cytokines/chemokines [20,21]. We, along with others, have previously shown that gp120 increases the expression of IL-6 and CXCL-8 in human astrocytes [22,23]. As demonstrated by these reports, gp120-mediated effects on neurons and glia involve numerous mechanisms which contribute to the pathogenesis of HIV encephalitis and neuronal apoptosis. Thus, the effects of gp120 on the brain, and the mechanisms that underlie these effects, may offer important insights into the pathogenesis of neuroAIDS as well as the determination of potential therapeutic targets.

In this study, we sought to address the role of gp120 in regulation of CCL5 in astrocytes, because this chemokine has been shown to exacerbate the inflammatory response. In order to determine the mechanism underlying gp120-mediated CCL5 induction, we explored the NF- κ B pathway by using a pharmacological inhibitor as well as siRNA.

Materials and Methods

Cell culture, transfection, and reagents

SVGA, a clone of human astrocytes SVG [24], were cultured as previously described [23,24]. Transfections with HIV gp120, pSyn gp120 JR-FL (Catalog # 4598, NIH AIDS research and reference reagent program [25]) and treatments with SC514 (Cayman Chemicals, Ann Arbor, Michigan), an antagonist specific for the NF- κ B pathway were performed as previously described [23,24]. Specific siRNA against gp120 were designed using the Ambion siRNA design tool and then synthesized by Ambion (Applied Biosystems/Ambion, Austin, TX). Pre-designed and validated siRNA against p50 (P/N AM51331; id 5213) and negative silencer control (#AM4611) were obtained from Ambion Inc. (Applied Biosystems/Ambion, Austin, TX). Rabbit Anti-CCL5 PAb (#P230E) and Mouse Anti-GFAP (GF5) (# ab10062) were obtained from ThermoScientific (Rockford, IL) and Abcam (Cambridge, MA), respectively. The fluorescently labeled antibodies against rabbit IgG (#4412) and mouse IgG (#4409) were obtained from Cell Signaling (Danvers, MA).

Experiments using siRNA targeted against gp120 and NF- κ B were performed as previously described (23, 24).

Realtime RT-PCR

The mRNA expression of CCL5 was measured by real time RT-PCR. Total mRNA was extracted from the astrocytes using RNeasy mini kits (QIAGEN, Valencia, CA) as per the manufacturer's protocol. The mRNA was reverse transcribed at 50°C for 30 minutes. This

was followed by a denaturation step at 95°C for 15 minutes and then amplified for 45 cycles (95°C for 15 sec, 54°C for 30 sec and 76°C for 30 sec) using CCL5 primers (5' ACC AGT GGC AAG TGC TCC A-3' for forward and 5' ACC CAT TTC TTC TCT GAG TTG GCA-3' for reverse) (Integrated DNA Technology, Coralville, IA) and Sybr green dye. The expression of CCL5 was calculated using the $2^{-\Delta\Delta C_t}$ method [26]. The C_t values were normalized using HPRT as a house-keeping gene and fold-expression was calculated with respect to appropriate controls.

Multiplex cytokine assay

The expression of CCL5 protein was measured in the cell culture supernatants of the astrocytes. The supernatants were collected at the times indicated and centrifuged twice at 2000×g for 5 minutes to eliminate debris and stored at -86°C until used. The concentrations of CCL5 were measured using the Bio-Plex multiple cytokine assay kits (Bio-Rad, Hercules, CA) according to the manufacturer's protocol as per our previous reports [22,23].

Immunocytochemistry

3×10^5 SVGA cells were cultured on glass cover slips in a 12-well plate and transfected with gp120. The culture media was supplemented with 1 mg/ml GolgiStop™ (BD Biosciences, San Jose, CA) in order to prevent the release of CCL5. At 6 hours post-transfection, the cells were fixed with 1:1 ice-cold methanol:acetone for 20 minutes at 20°C. The cells were rinsed 3X with cold PBS and permeabilized with 0.1% TritonX-100 containing PBS (PBST). The cells were washed 3X with PBS followed by blocking with 1% BSA in 0.1% PBST for 30 min at room temperature. All the antibodies were diluted in blocking buffer. After blocking, the cells were incubated with a cocktail of rabbit PAb anti-RANTES (1:200) and mouse MAb anti-GFAP (GH5) (1:1500) at 4°C overnight in humidified chamber. After 3 X washes for 5 min each with PBS, the cells were probed in the dark for 1 hour at room temperature with anti-mouse conjugated with Alexa Fluor 555 (1:1000) and anti-rabbit conjugated with Alexa Fluor 488 (1:1000) antibodies in blocking buffer. Finally, the cells were washed 3 X with PBS for 5 minutes each and mounted on a slide with 10 µl of Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). The fluorescence was observed using a fluorescent Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). The images were captured using a 60X zoom lens. The cells were also stained with individual antibodies to check the specificity of each antibody (data not shown).

