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Neuroprotective Effects of IGF-I against TNFα-Induced Neuronal Damage in HIV-Associated Dementia

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Human immunodeficiency virus type 1 (HIV-1) infection often results in disorders of the central nervous system, including HIV-associated dementia (HAD). It is suspected that tumor necrosis factor-α (TNFα) released by activated and/or infected macrophages/microglia plays a role in the process of neuronal damage seen in AIDS patients. In light of earlier studies showing that the activation of the insulin-like growth factor I receptor (IGF-IR) exerts a strong neuroprotective effect, we investigated the ability of IGF-I to protect neuronal cells from HIV-infected macrophages. Our results demonstrate that the conditioned medium from HIV-1-infected macrophages, HIV/CM, causes loss of neuronal processes in differentiated PC12 and P19 neurons and that these neurodegenerative effects are associated with the presence of TNFα. Furthermore, we demonstrate that IGF-I rescues differentiated neurons from both HIV/CM and TNFα-induced damage and that IGF-I-mediated neuroprotection is strongly enhanced by overexpression of the wt IGF-IR cDNA and attenuated by the antisense IGF-IR cDNA. Finally, IGF-I-mediated antiapoptotic pathways are continuously functional in differentiated neurons exposed to HIV/CM and are likely supported by TNFα-mediated phosphorylation of $I_{\kappa}B$. All together these results suggest that the balance between TNFα and IGF-IR signaling pathways may control the extent of neuronal injury in this HIV-related experimental setting.

Key Words: HIV associated dementia; neuronal damage; neuronal survival; IGF-I; TNFα.

INTRODUCTION

Human immunodeficiency virus (HIV) infection often involves clinical manifestations including cognitive motor disorders as well as behavioral and cognitive abnormalities, which are observed in at least two-thirds of AIDS patients (Gabuzda and Hirsch, 1987). The brains at autopsy often exhibit microglial nodules containing multinucleated giant cells, increased numbers of perivascular macrophages, and activated macrophages/microglia in brain parenchyma (Budka *et al.,* 1987). Although monocyte and T cell infiltration of the central nervous system appears relatively early during HIV infection, and the initial invasion is usually cleared, neurological changes occur much later during the course of the disease. Therefore, the presence of HIV-infected cells within the brain tissue may not be the sole cause of neuronal cell dysfunction and death (Price, 1988; Price *et al.,* 1988). During the course of HIV infection, macrophages/microglia, as well as other cells in the brain, can be exposed to viral proteins such as gp120 and Tat. This in turn may activate expression and secretion of several cytokines, including tumor necrosis factor-α (TNFα), which via auto- or paracrine mechanisms may dysregulate even further metabolism in the brain tissue (Rieckmann *et al.,* 1991; Rimaniol *et al.,* 1997). TNFα mRNA and protein expression are increased in HIV-induced neurological disorders (Kaul and Lipton, 1999; Sippy *et al.,* 1995; Tan *et al.,* 1996). In addition to the deleterious effects of TNFα production, neuronal injury may be further promoted by parallel decreases in neuroprotective factors. In HIV patients with wasting syndrome and in children with Failure to Thrive (FTT), reduced insulin-like growth factor I (IGF-I) serum levels have been reported (Jain *et al.,* 1998; Laue *et al.,* 1990).

The receptor for IGF-I (IGF-IR) is a membrane-associated multifunctional tyrosine kinase with activities in many cell types, including cells from the central nervous system. IGF-IR signaling protects neurons from ischemic injuries (Gluckman *et al.,* 1992), inhibits low potassium-induced apoptosis of cerebellar granule neurons (D'Mello *et al.,* 1993), and rescues brain tumor cell lines from apoptosis induced by anchorage-independent culture conditions (Wang *et al.,* 2001). At least three pathways may contribute to the IGF-IR-mediated cell protection from apoptotic death. Two of these pathways depend upon Akt activation and may result in Bad (Peruzzi *et al.,* 2001; Zha *et al.,* 1996) and/or procaspase 9 phosphorylation (Cardone *et al.,* 1998), events that are thought

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to counteract apoptosis. The third pathway, in contrast, appears to be Akt-independent and results in Raf phosphorylation and its translocation to the mitochondrial compartment (Peruzzi *et al.,* 2001).

