p75^{NTR} Mediates Neurotrophin-Induced Apoptosis of Vascular Smooth Muscle Cells

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**The development of atherosclerotic lesions results
from aberrant cell migration, proliferation, and ex-
tracellular matrix production. In advanced lesions,
however, cellular apoptosis, leading to lesion remod-
eling, predominates. During lesion formation, the
neurotrophins and the neurotrophin receptor ty-
rosine kinases, trks B and C, are induced and mediate
smooth muscle cell migration. Here we demonstrate
that a second neurotrophin receptor, p75^{NTR}, is ex-
pressed by established human atherosclerotic lesions
and late lesions that develop after balloon injury of
the rat thoracic aorta. The p75^{NTR}, a member of the
tumor necrosis factor/FAS receptor family, can mod-
ulate trk receptor function as well as initiate cell death
when expressed in cells of the nervous system that
lack kinase-active trk receptors. p75^{NTR} expression
colocalizes to neointimal cells, which express smooth
muscle cell α-actin and are expressed by cultured
human endarterectomy-derived cells (HEDC). Areas
of the plaque expressing p75^{NTR} demonstrate in-
creased TUNEL positivity, and HEDC undergo apopto-
sis in response to the neurotrophins. Finally, neuro-
trophins also induced apoptosis of a smooth muscle
cell line genetically manipulated to express p75^{NTR},
but lacking trk receptor expression. These studies
identify the regulated expression of neurotrophins
and p75^{NTR} as an inducer of smooth muscle cell apo-
ptosis in atherosclerotic lesions.   *(Am J Pathol 2000,
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The neurotrophins are a family of highly conserved
growth factors best characterized for their critical roles in
the differentiation and survival of neurons, via activation
of two classes of receptors. The trk receptor tyrosine
kinases, including trk A, trk B, and trk C, bind selectively
to distinct neurotrophins, with nerve growth factor (NGF)
binding trk A, brain-derived neurotrophic factor (BDNF)
binding trk B, and neurotrophin-3 (NT-3) binding trk C.

Neurotrophin activation of trk receptors confers survival
functions in neurons[1] and migration and proliferation
functions in other cell types.[2–5] In addition to kinase-
active isoforms, trk B and trk C genes encode truncated
isoforms, generated by alternative splicing,[6,7] which in-
hibit intracellular signaling when coexpressed with the full
length trk isoform.[8] This observation has been extended
to *in vivo* studies, as transgenic mice overexpressing the
truncated trk C receptor exhibit a phenotype similar to
that observed with targeted deletion of kinase active trk C
or NT-3.[9]

All neurotrophins can also bind to the second class of
neurotrophin receptor, p75^{NTR}, a member of the tumor
necrosis factor (TNF) receptor superfamily, whose mem-
bers also include the p55 TNF receptor (TNF-R1) and
FAS. Like the TNF and FAS receptors, p75^{NTR} has a
cysteine-rich extracellular domain and an intracellular do-
main that lacks enzymatic activity but encodes a so-
called death domain, a structural domain necessary for
TNF- and FAS-induced apoptosis.[10] Two opposing bio-
logical activities have been proposed for p75^{NTR}. First,
p75^{NTR} can act as an accessory receptor for trk A, in-
creasing the binding of NGF to trk A[11] and thereby en-
hancing NGF-mediated neuronal survival. Alternatively,
ligand-induced activation of p75^{NTR} can induce apopto-
sis, or programmed cell death, of certain classes of neu-
ronal and glial cells, under conditions where trk activation
is reduced or absent.[12–14]

In addition to their well characterized expression and
function in neurons, neurotrophins and trk receptors are
also expressed in non-neuronal tissues,[2,15,16] where their
biological actions are less well defined. Their activities
have been best described in the developing cardiovas-
cular system, where reduced trk C and trk B signaling
results in abnormal cardiac[16] and intramyocardial vessel
development, respectively.[49] Moreover, neurotrophins
and trk receptors are expressed in human atherosclerotic
lesions and in early lesions that develop after balloon
de-endothelialization of the rat aorta, where they mediate

chemotactic actions on vascular smooth muscle cells.[2,5] In the rat model of vascular injury, however, a switch from kinase-active trk isoforms to truncated trk isoforms is observed in later lesions,[2] which may limit the chemotactic activity of kinase-active trk receptors, and modulate neurotrophin responsiveness as the lesion continues to develop.

In response to vascular injury, medial smooth muscle cells migrate into the intima, where they proliferate and secrete extracellular matrix, resulting in the formation of a neointimal lesion. As the lesion continues to develop, however, areas of decreased cellularity are evident within the sclerotic regions of the plaque, with little appearance of cellular debris.[17] It is now recognized that apoptosis contributes to the extensive remodeling that occurs in vascular lesions; ultimately, the size and cellularity of vascular lesions will be dependent on the migration and proliferation of smooth muscle cells countered by their subsequent apoptosis. However, the molecular mechanisms regulating apoptosis in vascular lesions are incompletely understood.

Although the expression of trk receptors and the neurotrophins in vascular lesions has been examined,[2] the expression and biological actions of p75[NTR] have not been assessed. Thus, we sought to determine whether p75[NTR] is expressed in neointimal lesions and to define the biological consequences of p75[NTR] activation in vascular smooth muscle cells. Our results demonstrate that p75[NTR] is expressed exclusively in neointimal smooth muscle cells and that binding of the neurotrophins to p75[NTR] induces smooth muscle cell apoptosis. Thus, we have identified a new receptor regulating apoptosis of neointimal smooth muscle cells and contributing to the remodeling of atherosclerotic lesions.

Materials and Methods

Animal Surgery and Tissue Preparation

In Institutional Animal Care and Use Committee-approved studies, 3-month-old male Sprague-Dawley rats were anesthetized by intraperitoneal injection of ketamine/xylazine. Injury to the abdominal and thoracic aorta was performed using a balloon embolectomy catheter, as previously described,[2] using 3 to 5 animals per experimental group. In brief, a 2 French catheter was introduced into the left carotid, advanced to the abdominal aorta, inflated, and withdrawn. This procedure was repeated three times. Control animals were subjected to anesthesia and preparative surgery without vascular injury. After 5 and 14 days, the animals were sacrificed and tissues were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. Human endarterectomy specimens were obtained as waste surgical material, under Institutional Review Board-approved protocols, from patients undergoing surgical endarterectomy at the New York Presbyterian Hospital. Specimens were cryopreserved in 30% sucrose/OCT (1:1) within 1 hour of retrieval from the patient. Investigators were unaware of

patient identifiers. Five specimens were analyzed and gave similar results.

Cell Culture

Mouse smooth muscle cells (TsTmSMC) were cultured from aortic explants of a transgenic mouse line expressing a temperature-sensitive SV40-T antigen, under the control of the promoter for smooth muscle cell α-actin.[5] TsTmSMC grown at 33°C were transfected, using the CaPO$_4$ method, with pMex vector containing the cDNA for human p75[NTR].[18,19] After selection in G418 (1 mg/ml; Gibco BRL, Rockville, MD), colonies were subcloned, expanded, and tested for stable expression of p75[NTR] by Western blot analysis (see below). Purified clonal populations of TsTmSMC expressing p75[NTR] were isolated by Dynal-bead selection using anti-p75[NTR] antisera. In brief, cells were incubated with a monoclonal antibody directed against the extracellular domain of p75[NTR] (ME 20.4[20]) for 1 hour at 4°C. After washing, the cells were incubated with goat anti-mouse IgG bound to superparamagnetic polystyrene beads (M45; Dynal, Lake Success, NY) for 20 minutes at 4°C. p75[NTR]-expressing cells were selected using a Dynal magnetic particle concentrator. In all experimental protocols, the cells were seeded and cultured overnight at 33°C, after which the cells were cultured for 3 days at 39.5°C in media containing 10% fetal calf serum (FCS). A previously established cell line of TsTmSMCs stably expressing trk A (TrkA48-TsTmSMC), generated by transfection of TsTmSMC with the pMEX vector containing the cDNA for trk A, was cultured as described.[5]