Statistical Analysis

Statistical analysis was performed using standard student's T-test. Each experiment was performed at least 3 times with each treatment in triplicate. The results were considered statistically significant if p-values ≤ 0.05 .

Results and Discussion

HIV-1 gp120 induces the expression of CCL5 in time-dependent manner

The neurotoxic effects of gp120 have been reported by several groups [27,28,29,30]. In tissue culture, gp120 has been shown to induce neuronal apoptosis via oxidative stress [27,28]. Transgenic mice expressing gp120 show a pathological profile similar to that seen in brains of patients afflicted with neuroAIDS [31]. In addition to viral entry into CNS, gp120 is also responsible for loss of tight junction proteins and BBB integrity via the STAT1 signaling mechanism, which leads to increased expression of various cytokines, leukocyte adhesion and transmigration through the BBB [32,33,34,35]. We recently showed that gp120 can alter pro-inflammatory cytokines and chemokines in human fetal astrocytes

[22,23]. Thus, it is important to study the effect of gp120 on other inflammatory modulators to fully understand its role in neuroinflammation. The role of CCL5 in inflammation has been well documented and this chemokine has been shown to increase HIV-1 infection in several reports [36,37]. Although the role of CCL5 in HIV-1 infection appears to be multifaceted, CCL5 can oligomerize on the cell surface and function as a potent modulator of inflammation [38,39]. Furthermore, Eugenin et al. demonstrated the role of CCL5 in inducing CCL2 expression, which can mediate transmigration of HIV-1 infected leukocytes across the BBB [40]. In order to determine the effect of gp120 on CCL5 expression, we transfected SVGA astrocytes with a plasmid expressing gp120. The transfection efficiency was found to be 50–70 % and expression levels were normalized to mock-transfected astrocytes. As shown in figure 1a, a high level of induction of CCL5 was observed at 6 hours (24.6 ± 2.67 fold), and gradually declined until levels near those of mock-transfected cells were reached at 72 hours (14.3 ± 1.2 fold at 12 hours, 6.2 ± 0.2 fold at 24 hours, 0.9 ± 0.0 fold at 48 hours and 1.4 ± 0.1 fold at 72 hours). Clearly, the mRNA expression of CCL5 due to gp120 was time dependent. Similarly, when measured in the culture supernatants, CCL5 protein levels increased as early as 6 hours post transfection (0.5 ± 0.21 ng/ml) with a peak response at 48 hours (3.0 ± 0.5 ng/ml) due to gp120. These results do not confirm the earlier reports by Fitting et al., who showed that gp120 had a limited effect on the basal levels of CCL5 in astrocytes obtained from different regions of mouse brain [41]. The differences in these results can be attributed to the astrocytes, which were of human origin in this study and therefore more relevant to clinical scenario. Earlier, Ubogu et al showed that increased levels of CCL5 can promote migration of monocytes through BBB, which may further aggravate neuroinflammatory disorders [42]. Also, protective role of CCL5 in the context of HIV-1 infection is observed at concentrations of 10 nM (equivalent to 78.76 ng/ml) [43]. However, the CCL5 levels induced by gp120 in the present study are only 3 ng/ml at maximum levels of induction, and thus unable to achieve the protective concentration. Fowler et al. showed that *N. meningitidis* infection could increase the CCL5 expression upto 5–10 ng/ml in meningioma cell [44], which were consistent with those found in CSF of patients with meningitis caused by *N. meningitidis*. It may be that the suboptimal concentrations of CCL5 induced by gp120, similar to those levels observed with bacterial meningitis [44] may recruit T-cells and monocytes and play a role in neurotoxicity. In order to confirm our findings regarding CCL5 induction by gp120, gp120-transfected cells were immunostained with fluorescently labeled antibodies. Distinct accumulation of CCL5 in the cellular processes of astrocytes was observed (Figure 2A–I) in astrocytes transfected with gp120 as opposed to mock-transfected or untransfected controls. Clearly, gp120-mediated increases in CCL5 expression could be one of the mechanisms responsible for a neuroinflammatory response.

Small interfering RNA targeted against gp120 abrogated CCL5 expression

In order to further confirm our hypothesis, we designed 4 different siRNA against gp120 the sequences of which were reported in our earlier studies [22,23]. All but one of the siRNAs showed significant abrogation of gp120-mediated CCL5 expression (Figure 3A–3B). siRNA 2 was found to be the most efficient in reducing gp120-mediated CCL5 expression. The abrogation of protein levels of CCL5 was also observed to be similar when compared with that of mRNA levels. Taken together these results, confirmed that the increase in CCL5 expression is specific to gp120.

Specific inhibitors and siRNA against NF-κB prevented gp120 mediated CCL5 expression

In order to further dissect the mechanisms involved in increased expression of CCL5 due to gp120, we investigated the potential role of the NF-κB pathway. Activation of the NF-κB pathway is involved in the pathophysiology of various inflammatory disorders including Alzheimer's disease [45], Parkinson's disease [46] and rheumatoid arthritis [47].

