Inhibition of p75 Tumor Necrosis Factor Receptor by Antisense Oligonucleotides Increases Hypoxic Injury and β-Amyloid Toxicity in Human Neuronal Cell Line*

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Recent evidence indicates that tumor necrosis factor-α (TNF-α) is up-regulated following brain injury and in neurodegenerative disorders such as stroke, multiple sclerosis, Parkinson’s disease, and Alzheimer’s disease. TNF-α elicits its biological effects through two distinct TNF receptor (TNFR) subtypes: p55 TNFR (TNFR1) and p75 TNFR (TNFR2). Studies have demonstrated that the p55 TNFR contributes to cell death, whereas the role of the p75 TNFR in neuronal viability is unclear. To better understand the role of p75 TNFR, we treated human neuronal SH-SY5Y cells with phosphorothioate-modified antisense oligonucleotides (ASO) for p75 TNFR and established that ASO inhibited p75 TNFR expression. Treatment of SH-SY5Y cells with ASO alone did not affect cell viability, whereas treatment with both ASO and human TNF-α significantly increased cell death relative to treatment with TNF-α alone. Moreover, addition of ASO significantly increased the level of cell injury observed following hypoxic conditions or exposure of β-amyloid peptide. These results indicate that inhibition of p75 TNFR using ASO increases the vulnerability of neurotypic cells to insults and suggest that the p75 TNFR may not be required for normal neuronal cell viability but rather plays a protective role following injury.

Tumor necrosis factor-α (TNF-α), an inflammatory mediator, is recently reported to be up-regulated following brain trauma (1, 2) and ischemic injury (3, 4), and in multiple sclerosis (5, 6), Parkinson’s disease (7), and Alzheimer’s disease (8). Studies have shown that TNF-α can elicit either a trophic or toxic effect, which is dependent on the target cell type. For example, TNF-α is toxic to human oligodendrocytes (9) and neuronal cells (10, 11), but it is trophic to rat hippocampal neurons (12). Since TNF-α elicits its biological effects through activation of two distinct receptors, p55 TNFR and p75 TNFR, both of which have been cloned (13–15), we hypothesized that the p75 TNFR might play a protective role following neuronal insult.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human neuronal-like cells, SH-SY5Y, were cultured in a 1:1 ratio of minimum Eagle’s medium/F-12 medium, 15% fetal calf serum (Life Technologies, Inc.), and 10 μM retinoic acid (RA, Sigma). The cells were seeded at 25,000 cells/well in a 24-well plate for cytotoxicity assays or 3 × 10⁴ cells in Petri dishes for radioligand-binding studies. The medium was replaced every 3 days. Treatments were given to the cells in serum-free medium with 1:100 N2-supplement (Life Technologies, Inc.) 50% minimum Eagle’s medium and 50% F-12.

RT-PCR—The PCR technique was used to amplify cDNA derived from RNA extracted from RA-differentiated SH-SY5Y cells as described previously (17). The PCR-forward oligonucleotide primer for p75 TNFR was 5′-GGTCAACAAATGCTTTTCT-3′, and backward oligonucleotide primer for p75 TNFR was 5′-GCCGTGATCTCCAGTATG-3′. PCR-forward oligonucleotides primer for p55 TNFR was 5′-CGGTTTATGGTGGTGG-3′, and backward oligonucleotides primer for p55 TNFR was 5′-AAAGCAATGCGGGCTCA-3′. After an initial denaturation step at 94°C for 5 min, the cycle was initiated, which consisted of denaturing for 1 min at 94°C, annealing for 2 min at 53°C, and extending for 2 min at 72°C. The cycle was repeated 35 times. Control reactions in the absence of RT with RNA template yielded no detectable product. A proportion (25%) of the reaction mixture was run in an agarose gel (1.2%).

Preparation of p75 TNFR Antisense Oligonucleotides—Both sense and antisense p75 TNFR oligonucleotide phosphorothioates were synthesized on an Applied Biosystems model 392 DNA synthesizer using phosphoramidite chemistry. Oligonucleotides were purified by reverse-phase chromatography using Oligo-Pak oligonucleotide purification columns (Milligen) as reported (18). The p75 TNFR antisense oligonucleotide sequence was 5′-GGTCAACAAATGCTTTTCT-3′ overlapping to the code for the initiation site of methionine on the human p75 TNFR gene (19). The p55 TNFR antisense oligonucleotide sequence was 5′-CCACCTCTCCGGGTACGG-3′ overlapping to the code for the initiation site of methionine on the human p55 TNFR gene (20). The sense oligonucleotides for each receptor subtype were the exact inverse complement of the antisense oligonucleotides. Liphospholipid oligonucleotides were dissolved in sterile water to prepare stock solutions, which were stored at −70°C until use.

