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Gene Transfer of Baculoviral p35 by Adenoviral Vector Protects Human Cerebral Neurons from Apoptosis

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ABSTRACT

Apoptosis plays an important role in neuronal cell death in both chronic and acute human neurological diseases, including ALS, Huntington's disease, cerebral ischemia, and HIV encephalopathy. We evaluated the ability of an extremely powerful antiapoptotic agent, *baculoviral p35*, to prevent apoptosis and cell death of human cerebral neurons that undergo severe neurotoxic changes in a culture system when treated with agents that are implicated in human neurological disorders, that is, tumor necrosis factor (TNFα) and the HIV proteins Tat and gp120. P35 is a potent broad-spectrum antiapoptotic protein derived from baculovirus, that inhibits nearly all caspases, and has other antiapoptotic actions as well. Adenoviral vectors expressing p35 (Ad.p35) or a control gene (lacZ) efficiently transduced human neurons. Treatment of control cultures with the toxic agents TNFα, TNFα plus Actinomycin D, or Tat and gp120, induced neurotoxicity and death of neurons. Transduction of neurons with Ad.p35 blocked apoptosis, and eliminated cell death due to TNFα, or Tat and gp120. Viral vector transfer of the p35 gene efficiently protects human neurons from TNFα, or Tat and gp120-induced apoptosis and cell death. These results suggest that p35 transduction of neurons by viral vectors could be therapeutically useful in the treatment of human neurodegenerative diseases.

INTRODUCTION

APOPTOSIS PLAYS AN IMPORTANT ROLE in the neuronal cell death that occurs not only in chronic degenerative neurological diseases, such as amyotrophic lateral sclerosis (ALS), Huntington's disease, and Alzheimer's disease (Martin, 2001; Friedlander, 2003), but also in a variety of subacute and acute disorders of the nervous system, including HIV encephalopathy (Magnuson *et al.*, 1995; Nath *et al.*, 1996; Turchan *et al.*, 2003) and cerebral ischemia (Martin, 2001; Friedlander, 2003), for example. It is well established that caspases (cysteine aspartate proteases) participate in these processes, and agents that inhibit caspases have shown promise in the prevention and treatment of cell death in various disease models. Inhibition of caspases by pharmacological agents such as ZVAD-fmk (Li *et al.*, 2000), or by transgenic overexpression of Bcl-2 (Kostic *et al.*, 1997) has provided some benefit in the transgenic mouse model of ALS, but neither of these methods would be appropriate for treatment of human neurodegenerative diseases. We undertook the present study to evaluate the ability of the potent antiapoptotic agent, baculoviral p35, to prevent apoptosis of human neurons, when they are transduced by a viral vector carrying the p35 gene. To evaluate the efficacy of p35, we used a human fetal brain culture system in which neurons normally survive in a stable condition for months, but rapidly undergo severe apoptotic changes and cell death when treated with either of two agents that are related to human neurodegenerative diseases: tumor necrosis factor alpha (TNFα), or the HIV Tat and gp120 proteins. P35 is an extremely powerful broad-spectrum antiapoptotic agent derived from baculovirus, that is known to inhibit nearly all caspases, and to have other antiapoptotic actions as well (Rabizadeh *et al.*, 1993; Bump *et al.*, 1995; Luo *et al.*, 2001; Date *et al.*, 2002). Our results demonstrate that gene transfer of p35 to human brain cells by means of an adenoviral vector produced highly significant prevention of neurotoxicity and cell death.

MATERIALS AND METHODS

Adenoviral vectors

To generate the Ad.p35 vector we inserted cDNA for the baculoviral protein p35 (Upstate Biotechnology, Waltham, MA) un-

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der control of a CMV promoter into a replication-incompetent (E1/E3 deleted) human adenoviral type 5 (Ad5) genome, using the "Adeno-X" cloning system (BD Biosciences Clontech, Palo Alto, CA). Ad.lacZ, an Ad5 control vector expressing the β-galactosidase gene under control of a CMV promoter, was provided by BD Biosciences Clontech. Both viral vectors were propagated in the HEK293 packaging cell line (Clontech) and purified by centrifugation on a continuous $CsCl_2$ gradient (Current Protocols in Human Genetics, John Wiley & Sons Inc., p. 12.4.1). The concentrations of purified viral vectors were determined using the AAdeno-X rapid titer kit[7] (Clontech). Titers for Ad.lacZ and Ad.p35 were 8×10^{13} and 7.4×10^{11} infectious units per milliliter (IU/ml), respectively.