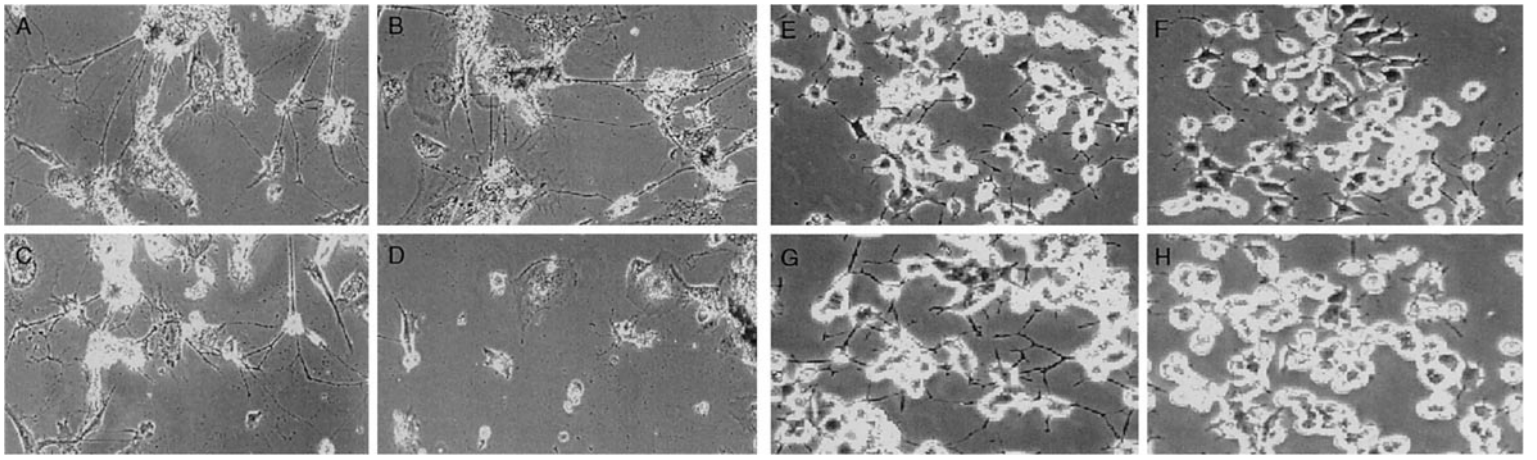
Similar to IGF-I, TNFα induces a broad range of biological events (Locksley *et al.,* 2001). TNFα is an inflammatory cytokine produced by activated macrophages and other cell types, including lymphocytes, hepatocytes, and fibroblasts (Natoli *et al.,* 1998; Wallach, 1997). It binds with high affinity and activates two receptors, TNFR1 and TNFR2, which belong to a large superfamily including more than 20 surface proteins involved in a variety of biological responses including host defense, inflammation, apoptosis, and differentiation (Locksley *et al.,* 2001). Although TNFR2 is able to activate some cellular responses mostly in hematopoietic cells, the TNFR1 is thought to be the major receptor responsible for TNFα signaling in the majority of cells including neurons (Gary *et al.,* 1998; Natoli *et al.,* 1998). The receptor does not reveal any known enzymatic activities; however, it engages into signaling pathways by recruiting other adaptor proteins. Following ligand binding, receptor molecules trimerize creating internal complexes with signaling proteins such as TNFR-associated death domain containing protein (TRADD), TNFR-associated factor 2 (TRAF2) and/or Fas-associated death domain protein (FADD) (Natoli *et al.,* 1998). Present understanding of the signaling events includes TRADD recruitment into C-terminus of TNFR1 and subsequent binding of FADD, TRAF2, and/or receptor interacting protein (RIP) into the complex. Depending on the circumstances, which are not well defined and could depend on the activation of other growth factor receptors and/or availability of the substrate(s), the TNFR1 receptor may send contradictory signals. The first one is antiapoptotic and involves preferential binding of TRAF2 into ligand-activated receptor. Two signaling branches can diverge from here: $NF_{\kappa}B$ translocation to the nucleus (Lieberson *et al.,* 2001) and JNK/p38-mediated phosphorylation of transcription factors including AP-1, ATF-2, and Elk (Boone *et al.,* 1998; Yuasa *et al.,* 1998). Both pathways lead to the rearrangement of the transcriptional activity of the cell, which in turn could render resistance to apoptosis via expression of antiapoptotic molecules such as FLIPs (Micheau *et al.,* 2001) and IAPs (Furusu *et al.,* 2001). The second one is purely proapoptotic and involves predominant recruitment of FADD, its direct association with the CARD (caspase recruitment domain) of caspase 8 or caspase 10 (initiators), and subsequent cleavage and activation of executioner caspases 3, 6, and 7, leading to apoptotic death of the cell (Boone *et al.,* 2000; Kubota *et al.,* 2001).

Since IGF-IR and TNFR1 may affect neuronal function and survival by engaging opposing signaling pathways, here we evaluate the contribution of TNFα and IGF-I in determining the fate of differentiated neurons upon exposure to the conditioned medium from HIV-infected macrophages. Our results demonstrate that in the absence of IGF-I stimulation, TNFα strongly contributes to the neuronal damage inflicted by the conditioned medium from HIV-infected macrophages (HIV/CM). IGF-I treatment rescues differentiated neurons from both HIV/CM and TNFα-induced damage, and the neuroprotection is proportional to the level of IGF-IR expression in the affected neurons. In the presence of IGF-I differentiated neurons retain the ability to survive the treatment with HIV/CM by activating antiapoptotic molecules from the IGF-IR.

RESULTS

Neurotoxic effect of the conditioned medium from HIV-infected macrophages (HIV/CM)

We have utilized differentiated cultures of PC12 and P19 neurons to evaluate potential toxic effects of the conditioned medium collected from HIV-infected macrophages (HIV/CM). P19 mouse teratocarcinoma cells proliferate in the absence of the differentiation signal and can be efficiently transduced with retroviral expression vectors. Treatment of the cells with retinoic acid results in the expression of neuronal cell markers and the development of axon-like and dendrite-like cellular processes (Jones-Villeneuve *et al.,* 1982). Treatment of differentiated P19 or PC 12 neurons with HIV/CM caused a drastic difference in the morphology of the cells. Shown in Fig. 1 are representative morphological changes in differentiated cultures of P19 cells before (A and B) and at 72 h following treatment with either the HIV/CM (D) or with the control conditioned medium (CCM) (C). Note that images in Figs. 1A and 1B and images in Figs. 1C and 1D were taken from the same microscopic fields at two different time points, respectively. Figure 1 also demonstrates representative alterations in PC 12 cells upon the treatment with CCM (compare Figs. 1E and 1G) or HIV/CM (compare Figs. 1F and 1H). The quantitative assessment of the observed differences for PC 12 and P19 cells is shown in Figs. 1I and 1J, respectively. We have determined the baseline for the number of the neurite-bearing cells present in three randomly chosen microscopic fields per plate at 3 days after NGF treatment (TO). The number of neurite-bearing cells in the same microscopic fields was monitored at 24 and 96 h after the HIV/CM treatment. In the absence of the conditioned media (No CM), the number of PC 12 cells bearing cellular processes increased with time. In contrast, a dose- and time-dependent decrease in the number of differentiated neurons was observed following the treatment with increasing concentrations of the HIV/CM. Although control conditioned medium (CCM) collected from uninfected macrophages also affected the number of neurite-bearing cells, the effects were much less pronounced than those seen upon exposure of the cells to HIV/CM. Since the observed difference between HIV/CM