Hypoxic Exposure—The cells were first transferred into a serum-free medium and then placed in a modular incubator containing 5% carbon dioxide and 95% nitrogen at 37°C. Following desired exposure (6 h), cultures were returned to a normoxic atmosphere of 5% carbon dioxide and 95% room air at 37°C.

Preparations of Human Tumor Necrosis Factor-α and Aβ—Human tumor necrosis factor-α (R & D Systems) was aliquoted into 1 μg/ml of medium and then diluted into 20 ng/ml with serum-free N2 medium. Aβ (1–42) was dissolved in dry MeSO at 6.5 μM and then diluted with serum-free N2 medium.

Immunoprecipitation of p75 TNFR—RA-differentiated SH-SY5Y cells (3 × 10⁴) were cultured with the medium without methionine and
cysteine for 2 h followed by incubation with [35S]methionine and [35S]cysteine (50 μCi/ml, DuPont NEN) in methionine- and cysteine-free medium at 37 °C. Fifteen hours later, cells were washed twice for 15 min each. Cell lysates were harvested and centrifuged at 45,000 rpm at 4 °C for 30 min. incubated with p75 TNFR antibody (polyclonal detection antibody, R & D Systems, 1:500) for 16 h at 4 °C, and precipitated with Staphylococcus protein A (Boehringer Mannheim). The immunoprecipitates were resolved in a 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, gels were fixed in 10% acetic acid and 50% methanol solution and then incubated in Enlightning (DuPont) for 30 min with rocking. Bands in dried gels were detected by autoradiography.

Western Blotting Procedure—RA-differentiated SH-SY5Y cells were cultured without fetal calf serum for 24 h. Cells were then lysed with deoxycholate (5 mg/ml), phenylmethylsulfonyl fluoride (0.175 mg/ml), and aprotinin (100 kallikrein-inactivating units/ml). The lysate was analyzed by SDS-polyacrylamide gel electrophoresis (Enprotech) 10–50% methanol solution and then incubated in Enlightning (DuPont) for 30 min with rocking. Bands in dried gels were detected by autoradiography.

RESULTS AND DISCUSSION

Establishment of p75 TNFR Protein Expression in Human Neuronal Cells and Its Inhibition by ASO—We used RA-differentiated SH-SY5Y cells, considered to represent human neuronal-type cells, as our in vitro model. Results from RT-PCR demonstrated that both the p55 and p75 TNFRs were detected at the message level (Fig. 1) in RA-differentiated SH-SY5Y cells. The sequences of two excised DNA bands of both TNFRs are identical to published ones (data not shown). Western blotting or immunoprecipitation studies have shown that these receptors were expressed at the protein level (Fig. 2a, p75 TNFR; Fig. 2b, p55 TNFR). These results are consistent with a report that another human neuronal-like cell line (SKNBE) expresses endogenous TNFRs (21). To investigate a potential role of p75 TNFR in neuronal cell survival, we synthesized phosphorothioate-modified ASO for p75 TNFR in which the nucleotide sequence overlaps the code for the methionine initiation site on the human p75 TNFR gene (14). We confirmed by immunoprecipitation that treatment of RA-differentiated SH-SY5Y cells with ASO (5–10 μM) directed at the p75 TNFR blocked expression of p75 TNFR (Fig. 2a, ASO-1 and ASO-2). Expression of p75 TNFR was almost completely inhibited by ASO at 10 μM (Fig. 2a, ASO-2) compared with that at 5 μM (Fig. 2a, ASO-1). Moreover, a number of studies were conducted to establish the specificity of the ASO treatment (22, 23). Control experiments in RA-differentiated SH-SY5Y cells showed that expression of p75 TNFR protein was not affected in cells treated with hypoxia or Aβ(1–42) alone, sense oligonucleotides (SO) alone, or co-treated with SO and hypoxia or Aβ(1–42) (Fig. 2a). Furthermore, protein expression of the p55 TNFR was not affected in RA-differentiated SH-SY5Y cells following treatment with the p75-directed ASO (Fig. 2b).

Increased Cell Degeneration Induced by hTNF-α—To assess whether oligonucleotide treatments altered cell viability under basal conditions, the levels of LDH released from RA-differentiated SH-SY5Y cells treated with ASO and from cells treated with the corresponding SO for p75 TNFR were compared to non-treated control levels. No significant differences in LDH levels were detected following either ASO or SO for p75 TNFR treatment (10 μM) compared with the no treatment control (Fig. 3). Furthermore, no significant differences in LDH levels were observed in the cells treated with either ASO or SO for p55 TNFR treatment (10 μM) compared with the no treatment control (data not shown). Moreover, co-treatment of RA-differentiated SH-SY5Y cells with ASO for p75 TNFR (10 μM) and hTNF-α (20 ng/ml) produced significantly higher levels of LDH release compared to hTNF-α treatment alone (Fig. 3). Co-treatment with SO and hTNF-α did not affect the level of LDH release relative to hTNF-α treatment alone (Fig. 3). To support the findings that inhibition of p75 TNFR expression increases vulnerability of the cell to hTNF-α, we added anti-human p75 TNFR monoclonal neutralizing antibody into the cells to neutralize the biological activity of human p75 TNFR but not p55 TNFR (21, 22) and then treated the cells with hTNF-α (20 ng/ml). We found a similar increase in LDH release relative to that observed following treatment with ASO for p75 TNFR (Fig. 3).

