

# NGF exhibits a pro-apoptotic activity for human vascular smooth muscle cells that is inhibited by TGF $\beta$ 1

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**Abstract** Apoptosis of vascular smooth muscle cells (SMCs) has been described in culture and also during remodelling of the artery following injury. However, the mediators that regulate apoptosis in SMCs are unknown. Because neurotrophins, a family of related polypeptide growth factors, including nerve growth factor (NGF) and its cognate receptor TrkA have been shown to be strongly expressed in atherosclerotic lesions, the present study was undertaken to evaluate *in vitro*, the activity of NGF with regard to apoptosis of confluent cultures of human aortic SMCs. We report here that NGF induced apoptosis of SMCs in a dose-dependent manner. This effect was detected from the concentration of 1 ng/ml and reached a maximum at 100 ng/ml. The concentration that induced a half-maximum effect was 8.8 ng/ml. The pro-apoptotic activity of NGF was time dependent and was significant after 3 h of incubation. The pro-apoptotic activity of NGF was blocked in a dose-dependent manner by K-252a, an inhibitor of TrkA tyrosine phosphorylation, suggesting that a NGF/TrkA signal transduction pathway could activate apoptotic cell death programs in human SMCs. Significantly, NGF-induced apoptosis was inhibited by wortmannin and PD 98059, showing that both PI3 kinase and MEK kinase were involved. At a NGF concentration that strongly induced apoptosis (100 ng/ml), TGF $\beta$ 1 which has been identified several times as a protective factor, dose dependently inhibited the pro-apoptotic effect of NGF. The IC<sub>50</sub> value was 1.5 ng/ml. These results indicate that, at least *in vitro*, TGF $\beta$ 1 can inhibit the pro-apoptotic activity of NGF for SMCs therefore suggesting that TGF $\beta$ 1 has the capacity to diminish the deleterious consequences of an excitotoxic or ischemic injury that might occur during atherogenesis or following angioplasty.

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**Key words:** Smooth muscle cell; Apoptosis; NGF; TGF $\beta$ 1

## 1. Introduction

Apoptosis is a physiological non-inflammatory mechanism of cell death regulating mass and cyto-architecture of a wide variety of tissues. In particular, apoptosis plays an important role in embryogenesis, morphogenesis, normal cell turnover and hyperplasia [1]. Cell death is also a prominent component of the human atherosclerotic plaque with areas of 'necrosis' being present in over 80% of primary lesions [2]. Cell death has also been detected at all stages of lesion development in animal models of atherosclerosis [3,4]. Cell death in the vasculature has previously been thought to be due to direct toxic insult [5] but recent evidence has indicated that cell death of endothelial cells, vascular smooth muscle cells (SMCs) and macrophages may occur via a highly regulated process [2,6].

The mechanisms that trigger apoptosis in atherosclerotic lesions remain largely unknown but several authors hypothesised that growth factors and cytokines might contribute to apoptosis of vascular SMCs during atherogenesis [2,7]. Among these mediators, recent studies have suggested that nerve growth factor (NGF) plays an important role in regulating the response of vascular SMCs to injury [8]. Indeed, increased expression of NGF and its receptor TrkA occurred following vascular injury of the rat carotid artery suggesting that NGF acts as an autocrine or paracrine mediator during SMC hyperplasia [8]. These findings have tended to interpret the role of NGF as a trophic factor that promotes innervation, but the expression of a neurotrophin receptor mRNA in vessel mesenchyme of mouse embryos [9] suggested that neurotrophins could be acting directly on SMCs.

Among the growth factors and cytokines which participate in the pathogenesis of atherosclerosis has been suggested, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) is a potent inhibitor of endothelial and SMC proliferation, migration and protease secretion [10]. The precise molecular changes that yield these activities of TGF $\beta$ 1 has been tentatively attributed to a decrease of cell-mediated proteolysis and to the subsequent deposition of a new basement membrane and the formation of junctional complexes between cells [11]. Moreover, very recently it has been suggested that this protective activities might be attributed to the potent inhibitory effect of TGF $\beta$ 1 with regard to apoptosis [12].