Human endarterectomy-derived cells (HEDC) were cultured from explants of primary human endarterectomy lesions as previously described.[21–23] Total RNA was isolated from cells within 2 to 4 passages (4–6 weeks) by extraction in guanidinium isothiocyanate followed by phenol/chloroform extraction.[24,25]

Immunohistochemistry

Immunohistochemistry was performed after selected slides were stained with hematoxylin and eosin to identify lesions. Serial sections of rat aortic lesions were deparaffinized and endogenous peroxidase activity quenched with 3% H$_2$O$_2$ in methanol for 15 minutes at room temperature. Cryopreserved sections of human endarterectomy were air-dried onto microscope slides and prefixed with 3% paraformaldehyde for 5 minutes. Endogenous hydrogen peroxidase was quenched as above. Adjacent sections were then incubated with either the anti-p75[NTR] antibody (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), anti-full length trk B antibody (goat polyclonal; directed against the carboxy terminus of trk B); anti-truncated trk B antibody (rabbit polyclonal; Santa Cruz Biotechnology); anti-full length trk C antibody (rabbit polyclonal directed against carboxy terminus against trk C; Santa Cruz Biotechnology) anti-truncated trk C antisera (rabbit polyclonal[2]), anti-smooth muscle cell α-actin antibody (monoclonal, Clone 1A4, Dako Corp., Carpinte-

ria, CA), anti-CD31 antibody (Hec-7[26] kindly provided by Dr. William Muller, Weill Medical College of Cornell University) or anti-macrophage antibody, (HAM 56 mouse monoclonal; DAKO Corp.). Sections were incubated with antibody preabsorbed with blocking peptide (anti-p75^{NTR}, anti-full length trk B and trk C and anti-truncated trk B), preimmune serum (anti-truncated trk C) or purified mouse IgG (anti-smooth muscle cell α-actin, anti-CD 31 and HAM 56; Santa Cruz Biochemicals) as a negative control. Following incubation with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), immunoreactive proteins were detected using an avidin-biotin-based horseradish peroxidase system using Vector VIP solution as a chromogenic substrate (Vector Laboratories), followed by counterstain with hematoxylin.

Detection of Apoptotic Smooth Muscle Cells in Human Atherosclerosis

Cryopreserved sections of human endarterectomy were air-dried onto microscope slides and postfixed with 3% paraformaldehyde for 10 minutes, followed by incubation with 0.1% sodium citrate containing 0.1% Triton at 4°C. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells were detected using a kit purchased from Roche Biochemicals (Indianapolis, IN). Briefly, sections were incubated at 37°C for 1 hour with fluorescein-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Control sections were incubated with fluorescenated dUTP in the absence of TdT. The sections were then incubated with anti-fluorescein antibody conjugated with alkaline phosphatase for 30 minutes at 37°C. TUNEL-positive cells were detected using Fast Red (Roche Biochemicals) as a chromogenic substrate.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One microgram of total RNA isolated from HEDC was subjected to reverse transcription using murine leukemia virus-reverse transcriptase (Perkin-Elmer, Norwalk, CT). Total RNA not incubated with reverse transcriptase was used as a negative control. The cDNA products were incubated with Ampli*Taq* polymerase (Perkin-Elmer) and primers specific for human p75^{NTR} (Whitehead Institute for Biomedical Research, Cambridge, MA). Primers were designed using the Primer 3 program and published sequences.[19]

p75^{NTR} (human) sense 5′ AGC CTT CAA GAG GTG GAA CA 3′

p75^{NTR} (human) antisense 5′ CTG CAC AGA CTC TCC ACG AG 3′

After a 95°C hot start, cycling proceeded for 40 cycles of 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C. Products were resolved by electrophoresis in a 1% agarose gel, followed by visualization with ethidium bromide. The primer pair yields an oligonucleotide product

of 463 bp. PCR using primer pairs against the unique exon expressed at the carboxy terminus of either truncated trk B, truncated trk C, or primer pairs directed against the kinase domains of trk B or trk C was performed as described.[27] RNA isolated from either p75-TsT-mSMC or adult bone marrow cells was used as a positive control for p75^{NTR} and trk receptors,[27] respectively.

Western Blot Analysis

Native or p75-expressing TsTmSMC were lysed in radio-immunoprecipitation assay buffer containing phenyl-methylsulfonyl fluoride (1 mmol/L), aprotinin (1 mg/ml), and leupeptin (10 mg/ml).[28] Lysates were clarified by centrifugation at 14,000 rpm (Beckman Microfuge, Palo Alto, CA) at 4°C, and the protein content of the supernatant determined by Biorad protein assay (Bio-Rad Laboratories, Richmond, CA). Lysates containing equivalent amounts of protein were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and Western blot analysis was performed using either anti-p75^{NTR} rabbit polyclonal antisera (Promega, Madison, WI), a rabbit polyclonal antiserum that recognizes all full length trk isoforms (203 antisera[29]) or a rabbit polyclonal antibody specific for murine FAS receptor (M-20, Santa Cruz Biotechnology). Immunoreactive proteins were detected using enhanced chemiluminescence (ECL) detection (Amersham Corp., Arlington Heights, IL).

