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Interaction of HIV-1 Core p24 Antigen with Human Monocytic Cell Line THP1 Results in TNF-α Dependent Secretion of Matrix Metalloproteinase-9

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Immunological mechanisms involving the release of inflammatory factors by HIV-1 infected microglia in the brain have been implicated in the pathogenesis of HIV dementia (HIVD). Since the regulation of matrix metalloproteinases (MMPs) activity can be influenced by variety of inflammatory mediators, this study was undertaken to look for a correlation between the MMP-9 release and the production of TNF-α in response to HIV-1 p24 in the human monocyte cell line THP-1 as a model for microglia. First, it was shown that HIV-1 core p24 antigen induced THP-1 to secrete MMP-9 in a dose response manner while it elicited a little effect on MMP-2 release in human astroglial cell line T98G. Next, it was found that p24 induced THP-1 to secrete TNF-α without prior differentiation into macrophages by phorbol myristate acetate (PMA) treatment. Furthermore, anti-TNF-α neutralizing antibodies significantly blocked p24-induced MMP-9 release in a dose dependent manner. Our data indicate that p24 antigen induces monocytic MMP-9 release by triggering up-regulation of TNF-α secretion.

Key Words: AIDS dementia complex (ADC), Matrix metalloproteinase 9 (MMP-9), Tumor necrosis factor α (TNF-α), Phorbol myristate acetate (PMA), THP-1, Human immunodeficiency virus-1 (HIV-1)

INTRODUCTION

A significant proportion of Human immunodeficiency virus-1 (HIV-1) infected individuals develop a devastating CNS complication consisting of dementia and motor deficits termed HIV dementia (HIVD) or the AIDS dementia complex (ADC) (14, 23). Although the exact mechanism(s) responsible for ADC are being investigated, it is widely believed that indirect mechanisms which involve viral gene products and cytokine dysregulation and other cellular factors released by HIV-1 infected brain macrophages/microglia as well as reactive astrocytes are primarily responsible for the severity of neurological impairment which occurs in ADC (5, 9, 13, 17, 20). Earlier data reported that the HIV-1 envelope proteins such as gp120 and gp41 could contribute to neuronal degeneration

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by excitotoxic mechanism and nitric oxide production (1, 31). However, a direct relationship was noted between detection frequency of p24 antigen in blood/CSF and the degree of cognitive impairment (24). This observation strongly suggests that core protein p24 could be a key determinant in neurodegenerative cascade and that more closely correlated with the extent of dendritic and neuronal damage in HIV encephalitis.

Earlier *in vitro* studies implicated that dysregulation of the synthesis and the release of matrix metalloproteinases (MMPs) by HIV infection of lymphocytes, monocyte derived macrophages may contribute to viral dissemination and tissue damage as seen in patients during the progression of AIDS (3, 12, 29). Furthermore, recent report demonstrating that the level of MMP-9 is elevated in the CSF of HIV-infected patients strongly supports a possible pathogenic role of MMPs in HIVD neuropathology (28). Expression of MMPs can be influenced by a variety of mediators such as proinflammatory cytokines which were implicated to be involved in HIV-associated brain injury (17, 26). In fact, TNF-α has been implicated as a potent neurotoxic agent which was elevated in CSF of HIVD patients (10, 26). However, little is known about the viral or cellular factors that are responsible for the production of TNF-α and MMPs in HIV-1 infected brains.

The purpose of this study was to investigate the interaction of HIV-1 core antigen p24 with the human monocyte cell line THP-1 as a model for microglia and the human astroglioma cell line T98G in order to determine whether p24 could modulate MMP-9 production and whether there would be an association between MMP-9 secretion and TNF-α production. Here, we show that THP-1 upon exposure to p24 antigen can potently induce MMP-9 production in time and dose dependent man ners while T98G failed to increase MMP-2 secretion in response to p24. The soluble mediator, TNF-α is a main mediator of this effect because blocking the endogenous TNF-α activity with neutralizing anti-TNF-α antibodies blocked p24-induced augmentation of MMP-9 release.

