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Tat Mediates Apoptosis *in Vivo* in the Rat Central Nervous System

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The HIV-1 transactivator of transcription, Tat, mediates apoptosis in neurons and may cause AIDS-associated neurologic disorders, which include dementia and loss of motor control. Here we investigate the ability of Tat to stimulate apoptosis in the rat central nervous system *in vivo.* Using the TUNEL assay, treatment of rat pheochromocytoma (PC12) cells with 1.0 μg/ml of Tat for 24 h was found to stimulate apoptosis. Further, electrophoresis of DNA from Tat-treated PC12 cells demonstrated fragmentation. To investigate Tat mediated apoptosis *in vivo,* 20 μg of Tat was infused into the striatum of Sprague-Dawley rats. Histochemical analysis (TUNEL) of the Tat-infused area demonstrated apoptosis. Further, these rats demonstrated postural deviation ipsilateral to the infusion. These data demonstrate that Tat stimulates apoptosis *in vivo* and causes neurologic dysfunction in the intact animal.

Infection with the human immunodeficiency virus (HIV) is often with associated neurological disorders. At least 10% of all AIDS cases involve neurological symptoms; however, in populations with progressed disease involvement of the central or peripheral nervous system occurs in 30% to 63% of patients (reviewed in 1). Some examples of AIDS associated neurological disorders include aseptic meningitis, inflammatory demylinating polyradiculoneuropathy, sensory neuropathy, cognitive dysfunction, and dementia (AIDS dementia complex or ADC) (reviewed in 2). ADC is reported in approximately 30% of adult and 50% of pediatric patients and leads to loss of independence and a shortened life expectancy (3, 4). HIV-1 associated dementia is the most frequent cause of neurologic disease in young adults in the United States and occurs at an annual rate of 7% in all people with AIDS (reviewed in 5). Although rare, neurological diseases can occur prior to profound immunosuppresion (4). For example, the brain may be devastated by bland encephalitis in AIDS patients whose immune function is relatively normal (reviewed in 1). In this regard the AIDS-associated neurological disorders represent a set of tissue specific syndromes which are not clearly linked to the immunodeficiency caused by HIV infection.

Relatively little is known about the pathological mechanisms of AIDS-associated neurological disorders. Possible mechanisms of AIDS-related central nervous system damage include release of neurotoxins from HIV infected macrophages, tissue destruction mediated by cytokines or neurotoxicity of HIV proteins (reviewed in 6). Abnormalities in the blood-brain barrier may be important both in facilitating viral entry into the brain and in mediating some of the tissue damage that accompanies HIV infection (reviewed in 6). In addition to direct infection of cells in the central nervous system, neurotoxic factors associated with HIV and cellular responses to HIV infection include the HIV-1 proteins gp120 and Tat, and elevated levels of cellular metabolites which include eicosonoids (i.e., arachidonic acid and its metabolites), platelet activating factor, tumor necrosis factor alpha and quinolic acid (reviewed in 5). The HIV-1 protein, Tat has been shown to be neurotoxic *in vivo* and lethal when injected into the intracerebroventricular space in mice (7) or into the rat striatum (8). While the neurotoxicity of Tat is well established, the mechanisms of Tat neurotoxicity *in vivo* are yet to be elucidated.

Recent evidence suggests that Tat can cause apoptosis in cultures of neuronal cells (9). The Tat protein has been shown *in vitro* to be secreted by HIV-1 infected lymphocytes and endocytosed by a variety of cell types (10). Neuronal cell endocytosis of Tat may be receptor-mediated since neuronal cells bind to the Tat basic domain through a 90 kDa cell surface protein (11). While the neurotoxicity of Tat *in vitro* has been well established, the mechanism of Tat toxicity *in vivo* has not yet been demonstrated. Here we confirmed that Tat causes apoptosis in neurons *in vitro* and extended these investigations to the rat central nervous system *in vivo.*

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MATERIALS AND METHODS

Materials. Recombinant Tat was purchased from Advanced Biotechnology's (Columbia, MD). The TUNEL assay was performed using Oncor's (Gaithersburg, MD) ApopTag peroxidase kit (catalog #:S7101-KIT) and counterstained with methylene green purchased from Fisher Scientific. The DNA ladder (50–2000 bp) used in gel electrophoresis was gotten from Bio-Rad (Hercules, CA). PC12 cells were obtained from Dr. Gordon Guroff (NICHD, NIH) and cultured in Dulbecco's modification of Eagle's medium (DMEM) containing 7.5% horse serum; 7.5% fetal bovine serum. The cells were cultured in a CO_2 incubator at 37.5°C.

