MOLECULAR AND CELLULAR BIOLOGY, May 1995, p. 2359–2366 Vol. 15, No. 5
0270-7306/95/$04.00+0

Tumor Necrosis Factor Alpha-Induced Apoptosis in Human Neuronal Cells: Protection by the Antioxidant *N*-Acetylcysteine and the Genes *bcl-2* and *crmA*

ANGELA K. TALLEY,[1] STEPHEN DEWHURST,[2,3] SETH W. PERRY,[1] SHEILA C. DOLLARD,[2] SURYARAM GUMMULURU,[3] STEVEN M. FINE,[2,4] DEBORAH NEW,[5] LEON G. EPSTEIN,[1,2,6] HOWARD E. GENDELMAN,[7,8] AND HARRIS A. GELBARD[1,6]*

Departments of Neurology (Child Neurology Division),[1] Microbiology and Immunology,[2] Medicine (Division of Infectious Diseases),[4] Neurobiology and Anatomy,[5] and Pediatrics[6] and Cancer Center,[3] University of Rochester Medical Center, Rochester, New York 14642, and Departments of Medicine[7] and Pathology and Microbiology,[8] The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198

Received 23 November 1994/Returned for modification 23 December 1994/Accepted 26 January 1995

Tumor necrosis factor alpha (TNF-α) is a candidate human immunodeficiency virus type 1-induced neurotoxin that contributes to the pathogenesis of AIDS dementia complex. We report here on the effects of exogenous TNF-α on SK-N-MC human neuroblastoma cells differentiated to a neuronal phenotype with retinoic acid. TNF-α caused a dose-dependent loss of viability and a corresponding increase in apoptosis in differentiated SK-N-MC cells but not in undifferentiated cultures. Importantly, intracellular signalling via TNF receptors, as measured by activation of the transcription factor NF-κB, was unaltered by retinoic acid treatment. Finally, overexpression of *bcl-2* or *crmA* conferred resistance to apoptosis mediated by TNF-α, as did the addition of the antioxidant *N*-acetylcysteine. These results suggest that TNF-α induces apoptosis in neuronal cells by a pathway that involves formation of reactive oxygen intermediates and which can be blocked by specific genetic interventions.

Until recently it was unknown whether neurons die by apoptosis or necrosis during human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system. Studies from our laboratory using an in situ technique demonstrated that apoptotic neurons were infrequently observed in cerebral cortex and basal ganglia of pediatric patients that had HIV-1 infection but no evidence of encephalitis or neurologic dysfunction. In contrast, patients who had HIV-1 encephalitis and neurologic dysfunction had up to 30% of neurons with apoptotic nuclei in proximity to perivascular infiltrates of HIV-1-infected macrophages and reactive microglia in cerebral cortex and basal ganglia (14). Because only latent infection of neurons by HIV-1 has been demonstrated in a single postmortem case (40), these findings suggest that HIV-1-induced diffusible factors may mediate neuronal apoptosis in HIV-1 encephalitis.

A plausible explanation for how HIV-1 causes neuronal dysfunction and cell loss when only a small number of brain microglia and macrophages support productive viral infection is production of soluble neurotoxic factors from HIV-1-infected macrophages and microglia (16, 18, 19). Neuronal death can occur after exposure of neurons to supernatants from HIV-1-infected monocytes or monocytoid cell lines or during cell-to-cell contact of virus-infected monocytes and astroglioma cells (16, 18, 19). The most widely studied gene product of HIV-1 is the envelope protein gp120. In picomolar amounts, in the presence of sublethal concentrations of glutamate, it has been shown to be neurotoxic to rat retinal ganglion cells in vitro (8, 29). However, gp120-induced neurotoxicity disappeared when neuronal cultures were depleted of macrophages (27, 28). Giulian et al. (20) have shown that gp120 can activate purified cultures of rat microglia to produce a low-molecular-weight neurotoxin that activates *N*-methyl-D-aspartate receptors and results in neuronal loss. These findings suggest that gp120 may not be directly toxic to neurons but rather may activate either HIV-1-infected or uninfected macrophages or microglia in the brain to kill neurons through an excitotoxic mechanism. Lethal excitotoxic damage to neurons from overstimulation of *N*-methyl-D-aspartate receptors by the neurotransmitter glutamate has been implicated in the neuropathogenesis of AIDS (9, 29). In vitro models suggest that chronic exposure to glutamate can result in neuronal apoptosis (41, 42).

Indeed, gp120 is not the only neurotoxic factor produced by HIV-1 infection of macrophages or microglia. Genis et al. (18) demonstrated that HIV-1-infected macrophages could be activated by coculture with astrocytoma cells to produce neurotoxic factors. Conditioned medium from such macrophages has been shown to contain high levels of the cytokine tumor necrosis factor alpha (TNF-α), as well as platelet-activating factor (PAF) and various arachidonic acid metabolites. PAF is in fact a regulator of TNF-α biosynthesis (49), and both PAF and TNF-α have been shown to be neurotoxic to cultured human fetal neurons at physiologically relevant concentrations (13, 16). Importantly, mRNA for TNF-α is present at higher levels in the brains of patients with HIV-1 associated dementia than in brains of patients with HIV-1 infection but without dementia (50). These studies strongly suggest that TNF-α may play a major role in the pathogenesis of HIV-1-associated dementia.

TNF-α has been well characterized as a mediator of oxidative stress. TNF-α stimulates the production of reactive oxidative intermediates (ROIs) in T cells (26, 33). TNF-α-mediated

* Corresponding author. Mailing address: Division of Pediatric Neurology, Box 631, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Phone: (716) 275-4784. Fax: (716) 275-3683.

oxidative stress, in turn, produces apoptosis in nonneural cells (5, 32). This has been proposed as a mechanism for depletion of $CD4^+$ T cells in AIDS (1, 21). Because TNF-α acts in a neurotoxic fashion in primary human neuronal cultures and apoptotic neurons have been identified in the brains of patients with HIV-1 encephalitis and dementia, we sought to determine whether TNF-α could induce apoptosis in neuronal cells. In this report, we describe the ability of TNF-α to induce apoptosis and loss of viability in a dose-dependent fashion in the human neuroblastoma cell line SK-N-MC if cells were first differentiated to a neuronal phenotype by using retinoic acid (RA); undifferentiated SK-N-MC cells were not killed by TNF-α. We further demonstrate that ROIs are required for TNF-α-mediated neuronal apoptosis, since cell death can be blocked by the antioxidant *N*-acetylcysteine (NAC). Finally, we show that overproduction of specific antiapoptotic gene products (encoded by *bcl-2* and *crmA*) can protect neuronal cells from the TNF-α-mediated cytotoxicity.