In the present study, we have determined the effect of NGF on apoptosis of human SMCs and evaluated the effect of TGF $\beta$ 1 with regard to this process.

## 2. Materials and methods

### 2.1. Materials

Human aortic SMCs were purchased from Clonetics (Tebu, Le Perray, France). Dulbecco's modified Eagle medium (DMEM) and phosphate buffered saline (PBS) were from Gibco-BRL (Cergy-Pontoise, France). Fetal calf serum (FCS), penicillin, streptomycin sulfate and glutamine were from Boehringer Mannheim (Meylan, Claix, France). Human recombinant NGF and wortmannin were obtained from Sigma Chemical Co. (L'Isle d'Abeau, France). Human TGF $\beta$ 1 was from Genzyme S.A. (Cergy Saint Christophe, France). PD 98059 and K-252a were from Calbiochem (Meudon, France).

### 2.2. Cell cultures

SMCs were routinely cultured in DMEM medium supplemented with 10% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin sulfate and 4 mM glutamine. Cells were used between the third and 10th passage.

### 2.3. Measurement of apoptosis

SMCs were seeded in 35 mm Petri dishes ( $5 \times 10^5$  cells/well) in DMEM+10% FCS and grown to confluence for 3 days. Culture medium was then aspirated, cells were rinsed and fresh medium+20% FCS was added in the presence of saline, TGF $\beta$ 1, NGF, K-252a,

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wortmannin or PD 98059. Twenty-four hours later, apoptosis was measured with a photometric enzyme immunoassay (cell detection ELISA, Boehringer Mannheim, Mannheim, Germany) for the quantitative detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Apoptosis was expressed as mean number of oligonucleosomes/ $10^5$  cells  $\pm$  S.D. Results were from three different experiments performed in triplicate.

### 3. Results and discussion

#### 3.1. Pro-apoptotic effect of NGF on human ASMCs

NGF is the most well-studied representative of neurotrophins, a family of trophic factors. NGF has pleiotrophic effects including the ability to induce differentiation, support cell survival, and prevent apoptosis in neuronal progenitor cells and immature neurons of the central and peripheral nervous system [13,14]. However, recent studies have shown that NGF induces apoptosis in human medulloblastoma cell lines [15] and in neural tumour cells [16]. Two different receptors for NGF have been identified including the P75 receptor [17] and the TrkA receptor, a transmembrane protein receptor with a cytoplasmic tyrosine kinase domain [18]. Recent studies have shown that TrkA is of major importance for NGF-mediated signal transduction [18,19]. NGF and its cognate receptor the trkA tyrosine kinase have been recently identified in human and rat vascular SMCs [8]. In these cells, NGF seemed to behave as an autocrine or local paracrine mediator of cell migration, therefore suggesting that neurotrophins play an important role in regulating the response of SMCs to injury.

On the basis of these various findings, we determined the effect of NGF with regard to the apoptosis of human SMCs in vitro. In culture, many cell types have been shown to undergo apoptosis when deprived of essential growth factors [20]. These include vascular endothelial cells deprived of bFGF [21], mouse embryo cells after removal of epidermal growth factor [22], rat pheochromocytoma PC12 cell line and sympatric neurons deprived of NGF [23], hormone-dependent cells of the breast or prostate deprived of steroid [24] and glial cells deprived of platelet derived growth factor [25]. The present study demonstrates that, on confluent human SMCs, removal of FCS resulted after 24 h in a dose-dependent pro-apoptotic effect (Fig. 1). In the presence of 20% FCS apoptosis of human SMCs was very low but increased significantly when the amount of FCS in the culture medium de-