Determination of Tat toxicity. Tat toxicity was determined by plating 6.0×10^5 PC12 cells in 0.5 ml of DMEM in each well of a 24 well culture cluster plate (Costar). Tat was then added at the following final concentration to each of triplicate wells in duplicate plates: 0.01 μg/ml, 0.01 μg/ml, 0.1 μg/ml, 1.0 μg/ml, and 10 μg/ml. Triplicate wells were also left untreated. The plates were then allowed to incubate at in a CO_2 incubator at 37.5°C for three (plate 1) or twenty-four hours (plate 2). After three and twenty-four hours the cells in each well were harvested and stained with trypan blue. Viability was then determined by dividing the number of trypan excluding viable cells by the total number of cell counted and multiplying this number by 100. The mean viability and the standard error of the mean for each Tat concentration was determined.

Assay for apoptosis. PC12 cells (5×10^3) were plated in the wells of an 8 chamber slide (NUNC). The cells were then either left untreated or treated with a final Tat concentration of 1.0 μg/ml for 24 h. The medium in each well was then gently removed and the cells were fixed 4% neutral buffered formalin at room temperature for twelve minutes. The cells were then briefly rinsed with Dulbecco's phosphate buffered saline (PBS) treated with 3% hydrogen peroxide for five minutes and again rinsed with PBS. The cells were and subjected to the TUNEL assay using Oncor's Apoptag kit which detects digoxigen-dUTP end labeled DNA fragments. Briefly, terminal deoxynucleotidyl transferase (TdT) enzyme was utilized to incorporate digoxigen-dUTP onto the 3′ ends of DNA fragments generated by apoptosis. Then the cells were incubated with an HRP-conjugated antibody directed against the digoxigen-dUTP complex. Antibody binding was then detected using 3,3′-diaminobenzidine tetrachloride (DAB). After DAB staining, the chambers were removed from the slide and the slide was washed in distilled water in three separate washes of water for 1 min intervals and a final wash for 5 min. The slide was then counterstained with methylene green for 10 min at room temperature and viewed and photographed at 80× magnification with a light microscope.

Interstitial infusion of Tat into rat brain. Stereotaxic coordinates used for infusion of Tat were anterior 0, lateral 3 mm, and ventral 5 mm with respect to the bregma and dura mater. Infusion cannulas were made from polyether ether ketone tubing (inner diameter 250 μm; Thomson Instrument Company, Springfield, VA) and a fused silica needle (inner diameter 100/170 μm). Rats were infused 20 μg of Tat in sterile PBS. The cannulas were inserted into the center of the striatum using the streotaxic technique. After 15 min, 20 μl of solution containing 20 μg Tat, was infused using a syringe infusion pump (Harvard Apparatus, South Natick, MA) at 0.1 μl/min, for 200 min. Fifteen minutes after infusion, the cannulas were removed, skin sutured closed, and the rats allowed to recover. After 3 days the animals were anesthetized and perfused transcardially with 4% formaldehyde, 0.075 M sodium phosphate, and 1.5% methanol. The brains were cut on a vibratome. Fifty-micrometer coronal sections placed on microscope slides and assayed for apoptosis using Oncor's Apoptag kit as described above.

DNA electrophoresis. PC12 cells (3×10^6) were cultured for twenty-four hours in the presence of 1.0 μg/ml or left untreated. The cells were then collected and washed free of serum using PBS. The collected cells were then lysed with 500 μl 10 mM Tris-HCl (pH 6.0) containing 1 mM EDTA and 0.2% Triton X-100. After centrifugation at 13,000 × g for 10 min the supernatant was removed into a new 1.5-ml tube containing 100 μl of 5 M NaCl and 700 μl of ice cold 2-propanol. The tube was then introverted several times to precipitate the DNA. The sample was then centrifuged for one min at 12,000 × g and the supernatant was removed with a transfer pipette. A volume of 500 μl of 70% ethyl alcohol was then added and the sample was vortexed. The sample was again centrifuged for one minute at 12,000 × g and the supernatant was removed. The sample was then dried in a speed-vacuum and the DNA was hydrated in 50 μl of gel loading buffer. The DNA from both untreated cell and Tat treated cells were then subjected to electrophoreses on a 1.5% agarose gel at 100 volts for 1 h. The DNA was then visualized by EtBr staining and the gels were photographed.