Annexin V Binding

Native TsTmSMC, trk A, or p75-expressing TsTmSMC (100,000 cells/well) or HEDC (30,000/well) were seeded in six-well tissue culture plates and cultured for 2 to 3 days in 10% FCS media at 39.5°C (TsTmSMC cell lines) or 37°C (HEDC). Native p75 and trk A expressing TsTmSMC were cultured overnight in 1% FCS media, then treated with either NGF, NT-3, BDNF (each at 2–4 nmol/L), or a monoclonal antibody which cross-links and activates the murine FAS receptor (Clone Jo2 100 ng/ml; PharMingen, San Diego, CA).[30] HEDC were cultured in 0.5% FCS in the presence or absence of either the neurotrophins or an antibody which binds and activates human FAS (Clone CH11 100 ng/ml; Upstate Biotechnology, Lake Placid, NY).[31] At the indicated time points, cells were suspended using PBS/EDTA and washed in serum-free Dulbecco's modified Eagle's medium (DMEM). Annexin V binding was determined by incubating the cells with fluorescein isothiocyanate (FITC)-conjugated annexin V (Immunotech, Miami, FL) in DMEM containing 1.5 mmol/L Ca^{2+} on ice for 10 minutes. After washing to remove unbound annexin V, the cells were incubated with propidium iodide and the cell samples were analyzed by flow cytometry using a Coulter Elite Flow Cytometer. Optimal parameters for flow cytometric analysis were set using control cells not incubated with FITC-annexin V and propidium iodide.
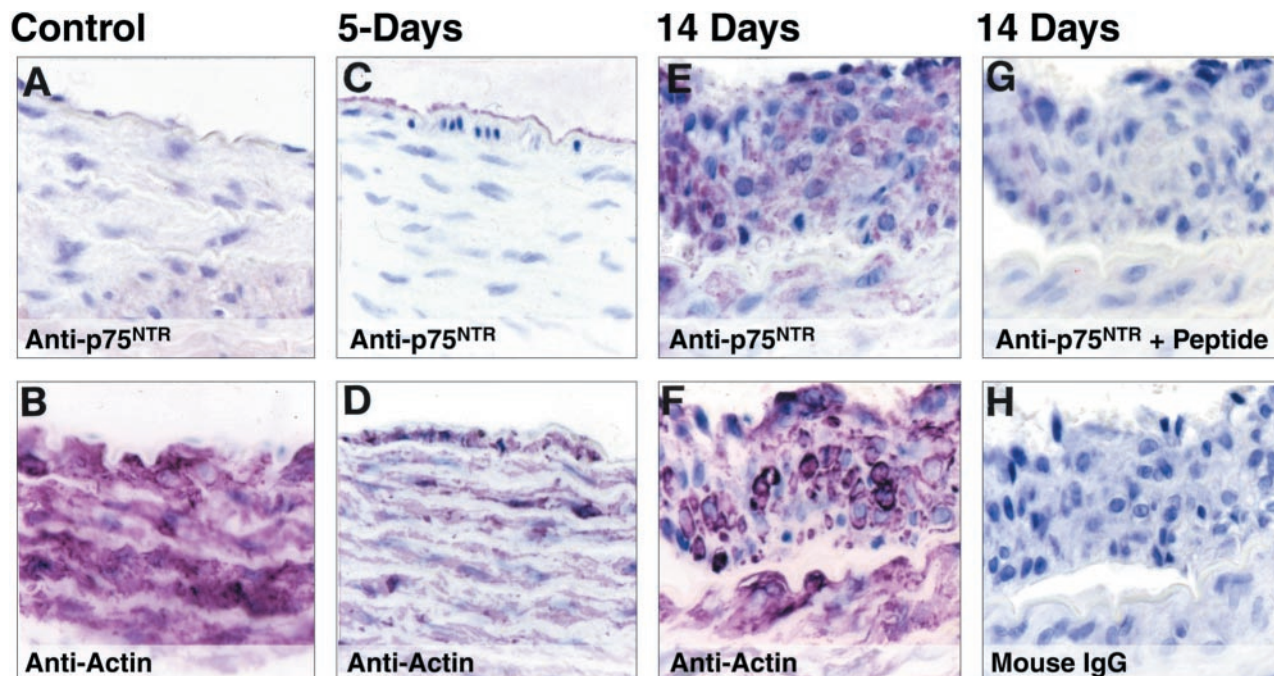
Figure 1. Immunohistochemical analysis of p75^NTR in vascular lesions of the rat thoracic aorta 2 weeks after balloon de-endothelialization. Sections of rat thoracic aorta from either control animals (**A** and **B**), or 5 (**C** and **D**) and 14 days (**E** and **F**) after balloon de-endothelialization were incubated with the indicated antibodies. Immunoreactive proteins were visualized using avidin-biotin-based horseradish peroxidase kit using Vector VIP as a chromogenic substrate; the sections were counterstained with hematoxylin. Original magnification, ×90.

TUNEL Staining

Native TsTmSMC, p75-TsTmSMC, or trk A48-TsTmSMC were seeded on 8-chamber permanox slides and cultured for 3 days at 39.5°C. HEDC were seeded on 8-chamber glass slides and cultured overnight at 37°C. The cells were rinsed with PBS and treated with NGF, NT-3, or BDNF (each at 4 nmol/L) or anti-FAS activating antibody (Jo2; 100 ng/ml, murine cells only) in DMEM containing 0.5 to 1% FCS. After 24 hours, the cells were fixed in 3% paraformaldehyde and the TUNEL assay was performed by incubation with fluoroscein-labeled dUTP in the presence of TdT (Boehringer Mannheim, Chicago, IL). Using fluorescent microscopy, the results were quantified by counting the number of TUNEL-positive cells per high-power field in 10 to 15 fields, to assess 1000 cells per well.

Caspase Activation

p75-TsTmSMC were seeded and treated as for TUNEL staining. Immunodetection of activated caspase 3 was performed using a rabbit antibody directed against the p20 subunit[32] (provided by Idun Pharmaceuticals, La Jolla, CA) and a goat anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes, Eugene, OR). The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Sigma Chemical Co., St. Louis, MO) and fluorescence was detected at 488 nm (FITC; Alexa green) and 368 nm (DAPI).

Results

Expression of p75^NTR in Neointimal Lesions

Although trk receptors and neurotrophins are up-regulated after vascular injury,[2] the expression of p75^NTR has not been examined in neointimal lesions. No immunodetectable p75^NTR was present in uninjured adult rat vessels (Figure 1A). p75^NTR was also not expressed in the small lesions which developed 5 days after balloon injury (1–2 cell layers composed of smooth muscle cells, as assessed by expression of smooth muscle cell α-actin; Figure 1, C and D). However, at 2 weeks after balloon injury, significant expression of p75^NTR was detectable in smooth muscle cells within a highly organized neointimal lesion (Figure 1, E and F) and in medial smooth muscle cells just adjacent to the lesion. No immunoreactive proteins were detected in adjacent sections incubated under control conditions (Figure 1, G and H). Thus, in contrast to the early expression of trk receptors after balloon injury[2] p75^NTR expression is up-regulated later in lesion development.

To assess p75 expression following long-standing vascular injury, atherosclerotic lesions obtained from human endarterectomy specimens were examined. Figure 2A shows a section of endarterectomy lesion stained by hematoxylin and eosin, demonstrating the presence of the medial smooth muscle cell layer (as determined by morphological assessment and by immunohistochemical analysis of smooth muscle cell α-actin; upper right inset labeled B-D of Figure 2, A and B), surrounding
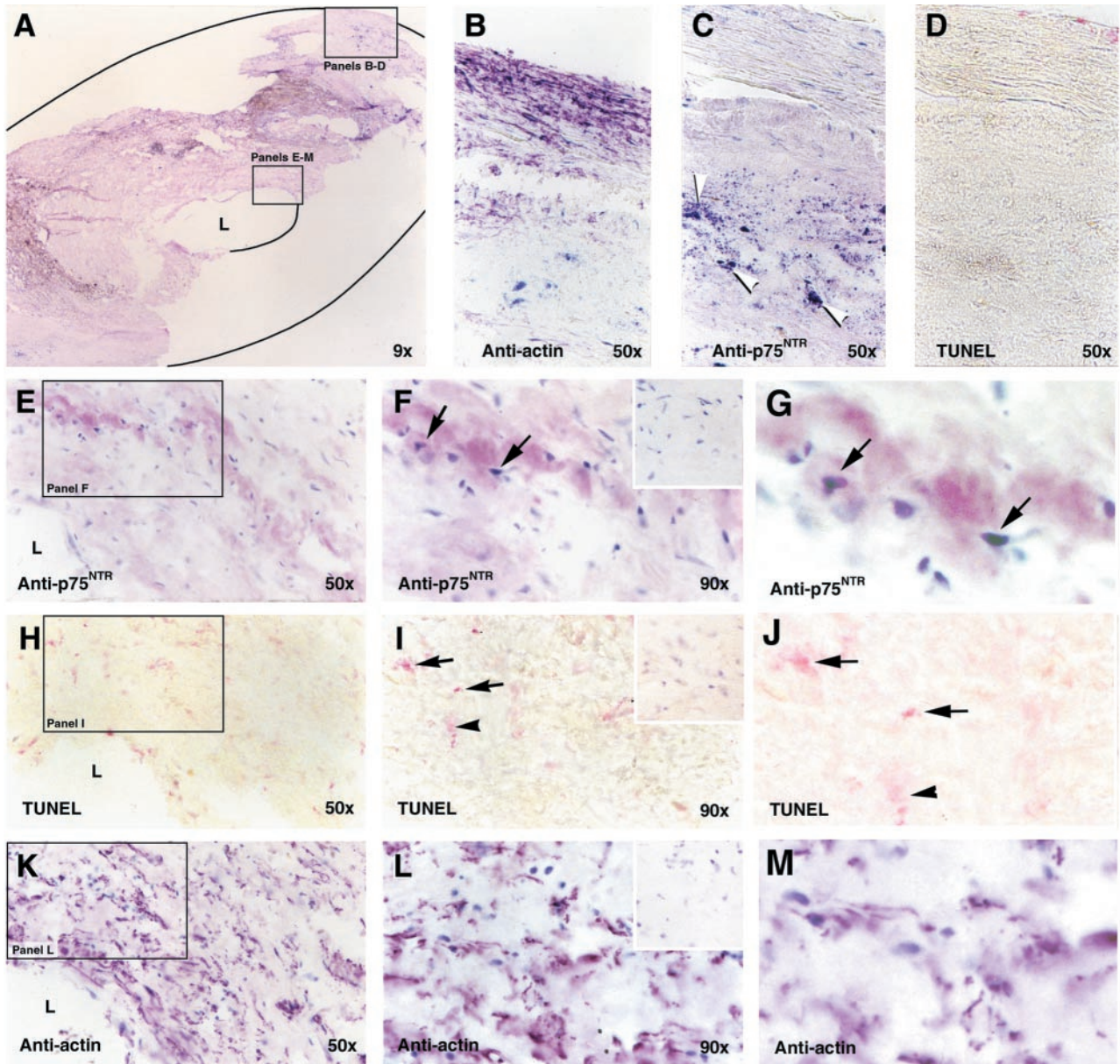
Figure 2. Immunohistochemical analysis of p75^{NTR} expression and *in situ* TUNEL analysis in vascular lesions of human atherosclerotic lesions. **A:** Hematoxylin and eosin stain of atherectomy specimen derived from a carotid artery. Panels represented by the insets are indicated. The outer black line represents where the medial smooth muscle layer would extend to form the entire cross-section of the vessel. The inner black line represents the potential boundary of the lumen of the vessel. Panels **B-M** were treated as indicated. Immunoreactive proteins were detected as in Figure 1. Areas marked off in **E, H,** and **K** are shown at higher magnification in **F, I,** and **L,** respectively. **Open arrowheads** in **C** are calcifications stained with hematoxylin. **Arrows** in **F,** cells expressing high levels p75^{NTR}, shown at higher magnification in **G; arrows** in **I,** nuclei of TUNEL-positive cells, shown at higher magnification in **J; arrowhead** in **I,** nuclei of TUNEL-positive cells sectioned obliquely, shown at higher magnification in **J. M,** magnified area of **L** represented in **G** and **J. Insets: F,** control section incubated with anti-p75^{NTR} preabsorbed with blocking peptide; **J,** control section incubated with fluorescenated-dUTP in the absence of TdT; **L,** control section incubated with mouse IgG.