Fetal brain cultures

Brain specimens were obtained from human fetuses of 12 to 14 weeks gestational age with consent from women undergoing elective termination of pregnancy, and with the approval of the Johns Hopkins Institutional Review Board. Cells were isolated and cultured as described previously (Magnuson *et al.*, 1995; Nath *et al.*, 1996). Briefly, the cells were mechanically dissociated, suspended in Opti-MEM medium with 5% heat-inactivated fetal bovine serum, 0.2% N2 supplement (Gibco, Gaithersburg, MD), and 1% antibiotic solution (penicillin G 10^4 units/ml, streptomycin 10 mg/ml, and amphotericin B 25 μg/ml); and plated in tissue culture flasks. The cells were maintained in culture for at least 1 month before conducting the experiments. Cerebral cell cultures were prepared from four different human fetuses. To evaluate the possibility of differential susceptibility to neurotoxic damage, each specimen was genotyped for ApoE haplotypes by PCR (Corder *et al.*, 1998).

Adenoviral transduction and transgene expression analysis

Three days prior to transduction, cells were harvested and plated in poly-L-lysine coated 96-well plates. The cultures were then transduced by infection with adenoviral vectors at an MOI of 100. After 72 h the cells were used for evaluation of transgene expression, and experiments on induction and/or prevention of apoptosis. Expression of the β-gal reporter gene was detected using the "β-gal reporter gene detection kit" (Sigma, St. Louis, MO). Expression of p35 was evaluated by RT-PCR using the "SuperScript One-Step RT-PCR System" (Invitrogen, Carlsbad, CA) and p35-specific primers, as follows: p35 forward primer: 5′-ATGTGTGTAATTTTTCCGG-3′; p35 reverse primer: 5′-TTATTTAATTGTGTTTAATATTAC-3′.

Evaluation of neurotoxic cell damage

At the time of experimental treatment, the culture medium was replaced with Locke's buffer containing: NaCl (154 mM). KCl (5.6 mM), $CaCl_2$ (2.3 mM), $MgCl_2$ (1 mM), $NaHCO_3$ (3.6 mM), glucose (5 mM), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (5 mM; pH 7.2). Various concentrations of TNFα, TNFα plus Actinomycin D (which enhances the TNF effect (Chen *et al.*, 1985), or Tat plus gp120 were added to the medium. To quantify the level of neurotoxicity induction we determined the change in the mitochondrial membrane potential, as previously described (Turchan *et al.*, 2003). The data were calculated as the means ± SEM of optical measurements from eight replicate wells, and analyzed using the ANOVA statistical method. A decrease in the mitochondrial membrane potential is an early feature of apoptotic cell damage (Turchan *et al.*, 2001; Friedlander, 2003). To evaluate the viability of neurons we used the trypan blue dye exclusion method. After treatment with each of the toxic agents (i.e., TNFα, TNFα plus Actinomycin D, or Tat/gp120) cells were stained with trypan blue dye. Five randomly selected microscopic fields (400×) were photographed, and a blinded investigator determined neuronal counts. At least 200 neurons were counted in each field. Each experiment was done in replicates of four, and five independent experiments were conducted. The results are expressed as the mean percentage of trypan blue stained dead neurons ± SEM.

RESULTS

To ensure a high level of gene transfer and expression in human brain cells we generated an adenoviral expression vector. We cloned the gene for baculoviral protein p35 into replication-incompetent (E1/E3-deleted) human adenovirus type 5 (Ad5), and called the resultant vector Ad.p35. Expression of p35 cDNA was driven by a cytomegalovirus (CMV) promoter.