TABLE 1

The Toxicity of Tat to Cultures of PC12 Cells

Tat (μg/ml)	Viability (%)	
	3 h	24 h
0	95 ± 1.5	93 ± 0.7
0.01	91 ± 0.4	89 ± 3.5
0.1	90 ± 2.0	72 ± 4.7
1.0	77 ± 1.0	39 ± 4.0
10	0 + 0	0 + 0

Note. PC12 cells were cultured in the presence of the indicated concentrations of Tat for either 3 or 24 h in triplicate wells. Cells were collected at the indicated times and analyzed for trypan blue exclusion. Cells which excluded trypan were considered viable and the number of viable cells from each well was divided by the total number of cells counted and multiplied by 100 to give the percentage viability in the culture. The mean viability for the triplicate wells was determined and presented with the standard error of the mean (±).

RESULTS

In order to determine the appropriate concentrations of Tat to investigate mechanisms of toxicity, PC12 cells were treated with concentrations of Tat ranging from 0.01 μg/ml to 10 μg/ml for three and twenty-four hours and then stained with trypan blue. Untreated cells were 95%–93% viable over the twenty-four hour period. With three hours of treatment, 10 μg/ml Tat was toxic to all cells while 1.0 μg/ml was toxic to approximately 23% of the cells (Table 1). With twenty-four hours of treatment with 0.01 mg/ml Tat, toxicity to approximately 10% of the PC12 cells was observed, which was not a significant reduction in viability (Table 1). With a 0.1 mg/ml Tat treatment for twenty-four hours, PC12 cell viability was significantly reduced to 73% and a Tat treatment of 1.0 mg/ml led to a PC12 cell culture viability of 39% (Table 1). A Tat treatment of 10.0 μg/ml killed all PC12 cells after twenty-four hours (Table 1). These data demonstrate that Tat is toxic to 50% of PC12 cells at a concentration between 0.1 and 1.0 mg/ml.

In order to determine if Tat toxicity to the PC12 cells was the result of apoptosis, PC12 cells were treated

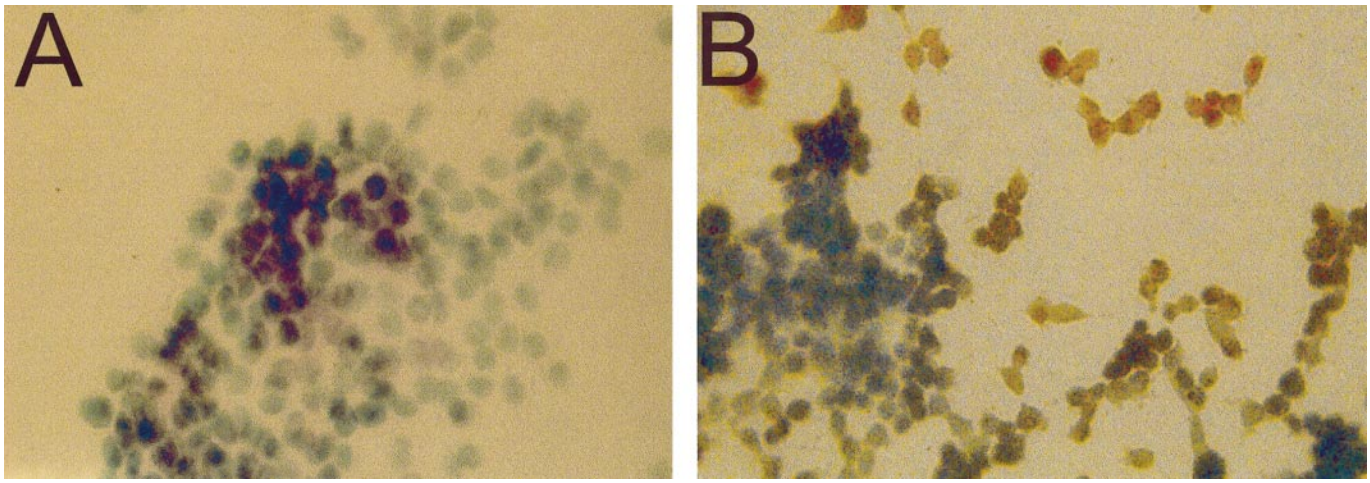

FIG. 1. Tat mediated apoptosis in PC12 cells. PC12 cells were seeded in wells of an eight-well chamber slide (5×10^3cells/well) and either untreated (A) or treated with 1 μg/ml Tat (B) for 24 h. The cells were then subjected to the TUNEL assay using the Oncor Apoptag kit. The cells were stained and counterstained with methylene green and photographed at 80× magnification. Areas where cells have piled up have a blue appearance. Only those cells treated with Tat in B show the positive brown staining for apoptosis.

with 1.0 mg/ml Tat for twenty-four hours and then analyzed by the TUNEL assay for the presence of fragmented DNA. Tat treated PC12 cells stained positive for fragmented DNA while untreated cells did not (Fig. 1). In order to directly visualize the Tat mediated DNA fragmentation, the DNA from Tat treated and untreated cells was analyzed by gel electrophoresis. Tat treated cells showed smears of DNA fragments on the gel, while untreated cells contained DNA which only slightly entered the gel indicating that it was intact (Fig. 2).