a highly cellular plaque. Using serial sections, p75^{NTR} immunoreactivity was present in regions of the plaque that exhibited prominent expression of smooth muscle cell α-actin; see Figure 2, E-G (anti-p75^{NTR}) and K-M (anti-α-actin). Little or no immunoreactivity for the endothelial cell marker, PECAM (Hec-7) or HAM 56, a macrophage marker (results not shown) was detected in areas that stained for p75^{NTR} or smooth muscle cell α-actin. Expression was highest in neointimal smooth muscle cells within the plaque, adjacent to the lumen area (lower inset labeled E-M in Figure 2A), with very little expression

within medial smooth muscle cells (Figure 2C). Among populations of neointimal smooth muscle cells, there was variability in the expression of p75^{NTR}, with some cells expressing high levels of p75^{NTR} (arrows, Figure 2, F and G), while others demonstrated less expression (Figure 2F). No immunoreactive proteins were detected in adjacent sections incubated under control conditions (Figure 2F, inset for p75^{NTR} and inset panel L for α-actin). Immunoreactivity for full length trkB and trkC was present in subpopulations of neointimal smooth muscle cells (results not shown) similar to our previously published re-
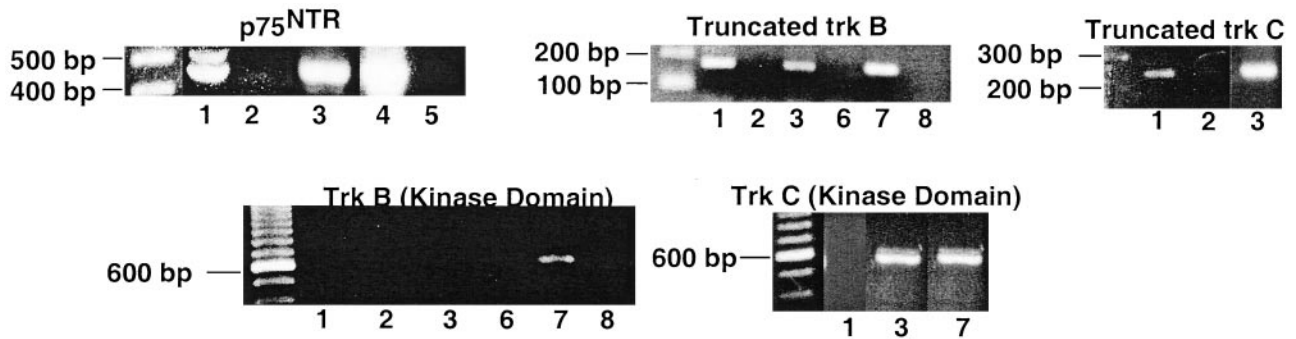
Figure 3. Expression of p75[NTR], truncated trk B, and truncated trk C mRNA in human atherectomy-derived smooth muscle cells. Total RNA was subjected to reverse transcription polymerase chain reaction and amplified products detected following cycling with p75[NTR]-, truncated trk B-, or truncated trk C-specific primer pairs and *Taq* polymerase. Sizes of amplified product: p75[NTR], 463 bp; truncated trk B, 161 bp;[27] truncated trk C, 158 bp;[27] kinase-active trk B, 571 bp;[27] kinase-active trk C, 571 bp.[27] **Lanes 1, 3–5,** and **7,** RNA subjected to reverse transcription. **Lanes 2, 6,** and **8,** RNA not subjected to reverse transcription. **Lanes 1** and **2,** HEDC-12. **Lanes 3** and **6,** HEDC-201. **Lane 4,** p75-TsTmSMC (positive control, see below). **Lane 5,** TsTmSMC. **Lanes 7** and **8,** bone marrow.

sults,[2] whereas immunostaining for either truncated trkB or truncated trkC showed little or no staining above sections incubated under control conditions (results not shown). Thus, similar to what was observed in the rat model of vascular injury, neointimal smooth muscle cells present in human atherosclerotic lesions express p75[NTR].

Colocalization of p75[NTR] with TUNEL-Positive Neointimal Smooth Muscle Cells

To determine whether p75[NTR] activation could contribute to apoptosis of neointimal smooth muscle cells, we first assessed the localization of p75[NTR] expression with TUNEL reactivity as a marker of apoptotic cells in human endarterectomy samples. TUNEL-positive smooth muscle cells were detectable throughout the neointimal lesion, but the area with the highest concentration of TUNEL-positive cells was the neointimal smooth muscle cells adjacent to the lumen, where p75[NTR] was expressed (compare Figure 2, E-G, with Figure 2, H-J). In analysis of 10 sections of 5 lesions, TUNEL-positive cells were detected in areas where p75[NTR] was expressed. In contrast, very little TUNEL positivity was detected in medial smooth cells or in acellular areas of the plaque just below the medial smooth muscle cell layer (Figure 2D). Sections incubated with fluorescentated dUTP in the absence of TdT confirmed the specificity of the TUNEL detection (Figure 2I, inset). These results suggest p75[NTR] is expressed in areas where neointimal smooth muscle cells are undergoing apoptosis in human atherosclerotic lesions.