To evaluate the appropriate conditions for adenoviral transduction of human fetal brain cells in culture, we used a control Ad vector expressing the β-gal reporter gene under control of a CMV promoter (Ad.lacZ). We added Ad.lacZ to the human brain cells cultures at multiplicities of infection (MOIs) of 10, 100, 1000, and 10,000 infectious units (IU) per cell. We evaluated the cultures for β-gal expression and for cellular integrity at intervals up to 72 h. We found that: (a) the cells tolerated very high concentrations of adenoviral vectors; only at the highest concentration (10,000 IU/cell) were there significant morphological changes in these cultures; (b) the minimal concentration used (10 IU/cell) was enough to transduce 70–90% of human brain cells *in vitro*. At the optimal concentration of 100 IU/cell, 100% of brain cells expressed the reporter LacZ gene product (Fig. 1a). To evaluate expression of p35 by Ad.p35-transduced primary human neurons we employed RT-PCR analysis using gene-specific primers. A reliable antibody for p35 protein is not currently available, which precludes direct detection of p35 protein. We demonstrated a high level of p35 mRNA expression in Ad.p35-transduced human cerebral cultures, by RT-PCR (Fig. 1b).

We next examined the conditions for induction of neuronal damage in the human fetal brain culture system by the neurotoxic agents TNFα, and HIV proteins Tat and gp120. As an indicator of neurotoxicity, we measured the change in mitochondrial membrane potential using fluorescent dye JC1, as previously described (Mancini *et al.*, 1997; Turchan *et al.*, 2003). A decrease in mitochondrial potential activity reflects the loss of mitochondrial membrane integrity in the cells, and is an early cellular event that leads to apoptosis (Friedlander, 2003). TNFα alone at a concentration of 10 ng/ml, or TNFα at only 1 ng/ml together with Actinomycin D (10 μg/ml), which enhances the effect of TNFα (Chen *et al.*, 1985), induced a marked loss of mitochondrial membrane potential in the neuronal cell cultures. The HIV proteins Tat (80 nM) plus gp120 (30 pM) have been reported to interact synergistically to induce neuronal apopto-