I

P19 neurons

% change in the length of neuronal processes over time

80 60 40 20 0 -20 -40 -60 -80 -100

	No CM	CM 1/2	CM 1/5	CM 1/10	CM 1/20	CCM 1/5	
□24	6	-72	-67	-43	-10	-24	0
■72	-4	-100	-100	-86	-18	-40	

HIV/CM dilutions

J

PC12 Neurons

% change in the number of neurite bearing cells over time

80 60 40 20 0 -20 -40 -60 -80 -100

	No CM	CM 1/2	CM 1/5	CM 1/10	CM 1/20	CCM1/5
□24h	13	-6	-9	0	4	3
■96h	56	-69	-55	-20	-18	-21

HIV/CM dilutions

FIG. 1. Effect of the HIV/CM on the preservation of neuronal processes. Phase-contrast images taken from P19 cells 5 days following the retinoic acid treatment (A and B), and from PC 12 cells 3 days following NGF treatment (E and F). Differentiated cultures of P19 and PC 12 cells were subsequently treated with the control-conditioned medium (CCM) (C and G, respectively); or with the HIV/CM (D and H, respectively). The HIV/CM incubation lasted 72 h for P19 cells, and 96 h for PC 12 cells. Original magnification, ×20. I and J illustrate in quantitative manner effects of the HIV/CM on retinoic acid differentiated P19 cells and NGF-differentiated PC 12 cells, respectively. Differentiated neurons were treated with a series of dilutions of the HIV/CM in fresh culture medium, and the effects on neuronal processes were evaluated at 24, 72, and 96 h following the HIV/CM treatment. For PC 12, results are expressed as percentage change in the number of neuritis-bearing cells. Since P19 cells form extremely long neuronal processes, the results reflect a percentage change in the average length of neuronal processes per field. The measurements of the length of neuron-like processes were taken from a series of pictures of corresponding microscopic fields and reflect an average length of neuron-like processes detected in a particular microscopic field. For simplicity, results for the control-conditioned medium (CCM) are given only for one dilution (1 vol of the HIV/CM and 5 vol of the fresh culture medium, 1:5). Results represent an average of two experiments consisting of two 35-mm plates per point from which measurements were taken in three separate microscopic fields ($n = 12$), and with an average of at least 500 cells counted. An asterisk indicates values that are statistically significantly different, $P < 0.001$. Comparisons were made between HIV/CM (1/5) and CCM (1/5) at 24 (**) and 72 h (*) for P19 cells; and at 24 (**) and 96 h (*) for PC 12 cells. Statistical significance for comparison between groups of data was determined by using the unpaired, two-tailed Student's t test.

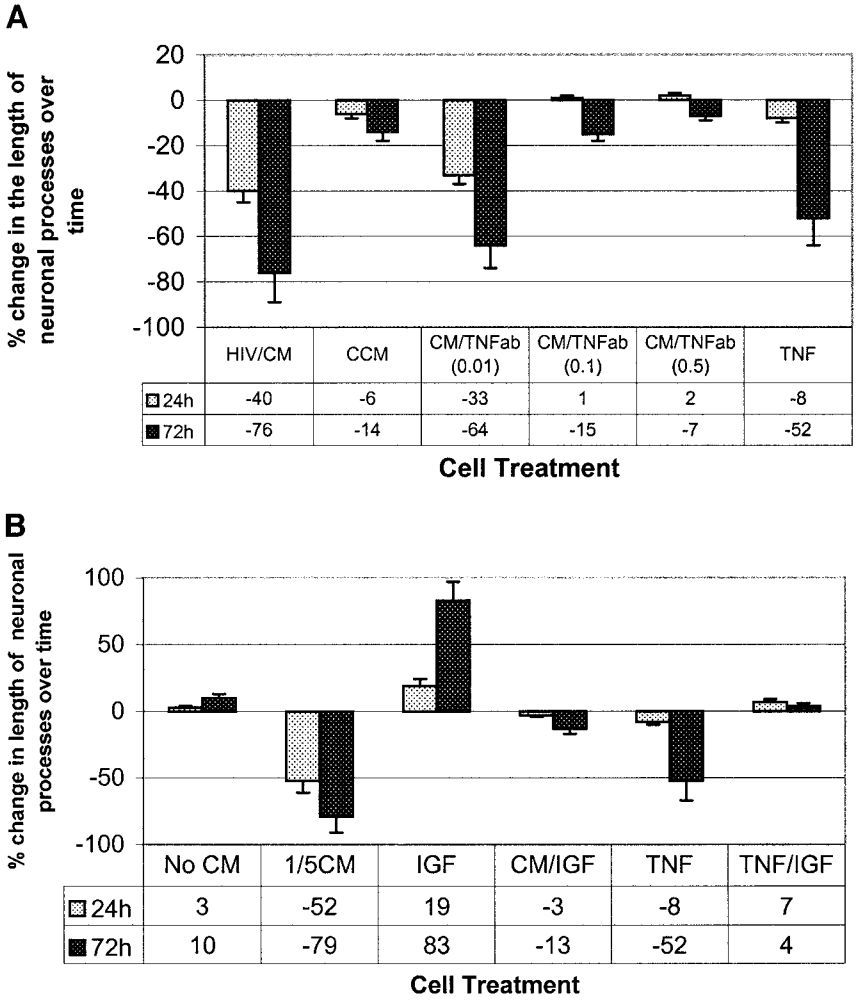


FIG. 2. Contribution of TNFα and IGF-I to HIV/CM-mediated neuronal damage. (A) Differentiated P19 cells were exposed either to HIV/CM or to control conditioned medium (CCM) both at 1:5 dilution with the fresh culture medium. In some samples, HIV/CM was modified by 2 h preincubation with increasing concentrations (from 0.01 to 0.5 μg/ml) of the anti-TNFα neutralizing antibody (R&D Systems; AF-210-NA). To imitate neurotoxic action of the HIV/CM, differentiated P19 cells were also treated with the recombinant TNFα (20 ng/ml). All other parameters of the experiment are described in the legend to Fig. 1. (B) P19 cells differentiated by retinoic acid were treated with IGF-I (50 ng/ml), TNFα (20 ng/ml), and the combination of either IGF-I and TNFα or IGF-I and HIV/CM (1:5 dilution). The results represent percentage change in an average length of neuronal processes in comparison to measurements taken before the treatment. This is an average of two experiments consisting of two 35-mm plates per data point, from which measurements were taken in three separate microscopic fields ($n = 12$).