MATERIALS AND METHODS

Cell culture. Human neuroblastoma SK-N-MC cells were obtained from the American Tissue Culture Collection (3) and cultured in plastic tissue culture dishes (Corning) without any additional substrates. Cells were grown in a humidified incubator at 37°C with a 5% CO_2–95% air atmosphere. Cells were fed every 2 days with complete minimal essential medium and differentiated to a neuronal phenotype, as necessary, by addition of 5 μM RA (Sigma, St. Louis, Mo.) for 4 to 5 days (this results in morphologic changes, which include the development of neuritic processes, as well as the expression of neuronal markers).

SK-N-MC subclones. To establish SK-N-MC cell lines that overexpress *crmA* and *bcl-2*, pJ415 and pJ436 (gift of J. Yuan) (35) were transfected into the amphotropic retroviral packaging cell line PA317 (American Type Culture Collection) (34) by using the Lipofectamine reagent (Gibco/BRL); the control vector pBabePuro (gift of H. Land) (36) was likewise transfected into PA317 cells. Supernatant from transiently transfected PA317 cells was used to infect SK-N-MC cells overnight, and resistant cells were then selected in 5 μg of puromycin per ml for approximately 20 days. Resistant colonies were cloned and checked for expression levels by using Western blots (immunoblots). In gene-modified populations of SK-N-MC cells (*bcl-2* or *crmA*), cells stopped growing within 1 day of RA treatment. Neuronal morphology was also observed with RA treatment.

HIV-1 infection of monocytes and preparation of conditioned media. Monocytes were recovered from peripheral blood mononuclear cells of HIV- and hepatitis B-seronegative donors after leukapheresis and purified by countercurrent centrifugal elutriation as previously described (18). Cells were cultured as adherent monolayers (10^6 cells per ml in 24-mm-diameter plastic culture wells) in Dulbecco modified Eagle medium (Sigma) with recombinant human macrophage colony-stimulating factor (Genetics Institute, Inc., Cambridge, Mass.) (17). The human brain astroglia tumor-derived cell line U251 MG was used in cocultivation assays with HIV-1-infected monocytes as described previously (18). These cells were grown as adherent monolayers in Dulbecco modified Eagle medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 50 μg of gentamicin per ml.

Monocytes were exposed to $HIV\text{-}1_{ADA}$ (GenBank accession number M60472) (38) at a multiplicity of infection of 0.01 infectious virion per target cell as previously described (17). The viral inocula were free of mycoplasma contamination (Mycoplasma Detection Kit III; Geneprobe, San Diego, Calif.). Macrophage colony-stimulating factor-treated monocytes were cultured as adherent monolayers 7 to 10 days prior to use as viral target cells. Under these conditions, 10 to 20% of the monocytes were infected 7 days after HIV-1 inoculation, as determined by immunofluorescence and in situ hybridization techniques (22). All cultures were refed with fresh medium every 2 to 3 days. Reverse transcriptase activity for HIV-1 was determined in culture fluids as previously described (22). Five to seven days after infection and during the peak of reverse transcriptase activity (10^7 cpm/ml) in HIV-1-infected monocytes, equal numbers of astroglial U251 MG cells were added, and the medium was analyzed for TNF-α by enzyme-linked immunosorbent assay (ELISA) as previously described (18).

Apoptosis assays and TUNEL immunostaining. The ApopTag peroxidase assay kit (ONCOR, Gaithersburg, Md.) was used to detect free 3′-OH ends of newly cleaved DNA in situ. In this method, called TUNEL (terminal deoxynucleotidyltransferase end labeling), digoxigenin-dUTP is added to 3′-OH ends of double- and single-stranded DNA by terminal deoxynucleotidyltransferase. An antidigoxigenin Fab antibody with conjugated peroxidase is then used to immunostain nuclei that have high concentrations of 3′-OH ends of cleaved DNA. Because the ApopTag antidigoxigenin antibody has no Fc portion and has less than 1% cross-reactivity with other endogenous steroids, nonspecific binding is very low.

Parallel sections from SK-N-MC cell cultures grown on poly-L-lysine-coated coverslips were counterstained with methyl green (0.5% [wt/vol] in 0.1 M sodium acetate [pH 4.0]) to label cells with intact nuclear DNA. All experiments with ApopTag reagent included a positive control (control neuronal SK-N-MC cells) that was incubated with endonuclease prior to assay (DNase I; 0.0002 U/μl; Promega, Madison, Wis.). This treatment nicks DNA in all cells and gives uniformly positive nuclear staining in all cell types present.

Digitized images of ApopTag-stained neuronal SK-N-MC cells in ≥15 microscopic fields were analyzed for numbers of positively stained nuclei per 50× field by using a densitometer (Imaging Research Inc., Ontario, Canada). Data were expressed as mean of positive nuclei ± standard error of the mean. Tests of statistical significance between control and experimental treatments were determined by paired *t* tests.

Acridine orange staining. Cell suspensions (10^6 cells per ml) of either vehicle (medium alone) or TNF-α (1 ng/ml [final concentration], 48 h) were mixed with acridine orange (100 μg/ml; Sigma) at a ratio of 1:25, placed on a microscope slide, coverslipped, and examined by fluorescence microscopy, using a 40× dry objective. Representative fields were photographed to examine morphologic changes indicative of apoptosis.

Gel electrophoresis of low-molecular-weight DNA. SK-N-MC cells treated with either vehicle (medium) or TNF-α (2 ng/ml [final concentration], 48 h) were harvested mechanically and lysed in 0.5 ml of 0.5% Triton X-100–5 mM Tris (pH 7.4)–5 mM EDTA at 4°C for 20 min. The suspension was then centrifuged at 27,000 × *g* for 15 min in an SW50.1 rotor (Beckman); the supernatant was extracted repeatedly with phenol-chloroform and precipitated in ethanol. The precipitates were resuspended in 10 mM Tris–1 mM EDTA, digested in 0.5 μg of boiled RNase A per ml for 1 h at 37°C, and submitted to electrophoresis through 1.8% agarose followed by staining with ethidium bromide.

Cytotoxicity assays. 3-(4,5-Dimethylthiazol-2 yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed by a modification of the method of Mosman (37). Briefly, viability of SK-N-MC cells grown in 96-well plates is determined by conversion of MTT (Sigma). Reduced MTT is solubilized with dimethyl sulfoxide as instructed by the manufacturer, and the color intensity is measured at 490 nm with an ELISA reader. In some experiments, cell viability was also assayed with SK-N-MC cell cultures containing a 0.4% solution of trypan blue, with blue (nonviable) cells and cells excluding trypan blue counted in a hemocytometer under phase-contrast microscopy.