MATERIALS AND METHODS

Cell culture and treatment for preparation of conditioned media. The human monocytic cell line THP-1 as a model for microglia and human glioblastoma cell line, T98G were obtained from ATCC (Rockille, MD) and maintained as described previously (4, 6, 8). Confluent cultures were exposed to p24 for indicated periods in the presence or absence of specific stimulators and inhibitors and the serum free conditioned media were prepared for zymographic analysis, TNF-α ELISA assay, and western blotting analysis. The recombinant proteins, p24 and gp41 were generous gifts from Green Cross Co., Seoul, Korea and HIV related synthetic peptides such as ID (env 598-613) or ISP (env 583-599) and its reverse peptide, PSI (env 599-583) were purchased from QCB (Hopkinton, MA). TNF-α and anti-TNF-α antibodies were purchased from R & D (Minneapolis, MN). Other chemicals and phorbol myristate acetate and (PMA) were purchased from Sigma chemical Co. (St. Lois, MO).

Zymographic analysis. The gelatinolytic activities in each sample, normalized for the equal amount of protein were determined using zymography with gelatin copolymerized with acrylamide in the gel according to previously published methods (3, 4). Briefly, conditioned media collected for gelatin zymography were electrophoresed on a 10% SDS-PAGE gel with 0.1% gelatin as a substrate without heat and reduction. After removing SDS in 2.5% Triton X-100 for 1 h, gels were incubated for 20 h at 37℃ in 50 mM Tris-Cl, pH 7.4 containing 10 mM $CaCl_2$ and 0.02% NaN_3 to allow digestion of the gelatin substrate. Finally, the gels were

stained for 1 h in 7.5% acetic acid/10% propanol-2 containing 0.5% Coomassie Brilliant Blue G250 and destained in same solution without dye. Positions of gelatinolytic activities are unstained on a dark stained background.

Measurement of TNF-α. THP-1 cells were seeded into 24 well culture plates ($5x10^5$/well) in 0.5 ml of RPMI-1640 medium. They were incubated for indicated time in the presence or absence of specific stimulators and inhibitors. The concentrations of TNF-α in cell-free supernatants was measured by an enzyme-linked immunoabsorbant assay (ELISA) using monoclonal antibodies and the procedure recommended by the supplier (Pharmingen, San Diego, CA). A standard curve using recombinant human TNF-α was set up for ELISA according to the manufacturer's instruction (R & D).

Western immunoblot analysis. The proteins in conditioned media were separated by 10% SDS-PAGE. Were then transferred to PVDF membranes and the blots were blocked by incubation with 5% nonfat dry milk in Tris-buffered saline containing 0.15% Tween-20 for 2 h. The blots were then probed for 2 h with mouse monoclonal IgG antibodies specific for MMP-2 or MMP-9 (Oncogene Science, Cambridge, MA), diluted 1:1000, followed by incubation for 1 h with a goat anti mouse IgG conjugated with horseradish peroxidase diluted 1:3000 (Amersham). The proteins were visualized by the addition of chemiluminescent substrate according to the protocol of manufacturer (ECL, Amersham).

Data analysis. All values of MMP-9/MMP-2 activities were determined based on at least three independent experiments. The clear bands on the zymograms were photographed on the negative (Polaroid's 665 film) and the signals were quantified by densitometric scanning using UltroScan XL laser densitometer (LKB, Model 2222-020) to determine the intensity of MMP activity. The arbitrary densitometric units were expressed or converted to a fold of the response of PBS treated control for each individual experiment.