and CCM at the 1:5 dilution (1 vol of the HIV/CM or CCM and 4 vol of the fresh culture medium) was highly significant ($P < 0.001$), this condition was used throughout the next series of experiments. Similar to PC 12, a massive neuronal loss was detected in differentiated cultures of P19 cells upon treatment with HIV/CM. These cells appear to be particularly sensitive to HIV/CM since a substantial loss of the neuronal processes was noticed as early as 24 h after the treatment, and virtually all differentiated P19 cells were lost within 72 h (Fig. 1J). In contrast, examination of the effects of HIV/CM on undifferentiated monolayer cultures of PC 12 and P19 cells revealed no significant effects on the cell death, as determined by the TUNEL assay and by counting of the cell number. Following the treatment, HIV/CM 1:5 dilution for 48 h, only 0.27% ($\pm$0.04) of P19 cells and 0.15% ($\pm$0.05) of PC12 cells show a positive labeling for DNA strand breaks. In control conditions, C/CM, 1:5 dilution for 48 h, the values were similar, showing 0.22% ($\pm$0.01) and 0.1% ($\pm$0.02) of apoptotic cells, respectively.

Opposing effects of TNFα and IGF-I on HIV/CM-mediated neuronal damage

To evaluate a potential contribution of TNFα in HIV-mediated neuronal cell damage, we utilized the approach in which the conditioned medium (HIV/CM) was pretreated with the antibody against TNFα. As shown in Fig. 2A, preincubation with anti-TNFα neutralizing antibody at 0.1 μg/ml for 2 h efficiently neutralized in a dose dependent manner toxicity of the HIV/CM. Conversely, addition of the recombinant TNFα, 20 ng/ml, into the

fresh medium markedly enhanced loss of neuronal processes in differentiated cultures of P19 cells, further supporting a role of this inflammatory cytokine in the HIV-mediated neuronal damage. To determine if activation of the IGF-I receptor restores and/or promotes formation of neuronal processes, P19 cells were cultured in media containing IGF-I (50 ng/ml). As shown in Fig. 2B, it is evident that IGF-I facilitates retinoic acid induced formation of neuronal processes in P19 cells and that the neurotoxic effects of HIV/CM and TNFα are substantially reduced in cells treated with IGF-I.

In the next set of experiments, we evaluated the ability of IGF-IR to enhance IGF-I-mediated neuronal cell protection. In parallel, we have evaluated the ability of antisense IGF-IR cDNA to suppress IGF-I-dependent neuronal outgrowth and to sensitize cells to neurodegenerative effects of the HIV/CM. We have generated stable cell lines by transducing P19 cells with retrovirus vectors expressing human IGF-R cDNA in sense or antisense orientations. To avoid clonal variability, mixed populations of transduced cells were collected and utilized in the study. Figure 3A indicates the levels of IGF-IR (top) and TNFR1 (bottom) in parental P19 cells, in P19 cells stably transduced with the wild-type IGF-IR cDNA (P19/IGF-IR), in cells expressing antisense IGF-IR cDNA (P19/asIGF-IR), and in control R600 fibroblasts, which express 3×10^4 IGF-IR molecules per cell (Reiss *et al.,* 1998). Densitometric assessment revealed that P19/IGF-IR cells are characterized by a threefold increase in the IGF-IR protein level when compared with the parental P19 cells. Conversely, more than 50% decrease in the level of IGF-IR was detected upon transducing cells with a retrovirus vector expressing antisense IGF-IR. The same blot was utilized to examine the level of housekeeping gene product, GRB-2, to verify equal loading of the proteins in various samples. The observed differences in the levels of IGF-IR in transduced cell populations correlated with the growth responses of the cells to IGF-I, as determined by anchorage-independence assays. This assay is useful for evaluating the protective effects of IGF-I under conditions where the ability of cells to interact with extracellular matrix is restricted (Valentinis *et al.,* 1998). Under these conditions parental P19 cells exhibited a modest ability to proliferate in response to IGF-I (Fig. 3B). As expected, P19/IGF-IR cell proliferation was noticeably improved in anchorage-independence, and the cells in which the endogenous IGF-IR level was reduced due to the expression of antisense IGF-IR cDNA lost the ability to respond with cell proliferation following the IGF-I stimulation. The cells, which were utilized in the above experiments, were further analyzed for the protective effects of IGF-I. As mentioned above, differentiated P19 cells lose neuronal processes after the treatment with HIV/CM (Fig. 1), and the addition of IGF-I substantially protected their neuron-like phenotype (Fig. 2B). As illustrated in Fig. 3C, sensitivity of the cells to HIV/CM is greatly increased upon the down-regulation of the endogenous IGF-IR with the antisense cDNA (P19/asIGF-IR cells). In contrast, P19 cells which ectopically overexpress IGF-IR become resistant to the deleterious effects of the HIV/CM upon the treatment with IGF-I (50 ng/ml). Altogether, these observations strongly support the hypothesis that activated IGF-I system confers protection against neurotoxins present in the conditioned medium of HIV-infected macrophages.

Effects of the HIV/CM on immediate signaling responses from the IGF-IR

To evaluate whether the IGF-IR signaling pathways are affected by HIV/CM, we investigated phosphorylation levels for three signaling molecules (Akt, ERKs, GSK-3β) that are known to be activated by IGF-I stimulation and, further, suggested to play a role in neuronal differentiation and neuronal survival (Dudek *et al.,* 1997; Yuan and Yankner, 2000; Zheng *et al.,* 2000). In these studies, IGF-I stimulation was applied to retinoic acid differentiated P19 cells. Results in Fig. 4 demonstrate that in serum-free medium (SFM), P19 cells exhibit basal levels of phosphorylation of GSK-3p, Akt, and Erk-2. Under the same culture conditions, Erk-1 phosphorylation was not detected. Within 15 min following IGF-I stimulation (IGF-I), both phospho-GSK-3β and phospho-Akt were significantly increased; phospho-Erk-2 was not affected and phospho-Erk-1 remained undetectable. Having established basal and induced levels of phosphorylation in response to IGF-I in differentiated P19 cells, we examined whether pretreatment of the cells with the HIV/CM affects the phosphorylation levels of these signaling molecules. First of all HIV/CM by itself did not affect basal levels of the phosphorylation (CM/24 h). Preincubation of P19 cells with the HIV/CM did not affect IGF-I-mediated phosphorylation of GSK-3β and Akt at both 24 and 48 h time intervals. The only detectable change induced by HIV/CM was the enhancement of Erk-2 phosphorylation after the IGF-I treatment. Altogether, these results indicate that HIV/CM is not affecting both basal and IGF-I-stimulated levels of Akt, ERKs, and GSK-3β phosphorylation in differentiated 19 cells.