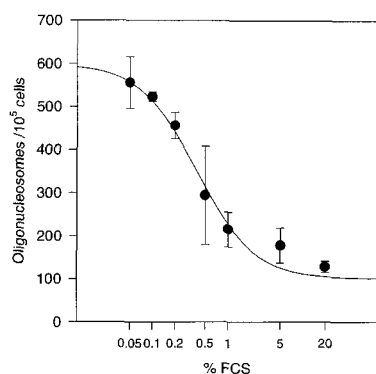


Fig. 1. Serum-induced apoptosis of human ASMCs. Confluent monolayers of human ASMCs cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing increasing concentrations of FCS. Apoptosis was measured with an ELISA kit as described in Section 2 and expressed as mean numbers of oligonucleosomes/ $10^5$  cells  $\pm$  S.D. ( $n=9$ ).

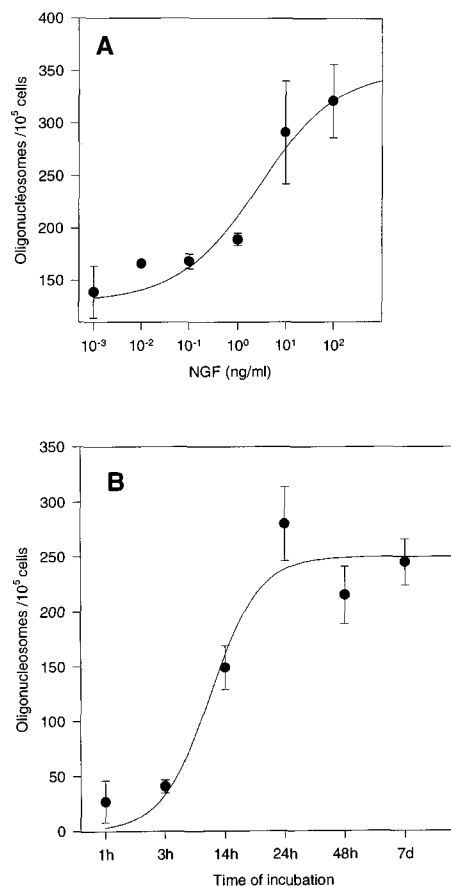


Fig. 2. Effect of NGF on apoptosis of human ASMCs. A: Dose-response: Confluent monolayers of human ASMCs cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing 20% FCS and increasing concentrations of NGF. B: Kinetics: Confluent monolayers of human ASMCs cultured in 35 mm dishes were incubated for different periods of time at 37°C in DMEM containing 20% FCS and 100 ng/ml NGF. Apoptosis was measured with an ELISA kit as described in Section 2 and expressed as mean numbers of oligonucleosomes/ $10^5$  cells  $\pm$  S.D. ( $n=9$ ).

creased. After a 24 h incubation in 0.05% FCS,  $560 \pm 60$  oligo/mononucleosomes were detected in  $10^5$  SMCs. Under experimental conditions which did not allow the cells to undergo apoptosis (i.e. in 20% FCS), NGF induced apoptosis of human SMCs in a dose-dependent manner (Fig. 2A). This effect was detected from the concentration of 1 ng/ml and reached a maximum at 100 ng/ml. The concentration that induced a half-maximum effect (ED50) was 8.8 ng/ml. The pro-apoptotic effect of NGF was time dependent (Fig. 2B) and was significant after 3 h of incubation. The activity of NGF reached a maximum after 24 h of incubation and persisted at the same level for at least 7 days. The maximum effect of NGF observed was similar to that observed when the cells were cultured for 24 h in 0.1% FCS. It is noteworthy that this pro-apoptotic activity of NGF occurred without any effect on cell growth (not shown).

In order to determine if NGF induced apoptosis of human SMCs in a TrkA-dependent manner, we evaluated the activity of K-252a, a specific inhibitor of TrkA tyrosine phosphorylation [15]. K-252a inhibited in a dose-dependent manner the pro-apoptotic effect of NGF (100 ng/ml) (Fig. 3). The IC50 value (concentration which inhibited 50% of the pro-apoptotic effect of NGF) was 140 nM. Similar results have been re-

ported concerning the effect of K-252a with regard to the pro-apoptotic effect of NGF for human neuroblastoma cell lines that express TrkA receptors [15].