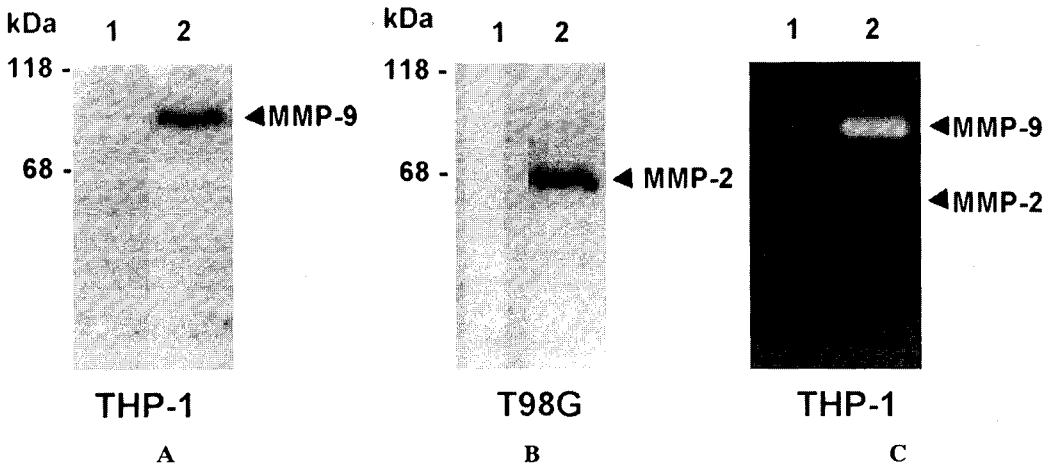

Figure 1. Representation of immunoblots of the 92-kDa gelatinase B and 72-kDa gelatinase A for verification of MMP-9 and MMP-2 respectively. Serum-free control media (lane 1) and serum free conditioned media from THP-1 treated with PMA (10 nM) for 18 h (lane 2) in **A** and serum-free control media (lane 1) and serum free conditioned media from T98G incubated for 18 h (lane 2) in **B** were subjected to SDS-PAGE and the proteins were blotted to PVDF and probed with monoclonal antihuman MMP-9 or MMP-2 sera. In **C**, zymographic analysis of gelatinolytic activities in conditioned media from THP-1 without (lane 1) and with PMA (10 nM) treatment for 18h (lane 2) was shown.

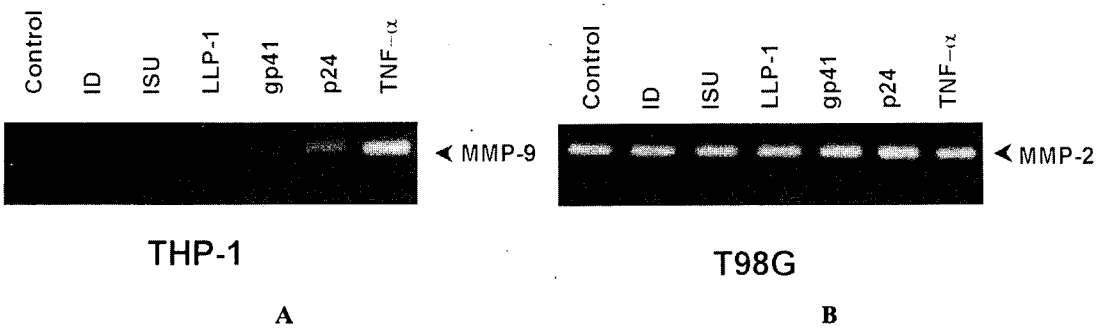

Figure 2. Effects of HIV-1 related peptides on MMP-9 and MMP-2 release. Zymographic analysis of gelatinolytic activities in conditioned media from the human monocytic cell line THP-1 (**A**) and the human astrocytic cell line T98G (**B**), after stimulation of the cells with HIV-1 related peptides at concentration of 1 μM except gp41 (0.5 μM) for 18 h was performed as described in materials and methods.