In order to determine if Tat could cause apoptosis in the central nervous system *in vivo,* Tat was infused into the rat striaum and section of the infused rat brain were subjected to the TUNEL assay using Oncor's Apotag kit. Apoptosis was detected throughout the infused volume of the brain (Fig. 3). The hemisphere of the brain which had not been infused with Tat showed no staining for apoptosis (Fig. 3). These data are quite important because they are the first to show that the ability of Tat to stimulate apoptosis *in vitro* translates to the *in vivo* environment and strengthens the hypothesis that Tat released into the central nervous system will lead to neurologic damage via apoptosis.

DISCUSSION

AIDS patients frequently suffer from a variety of neurological disorders. The mechanisms though which AIDS associated disorders develop are poorly understood. It has been suggested that HIV-1 infected monocytes carry HIV-1 into the brain where the HIV-1 protein, Tat, is released and causes nerve damage (reviewed in 12). Indeed, Tat has been shown to be highly neurotoxic and to stimulate apoptosis in cultured neurons. Here we investigated the ability of Tat to stimulate apoptosis in the neurons of the central nervous system of the intact animal. We have found that infusion of the rat striatum with Tat leads to neurologic dysfunction and apoptosis of the neurons at the site of infusion.

The mechanisms of Tat delivery to the brain in AIDS patients is still not known, however, HIV-1 is know to

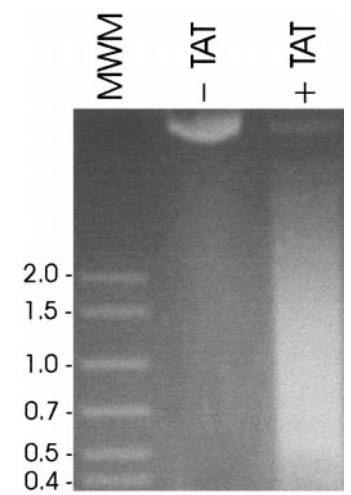

FIG. 2. Tat-mediated DNA fragmentation in PC12 cells. PC12 cells (3×10^6) were cultured for 24 h in the presence (+Tat) or absence (−Tat) of Tat. The cells were harvested and the DNA was collected as described under Materials and Methods. The DNA was then subjected to 1.5% agarose gel electrophoresis. Molecular weight markers (MWM) were also electrophoresed to give an indication of fragment size. The gels were stained with ethidium bromide and then photographed. The untreated (−Tat) cells show no indication of DNA fragmentation while the Tat-treated cells (+Tat) show fragmented DNA.