The mitogen-activated protein kinase (MAP kinase) cascade plays a crucial role in the transduction of extracellular signals governing cell growth and differentiation. The effects of a specific inhibitor of MAP kinase (PD 98059) and of wortmannin, a specific inhibitor of phosphoinositide-3' kinase (PI3 kinase), on NGF-induced apoptosis have also been examined. Wortmannin and PD 98059 inhibited in a dose-dependent manner the pro-apoptotic effect of NGF for human SMCs (Fig. 3). The IC<sub>50</sub> values were 7 nM and 40 nM respectively.

Therefore, as shown previously for other cell types [15,16], our studies provide compelling evidence that apoptosis is a consequence of the NGF/TrkA signalling pathways. Our results show that this effect of NGF, through TrkA occurs via activation of PI3 kinase and of the MEK/MAP kinase pathway. Moreover, these results are in agreement with a recent study showing that treatment of NIH 3T3 cells expressing the TrkA receptor with PD 98059 dramatically reversed the growth inhibitory activity of NGF for these cells [26].

### 3.2. Protective effect of TGFβ1 on NGF-induced apoptosis

In vitro studies with vascular SMCs have identified several growth factors and cytokines that regulate apoptosis. Along with platelet derived growth factor-BB and insulin growth factor-1, several cytokines such as interferon-γ, tumour necrosis-α and interleukin-1β are now considered to be likely candidates [2–4,27,28].

Although TGFβ1 has opposite effects on proliferation versus migration of SMCs [11,12,29], the precise molecular mechanisms for this dual role remain largely undefined but have been tentatively attributed to a decrease of cell-mediated proteolysis and the subsequent deposition of new basement membrane and formation of junctional complexes between cells [30]. In a recent study, we have shown that TGFβ1 inhibited the pro-apoptotic effect of deprivation of FCS in murine SMCs [12]. Furthermore, several studies have shown that

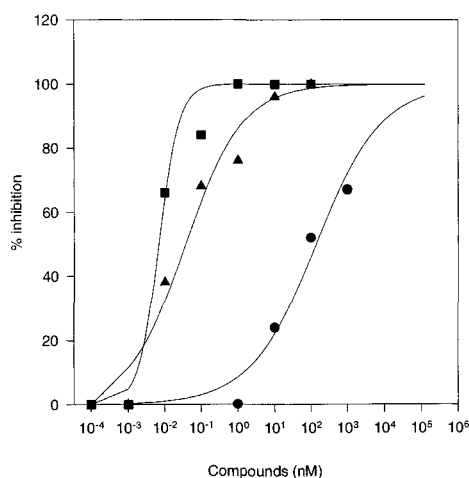


Fig. 3. Effect of K-252a, wortmannin or PD 98059 on NGF-induced apoptosis of human ASMCS. Confluent monolayers of human ASMCS cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing 20% FCS, 100 ng/ml NGF and increasing doses of K-252a (●), wortmannin (■) or PD 98059 (▲). Apoptosis was measured with an ELISA kit as described in Section 2. Data are reported as % inhibition of apoptosis compared to cells in 20% FCS+100 ng/ml of NGF ( $n=9$ ).