The $I_\kappa B$ protein binds to and prevents nuclear translocation of the transcription factor Rel/$NF_\kappa B$ (Baeuerle and Baltimore, 1988). The mechanism by which TNFR1 signaling activates $NF_\kappa B$ involves $I_\kappa B$ phosphorylation by IKKs, and subsequent proteosomal degradation of $I_\kappa B$ (Zandi *et al.,* 1998). Figure 5 shows that serum-starved, differentiated P19 cells have detectable levels of phosphorylated $I_\kappa B$. Following TNFα stimulation, levels of phosphorylated $I_\kappa B$ increase and remain elevated for at least 3 h. The same blot probed with antitotal $I_\kappa B$ antibody confirmed lack of expected down-regulation of $I_\kappa B$ at later time points following TNFα stimulation. When P19 cells were pretreated for 24 h with IGF-I (50 ng/ml),

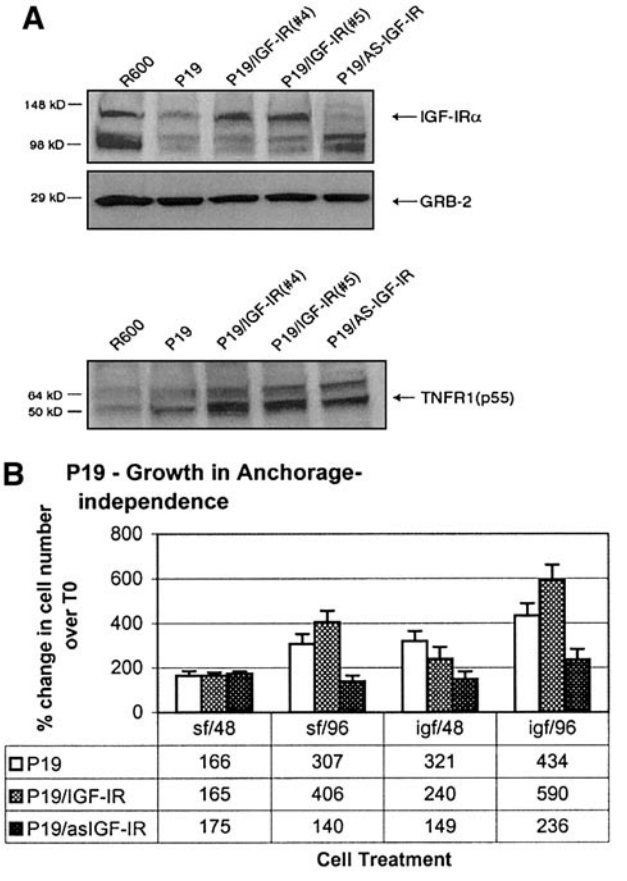


FIG. 3. IGF-IR-dependent protection of differentiated neurons from the HIV/CM. (A) Western blot analysis showing IGF-IR and TNFRI (p55) protein levels in R600 fibroblasts that express 3×10^4 IGF-IR molecules per cell; in parental P19 cells; and in P19 cells stably transduced with MSCV based retroviral vector expressing either wt human IGF-IR cDNA (P19/IGF-IR), or antisense IGF-IR (asIGF-IR). Proteins were isolated from transduced cell populations indicated above and 50 μg of each protein sample was applied to SDS–PAGE gels for Western blot analysis. After the transfer into nitrocellulose membranes, blots were probed with anti-IGF-IRα antibody (N-20; Santa Cruz) and with anti-TNFR1 antibody (H-271; Santa Cruz). To control equal loading conditions, the blots were stripped and reprobed with the antibody against GRB-2, as previously described (Peruzzi *et al.,* 1999). (B) Evaluation of the growth responses to IGF-I in the condition of anchorage-independence. Cell lines indicated above were detached from monolayer cultures and plated on PolyHema-coated dishes to prevent attachment. The suspension cultures were left without the treatment (SFM) or were stimulated with IGF-I (50 ng/ml). The cell number was evaluated before the treatment (TO) and at 48 and 96 h following the IGF-I stimulation. Data represent an average from three experiments. (C) Parental P19, P19 expressing IGF-IR cDNA (P19/IGF-IR), and P19 cells expressing antisense to IGF-IR (P19/asIGF-IR) were differentiated with retinoic acid and subsequently treated with the HIV/CM (dilution 1:5) in the presence of IGF-I (50 ng/ml). The percentage change in the number of neuronal processes was evaluated at 24 and 72 h following the treatment. The results represent percentage change in the average length of neuronal processes per field over TO (measurement taken before the treatment). This is an average of two experiments consisting of two 35-mm plates per point, from which measurements were taken in three separate microscopic fields ($n = 12$).