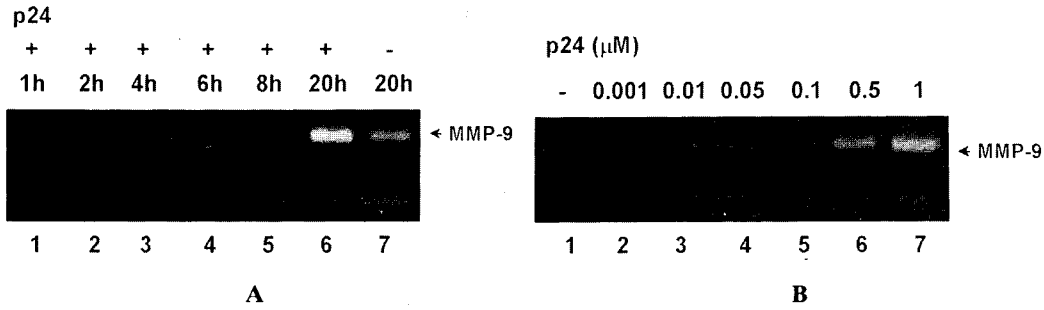

Figure 3. Time and dose dependent enhancement of MMP-9 secretion by p24 in THP-1. Time dependent MMP-9 release in response to p24 (1 μM) from THP-1 after treatment with increasing concentrations of p24 for 18 h were demonstrated in **A** and **B**, respectively. Results are representative of three independent experiments.

RESULTS

Analysis of the 92 and 66-kDa gelatinolytic activities as MMP-9 and MMP-2. Using a sensitive zymography method as shown in Fig. 1, we found that the unstimulated THP-1 cells did faintly display both 92 kDa and 66 kDa gelatinolytic bands (Fig. 1C, lane 1). However, PMA stimulation demonstrated that a 92 kDa gelatinolytic band was markedly induced in supernatants, while 66 kDa gelatinolytic band remained unchanged (Fig. 1C, lane 2). Western blot analysis using gelatinase B (MMP-9) and gelatinase A (MMP-2) specific human antibodies confirmed that 92 or 66 kDa are MMP-9 and MMP-2 respectively in the supernatants of PMA treated THP-1 cells and T98G cells (Fig. 1A and B, lane 2).

Induction of MMP-9 by HIV-1 p24. As shown in Fig. 2, HIV-1 p24 induced MMP-9 secretion in human monocytic THP-1 whereas they elicited a little effect on MMP-2 release in human astroglial cell line T98G (B). Similar effect was observed with gp41 while MMP-9 activity remained weak by other HIV-1 related peptides. We also confirmed that proinflammatory cytokine TNF-α significantly stimulated MMP-9 release from THP-1 cells. MMP-9 release induced by p24 was time and dose dependent (Fig. 3A and 3B) because MMP-9 proteolytic activity was significantly detected in the supernatant after 8 h and the level was increased to 4 fold by 20 h incubation compared