Apoptosis of Atherectomy-Derived Smooth Muscle Cells

As our prior studies indicate that neurotrophins are expressed by human endarterectomy lesions,[2] we assessed whether ligand-induced activation of p75[NTR] induces apoptosis of neointimal cells, using cultured cells derived from explants of HEDC.[2,21] Previous studies demonstrated the expression of smooth muscle cell α-actin by these cells under culture conditions.[21] HEDC ex-

pressed mRNA for p75[NTR], as well as truncated trk B and truncated trk C, as assessed by RT-PCR (Figure 3). Neither cell line expressed mRNA for kinase active trk B, whereas one of two cell lines expressed mRNA for kinase active trk C. Apoptosis was assessed by two established techniques: annexin V binding to phosphatidyl serine exposed on the outer membrane of apoptotic cells (Figure 4A) or TUNEL staining for fragmented DNA (Figure 4B). Flow cytometric analysis of annexin V binding revealed that HEDC cultured in media containing 0.5% FCS undergo low levels of spontaneous apoptosis, with 3% of the cells in the early stages (quadrant 4) and 10% in the later stages (quadrant 2) of the apoptotic pathway (Figure 4A). Twenty-four hours after treatment with either NGF, BDNF, or NT-3, (at 2 nmol/L [50 ng/ml] concentration) the number of apoptotic cells increased three- to fourfold for both early and late stage populations of apoptotic cells. A similar response was observed on activation of the FAS receptor. In contrast, cells cultured in 10% FCS exhibit a similar level of annexin V binding as cells cultured under control conditions. TUNEL assays to detect fragmented DNA *in situ* confirmed the apoptotic activity of the neurotrophins on HEDC, where in the absence of the ligand, 2.5 ± 0.7% of HEDC were TUNEL-positive, and this increased by two- to threefold after treatment with NGF (Figure 4B). These results demonstrate that nanomolar concentrations of neurotrophins can induce apoptosis of HEDC, potentially through binding and activation of p75[NTR].

Derivation of p75[NTR] Expressing TsTmSMC

Although primary cultures of HEDC undergo neurotrophin-induced apoptosis while expressing p75[NTR] and truncated trk receptors, we sought to further analyze this activity in smooth muscle cells with known patterns of expression of neurotrophins and neurotrophin receptor isoforms. Thus, we used an established, conditionally immortalized temperature-sensitive mouse smooth muscle cell line (TsTmSMC). Previous studies from our laboratory demonstrated that TsTmSMC expressed mRNA for NGF, BDNF and NT 4/5,[5] but do not express full length or
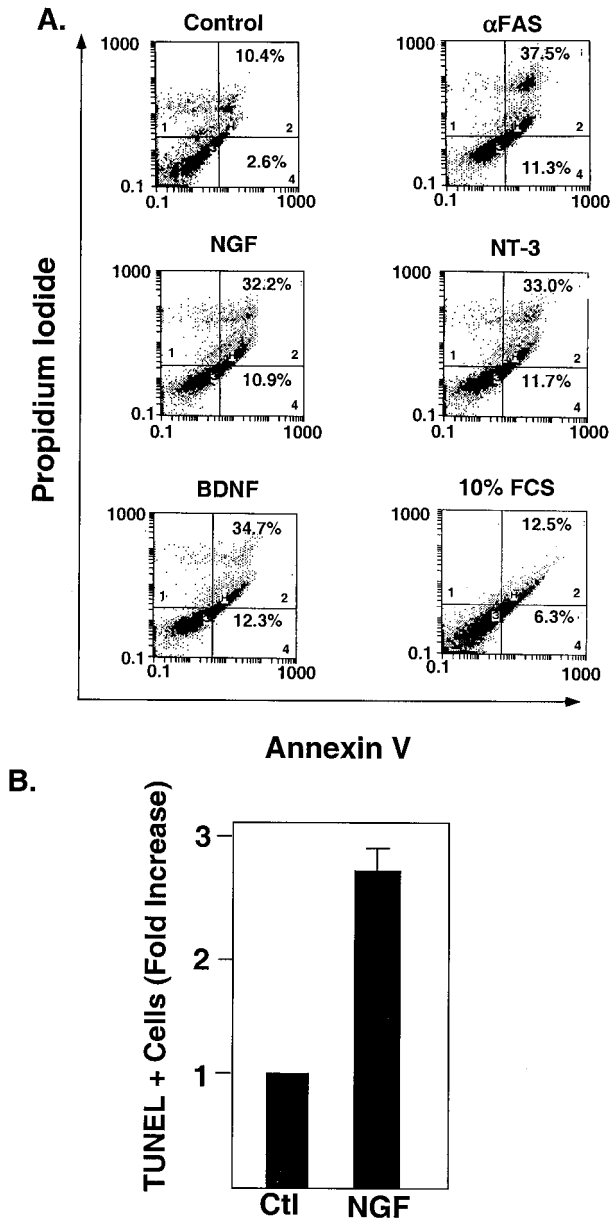
Figure 4. Neurotrophin-induced apoptosis of HEDC. **A:** Flow cytometric analysis of annexin V binding to HEDC. HEDC were treated with either NGF (2 nmol/L), NT-3 (2 nmol/L), BDNF (2 nmol/L), FAS-activating antibody (CH11; 100 ng/ml), or 10% FCS. After 24 hours, a cell suspension was prepared and the cells were stained with FITC-conjugated annexin V and propidium iodide as described in Methods, and flow cytometric analysis was performed. x axis, FITC-conjugated annexin V binding; y axis, propidium iodide incorporation. As has been previously demonstrated,[47,48] cells in quadrant 3 (lower left corner) represent live cells that do not bind annexin V or incorporate propidium iodide. Cells in quadrant 4 (lower right corner) represent cells in the early phases of apoptosis, in which annexin V binds to phosphatidyl serine exposed on the outer surface of apoptotic cells, but there is no incorporation of propidium iodide because membrane integrity is maintained. Cells in quadrant 2 (upper right corner) represent cells in the later stages of apoptosis, which bind annexin V and incorporate propidium iodide due to a loss of membrane integrity. Cells in quadrant 1 represent cells that have completed the apoptotic pathway. Histogram tracings from 1 of 3 duplicate experiments using 2 different HEDC culture lines is shown. Quadrants 1, 2, and 4 are labeled in the lower left (quadrant 1) and lower right (quadrants 2 and 4) corners. The percentage of the total cell population in either quadrant 2 (late stage, secondary necrotic) or quadrant 4 (early apoptotic phase) are given in the appropriate quadrant. **B:** Quantitative analysis of TUNEL for HEDC. In each experiment there were two replicates per treatment group. Data are the mean ± SE of 3 separate experiments using 3 different cell lines.
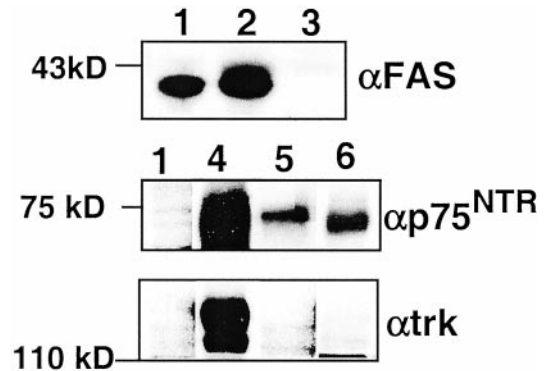
**Figure 5.** Western blot analysis of p75^NTR, trk, and FAS in native TsTmSMC and p75-TsTmSMC. For FAS Western blot analysis African green monkey kidney cells and the murine lymphocyte cell line A20 are used as a negative and positive control, respectively. The pheochromocytoma cell line PC12, genetically manipulated to overexpress trk receptors,[28] was used as a positive control for trk and p75^NTR Western blot analysis. This cell line expresses approximately 40,000 trk receptors and 40,000 p75^NTR cell. **Lane 1**, TsTmSMC. **Lane 2**, A20 cells. **Lane 3**, COS cells. **Lane 4**, PC12. **Lane 5**, p75-TsTmSMC. **Lane 6**, p75-TsTmSMC-2.

truncated trk receptors, or p75^NTR, as assessed by Western blot analysis ([5] and Figure 5). Additionally, native TsTmSMC expressed FAS (Figure 5). After transfection with a cDNA encoding human p75^NTR, 15 clones stably expressing p75^NTR were isolated. Clonal populations expressing 20–70,000 p75^NTR receptors/cell, were identified and expanded (Figure 5).[28] Purified populations of p75-TsTmSMC were obtained by immunoseparation to yield populations in which greater than 95% of the cells expressed high levels of p75^NTR, as assessed by immunohistochemistry (results not shown). The biological activity of the neurotrophins was examined using three stable-enriched clones expressing p75^NTR cultured for 3 to 4 days at 39.5°C to reduce the expression of T antigen and induce a less transformed phenotype, as previously described.[5] All 3 clones yielded similar results.