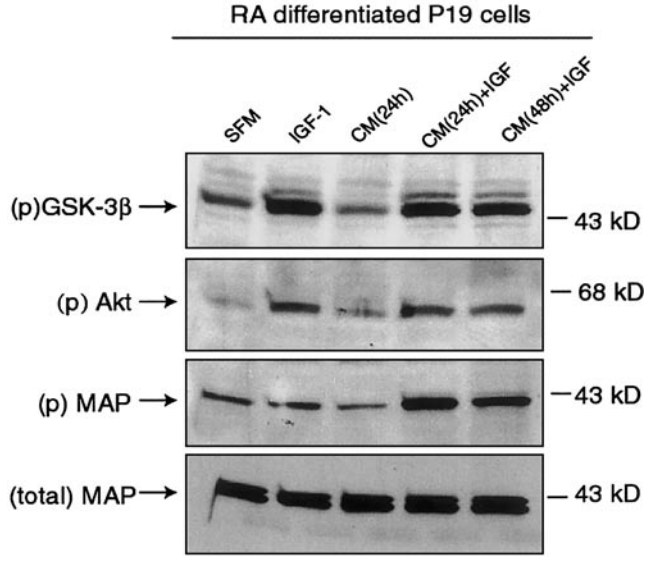


FIG. 4. Effects of the HIV/CM on immediate signaling responses from the activated IGF-IR. Western blots were processed with antibodies that recognize phosphorylated forms of GSK-3β, Akt, and MAP kinases (Erk-1 and Erk-2). All primary antibodies were obtained from Cell Signaling Technology, New England Labs. Retinoic acid (RA) differentiated P19 cells were starved in serum-free medium for 24 h and were subsequently incubated with HIV/CM (dilution 1:5) for 24 and 48 h. IGF-I stimulation (50 ng/ml) was executed in untreated cultures (IGF-I), or in cultures pretreated with the conditioned medium for 24 or 48 h (HIV/CM/24 h + IGF) or (HIV/CM/48 h + IGF). Cells which were not stimulated (SFM) or cells treated exclusively with the conditioned medium (HIV/CM/24 h) were used as reference samples. Protein extracts were collected within 15 min following IGF-I stimulation.

subsequent TNFα stimulation resulted in enhanced $I_\kappa B$ phosphorylation at 5 and 10 min after the treatment, and again total $I_\kappa B$ was not down-regulated. These results suggest that IGF-I cooperates with TNFα in respect to $I_\kappa B$ phosphorylation and indicate that the mechanism of $I_\kappa B$ degradation is impaired in differentiated P19 neurons.

DISCUSSION

HIV infection often results in neurological disorders of the central nervous system in a substantial number of AIDS patients (Gabuzda and Hirsch, 1987). The preva-

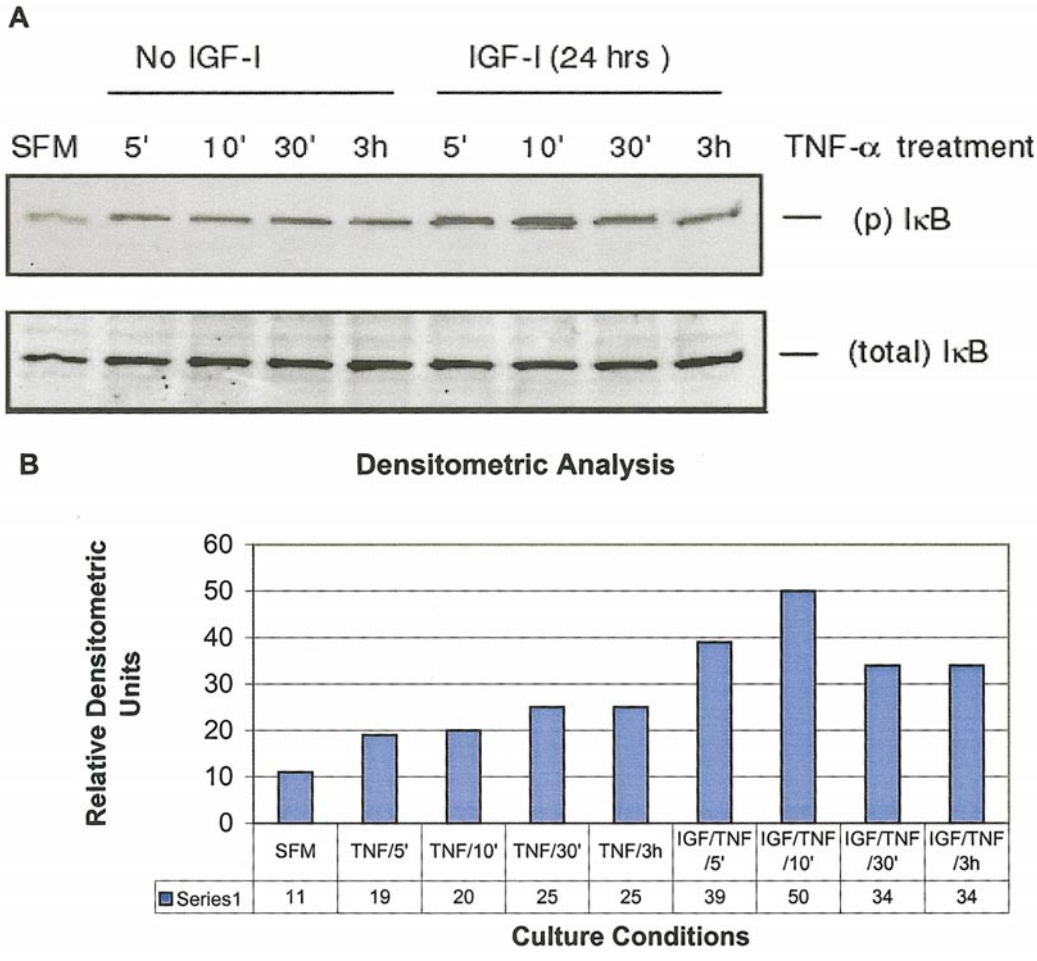


FIG. 5. Effects of TNFα and IGF-I on $I_{\kappa}B$ phosphorylation. (A) Western blots processed with antibodies that recognize phosphorylated (p) or total (total) $I_{\kappa}B$ (Cell Signaling Technology, New England Labs). Retinoic acid (RA) differentiated P19 cells were starved in serum-free medium for 24 h (SFM) and were subsequently incubated with IGF-I (50 ng/ml) for 24 h (IGF-I 24 h), or were left without treatment (No IGF-I). Subsequently cells were stimulated with TNFα (20 ng/ml) and total proteins were isolated at indicated time points. (B) Densitometric evaluation of the blot depicted in A.