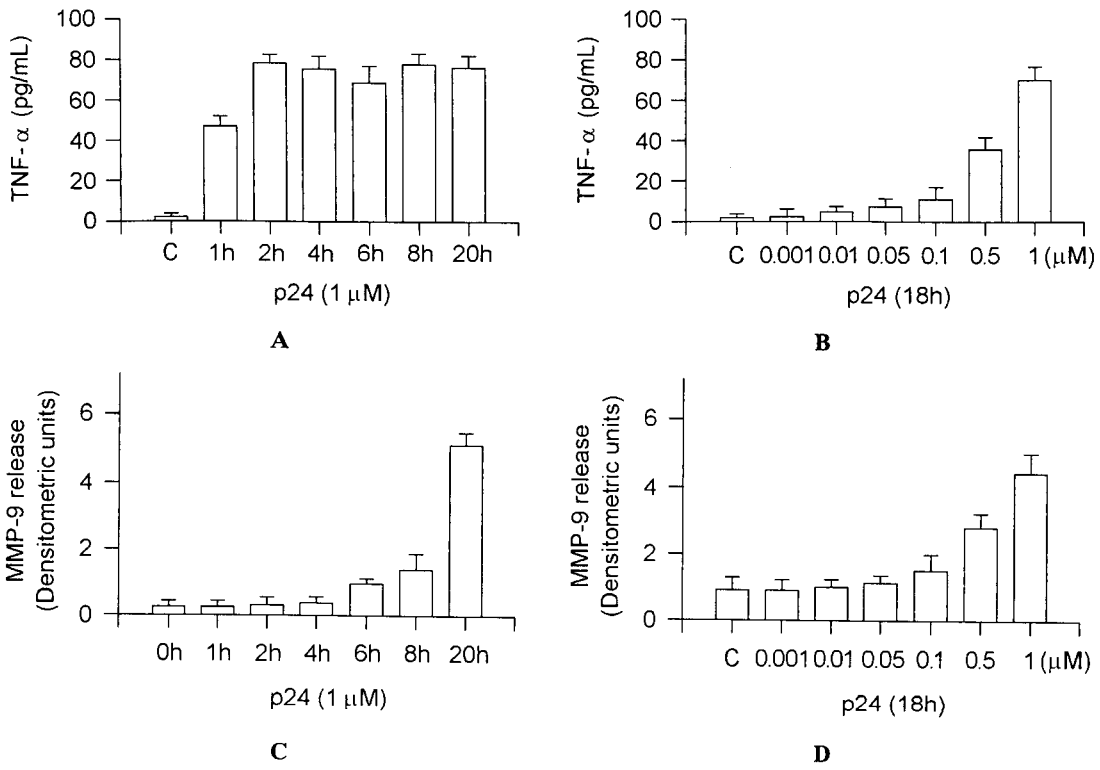

Figure 4. Time and dose dependent enhancement of TNF-α production p24 in THP-1. Time dependent TNF-α release in response to p24 (1 μM) after treatment with increasing concentrations of p24 for 18 h from THP-1 were demonstrated in **A** and **B**, respectively. The concentrations of TNF-α in the cell supernatants corresponded to the same experiments as in Figure 3 were assayed and expressed in pg/ml. Results were shown as means ± standard deviations for triplicate cultures. **C** and **D** represent the densitometric analysis of zymograms as shown in Figure 3 A and B respectively.

with that of the untreated control. Furthermore, increasing concentrations of p24 resulted in increases in MMP-9 release starting at 0.5 μM and the release was increased to 4.3 fold at 1 μM concentration compared with that of the untreated control. This p24-mediated induction of MMP-9 was mimicked by treatment with exogenous TNF-α (data not shown).

Stimulation of TNF-α production by HIV-1 p24 in human monocytic THP-1 cells. TNF-α production was studied in THP-1 cells over 20 h incubation with the same time course used in MMP-9 release assay (Fig. 3). TNF-α protein was undetectable in ELISA at the beginning of the incubation period. However, application of p24 at a concentration of 1 μM to undifferentiated THP-1 cells caused a significant increase in TNF-α secretion. The induction was acute since TNF-α release was detected at the beginning of culture at 1 h, and increased steadily to reach plateau by 2 h and sustained throughout the 20 h period (Fig. 4A). Furthermore, p24 caused a dose dependent increase of TNF-α production. TNF-α were increased at 0.01 μM concentration and further increases were observed at higher concentrations (Fig. 4B). Time course study demonstrated that TNF-α production preceded MMP-9 release as densitometric analysis of zymograms (Fig. 4C and D). It is noteworthy that the levels of secreted TNF-α by 0.01 to 1 μM of p24 antigen range from 6 to 70 pg/ml.