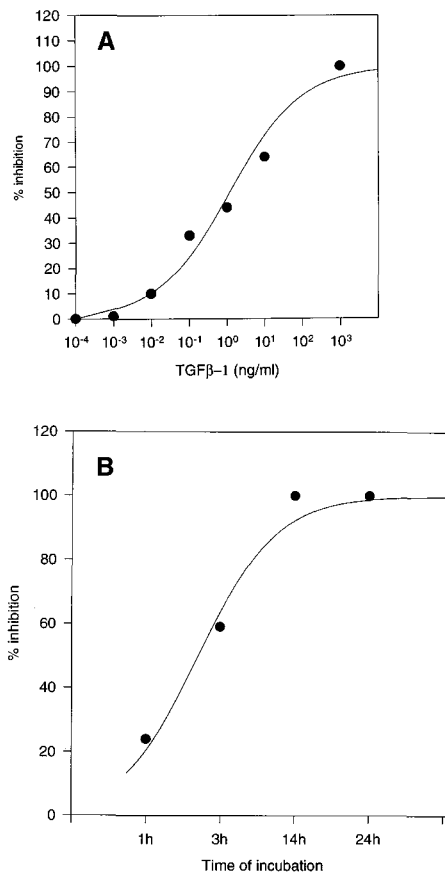


Fig. 4. Effect of TGFβ on NGF-induced apoptosis of human ASMCS. A: Dose-response: Confluent monolayers of human ASMCS cultured in 35 mm dishes were incubated for 24 h at 37°C in DMEM containing 20% FCS, 100 ng/ml NGF and increasing doses of TGFβ. B: Kinetics: Confluent monolayers of human ASMCS cultured in 35 mm dishes were incubated for different periods of time at 37°C in DMEM containing 20% FCS, 100 ng/ml NGF and 100 ng/ml TGFβ. Apoptosis was measured with an ELISA kit as described in Section 2. Data are reported as % inhibition of apoptosis compared to cells cultured in 20% FCS+100 ng/ml NGF ( $n=9$ ).

TGFβ1 exhibits a protective effect against neuronal degeneration caused by different stimuli [31]. In rat hippocampal neurons and in human foetal neurons, this effect seemed to be mediated by the ability of TGFβ1 to increase the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL [32].

In the present study, we showed that, when added to human SMCs, TGFβ1 inhibited in a dose-dependent manner the pro-apoptotic effect of NGF (Fig. 4A). This activity was significant from the dose of 10 pg/ml and reached a maximum at 100 ng/ml. The IC<sub>50</sub> value was 1.5 ng/ml. This inhibition of the pro-apoptotic activity of NGF was time dependent (Fig. 4B). The inhibitory activity of TGFβ1 was significant after 3 h and reached a maximum after 14 h of incubation with the cells. As shown by us previously [12], a similar effect was observed with regard to FCS depletion-induced apoptosis (not shown).

The mechanism(s) responsible for the potent anti-apoptotic activity of TGFβ1 remain largely unknown but can be compared with the protective effect of TGFβ1 observed on cultured hippocampal neurons, cardiac myocytes or endothelial cells [33]. In these experiments, TGFβ1 was shown to protect

these cells against a wide range of toxic insults (death induced by excitotoxicity, trophic factor removal or oxidative injury) by regulating two factors with great importance for cell viability: i.e. acute stabilisation of calcium homeostasis under conditions of physiological calcium overload but also via rapid induction of the Bcl2 oncoprotein [32,33]. Alternatively, TGF $\beta$ 1 may have indirect effects via induction of other neurotrophic agents such as bFGF [34] or insulin like growth factor [35]. TGF $\beta$ 1 has also been demonstrated to reduce the release of TNF $\alpha$  [36], a cytokine which strong pro-apoptotic activity for SMCs has been shown several times [37]. Thus, although not directly examined in the present study, it is conceivable that such mechanisms alone or in synergy might be responsible for the strong inhibitory activity of TGF $\beta$ 1 observed with regard to the pro-apoptotic effect of NGF for SMCs.

In conclusion, our work shows for the first time that the activity of NGF as a trophic factor for vascular SMCs might be attributed to its potent pro-apoptotic effect. The NGF/TrkA pathway was involved in this activity through an activation of PI3 kinase and the (MEK)/MAP kinase pathway. TGF $\beta$ 1 was a potent inhibitor of the pro-apoptotic effect of NGF suggesting that it might be an essential factor in the regulation of apoptosis of SMCs which occur following vascular injury or during atherogenesis.

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