Neurotrophins Induce Apoptosis of p75 TsTmSMC

Neurotrophin-induced apoptosis of p75-TsTmSMC was first assessed by annexin V binding. Cultured TsTmSMC, and clones stabilizing expressing either p75^NTR or, as a control, trk A, exhibited variable but similar rates of spontaneous apoptosis (Figure 6; ranges, 3.3–25.4%, 4.0–12.7%, and 12.5–19.2%, respectively; in 3 separate experiments per cell line). Treatment of p75-TsTmSMC with nanomolar concentrations of NGF or NT-3 caused a dose dependent increase in annexin V binding, in a response comparable to that elicited by activation of FAS (Figure 6). The addition of 4 nmol/L of either NGF or NT-3 resulted in an eight-to-ninefold increase in the number of cells in the early phases of apoptosis (quadrant 4), whereas 2 nmol/L caused a four- to fivefold increase. BDNF was less effective at inducing apoptosis of p75TsTmSMC, inducing only a four- to fivefold increase in annexin V binding in doses ranging from 1 to 4 nmol/L. All three neurotrophins increased by two- to threefold the number of cells in the later stages of apoptosis (quadrant 2). Treatment with 4
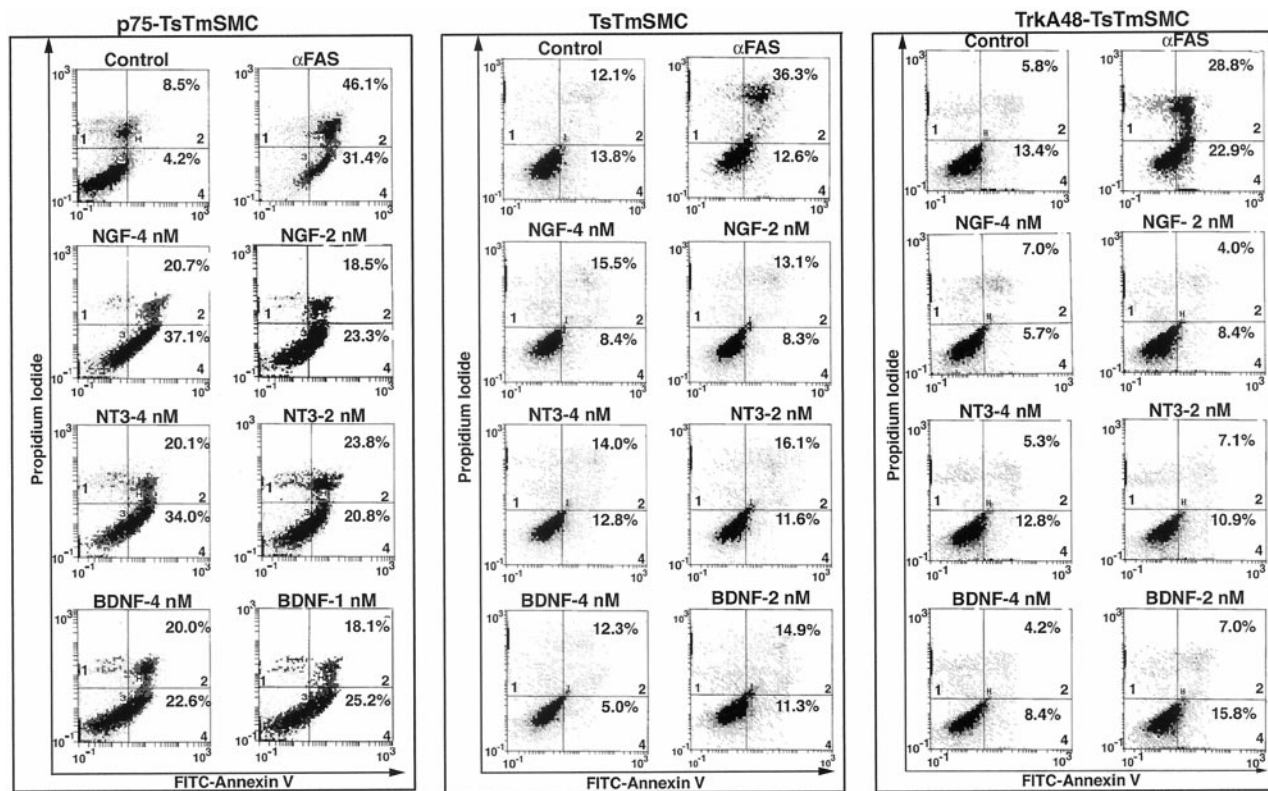
Figure 6. Flow cytometric analysis of annexin V binding to p75-TsTmSMC, native TsTmSMC, and trk48A-TsTmSMC. TsTmSMC cell lines were grown for 3 days at 39.5°C followed by treatment with the neurotrophins at the indicated doses. After 15 hours, a cell suspension was prepared and the cells were stained with FITC-conjugated annexin V and propidium iodide, as described in Methods, and flow cytometric analysis was performed. Histograms from one of three duplicate experiments for each cell line is shown. Quadrants 1, 2, and 4 are labeled in the lower left (quadrant 1) and lower right (quadrants 2 and 4) corners. The percentage of cells in the early and late stages of apoptosis are indicated in quadrants 4 and 2, respectively.

nmol/L of the neurotrophins for 24 hours increases the percentage of cells present in quadrant 1 (results not shown). In contrast, the neurotrophins did not induce an increase in annexin V binding to TsTmSMC (Figure 6), whereas the FAS activating antibody induced a similar effect as observed in p75-expressing TsTmSMC. Thus, the expression of p75^{NTR} results in neurotrophin-induced apoptosis of vascular smooth muscle cells.

To differentiate between p75^{NTR}-mediated apoptosis and the actions of neurotrophins on trk-expressing smooth muscle cells, we assessed the apoptotic activity of NGF using a previously established stable clone of trkA48-TsTmSMC.[5] TrkA48-TsTmSMC displayed a similar level of basal apoptotic activity as observed with either native or p75-TsTmSMC (Figure 6). However, although FAS activation induced a two- to fourfold increase in annexin V binding, similar to the results with native TsTmSMC, the neurotrophins did not increase annexin V binding, suggesting that neurotrophin-induced vascular smooth muscle cell apoptosis is dependent on p75^{NTR} expression and is not mediated by the trk receptor tyrosine kinase.

To further assess the apoptotic activity of the neurotrophins, TUNEL assays and immunofluorescence for detection of activated caspase 3 were performed. Treatment of p75-TsTmSMC with NGF, NT-3, or BDNF for 24 hours induced a two- to threefold increase in the number of TUNEL positive cells over control cells treated with media alone (Figure 7A). In contrast, neither native TsTmSMC or trkA48-TsTmSMC showed increases in TUNEL positivity after treatment with the neurotrophins (Figure 7, A and B), whereas FAS activation increased the number of TUNEL-positive cells three- to fourfold, in either native, trk A-, or p75-expressing TsTmSMC (results not shown). In separate experiments, similar increases in the number of apoptotic p75-TsTmSMC in response to the neurotrophins or anti-FAS activating antibody were observed using DAPI stain to assess chromatin condensation as index of apoptosis (Figure 8).