Antibodies and immunochemistry. Cell surface levels of TNF receptor (CD120a or TR55) were assessed on SK-N-MC sublines by flow cytometry, using a murine anti-human TR55 monoclonal antibody (clone M50; Genzyme). Flow cytometric analysis was performed with a Becton Dickinson FACScan with Lysis II software or an EPICS PROFILE flow cytometer. Cellular expression of *bcl-2* was determined by using a rabbit polyclonal antiserum (Pharmingen); expression of *crmA* was detected with a rabbit polyclonal antiserum (gift of J. Yuan) by Western blotting procedures (ECL system; Amersham).

Analysis of NF-κB activation. SK-N-MC cells were grown on six-well plates and were transfected with plasmid DNAs [$p(kB)_3$conaLUC and pGDMAD3 (3, 38, 46)] by using the Lipofectamine reagent (Gibco/BRL). Briefly, 2 μg of DNA plus 8 μl of Lipofectamine, in medium, was added to each well. Cells were then incubated for 5 h at 37°C, after which the medium was aspirated and replaced with fresh medium containing either RA (5 μM) or dimethylsulfoxide alone (vehicle). After 60 h, cells were harvested for luciferase assays. Twenty-four hours prior to harvest (36 h after addition of RA), exogenous TNF-α (Genzyme) was added to selected cultures at a final concentration of 10 ng/ml. All transfections were performed in triplicate, and cell lysates were assayed for luciferase activity by using commercially available reagents (Promega) in an LKB series 1250 luminometer.

RESULTS

Initial experiments demonstrated that conditioned media from HIV-infected macrophages, activated by coculture with astroglial cells to produce levels of TNF-α that ranged between 2 and 6 ng/ml (17), were neurotoxic to cultures of SK-N-MC cells differentiated to a neuronal phenotype with 1.5 μM RA (Fig. 1). After a 48-h exposure to conditioned medium from HIV-1-infected macrophages cocultured with astroglial cells, SK-N-MC neuronal cultures had a 50% decrease in viability determined by the MTT assay relative to medium control cultures. In contrast, conditioned medium from uninfected macrophages (Fig. 1), uninfected macrophages cocultured with astroglial cells, or HIV-1-infected macrophages alone (data not shown) had no significant effect on SK-N-MC neuronal cell viability.

Because recombinant human TNF-α is neurotoxic to human fetal cortical neuronal cultures in a dose-dependent fashion

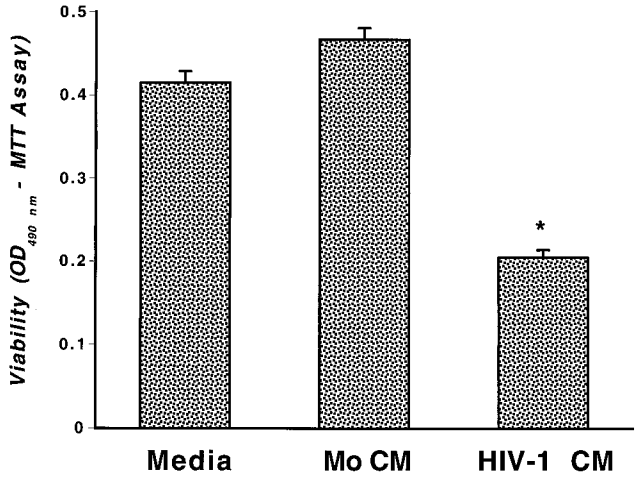

FIG. 1. Conditioned medium from HIV-1-infected monocytes, grown in the presence of astroglioma cells, is toxic to RA-treated SK-N-MC cells. Conditioned medium was prepared as described in Materials and Methods and applied for 48 h (1:10 [vol/vol]) to cultures of SK-N-MC cells grown to ~75% confluence prior to treatment with 5 μM RA. Conditioned medium from HIV-1-infected macrophages was obtained after 24 h of coculture of HIV-1-infected macrophages (7 days after HIV-1 infection) with U251 astroglial cells. After 48 h, SK-N-MC cells were observed for morphologic changes, then medium was removed, and cell viability of adherent SK-N-MC cells was determined by the MTT assay (eight replicates per condition). Values shown are means from a single representative experiment (this experiment was repeated two times, with similar results); the standard errors of mean values are marked by bars, and statistical significance (indicated by the asterisk) was taken at the $P < 0.01$ level. OD_{490} nm, optical density at 490 nm.

(13), we investigated whether TNF-α was toxic to undifferentiated (nonneuronal) or differentiated (neuronal) SK-N-MC cell cultures. Viability of undifferentiated SK-N-MC cells was unaffected after a 48-h exposure to doses of TNF-α of up to 10 ng/ml (Fig. 2). In contrast, neuronal SK-N-MC cultures had a dose-dependent decrease in viability.

Having established that TNF-α was toxic to neuronally differentiated SK-N-MC cells but not to control cells, we wished to determine the mechanism of TNF-α-mediated cell death. Previous studies have shown that TNF-α triggers apoptosis on nonneural cells (5, 32). We therefore examined whether exposure of neuronal SK-N-MC cells to TNF-α likewise resulted in apoptosis. TNF-α, at a dose of 2 ng/ml for 48 h, resulted in fragmentation of DNA into a 180- to 200-bp ladder resolved by gel electrophoresis (Fig. 3C). Exposure of neuronal SK-N-MC cells to vehicle did not cause any demonstrable DNA laddering (Fig. 3C). Exposure of neuronal SK-N-MC cells to 1 ng of TNF-α per ml for 24 h, followed by staining of trypsinized cells with the fluorescent DNA-binding dye acridine orange, revealed morphologic hallmarks of apoptosis, including chromatin condensation and membrane blebbing (Fig. 3B). In contrast, neuronal SK-N-MC cells exposed to vehicle for 24 h had evidence of intact chromatin structure, as demonstrated by acridine orange staining, without evidence of crescentic chromatin, spherical beading of chromatin, or membrane blebbing (Fig. 3A).