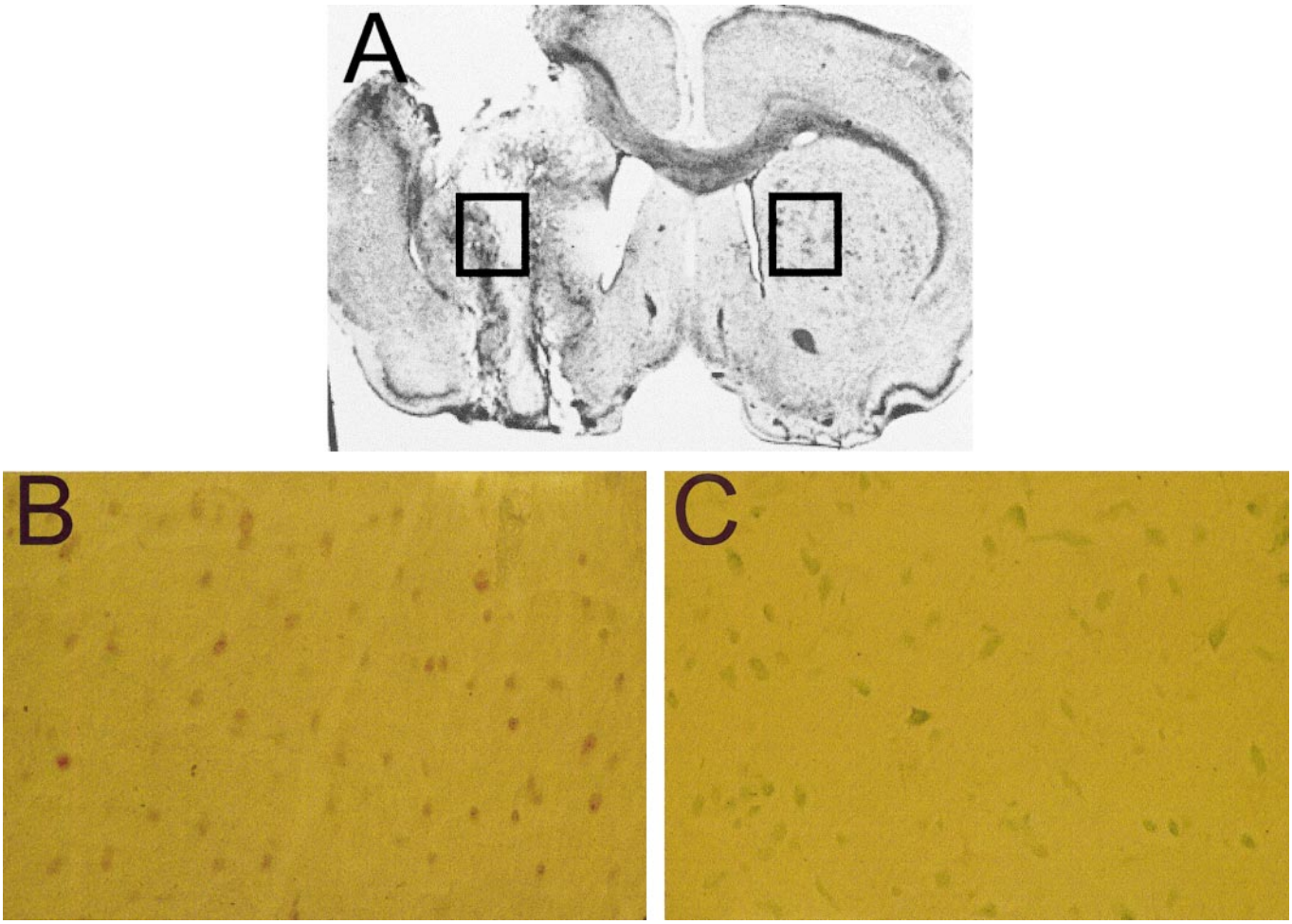

FIG. 3. Tat-mediated apoptosis in the rat central nervous system *in vivo.* Tat (20 μg) was infused into the rat striatum. After 3 days the brain was removed and serial sections were prepared as described under Materials and Methods. Serial sections were then stained with hematoxylin and eosin (A) or subjected to immunohistochemical analysis using the TUNEL assay for apoptosis (B and C). B and C are from the same section and are in series with the section shown in A. B is a photomicrograph taken within the serial section within the box on the left side of A. C is a photomicrograph taken within the serial section within the box on the right side of A. In A, the infusion of Tat was performed on the left side and widespread tissue damage is visible at the site of Tat delivery. B shows that the cells at the site of Tat (from left box in A) are apoptotic, while the cells in the stratum opposite to the site if Tat infusion are healthy (A) and are not apoptotic (C).

activate monocyte and T-cell invasiveness (reviewed in 12). Therefore infected cells are more likely to extravasate the blood brain barrier and localize in brain tissue. Infected leukocytes are known to secrete Tat (10) and neuronal cells express a 90 kDa receptor which recognizes the basic domain on Tat (11). It is not clear if Tat mediated apoptosis in neuronal cells requires endocytosis or if signaling through the receptor is sufficient to initiate the apoptosis cascade. In this regard, it should be noted that a synthetic peptide derived from the basic domain of Tat is not neurotoxic, yet this domain is required for neurotoxicity (8, 11). These observations suggest that at least two Tat domains are involved in activation of neuronal death and apoptosis.

In addition to Tat, several HIV-1 gene products have been shown to be neurotoxic (reviewed in 12). In addition HIV-1 infected cells have been shown to secreted neurotoxic level of cytokines and quinolic acid (reviewed in 12). Therefore it is likely that AIDS associated neurological disorders are caused by several molecules acting independently or in combination through several cytopathic mechanisms. Here we have shown that Tat can cause apoptosis in the brain *in vivo* and confirm that Tat may, at least in part, contribute to neurologic dysfunction in the whole organism by activating apoptosis in brain cells.

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