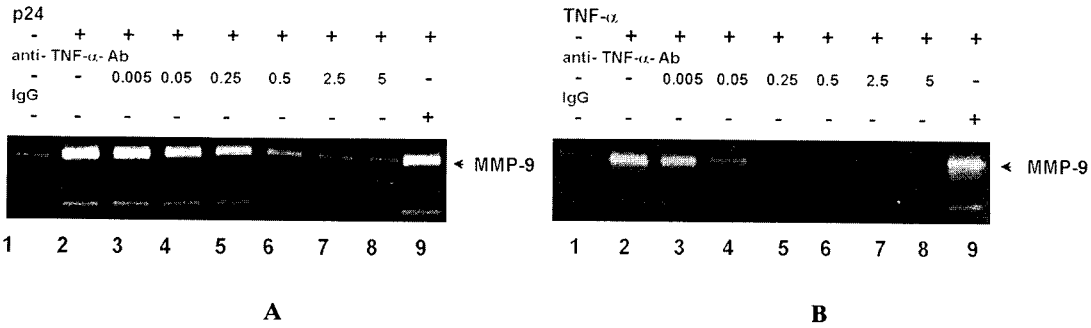

Figure 5. Effect of anti-TNF-α antibodies on p24-induced MMP-9 release in THP-1. In **A**, MMP-9 activities present in conditioned media from THP-1 after treatment with p24 in the absence or presence of increasing anti-TNF-α antibodies or preimmune IgG (5 μg/ml) for 20 h were analyzed. In **B**, TNF-α (10 ng/ml) were preincubated for 3h at 37℃ with the indicated concentration of anti-TNF-α antibodies before addition to the cell cultures and the media samples were analyzed after 24 h incubation. Results are representative of three independent experiments.

Evidence for a role of TNF-α in p24-stimulated MMP-9 release. To ensure that MMP-9 release was being stimulated directly by TNF-α production, experiments were conducted to investigate the effect of anti-TNF-α antibodies on MMP-9 release in response to p24. At the 0.5 μg/ml concentration of anti-TNF-α antibody used, the p24-mediated MMP-9 release was suppressed by more than 90% (Fig. 5A). Blocking TNF-α activity with this antibody at the 10 fold lower (0.05 μg/ml) concentration abolished the exogenous TNF-α induced MMP-9 release by 90% (Fig. 5B) while preimmune IgG (5 μg/mL) used as control had a little effect. Thus, anti-TNF-α antibodies efficiently suppressed both p24 and TNF-α induced MMP-9 release.

DISCUSSION

Earlier study implicated a direct relationship was noted between the degree of cognitive impairment and detection frequency of p24 antigen in blood/CSF (24). Furthermore, recent reports demonstrating that MMP-9 level elevated in the CSF of HIV-infected patients strongly suggests their pathogenic roles in HIV-induced inflammation leading to critically causing the destruction of tissues and cellular architecture or viral dissemination as seen in patients during the progression of AIDS (26, 28). We are here reporting that HIV-1 core antigen p24 induced human monocytic THP-1 to secrete MMP-9 in a dose-response and time-dependent manners. TNF-α production appears to be a main mediator of MMP-9 induction in response to p24 since increased MMP-9 production required treatment of monocytes with HIV-1 p24 more than 8 h while induction of TNF-α production rapidly occurred at 1 h treatment and this p24-mediated induction of MMP-9 was mimicked by treatment with exogeneous TNF-α. Furthermore anti-TNF-α neutralizing antibodies dose dependently blocked MMP-9 release induced either by p24 or by exogenous TNF-α. Recent study demonstrated that exposure of monocytes to regulatory protein HIV-1 Tat protein up-regulated the production of TNF-α and IL-1β which mediated induction of MMP-9 expression in autocrine fashion (15). These observations together suggest that the monocytes could be cellular sources responsible for increased production of TNF-α and MMP-9 in response to HIV-1 p24 or Tat and the mechanism by which these proteins activate monocytes to up-regulate MMP-9 activity is dependent on TNF-α production. Further experiments are required to examine a possible role

of IL-1β, which has been also implicated as a potent neurotoxic agent elevated in CSF of HIVD patients in addition to TNF-α (10, 26), in induction of MMP-9 activity in response to p24 although our data showed that anti-TNF-α neutralizing antibodies almost completely blocked p24-mediated MMP-9 induction.