Activation of the caspases from inactive proenzymes mediates many of the morphological and biochemical changes associated with apoptosis. To assess if caspases are activated in neurotrophin-treated p75-TsTmSMC undergoing apoptosis, immunostaining was performed using the CM-1 antibody, which recognizes only the activated form of caspase-3 but not the inactive 32-kd proenzyme.[32] Treatment of p75-TsTmSMC with either the neurotrophins or FAS-activating antibody (Figure 8) increased CM-1 immunoreactivity compared to control, nontreated cells. Moreover, 90 to 95% of those cells expressing activated caspase 3 exhibit chromatin condensation, as detected by Hoescht staining (Figure 8). These results confirm that neurotrophin activation of p75^{NTR} induces apoptosis of smooth muscle cells through a signaling cascade that activates caspase 3.

A.

B.
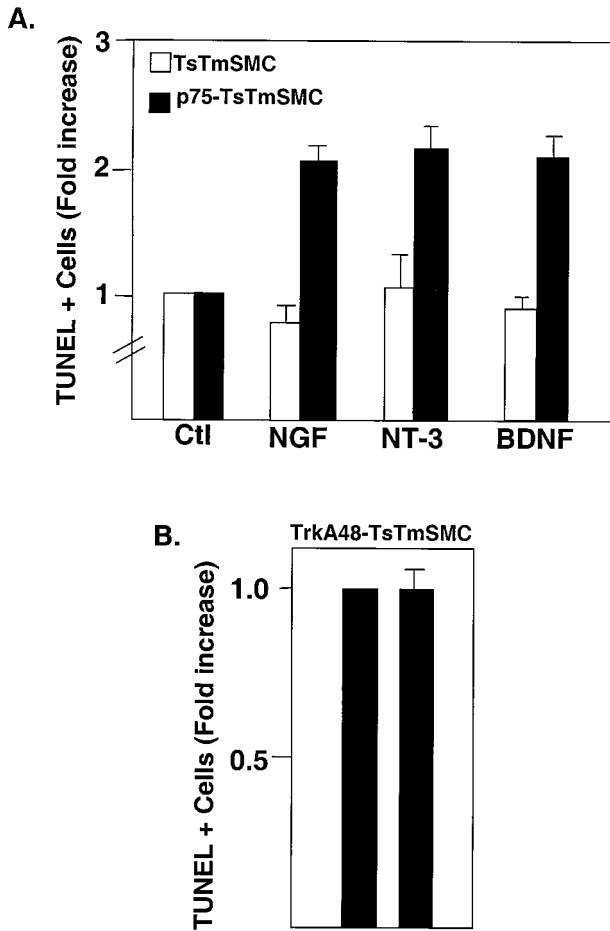
Figure 7. A: Quantitative analysis of the TUNEL assay for TsTmSMC and p75[NTR]-TsTmSMC after treatment with the neurotrophins (each 4 nmol/L). Data are presented as -fold increase of TUNEL-positive cells over control, nontreated cells and are the means ± SE of one of three duplicate experiments for both native TsTmSMC and p75-TsTmSMC. In each experiment, there were two replicates per treatment group. Percentage of TUNEL-positive cells in the absence of exogenous ligand: TsTmSMC = 2.1 ± 0.4%; p75TsTmSMC = 4.6 ± 0.9%. **B:** Quantitative analysis of TUNEL assay for trkA48-TsTmSMC. Data are presented as in **A** and are from one of two duplicate experiments, two replicates per experimental group. Percentage of TUNEL-positive cells in the absence of ligand = 1.9 ± 0.4%.

Discussion

The development of atherosclerotic and restenotic lesions is a dynamic process regulated by competing forces: the migration and proliferation of medial smooth muscle cells in the intima balanced by plaque remodeling through apoptotic cell death.[17,33] Few factors initiating apoptosis of smooth muscle cells in vascular lesions have been identified to date, although FAS is expressed in advanced atherosclerotic lesions and cultured medial smooth muscle cells undergo FAS-induced apoptosis *in vitro*[34,35] (and current results).

Our current results identify the p75 neurotrophin receptor as a novel regulator of apoptosis in neointimal lesions. First, in the rat model of vascular injury, p75[NTR] is temporally expressed only in the later stages of lesion development, when apoptosis is prominent and trk receptor activity is diminished.[2,17] Second, in human atherosclerotic lesions, p75[NTR] spatially localized to regions
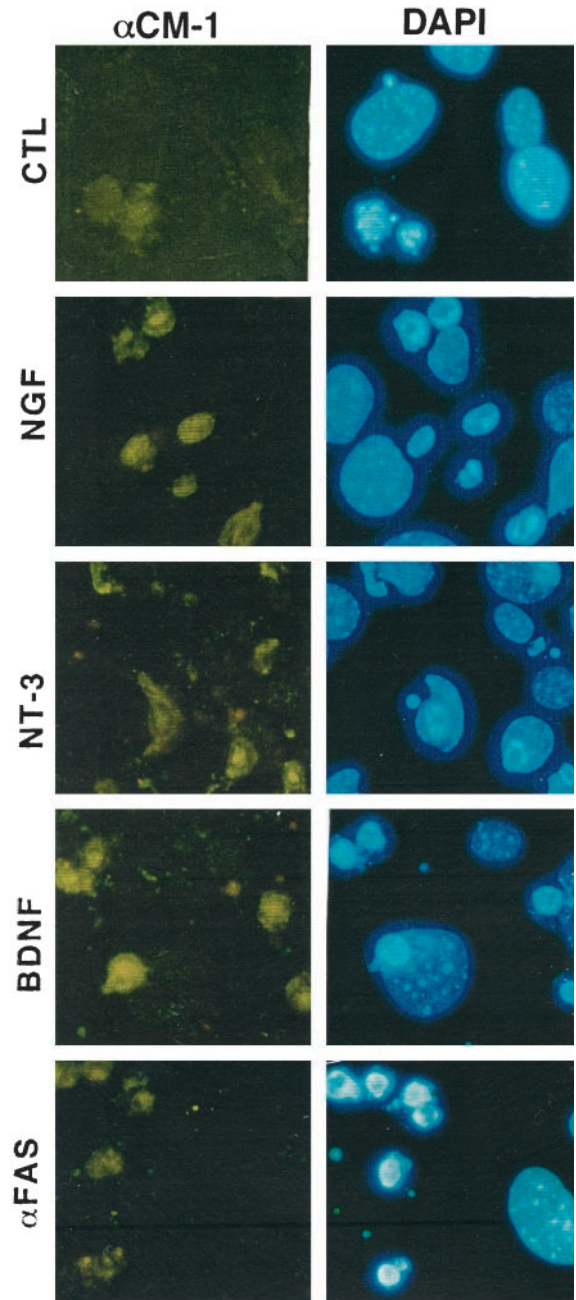
Figure 8. Immunofluorescent analysis for expression of activated caspases in p75-TsTmSMC. Cells were counterstained with DAPI for detection of condensed chromatin as an index of apoptosis.

of the neointima that demonstrate ongoing death. Moreover, neurotrophins at physiological concentrations induced p75[NTR] activation to initiate apoptosis of lesion-derived cells and stable cell lines that express p75[NTR], but not kinase-active trk receptors.