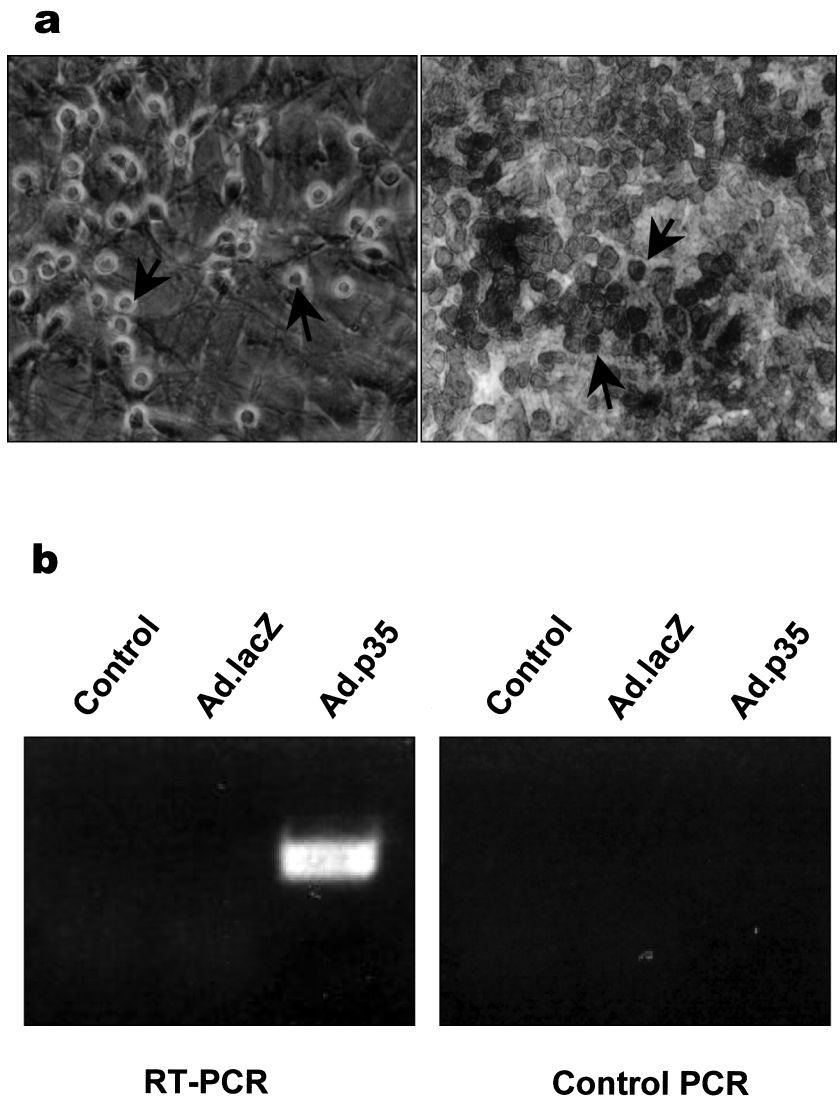

FIG. 1. Adenovirus-induced transgene-expression in primary human fetal brain cultures. (**A**) Expression of β-gal reporter gene in primary fetal brain culture. Left panel: Phase contrast photomicrograph (200×, reduced ~25%) of human fetal brain cells not transduced, but stained with X-gal (negative control). Right panel: Transmitted light photomicrograph (200×, reduced ~25%) of brain cells transduced with Ad.lacZ vector at MOI of 100 IU/cell for 72 h, stained with X-gal. Arrows indicate examples of typical neuronal cells (rounded), within representative fields containing many neurons as well as astroglia (flattened cells). Although not visible on this black and white print, 100% of the Ad.lacZ transduced neurons and astrocytes were stained blue, while there was complete lack of β-galactosidase staining of control (untransduced) brain cells. (**B**) RT-PCR analysis of primary human cerebral cultures transduced for 72 h with Ad.p35 vector (MOI 100 IU/cell). Negative controls consisted of uninfected cells (Control) and cells transduced with Ad.lacZ. To rule out undesirable amplification of viral DNA encoding the p35 gene, a control PCR reaction was performed using the same conditions, but omitting only the reverse transcriptase in the reaction mixture (Control PCR).

sis (Nath *et al.*, 2000), as shown in Figure 2a. Based on clinical evidence (Corder *et al.*, 1998) and preliminary unpublished results (Nath *et al.*), we suspected that expression of different isoforms of the LDL receptor ApoE would influence the susceptibility of neuronal cultures to apoptosis induced by Tat/gp120 or TNFα. Our results (Fig. 2b) showed that cells prepared from fetuses homozygous for the ApoE4 gene are more susceptible to neurotoxicity induced by the HIV proteins Tat + gp120 than fetal brain cells with other ApoE genotypes. However, the ApoE genotype had no influence on the susceptibility to TNFα-induced neurotoxicity (Fig. 2b).

To determine whether expression of p35 would have a neuroprotective effect, we transduced neuronal cells isolated from four different fetuses with Ad.p35 vector at the optimum MOI of 100 IU/cell. Seventy-two h later Ad.p35-transduced cells or untransduced control cells were treated with each of the neurotoxic agents (TNFα, TNFα + Actinomycin D, Tat plus gp120), and neurotoxicity was evaluated by measuring the change in mitochondrial membrane potential 6 hours later. Cultures that were infected with control Ad.lacZ virus, and cultures that were not infected showed a significant decline of the mitochondrial membrane potentials ($P < 0.04$). By contrast, the