lence of HIV associated dementia (HAD) has been estimated in the early 1990s to be as high as 20–30% of those patients with advanced HIV disease. It has been speculated that HAD remains the most common cause of dementia worldwide among people at ages 40 or less and is considered a significant independent risk factor for death due to AIDS (Glass and Johnson, 1996; Price, 1988; Sharer, 1992). The pathogenesis of HAD is believed to involve the release of toxic substances in the brain by activated and/or infected mononuclear phagocytes, leading to neuronal and astrocytic dysfunction and death (Price, 1988; Price *et al.,* 1988; Thompson *et al.,* 2001). The proinflammatory cytokine, TNFα, released by activated and/or HIV-1-infected brain macrophages and microglia, appears to provide a substantial contribution to neuronal cell damage (Rieckmann *et al.,* 1991; Rimaniol *et al.,* 1997). In addition to factors promoting neuronal injury, a decrease in levels/activity of neuronal protective mechanisms may also contribute to the overall injury process. In support of this notion, several studies suggest that IGF-I-mediated responses are impaired during the course of HIV infection (Jain *et al.,* 1998; Laue *et al.,* 1990), leaving neuronal cells with attenuated defense mechanism against proapoptotic cytokines and toxic metabolites. Reduced levels of serum IGF-I have been observed in HIV-infected patients, particularly those with wasting syndrome and in children with failure to thrive (Jain *et al.,* 1998; Laue *et al.,* 1990). As IGF-I is a principal mediator of the action of human growth hormone, its role in anabolic effects has prompted studies of IGF-I levels in HIV-infected patients and the use of both IGF-I and growth hormone in the treatment of cachectic patients (Frost *et al.,* 1996; Lo *et al.,* 2001; Mynarcik *et al.,* 1999, 2000). Although some improvements in body mass have been noted, these studies also suggest partial resistance to the effects of growth hormone and/or IGF-I in the setting of HIV-wasting syndrome (Jain *et al.,* 1998). Decreased levels of IGF-I in the central nervous system may promote neuronal apoptosis in HIV infection, or alternatively, by mechanisms which contribute to IGF-I resistance. The resistance may occur at the level of the receptor (low expression of the IGF-IR or its mutation), or

by a specific attenuation of the critical signaling factors such as Akt. Some of these effects may be due to the action of TNFα, which has been demonstrated to inhibit some of the known activities of the IGF-IR and insulin receptor (IR) in diabetes (Hotamisligil, 1999), and was shown to inhibit IGF-I-stimulated protein synthesis in myoblasts (Jain *et al.,* 1998). These TNFα-mediated effects have been suggested to involve serine phosphorylation and partial inactivation of two major signaling molecules of the IGF system, IRS-I and IRS-2 (Peraldi *et al.,* 1996; Venters *et al.,* 1999).

In the experimental setting proposed here, cellular responses to HIV/CM have been initially tested in exponentially growing monolayer cultures of P19 and PC 12 cells. Surprisingly, we observed no significant changes in the rate of cell proliferation and cell death, suggesting that the undifferentiated neuronal cell lines are resistant to the secreted neurotoxic factors from the HIV/CM. In contrast to the relative resistance of undifferentiated cells, differentiated neuronal cultures were highly sensitive to the injuries inflicted by the HIV/CM. We observed a greater proportional decrease in the number of neurite-like processes compared to the modest decrease in the cell number. This may imply that cells which lost neuronal processes did not undergo apoptosis or that cell proliferation occurred despite of the treatment. To avoid potentially complex issues related to the presence or absence of apoptosis versus proliferation in neuronal cultures, we expressed our results in terms of the number of neurite-bearing cells (PC 12 cells), or by the length of neurite-like processes per microscopic field (p19 cells). Results presented in this article demonstrate that differentiated neurons are not capable of maintaining neuronal processes when exposed to the conditioned medium from HIV-I-infected macrophages (HIV/CM). Further, it is evident that TNFα-activating TNFR1 (p55) participates in the process of HIV/CM-induced neuronal degeneration. This observation is in agreement with previous reports showing neuronal death, evaluated by LDH release, only in differentiated cultures of NT2N neurons treated with TNFα (Westmoreland *et al.,* 1996).

Since the IGF-IR efficiently rescues differentiated neurons from both HIV/CM and TNFα-induced damage of neuronal processes (Figs. 2 and 3), it is feasible that the mechanism of its neuroprotective action involves, at least partially, redirection of the signal from the TNFR1. Depending on the circumstances, which are not well characterized, TNFR1 may send contradictory signals in respect to cell survival: proapoptotic FADD-mediated recruitment of procaspase 8 or antiapoptotic Traf-2-mediated phosphorylation of $I_\kappa B$ (Natoli *et al.,* 1998). If the IGF-IR affects TNFR1 signaling on this level, the connection to procaspase 8 should be attenuated and the activation of $NF_\kappa B$ should be enhanced. In this respect our data demonstrate a strong TNFα-mediated phosphorylation of $I_\kappa B$ in P19 cells preconditioned by the IGF-I (Fig. 5). The IGF-IR could also switch TNFR1 into antiapoptotic mode by facilitating FLIP activity and subsequent inhibition of caspase 8. Although this possibility has never been shown for IGF-IR directly, signaling molecules that are known to be activated by the IGF-IR could do so. This includes Akt (Panka *et al.,* 2001) and/or Raf activation (Kataoka *et al.,* 2000). In addition, $NF_\kappa B$ has been also shown to facilitate FLIP expression (Micheau *et al.,* 2001; You *et al.,* 2001), suggesting another possible level of the synergy between IGF-IR and TNFR1. Importantly, the major signaling responses from the IGF-IR, including phosphorylation of Akt, MAP kinases, and Gsk-3β, are not attenuated in the presence of HIV/CM. Therefore, neuroprotective cross-talk between IGF-I and TNFα may include, in addition to the sustained Akt, ERK2, and GSK3β phosphorylation, TNFR1-mediated activation of $NF_\kappa B$ in this HIV-related experimental setting. This also indicates that molecular manipulations to increase effectiveness of the IGF-IR system may represent a potential therapeutic approach to rescue differentiated neurons in HIV-associated dementia.