To determine the time course of apoptosis in neuronal SK-N-MC cell cultures exposed to 0.2 ng of TNF-α per ml, we used TUNEL staining of free 3′-OH ends of cellular DNA to identify cells in situ with large amounts of fragmented nuclear DNA (i.e., apoptotic nuclei). Using this method, we detected significant numbers of apoptotic nuclei in neuronal SK-N-MC cell cultures 8 h (but not 1 or 4 h) after application of 0.2 ng of TNF-α per ml which increased to a maximum by 48 h after exposure (data not shown). We therefore examined the relationship between neurotoxicity and number of apoptotic nuclei in neuronal SK-N-MC cell cultures exposed to increasing doses of TNF-α for 48 h. Figures 4A and B demonstrate an inverse proportional relationship between dose-dependent loss of viability measured by the MTT (Fig. 4A) assay and trypan blue exclusion (Fig. 4B) and increasing doses of TNF-α ranging from 0.05 to 100 ng/ml in neuronal SK-N-MC cells. Figure 4C demonstrates a direct proportional relationship between increasing number of apoptotic nuclei and increasing doses of TNF-α ranging from 0.05 to 25 ng/ml in neuronal SK-N-MC cells. Figure 5 shows neuronal SK-N-MC cells TUNEL stained to demonstrate fragmented nuclear DNA. Even at low doses of TNF-α (0.05 [Fig. 5B] and 0.2 [Fig. 5C] ng/ml), significant numbers of nuclei are TUNEL stained. Using the TUNEL method, we observed no labeling in neuronal SK-N-MC cultures exposed to vehicle for 48 h (Fig. 4A).

TNF-α has been shown to mediate intracellular signals at least in part via the generation of ROIs (45), which have themselves been shown to be neurotoxic (23). Thus, we examined whether the antioxidant NAC would prevent TNF-α-mediated cytolysis of neuronal SK-N-MC cells (Fig. 6). NAC at a dose of 0.5 mM completely blocked TNF-α-mediated cytotoxicity at doses up to 20 ng/ml when coapplied to neuronal SK-N-MC cell cultures, as measured by the MTT assay. These results were also confirmed when measured by trypan blue exclusion (data not shown).

We also wished to determine whether known inhibitors of apoptosis could likewise block TNF-α-mediated neurotoxicity. Perhaps the best studied antiapoptotic gene is *bcl-2*, which has been shown to prevent apoptosis in several neural cell lines in response to a wide variety of stimuli, including agents that promote formation of free radicals (23). Accordingly, we overexpressed *bcl-2* in SK-N-MC cells using a retroviral vector (35)

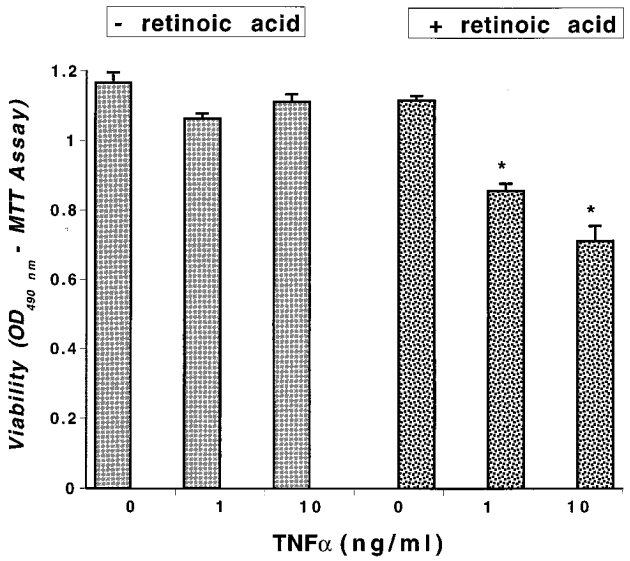

FIG. 2. Dose-dependent toxicity of TNF-α for neuronally differentiated SK-N-MC cells but not control cells. Cultures of SK-N-MC cells were grown in 96-well plates for 2 days and then treated with either medium alone or medium containing 5 μM RA for 4 days prior to treatment with increasing doses of TNF-α for 48 h. MTT assays were performed as described in Materials and Methods (eight replicates per datum point). Values shown are means values from a single representative experiment (this experiment was repeated two times, with similar results); the standard errors of mean values are marked by bars, and statistical significance (indicated by asterisks) was taken at the $P < 0.01$ level. OD_{490} nm, optical density at 490 nm.

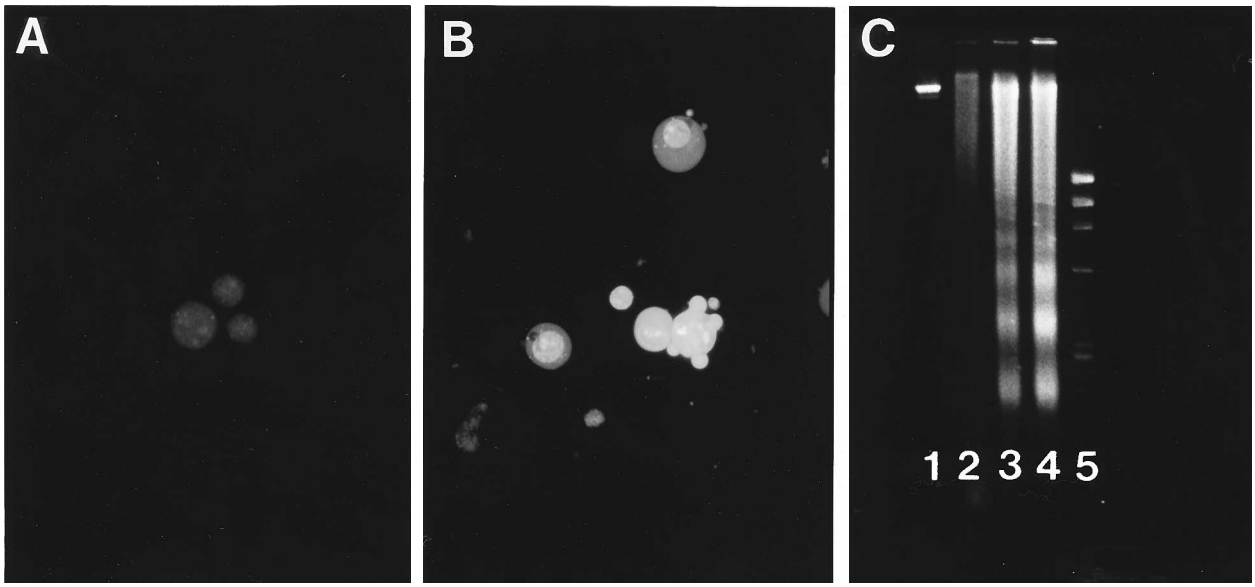

FIG. 3. TNF-α induces nuclear condensation in neuronally differentiated SK-N-MC cells. (A) SK-N-MC cells grown for 4 days in the presence of 5 μM RA, treated with medium containing RA for an additional 48 h, trypsinized, stained with acridine orange, and then examined by fluorescence microscopy using a 40× dry objective. (B) SK-N-MC cells grown for 4 days in the presence of 5 μM RA, treated with TNF-α (1 ng/ml) containing RA for an additional 48 h, trypsinized, stained with acridine orange, and then examined by fluorescence microscopy using a 40× dry objective. Note condensed chromatin and membrane blebbing, morphologic hallmarks of apoptosis. (C) Gel electrophoresis of low-molecular-weight DNA of SK-N-MC cells grown for 4 days in the presence of 5 μM RA and then treated with medium containing RA for an additional 48 h (lane 2), exposed to UV light for 20 min and then returned to the incubator for 6 h (lane 3, positive control for apoptosis), or treated with TNF-α (1 ng/ml) containing RA for an additional 48 h (lane 4). Marker lanes 1 and 5 are λ/*Hin*dIII and ϕX/*Hae*III, respectively.