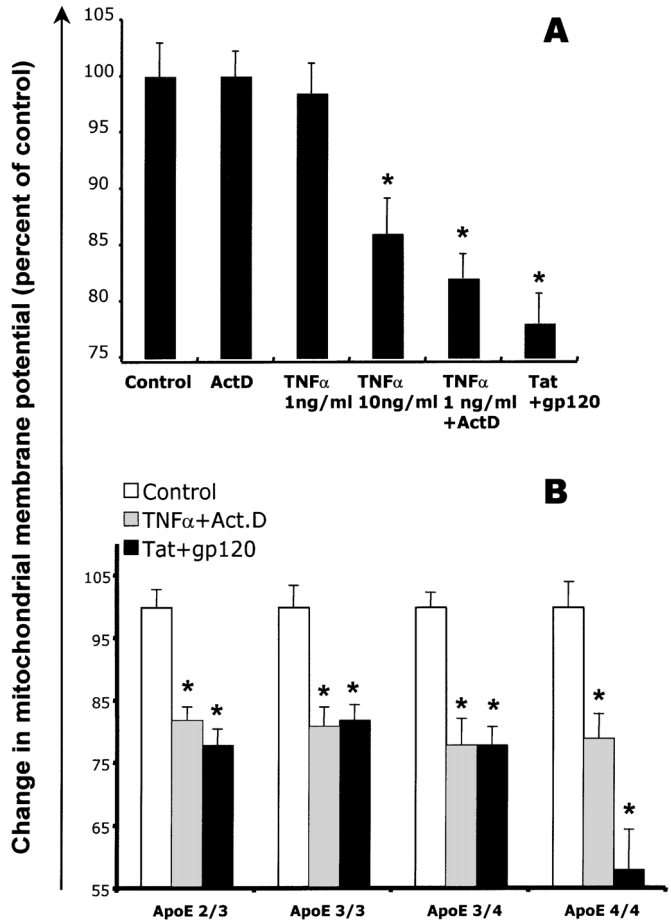

FIG. 2. Neurotoxic effects of various agents in human neuronal cell cultures. (**A**) Neurotoxicity of human fetal brain cells induced by TNFα, TNFα plus Actinomycin D, or HIV proteins Tat and gp120. Note that the addition of actinomycin D enhanced the toxicity of low dose TNFα. (**B**) Effect of ApoE genotype on susceptibility to neurotoxic damage. Cultures were treated with the indicated agents, and neurotoxicity was quantified by measurement of the change in the mitochondrial membrane potential. Human neurons isolated from ApoE 4/4 homozygous fetuses exhibit increased susceptibility to apoptosis induced by HIV proteins. *Indicates significant reduction of mitochondrial potential $P < 0.04$).

mitochondrial membrane potential of brain cells transduced with p35 remained unchanged throughout the duration of the experiments (Fig. 3). In the homozygous ApoE4 brain cells, the protection was slightly less pronounced than in cells of other genotypes.These results lead to the conclusion that expression of p35 efficiently protected human neuronal cells against apoptosis induced by TNFα or the neurotoxic HIV proteins Tat/gp120.

To evaluate specific neuronal protection further in Ad.p35-transduced cultures, we examined the *viability* of neurons by exclusion of trypan blue dye. We found that in control cultures not treated with the neurotoxic agents the background uptake of trypan blue was approximately 2% of neurons. Treatment with the neurotoxic agents resulted in a selective increase of neuronal cell death in a subset of neurons, ranging from 6 to 10% of the total, as previously described (Nath *et al.*, 2000). In contrast, Ad.p35-transduced cells showed no increase in neuronal cell death in the presence of TNFα and Actinomycin D or Tat plus gp120. Neuronal cell death remained at the background level of 2% in p35 transduced cultures, despite the neurotoxic treatments (Fig. 4). The differences were highly significant ($P < 0.001$).

DISCUSSION

Our findings demonstrate that the antiapoptotic agent baculoviral p35 potently protects human primary neuronal and astrocytic cells from toxic cell damage and cell death. Moreover, p35 can be efficiently transferred into these cells by the viral vector Ad.p35. We evaluated the ability of p35 to protect against damage induced by two different agents with known powerful neurotoxic effects, TNFα and the HIV proteins Tat and gp120, with strikingly beneficial effects. Both TNFα and the HIV proteins are believed to play important roles in human neurological diseases (Nath *et al.*, 2000; Martin, 2001; Friedlander, 2003; Turchan *et al.*, 2003). Indeed, this is the first demonstration that an anti-apoptotic agent can block the neurotoxic agents im-