MATERIALS AND METHODS

Cell lines

Cell-culture conditions for growth and differentiation of P19 mouse teratocarcinoma (ATCC No. CRL-1825), and PC12 rat pheochromocytoma (ATCC No. CRL-1721), were previously reported (Jones-Villeneuve *et al.,* 1982; Teng *et al.,* 1993). To induce neuronal differentiation, PC12 cells plated on poly-D-lysine culture dishes were treated in serum-free medium with NGF (20 ng/ml). Neuronal processes began to appear within the first 24 h following the treatment and could be observed in culture for at least 10 days. The P19 neuronal differentiation was induced by retinoic acid (RA) (500 nM; Sigma). The response to RA treatment required cell aggregation that was spontaneously induced when the cells were forced to grow in suspension. The aggregates were collected, resuspended in fresh culture medium (α-MEM + 7.5% CS + 2.5% FBS), and treated with the RA for an additional 48 h. Subsequently, large cellular aggregates were dissociated by trypsinization, and single cells and small cellular aggregates were plated on poly-D-lysine-coated culture dishes. Under these conditions, neuronal differentiation was observed within 48 h after the final plating. Long neuronal processes were maintained in culture for 2 weeks with the medium changed every second day. To avoid overgrowth with nondifferentiated cells, RA-treated cells were cultured either in serum-free medium (α-MEM + 0.1% BSA) or in serum-supplemented medium containing Ara-C (1 μM) (cytosine β-D-arabino-furanoside, Sigma). R600 fibroblasts, which express 3×10^4 of IGF-IR molecules/cell (Reiss *et al.,* 1998), were used as a reference sample to monitor the level of IGF-IR expres-

sion in parental P19 cells and in P19 cultures expressing IGF-IR cDNA in either sense or antisense orientation.

Preparation of conditioned media from HIV-I-infected T-lymphocytes

Suspension cultures of supT1 cells were infected with HIV-1 strain SF162 (or JRFL) with a multiplicity of infection (m.o.i.) of 0.1. Briefly, the cells were incubated with the virus inoculum for 2 h at 37°C in serum-free RPMI 1640. At the end of incubation period, the cells were centrifuged at 1000 rpm for 5 min at 4°C. The cell pellet was suspended in RPMI containing 2% fetal bovine serum, gentamicin (5 μg/ml), and incubated at 37°C. Four-fifths of the cell supernatant was harvested as conditioned media on alternate days up to day 24 of infection and stored in 10 ml aliquots at −80°C. For generation of virus-free conditioned media, one aliquot of the stored supernatant was thawed and then centrifuged at 3100 rpm at 4°C for 45 min in a centrifugal filter device (Biomax 10K NMWL membrane, Millipore Corp., MA). The filtrate was used as virus-free (evaluated by p24 assay) conditioned medium.

Growth in anchorage-independence

To determine this parameter, quiescent cells were detached from a culture dish with 0.02% disodium ethylenediamine tetraacetate (EDTA) and seeded on dishes coated with poly(2-hydroxyethyle methacrylate) [poly-(HEMA)] (Aldrich, Milwaukee, WI), prepared according to the methodology previously described (Reiss *et al.,* 1998). Cells were seeded in SFM at 1×10^4 cells/cm^2 and were treated either with 10% FBS or 50 ng/ml IGF-I or were left untreated. Twenty-four hours later cell suspensions were collected and dissociated with 0.25% trypsin, and cells were counted in a Brightline Hemocytometer. Results represent an average of three experiments and are expressed as a percentage change in cell number.

Detection of DNA strand breaks

DNA strand breaks were labeled with biotinylated dUTP (Boehringer Mannheim), using exogenous terminal deoxynucleotidyl transferase (TDT; Boehringer Mannheim). Cells were fixed on ice with a buffered 10% methanol-free formaldehyde (Polyscience, Inc.) for 20 min, incubated with 70% ethanol for 1 h at −20°C, and washed 3× with PBS. DNA strand breaks were detected by *in situ* labeling of free 3′-OH ends on DNA. Fixed cells were incubated with 50 μl of a reaction mixture (5 units TDT, 2.5 mM $CoCl_2$, 0.2 M potassium cacodylate, 25 mM Tris–HCl, 0.25% bovine serum albumin, and 0.5 nM biotin-16-dUTP) for 30 min at 37°C and incubated with staining solution [5 μg/ml of fluorescein isothiocyanate extravidin (FITC), 4× SSC, 0.1% Triton X-100, and 5% nonfat dry milk] for 30 min at 37°C. DNA strand breaks were visualized under a fluorescent microscope equipped with a set of excitation-emission filters for fluorescein (excitation blue, emission green).

Retroviral transduction

The MSCV.pac and MSCV.neoEB retroviral vectors, kindly provided by Dr. Hawley (University of Toronto, Canada), are described elsewhere (Hawley *et al.,* 1994). The self-inactivating (SIN) version of the MSCV.pac was engineered by deleting 299 bp in the 3′ long terminal repeat (LTR), as described elsewhere (Yu *et al.,* 1986). A general cloning strategy was based on PCR-amplified cDNA fragments of the wild-type IGF-IR cDNAs in the sense and antisense orientation. The PCR primers containing appropriate restriction sites in overhangs were previously described (Romano *et al.,* 1999; Valentinis *et al.,* 2000). Prior to cloning, each amplified fragment was verified by sequencing (Nucleic Acid Facility at Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA).

Western blot analysis

To determine levels of phosphorylated forms of IGF-I-induced signaling molecules, differentiated cultures of P19 cells were lysed on ice with 400 μl of lysis buffer [(50 mM HEPES); pH 7.5; 150 mM NaCl; 1.5 mM $MgCl_2$; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride (PMSF); 0.2 mM Na-orthovanadate, and 10 μg/ml aprotinin]. Protein concentration was determined by a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), and 50 μg of total proteins were separated on a 4–15% gradient SDS–PAGE (Bio-Rad) and transferred onto nitrocellulose membranes. Blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) and were probed with the appropriate primary antibodies. Total and phosphorylated forms of Akt/PKB, Erk1/Erk2, GSK-3β, and IκB were detected by utilizing PhosphoPlus antibody kits obtained from New England BioLabs, Beverly, MA.

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