and then measured TNF-α-mediated neurotoxicity. As anticipated, the cytolytic effects of TNF-α were completely abrogated in a bulk population of *bcl-2*-expressing neuronal SK-N-MC cells (Fig. 7B). We also derived a clonal subpopulation of SK-N-MC cells which expressed high levels of *crmA*, which is a cowpox virus gene product that has also been shown to prevent apoptosis in neural cells (11), presumably by suppressing the activity of interleukin 1β-converting enzyme (35). Again, TNF-α, at doses up to 12.5 ng/ml for 48 h, was not toxic to neuronal SK-N-MC cells that expressed high levels of *crmA* (Fig. 7B).

One explanation for the observation that TNF-α is toxic only to neuronal SK-N-MC cells, not to undifferentiated cells, is that the cellular differentiation triggered by RA is associated with an increase in TNF receptors or an alteration in receptor-mediated signalling pathways. To address these questions, we first determined by flow cytometry whether RA treatment led to elevated expression of the p55 TNF receptor, which is largely responsible for the cytotoxic properties of TNF-α (2, 47). No change was seen in the cell surface level of p55 receptors in the undifferentiated (non-RA-treated) versus the differentiated (neuronal; RA-treated) SK-N-MC cells (data not shown). We also determined whether RA treatment resulted in a perturbation of TNF signalling in SK-N-MC cells by measuring one of the downstream products of TNF receptor-mediated stimulation. Thus, we performed transient transfection analyses to quantitatively assess the activation of the cellular transcription factor NF-κB in TNF-α-treated SK-N-MC cells. To do this, SK-N-MC cells were transfected with an NF-κB responsive luciferase reporter plasmid [$p(kB)_3$conaLUC (38)] together with an equimolar amount of either pUC19 plasmid DNA or an expression construct which encodes a specific inhibitor of NF-κB, IκBα (pGDMAD3 [46]). As expected, TNF-α stimulation of SK-N-MC cells resulted in a greatly (17- to 18-fold) elevated level of luciferase expression from $p(kB)_3$conaLUC (Fig. 8). TNF-α-dependent enhancement of luciferase expression was equally pronounced in both RA-treated and non-RA-treated SK-N-MC cells (Fig. 8). Finally, this effect of TNF-α was shown to be due to activation of NF-κB since it was inhibited by coexpression of IκBα (Fig. 8). Thus, differentiation of SK-N-MC cells with RA does not change the relative extent of NF-κB activation in response to TNF-α. These results suggest that TNF-α-mediated neurotoxicity and apoptosis are due not to augmented signal transduction through TNF receptors but rather to an altered response of differentiated neuronal cells to such signalling compared with their undifferentiated counterparts.

DISCUSSION

The mechanism(s) by which neurons die in HIV-1 encephalitis remains unclear. Latent infection of neurons by HIV-1 has been reported in only one study (40) and thus remains an unlikely mechanism by which neurons die. However, many candidate neurotoxins produced by direct or indirect interactions between host cells have been studied. These neurotoxins, either by themselves or in combination with the neurotransmitter glutamate, have been shown to be neurotoxic to neurons in in vitro culture systems. These include the HIV-1 gene products gp120 (8, 29) and Tat (24, 25), leukotrienes and lipoxins (18), PAF (16, 18), and TNF-α (13). We have previously shown that TNF-α alone or in combination with an AMPA receptor agonist is neurotoxic to human fetal cortical neurons in primary cultures (13). In that report, electron microscopic studies of human fetal neurons exposed to low (0.2-ng/ml) doses of TNF-α were suggestive but not conclusive of some features of apoptosis (chromatin condensation). However, it has remained unclear whether TNF-α induces cell death by binding and activation of p55 or p75 receptors expressed on neurons or indirectly through receptors located on