Furthermore, LPS mediated induction of CCL5 (> 25 ng/ml) is shown to be mediated via NF- κ B activation [48]. Together, these reports reinforced our interest in investigating whether the NF- κ B pathway could be responsible for gp120-mediated CCL5 expression. In the present study, using an IKK-2 inhibitor SC514, we showed that gp120-mediated CCL5 expression is NF- κ B dependent. The cells were treated 1 hour prior to gp120 transfection with 10 μ M SC514. The concentration was optimized based on cell viability as monitored by visual observation (viability > 90%; data not shown). The expression levels of CCL5 were abrogated by 44.6 ± 4.2 % at the level of mRNA and 51.8 ± 5.0 % at the protein level (Figure 4A & 4B). In order to further confirm the role of NF- κ B, we knocked down NF- κ B expression using siRNA. siRNA specific for p50 was transfected 48 hour prior to gp120 transfection and mRNA and protein levels of CCL5 were measured. This abrogated the activation of NF- κ B and consequently reduced gp120-mediated CCL5 expression by 71.0 ± 6.8 % at the level of mRNA (Figure 4C) and 52.2 ± 3.2 % at the protein level (Figure 4D).

The results presented in the current study provide important information regarding the mechanism through which gp120 produces astroglial activation, which in turn further leads to neuronal loss and CNS complications. With the advent of successful antiretroviral therapy, the life span of HIV infected patients has been greatly extended. In order to develop new treatments for the CNS complications often associated with HIV infection, it is important to understand the mechanisms responsible for neuroinflammation. The results presented in this study explore these mechanisms and identify a potential target for therapeutic intervention for neuroAIDS.

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HIGHLIGHTS

- In this study we studied effect of HIVgp120 on CCL5 production in astrocytes.
- Gp120 up-regulated CCL5 production in astrocytes
- The increased CCL5 production could be partially abrogated by NF-kB specific inhibitor and siRNA.
- Gp120 mediated CCL5 increase could be interpreted only as mediator of inflammation.

Figure 1. HIV-1 gp120 induces CCL5 expression in a human astrocyte cell line in a time-dependent manner

Astrocytes were transfected with a plasmid encoding gp120 as described in Materials and Methods. The expression levels of CCL5 mRNA (A) and protein (B) were measured using real time RT-PCR and multiplex cytokine assays, respectively. The white bars represent mock-transfected controls and the black bars represent gp120-transfected samples. Each bar represents the mean $\pm$ SE of 3 experiments with each experiment performed in triplicate. The statistical significance was calculated using student's t-test and ** denotes p value of ≤ 0.01 .

Figure 2. Confocal images demonstrating gp120-mediated CCL5 expression in SVGA astrocytes SVGA transfected and grown as described in Materials and methods and then co-stained for GFAP (red) and CCL5 (green). Individual images for different fluorophores were captured using confocal microscopy and merged using EZ C1 confocal microscope software. The non-transfected control showed basal levels of both CCL5 and GFAP (A–C). Mock-transfected astrocytes exhibited a marginal increase in CCL5 expression as compared to the control (D–F), while gp120-transfected astrocytes demonstrated stronger accumulation of CCL5 in the processes of astrocytes that colocalized with GFAP (G–I). (Magnification - 60X in A–I)

A **B**

Figure 3. Small interfering RNA targeted against gp120 abrogated CCL5 expression

Cells were transfected with siRNA followed by transfection with a plasmid encoding gp120 as described in Materials and Methods. CCL5 mRNA (A) and protein (B) levels were measured after 6 hours and 48 hours respectively. Samples transfected with gp120 were used as a positive control and compared with those transfected with siRNA. Each bar represents mean $\pm$ SE of 3 experiments with each experiment done in triplicates. The statistical significance was calculated using student's t-test and ** denotes p value of ≤ 0.01 .

Figure 4. Specific inhibitors and siRNA against NF-κB prevented gp120-mediated CCL5 expression

8×10^5 SVGA astrocytes were treated with 10 μ M of NF-κB inhibitor SC514 for 24 hours followed by transfection with 2 μ g of pSyn gp120 JR-FL in the presence of SC514. mRNA (A) and protein (B) samples were collected after 6 and 24 hours of transfection and CCL5 expression was measured using real time RT-PCR and multiplex cytokine assays, respectively. Cells were transfected with NF-κB siRNA followed by transfection with pSyn gp120 JR-FL and samples were harvested for mRNA and protein at 6 hours and 48 hours post-transfection, respectively. The expression levels of CCL5mRNA (C) and protein (D). Cells transfected with scrambled siRNA and mock-transfected cells were used as controls. The cells transfected with gp120 were used as positive control to estimate % inhibition of CCL5 expression. Each bar represents the mean $\pm$ SE of 3 experiments with each experiment done in triplicate. The statistical significance was calculated using student's t-test and ** denotes p value of ≤ 0.01 .