Increased Cell Degeneration Induced by Hypoxic Injury and β-Amyloid—RA-differentiated SH-SY5Y cells exposed to oxygen deprivation for 6 h displayed morphological changes, e.g. swollen cell bodies (data not shown), and produced increased levels of LDH release relative to controls (Fig. 3). Treatment of ASO for p75 TNFR during the 6 h of hypoxic exposure resulted in a significant increase in LDH release compared with hypoxia alone or treatment with SO under the identical hypoxic condi-
Knockout of p75 TNF Receptor Increases Human Neuron Degeneration

![Graph 3. Inhibition of antisense oligonucleotides for p75 TNFR increased neuronal cell death induced by TNF-α and hypoxic injury. ASO, antisense oligonucleotides for p75 TNFR (10 μM); SO, sense oligonucleotides for p75 TNFR (10 μM); Ab, anti-p75 human TNFR neutralizing antibody (1.0 μg/ml, R & D Systems); hTNF-α, 20 ng/ml; Hypoxia, oxygen deprivation for 6 h; hTNF-α + SO, co-treated with hTNF-α (20 ng/ml) and sense oligonucleotides for p75 TNFR (10 μM); hTNF-α + Ab, co-treated with hTNF-α (20 ng/ml) and antisense oligonucleotides for p75 TNFR (10 μM). hTNF-α + Ab + SO, co-treated with hTNF-α (20 ng/ml) and anti-p75 human TNFR neutralizing antibody (1.0 μg/ml); Hypoxia + SO, cells were oxygen deprived for 6 h and treated with sense oligonucleotides for p75 TNFR (10 μM); Hypoxia + ASO, cells were oxygen deprived for 6 h and treated with antisense oligonucleotides for p75 TNFR (10 μM); Hypoxia + Ab, cells were oxygen deprived for 6 h and treated with anti-p75 human TNFR neutralizing antibody (1.0 μg/ml). SH-SY5Y cells were cultured and differentiated as described previously (12), and treatment was conducted in serum-free, N2-supplemented 50% minimum Eagle’s medium and 50% F-12 medium. LDH was measured at 48 h, and values represent the mean ± S.D. from five independent experiments each conducted in triplicate (asterisk, * p < 0.05 versus vehicle (where vehicle = no treatment or ASO, or SO treatment alone); double asterisk, ** p < 0.01 versus vehicle, analysis of variance). Preparations of ASO and hypoxic exposure are briefly described under “Experimental Procedures.”](image)

![Graph 4. Inhibition of antisense oligonucleotides for p75 TNFR increased neuronal cell death induced by Ab toxicity. ASO, antisense oligonucleotides for p75 TNFR (10 μM); SO, sense oligonucleotides for p75 TNFR (10 μM); Ab(1–42), 5 μM; Ab(1–42) + ASO, co-treated with Ab(1–42) (5 μM) and antisense oligonucleotides (10 μM); Ab(1–42) + SO, co-treated with Ab(1–42) (5 μM) and sense oligonucleotides (10 μM).](image)

homology suggests that distinct functions may be associated with each of these two receptors. Thus, TNF-α can elicit either a trophic or toxic response in neurons, which could be dependent on which TNFR receptor subtype is activated. Recent studies indicate that the p55 TNFR is responsible for cell death signaling through activation of phospholipase A2 and NF-κB (28). Recent studies reported that knocking out both p55 and p75 TNF receptors increased neurodegeneration induced by ischemia in mouse brain (29). Our results are the first to indicate that the p75 TNFR may protect human neurons against insults, e.g. exposure to hypoxia or β-amyloid. Thus when a neuron is insulted, TNF-α may be released to activate the p75 TNFR (30, 31), which in turn may trigger a series of cellular events, e.g. secretion of trophic factors (32), attenuation of calcium disruption, or free radical formation (12), that enables the cell to defend itself against the insult. Therefore, p75 TNFR may be necessary for neuronal cell survival under pathological conditions following injury, and conditions that attenuate levels or functioning of the p75 TNFR may render cells more vulnerable to insult and disease.

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Neurons, akin to a number of other cell types, can express both p55 and p75 TNFRs (19, 26, 27); the genes for these receptors are located on human chromosomes 12 and 1, respectively. Based on amino acid sequencing, there is a 28% overall homology between p55 and p75 TNFRs. The amino acid sequences in the extracellular domains, transmembrane region, and intracellular domains between these two TNFR subtypes share 22, 28, and 10% homologies, respectively. The low level of
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