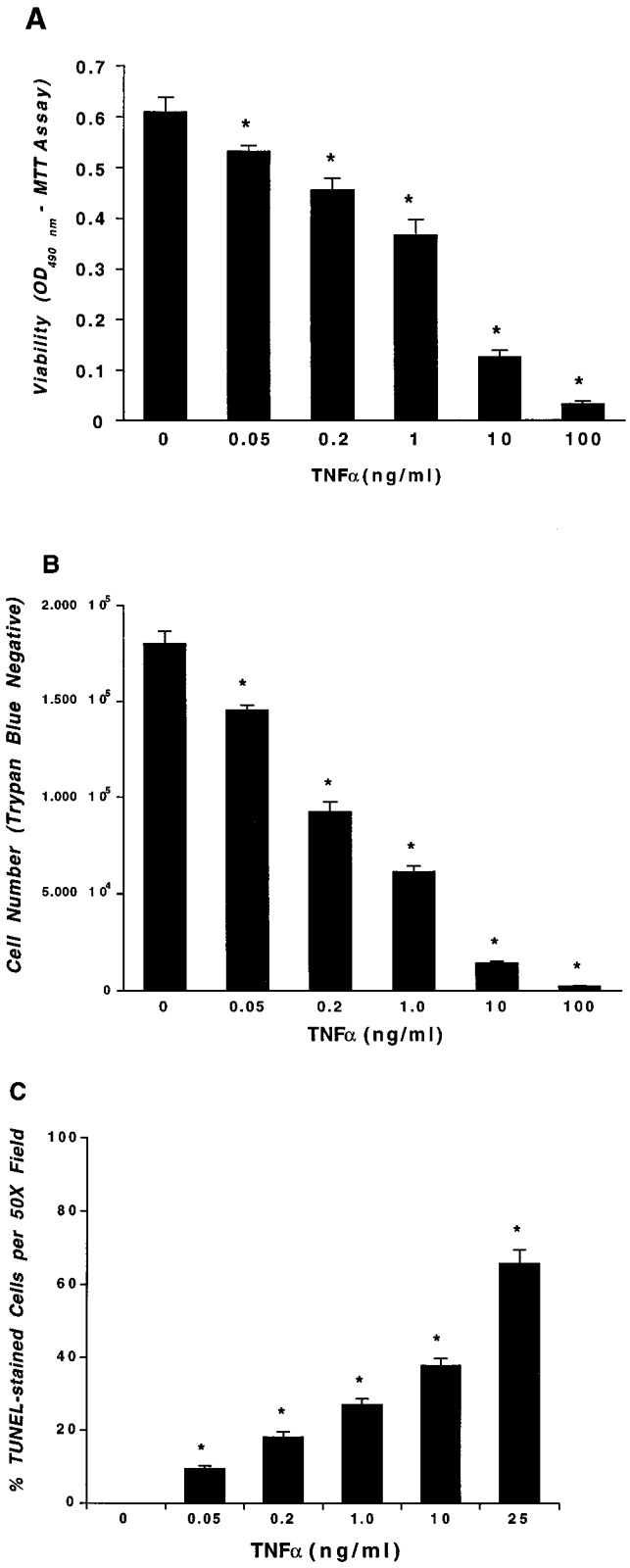

FIG. 4. TNF-α-mediated cytolysis in neuronally differentiated SK-N-MC cells is dose dependent and associated with an increase in apoptosis. (A) Dose-dependent decrease in viability, as measured by the MTT assay, for SK-N-MC cells grown for 4 days in the presence of 5 μM RA and then treated with TNF-α (0.05 to 100 ng/ml). (B) Dose-dependent decrease in viability, as measured by the trypan blue dye exclusion assay, for SK-N-MC cells grown for 4 days in the presence of 5 μM RA and then treated with TNF-α (0.05 to 100 ng/ml). (C) Dose-dependent increase in apoptotic nuclei, identified by TUNEL staining, for SK-N-MC cells grown for 4 days in the presence of 5 μM RA and then treated with TNF-α (0.05 to 25 ng/ml) for an additional 48 h. All assays were performed as described in Materials and Methods (eight replicates per datum point). Values shown are means from a single representative experiment (experiments were repeated two times, with similar results); the standard errors of mean values are marked by bars, and statistical significance (indicated by asterisks) was taken at the $P < 0.01$ level. OD_{490} nm, optical density at 490 nm.

glial cells or cells of macrophage lineage. Because TNF-α induces apoptosis in nonneural cells (5, 32), we investigated whether TNF-α induces apoptosis in human neuronal cells. The human neuroblastoma SK-N-MC cell line was chosen in part to determine whether TNF-α is directly neurotoxic to human neuronal cells, since neuronal SK-N-MC cells lack glutamate receptor subtypes (15).

Conditioned medium from HIV-1-infected macrophages activated by coculture with astroglial cells contains TNF-α at levels in the nanograms-per milliliter range (18) and is neurotoxic to differentiated (neuronal) SK-N-MC cells (Fig. 1). In contrast, conditioned medium from uninfected macrophages, uninfected macrophages cocultured with astroglial cells, or HIV-1-infected macrophages alone contains roughly 1,000 times less TNF-α (18) and is not neurotoxic to neuronal SK-N-MC cells (Fig. 1 and data not shown). Although HIV-1-infected macrophages, activated by coculture with astroglial cells, express other neurotoxic substances, including PAF, that may induce apoptosis in neuronal cells (data not shown), we focused on TNF-α's ability to induce apoptosis in neuronal cells for the following reasons: activated HIV-1-infected macrophage expression of TNF-α is correlated with neurotoxicity in primary neuronal cultures (13, 18), and levels of mRNA for TNF-α are correlated with dementia in patients with HIV-1 infection of the central nervous system (50).

Our data show that TNF-α is toxic to differentiated neuronal SK-N-MC cultures (Fig. 2) but not to control, undifferentiated, cells. We therefore investigated whether neuronal cell death was the result of apoptosis. Gel electrophoresis of low-molecular-weight DNA (Fig. 3C), acridine orange staining of DNA (Fig. 3A and B), and TUNEL staining of free 3′-OH ends of fragmented DNA (Fig. 5) confirmed by molecular and morphologic criteria that application of TNF-α in doses that ranged from physiologic (0.05 ng/ml) to pharmacologic (≤100 ng/ml) resulted in neuronal apoptosis.

The question remained whether TNF-α could trigger apoptosis in neuronal SK-N-MC cells through up-regulation of TNF-α receptors or alterations in TNF-mediated intracellular signalling. Clearly, only SK-N-MC cells differentiated to a neuronal phenotype were susceptible to TNF-α-mediated toxicity (Fig. 2). However, flow cytometry studies demonstrated that p55 TNF receptors were not up-regulated in neuronal SK-N-MC cells. Likewise, the relative magnitudes of TNF-α-mediated NF-κB activation in neuronal SK-N-MC cells and their undifferentiated counterparts were equivalent (Fig. 8). These results suggest that the neuronal SK-N-MC cell is susceptible to TNF-α-mediated programmed cell death because it is terminally differentiated, not because TNF-α-mediated signal transduction is augmented in neuronal cells.

In nonneural cells, application of TNF-α results in increased intracellular levels of ROIs (26, 33) which have been associated with oxidative stress-induced apoptosis. Since the thiol antioxidant NAC was able to prevent TNF-α-mediated neurotoxicity (Fig. 6), it is likely that increased formation, or decreased scavenging, of ROIs is important in initiating apoptosis in neuronal SK-N-MC cells exposed to TNF-α (5).