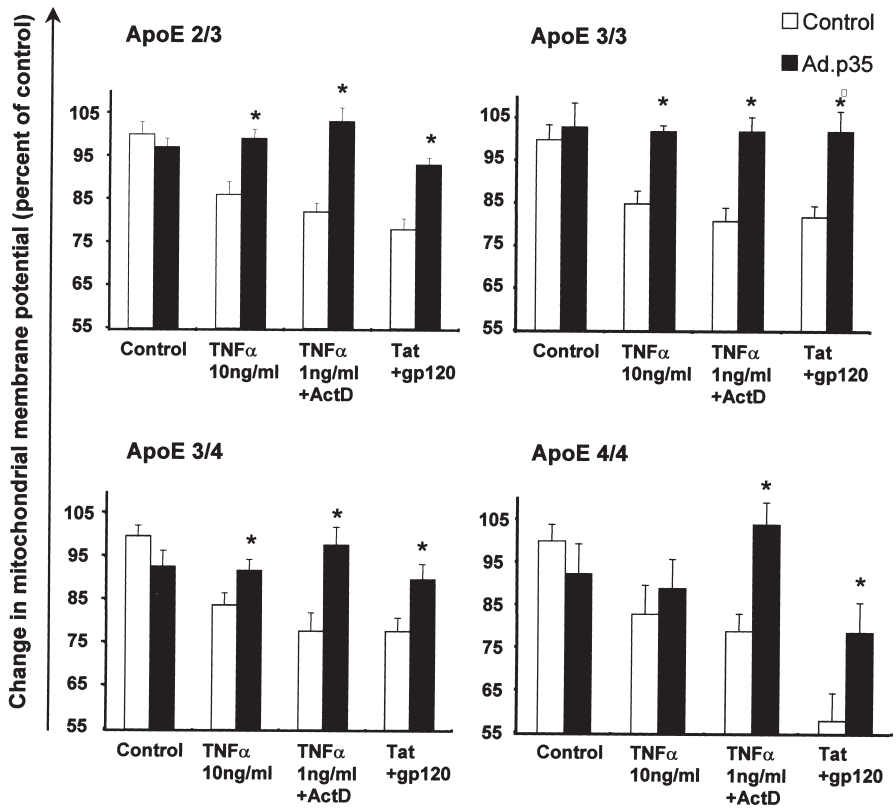

FIG. 3. Adenovirus induced expression of baculoviral protein p35 protects primary human neuronal cultures from apoptosis. Ad.p35 transduced, or untransduced control cultures, were treated with the indicated reagents for 6 h. Apoptosis induction was quantified by measurement of the change in the mitochondrial membrane potential. Cultures were prepared from four different fetuses. Results of ApoE genotyping are shown in each diagram. *Indicates statistically significant protection ($P < 0.04$).

plicated in HIV encephalopathic dementia. Most importantly, these agents induce neurotoxicity by activating the caspase cascade. We used the measurement of mitochondrial membrane potential as an indicator of overall toxicity of the brain cells in culture. A reduction in the membrane potential of mitochondria is recognized as a feature of apoptosis (Friedlander 2003).

Our choice of p35 as a neuroprotective agent is based on its broad spectrum and highly potent antiapoptotic effect. It is known to interfere with caspases of the "initiator", "executioner", and "cytokine processor" classes, including all but caspases 5 and 9 (Rabizadeh *et al.*, 1993; Bump *et al.*, 1995; Zhou *et al.*, 1998; Luo *et al.*, 2001; Date *et al.*, 2002). Its caspase blocking mechanism is due to its ability to bind tightly to all of these caspase enzymes. P35 has the additional advantage of blocking cell damage by "mopping out" reactive oxygen species (Sah *et al.*, 1999), which are thought to participate in cell damage in various neurodegenerative disorders. If the apoptotic damage of neurons can be interrupted at the level of the caspase system, it could potentially slow or halt neurodegenerative diseases, regardless of whether the specific factor or factors inducing apoptosis are known. We have demonstrated that the viral vector Ad5 can efficiently transduce neuronal cells. If adenovirus or other viral vectors expressing p35 are appropri-

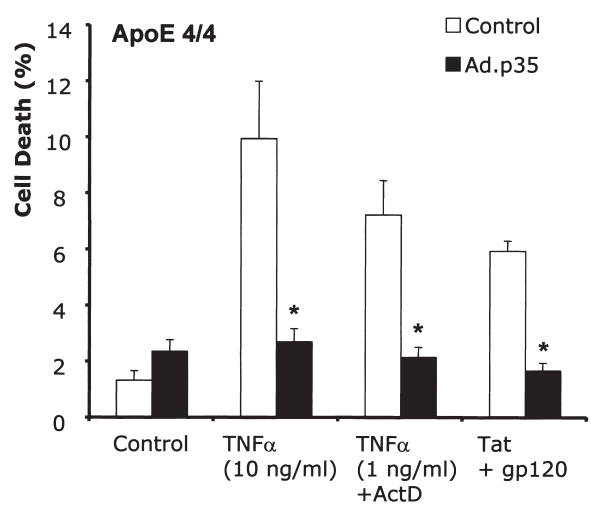

FIG. 4. p35 expression inhibits neuron-specific cell death. Ad.p35 transduced, or untransduced control cells, were treated with the indicated cytotoxic agents for 12 h. Neuron-specific cell death was evaluated by trypan blue exclusion assay, as described. *Indicates highly significant protection from cell death of p35 transduced neurons $P < 0.001$.

ately targeted to the cells at risk, they should be therapeutically beneficial in human neurodegenerative diseases in which apoptosis occurs.

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