The coexpression of neurotrophins[2] and p75[NTR] by neointimal muscle cells represents a local mechanism for induction of apoptosis in atherosclerotic lesions that contrasts with proposed models of FAS-induced apoptosis in atherosclerotic lesions. FAS-mediated apoptosis is dependent on the colocalization of smooth muscle cells with infiltrating macrophages and T cells,[34,36] which release

cytokines to increase the expression of FAS by smooth muscle cells and are themselves a potential source of FAS ligand.[34] Not all apoptotic smooth muscle cells in atherosclerotic lesions express FAS receptors,[34,36] however, and other mechanisms must exist which initiate smooth muscle cell apoptosis in vascular lesions. Our data indicate that the neurotrophins represent an additional ligand receptor system to initiate smooth muscle cell apoptosis which is independent of lymphocyte/macrophage colocalization, as p75[NTR] colocalized to areas where apoptotic smooth muscle cells were present but macrophages were absent.

Alternatively, little is known about the signal transduction pathways regulating apoptosis of smooth muscle cells in response to ligand-induced activation of members of the TNF receptor superfamily and how these pathways are regulated in response to injury. It is well established, in Jurkat-T cells, that FAS-killing is mediated through activation of the caspase cascade. Binding of ligand to the FAS receptor leads to the recruitment of FAS-associated death domain (FADD), an adapter protein encoding a death domain, to the death domain of FAS. FADD then recruits and activates the initiator caspase, caspase 8, which, in turn, activates other caspases, eventually leading to activation of the effector caspase, caspase 3. In contrast, in oligodendrocytes, NGF activation of p75[NTR] induces the cleavage and activation of caspase 1 and caspase 3, but not caspase 8,[37] suggesting that p75[NTR] activates the caspase cascade by a mechanism distinct from that of FAS. The nuclear magnetic resonance (NMR) structure of the p75[NTR] death domain has distinctive differences from the death domain of FAS,[38] where, the death domain of p75[NTR] does not self-associate in solution, as the death domains of the FAS and TNFR1 receptors are known to do, nor does it bind to any of the known death domain adapter proteins.[38] These structural studies suggest that p75[NTR] may use alternative adapter proteins and signaling molecules to initiate caspase activation. The newly identified adapter protein neurotrophin receptor interacting factor (NRIF) has recently been described as a p75[NTR]-binding protein transducing apoptotic signals during embryonic development.[39] In addition, the adapter protein TRAF-6 interacts with the p75[NTR]-juxtamembrane domain and mediates p75-[NTR] induced nuclear factor κB activation.[40] Other studies have demonstrated that the increased expression of the pro-apoptotic molecules, p53 and BAX, mediates neurotrophin-induced apoptosis by p75[NTR], although the signaling pathways leading to the increase in their expression in response to the neurotrophins are not completely understood.[41] Further studies will be necessary to determine whether there is regulation of expression of these or other, as yet unidentified signaling molecules in atherosclerotic lesions, which could regulate the apoptotic activity of p75[NTR]. Ultimately, however, although the upstream signals activated by p75[NTR] and FAS to initiate the caspase cascade may differ, both lead to the activation of the execution caspase, caspase 3, and our results demonstrate that a similar mechanism mediates smooth muscle cell apoptosis in response to p75[NTR] and FAS activation.

p75[NTR] activation has been implicated in mediating cell death in the nervous system, both during embryonic development and after neuronal injury. In mice carrying a deletion in the p75[NTR] gene, cell death is reduced in the retina and spinal cord, areas of the nervous system that express high levels of p75[NTR] during development.[42] In models of neuronal injury, p75[NTR] is expressed by hippocampal neurons undergoing apoptosis after seizures and by both oligodendrocytes and microglia in areas undergoing apoptosis in lesions from patients with multiple sclerosis.[43] NGF can induce apoptosis of cultured oligodendrocytes and of motor neurons in explants of rat embryonic spinal cords,[44] but only at high concentrations (>4 nmol/L). Neither BDNF nor NT3 however, has apoptotic activity on cultured oligodendrocytes,[13] and only BDNF has been reported to cause apoptosis of sympathetic neurons.[14] These results suggest that neurotrophin-induced apoptosis mediated by p75[NTR] may be a highly regulated process dependent on the cellular context in which it is induced. The ability of the neurotrophins to induce neuronal and glial cell death occurs in cells that express p75[NTR], but not active trk receptors,[12,13,45] or in cells in which trk activity is reduced.[14,41] When both receptors are expressed and fully functional, signaling by trk receptors predominates, resulting in cell survival, mechanistically similar to the current study.

The present studies demonstrate a unique system, where in the presence of truncated and full length trk receptors, p75[NTR] mediates apoptosis of plaque-derived smooth muscle cells. Cultured HEDC expressed mRNA for p75[NTR] and kinase active trks, but in addition also expressed mRNA for truncated isoforms. This suggests that in HEDC, trk activity is reduced and neurotrophin-induced apoptosis is mediated by activation of p75[NTR]. This was confirmed in experiments where NGF induces apoptosis of p75-TsTmSMC but not trk A-TsTmSMC. Our results conflict with those of Bono et al,[46] who suggested that NGF-mediated apoptosis of cultured human medial smooth muscle cells was mediated by trk A. However, their conclusions were based on studies using pharmacological inhibitors of trk A receptor activation and downstream signaling components. Neither Trk A activation by NGF nor the expression of p75[NTR] was assessed. Thus, the role of trk A-mediated apoptosis in their studies is unclear. The expression of truncated trk isoforms by HEDC but the inability to detect them by immunohistochemistry in frozen tissue sections suggests that their expression *in situ* is below the limit of sensitivity of the antibodies.

Our studies are in agreement with those performed in neuronal cell systems demonstrating that the biological responsiveness of the neurotrophins is dependent on the class of receptor that is expressed (Figure 9). Early in lesion development, in smooth muscle cells expressing full length, kinase active isoforms of trk, the neurotrophins confer a migratory response (Figure 9A),[2,5] whereas in neointimal cells, which express p75[NTR] but in which kinase active trk activity is potentially reduced due to the coexpression of full length and truncated trk receptors,
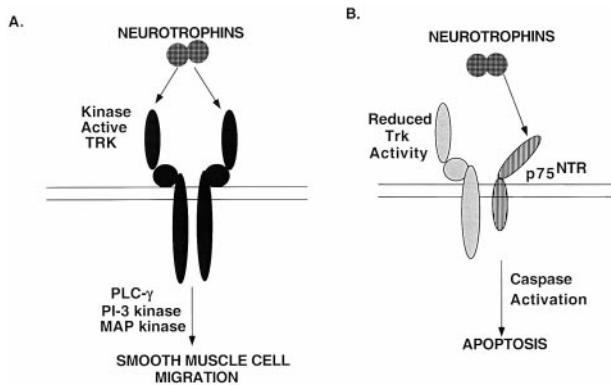
Figure 9. Model to assess the role of the neurotrophins in vascular lesion development. **A:** Migratory response of the neurotrophins on smooth muscle cells expressing full length, kinase-active trk receptors. **B:** Ligand-induced activation of p75^{NTR}, in the presence of reduced trk activity, results in apoptosis of smooth muscle cells.

the apoptotic activity of the neurotrophins predominates (Figure 9B). Thus, the ultimate responsiveness of neointimal smooth muscle cells to neurotrophins will depend on the dynamic regulation between the expression of full length isoforms, and potentially truncated isoforms of trk in the presence of p75^{NTR}. Little is known about the regulation of trk receptor and p75^{NTR} expression, or about how isoform switching from kinase-active to truncated trk receptors is modulated in response to injury. The coordinate regulation of both trk and p75^{NTR} genes to promote neointimal lesion progression and later lesion regression suggests that complex regulatory signals are used to modulate lesion development in atherosclerosis, where reduced trk activation unmasks the apoptotic activity of the neurotrophins through p75^{NTR} activation and may represent a mechanism for controlling apoptosis in vascular lesions.

Acknowledgments

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