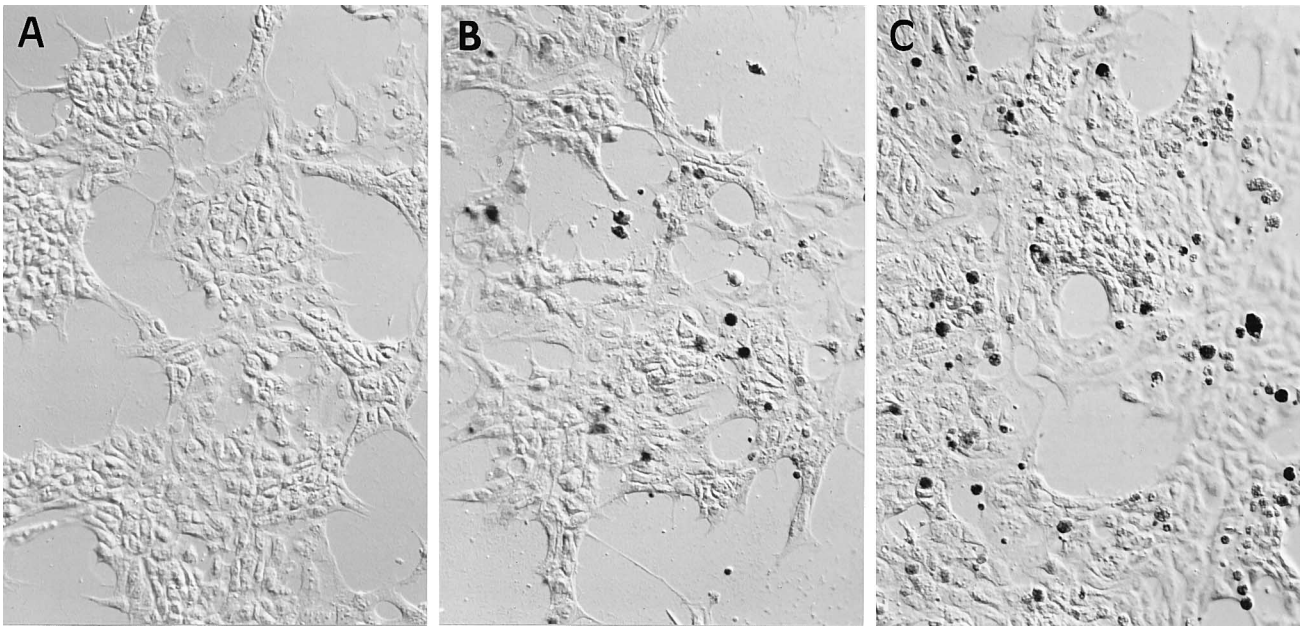

FIG. 5. TNF-α causes a dose-dependent increase in apoptosis in neuronally differentiated SK-N-MC cells. (A) TUNEL staining for SK-N-MC cells grown for 4 days in the presence of 5 μM RA and then treated with vehicle alone for an additional 48 h. (B) TUNEL staining for SK-N-MC cells exposed to TNF-α at a dose of 0.05 ng/ml for 48 h. (C) TUNEL staining for SK-N-MC cells exposed to TNF-α at a dose of 0.2 ng/ml for 48 h.

The finding that overexpression of *bcl-2* or *crmA* in neuronal SK-N-MC subclones was able to completely prevent TNF-α-mediated neurotoxicity (Fig. 7) confirms that TNF-α leads to neuronal cell death via an apoptotic pathway. It is noteworthy that *bcl-2* can also prevent necrotic cell death mediated by glutathione depletion in at least one neural cell line (23).

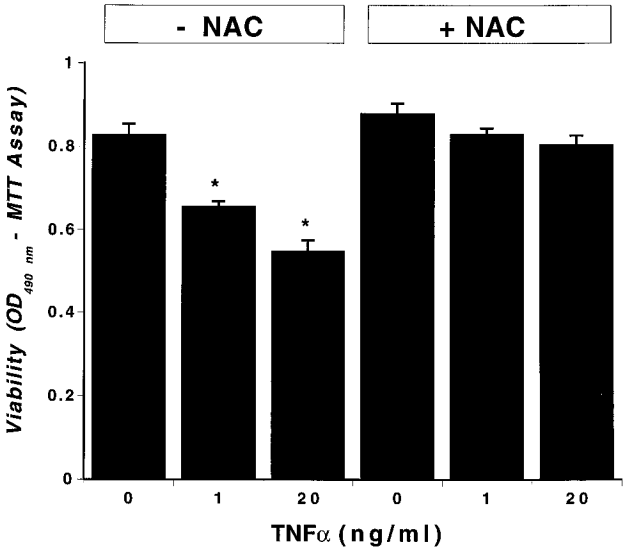

FIG. 6. TNF-α-mediated killing of neuronally differentiated SK-N-MC cells is inhibited by addition of antioxidant. MTT assays for cell viability of SK-N-MC cell cultures grown for 4 days in the presence of 5 μM RA and then treated with increasing doses of TNF-α in the presence or absence of 500 μM NAC for 48 h were performed as described in Materials and Methods (eight replicates per datum point). Values shown are means from a single representative experiment (this experiment was repeated twice, with similar results); the standard errors of mean values are marked by bars, and statistical significance (indicated by asterisks) was taken at the $P < 0.01$ level. OD_{490} nm, optical density at 490 nm.

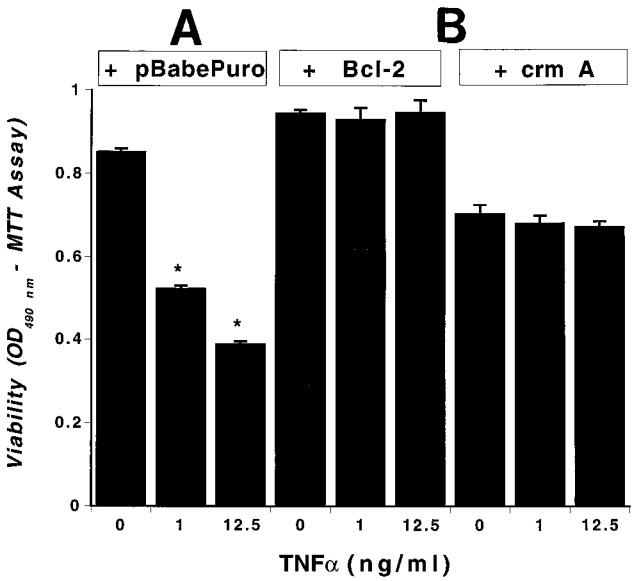

FIG. 7. Overexpression of *bcl-2* and *crmA* confers resistance to apoptosis mediated by TNF-α in neuronally differentiated SK-N-MC cells. (A) MTT assay for cell viability of a bulk population of puromycin-resistant SK-N-MC cells transduced with the control retroviral vector pBabePuro (see Materials and Methods). Cells were grown in the presence or absence of 5 μM RA for 4 days and then exposed to increasing doses of TNF-α. (B) MTT assay for cell viability of RA-treated SK-N-MC cells which overexpress *bcl-2* (bulk population of cells) or *crmA* (clonal cell population). In both populations of SK-N-MC cells (*bcl-2* or *crmA*), cells stopped growing within 1 day of RA treatment. Neuronal morphology was also observed with RA treatment. Expression of *bcl-2* or *crmA* was confirmed by Western blotting (data not shown). Values shown are means from a single representative experiment performed in triplicate (this experiment was repeated twice, with similar results); the standard errors of mean values are marked by bars, and statistical significance (indicated by asterisks) was taken at the $P < 0.01$ level. OD_{490} nm, optical density at 490 nm.