In contrast, application of either p24 or exogenous TNF-α elicited a little effect on MMP-2 production both in monocytic THP-1 and astroglial T98G cells. Similarly, p24 was ineffective to induce MMP-9 activity in astroglial T98G cells. In agreement with this data recent study demonstrated that there was no change in the constitutive activity of MMP-2 in the cultured human astrocytes after treatment with phorbol ester (2) and in response to cytokines such as IL-1β, LPS, and TNF-α in the cultured rat astrocytes (7). These observations together suggest a complex regulation of the expression of MMPs and the possibility for differences in their production dependent on cell types and species. The activity of MMP-2 was prominently expressed in T98G whereas a relatively weak level of MMP-9 activity was detected only after 3 day of incubation in these cells (data not shown). The higher level of constitutive activity of MMP-2 detected in a glial cell line T98G could be associated with its extensive cellular invasion property (19). Further studies will elucidate the mechanism involved in down-regulation of MMP-9 activity in T98G cells and defects on TNF-α production or signal transduction pathway responsible for MMP-9 induction.

Whether production of MMP or the lack thereof is involved in the pathological processes of ADC is unknown. However, specific inhibition of MMPs *in vivo* blocks edema and pathologic tissue damage of several inflammatory diseases may support the potential cytopathic effect of MMPs in HIV-I infected brains. Breakdown of the blood-brain barrier in the brains of highly demented HIV-1 patients (18, 21) in addition to its potential roles in viral dessimination, tissue damage (12, 22, 29) further suggest that MMP-9 could be associated with disease process in HIV-I infected brains. Increased activity of MMPs may be also associated with Fas-ligand deficiency as seen in PBMCs from HIV-infected patients (16, 27), possibly leading to decrease antiviral immune state during disease progression. In addition, our data confirm previous studies demonstrating induced expression of MMP-9 in response to proinflammatory cytokine TNF-α in the cultured rat astrocytes or in human macrophages and constitutive expression of MMP-2 (7, 25).

The levels of TNF-α induced by p24 in our *in vitro* study are physiologically relevant to the elevated levels of this cytokine present in HIV-1 infected patients suffering from ADC since the levels of TNF-α *in vivo* detected in AIDS CSF have been shown to range between 50 and 100 pg/ml (11). Induction of monocytic MMP-9 release through autocrine TNF-α production could be a key event for migration of monocytes and maturation into macrophages during inflammation process. Thus, the higher level of TNF-α induced by exposure to p24 certainly play a role through stimulation of MMP-9 release as observed in this study in regulating the development of HIV-related encephalopathy from penetration of infected monocytes into the brain to cause severe neuronal dysfunction leading to dementia. The importance of TNF-α in the pathological cascade is further supported by recent reports showing stimulation of viral replication in macrophages which are the only source of viral production in the brain (21, 30). Present study could imply that monocytes induced to differentiate into microglia-like cells by the CNS environment (10) could be a major source for MMP-9 and TNF-α production possibly linked to exposure to p24 in the HIV infected brains.

CONCLUSION

During the progression of AIDS, a significant number of patients develop neurological complications characterized by cognitive, motor, and behavioral changes. The biological functions of HIV-1 p24 in addition to HIV-1 env proteins (1, 12, 28) remains critical to unraveling the neuropathogenic mechanisms of ADC with regard to the development of new therapeutic modalities for the treatment and/or prevention of ADC. Data presented in this work suggest that monocytes could significantly enhance MMP-9 secretion by triggering up-regulation of autocrine TNF-α production in response to HIV-1 p24. Therefore, we propose the ability of HIV- p24 to up-regulate TNF-α level acting as a main mediator responsible for subsequent increase of MMP-9 activity. High levels of TNF-α and MMP-9 activity which were shown in CNS of demented HIV-1 infected patients (15, 23, 25) can contribute to brain injury associated with neurodegenerative process of AIDS dementia as well as viral replication/dissemination.

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