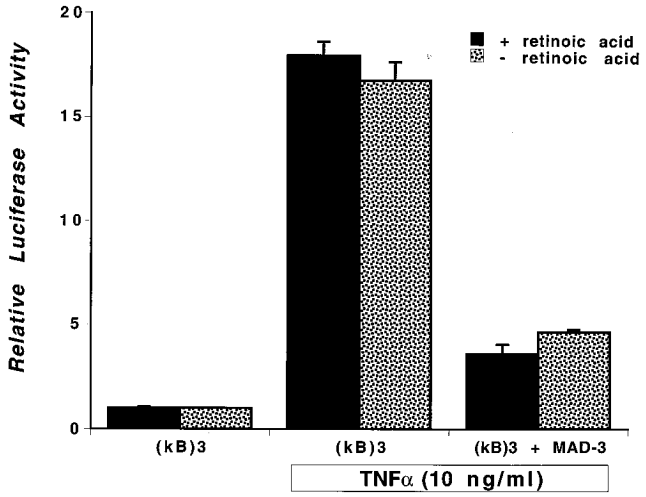

FIG. 8. Neuronal differentiation of SK-N-MC cells does not alter TNF-α-mediated activation of NF-κB. SK-N-MC cells were transfected with an NF-κB-responsive luciferase reporter plasmid [$p(kB)_3$conaLUC (38); bars marked $(kB)_3$] in the presence of a 0.5 M amount of an IκBα expression vector (pGM-MAD3; bar marked MAD-3) or vector alone (pUC19). Cells were then treated with RA or vehicle (dimethylsulfoxide) alone for 60 h prior to harvest and analysis of luciferase activity. TNF-α (10 ng/ml) was added to the indicated cultures 24 h prior to harvest. For both neuronally differentiated (RA-treated) SK-N-MC cells and their undifferentiated (untreated) counterparts, baseline levels of luciferase activity in cells not exposed to TNF-α were assigned a value of 1. Luciferase levels in TNF-α-treated cells are expressed relative to this value. Values shown are means of a single representative experiment which was performed in triplicate (this was repeated twice, with similar results); the standard errors of mean values are marked by bars.

However, we have shown by TUNEL staining, DNA laddering, and acridine orange binding that TNF-α-mediated death of neuronal SK-N-MC cells is apoptotic. Thus, expression of *bcl-2* in neuronal SK-N-MC cells is able to prevent programmed cell death due to TNF-α. Furthermore, since RA treatment induced growth arrest and neuronal morphology in all gene-modified derivatives of SK-N-MC cells, it is unlikely that transfection with *bcl-2* or *crmA* prevented TNF-α-mediated apoptosis by preventing differentiation. These findings are consistent with findings that *bcl-2* can prevent TNF-α-mediated cytotoxicity in HeLa cells (7) and suggest that it may ultimately be possible to design gene-based strategies to prevent neuronal loss in AIDS dementia complex and thereby ameliorate or halt this progressive neurodegenerative disorder. The data do not, however, reveal the precise molecular basis by which TNF-α triggers neuronal apoptosis. Although overexpression of *crmA* in neuronal SK-N-MC cells was apparently as efficacious as *bcl-2* expression in preventing the dose-dependent cell death induced by TNF-α, it is unclear whether *crmA* and *bcl-2* affect the same metabolic pathway, i.e., activation of putative death genes for interleukin 1β-converting enzyme or Ich-1 (48). Further studies will be required to determine the nature and abundance of pro- and antiapoptotic gene products in TNF-α-treated neuronal SK-N-MC cells. One possibility is that terminally differentiated SK-N-MC cells are vulnerable to oxidative stress induced by TNF-α and respond by aberrant activation of cyclin D1 (10).

Interestingly, TNF-mediated neurotoxicity is not seen in other experimental systems utilizing neuronal cultures. Cheng et al. demonstrated that pharmacologic doses (10 to 100 ng/ml) of TNF-α and TNF-β were protective against *N*-methyl-D-aspartate-mediated excitotoxicity or glucose deprivation injury to rat hippocampal neuronal cultures only when cultures were pretreated 24 h prior to the insult (6). One possible explanation for this apparent discrepancy is that there is a species difference in response. In previous studies of TNF-α-mediated neurotoxicity, we used primary human cortical neuronal cultures (13). In the present study, we used a monoclonal cell line derived from a human neural tumor. Another explanation for this paradox is that in cellular systems governed by redox regulation, opposite biological effects can be obtained depending on the level of oxidant stress. For example, nitric oxide can both induce and inhibit apoptosis in certain cell types (31, 44).

With respect to HIV-1 encephalitis and neurologic dysfunction, including progressive encephalopathy in children and dementia in adults, these studies are important for several reasons. First, only latent infection of neurons by HIV-1 has been demonstrated in a single postmortem case (40); hence, soluble HIV-induced neurotoxic factors are likely to induce cell death. Second, levels of mRNA for TNF-α are elevated in brain tissue of adults who had HIV-1-associated dementia (50). Third, TNF-α synthesis is markedly elevated in HIV-1-infected macrophages in vitro upon activation of cells via antigenic stimuli or contact with astroglial cells (18). Fourth, apoptotic neurons are present at high levels in brain tissues from children who died with HIV-1 encephalitis and encephalopathy, and these cells are located in close proximity to HIV-1-infected macrophages (14). In the present report, we present a possible explanation for these seemingly disparate observations by showing that TNF-α, a candidate HIV-1-induced neurotoxin, activates programmed cell death in human neuronal cells. We also demonstrate that this neurotoxicity can be ameliorated by the antioxidant NAC, and we show that overexpression of *bcl-2* and *crmA* can also block this process. These findings suggest novel therapeutic approaches to the treatment of neurologic dysfunction in HIV-1 infection of the central nervous system.

ACKNOWLEDGMENTS

These studies were funded in part by Pediatric AIDS Foundation grant 500278-14-PGR (H.A.G.), NIH grant PO1 NS31492-01 (H.A.G., H.E.G., and L.G.E.), NIH/NIAID grant KO4-AI01240 (S.D.), NIH/NIAID grant F32-AI08894 (S.C.D.), PHS grant T32-AI08894 (S.F.), and a generous grant from the Charles A. Dana Foundation (H.A.G., H.E.G., and L.G.E.). H.E.G. is a Carter-Wallace Fellow of the University of Nebraska.

We thank Junying Yuan for the gift of retroviral vectors expressing *crmA* and *bcl-2* (pJ415 and pJ436, respectively) and *crmA* antibodies, Hartmut Land for the control vector (pBabePuro), Alain Israël for the NF-κB-dependent reporter construct [$p(kB)_3$conaLUC], and David Baltimore and Gary Nolan for the IκBα expression construct (pGD-MAD3). We thank Peter Keng and Norb Roberts for assistance with flow cytometry and Edith Lord for generously sharing reagents.

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