

Nerve growth factor–endothelial cell interaction leads to angiogenesis in vitro and in vivo¹

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SPECIFIC AIMS

Nerve growth factor (NGF) plays important functions during embryonic development and on various tissues and organs under normal and pathological conditions. Recently, NGF has also been reported to modulate angiogenesis in the developing CNS. The present work addressed possible specific receptor-mediated, angiogenic effects of NGF in vitro in human umbilical vein endothelial cells (HUVEC) and in vivo in the chick embryo chorioallantoic membrane (CAM). The study was aimed to unravel mechanisms of NGF-dependent angiogenesis, providing potential tools for novel therapeutic approaches in antiangiogenic therapy.

PRINCIPAL FINDINGS

1. HUVECs express $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} mRNAs and proteins

Subconfluent cultures of HUVECs were assessed for expression of the NGF receptors $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} . Both $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} transcripts were detected in HUVECs by RT-PCR analysis. Accordingly, immunoprecipitation of the cell extracts followed by Western blot showed that $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} proteins are present in HUVECs, a result confirmed by immunocytochemistry experiments performed on the same batch of HUVEC cultures.

2. NGF/ $\text{trkA}^{\text{NGFR}}$ interaction triggers a mitogenic response in HUVECs

In the attempt to elucidate the biological significance of NGF receptor expression in endothelial cells, we evaluated the capacity of NGF to exert a mitogenic response in HUVECs. NGF promoted HUVEC proliferation in a concentration-dependent manner either in the presence or absence of the sustaining factor ECGS. NGF induced a rapid increase of $\text{trkA}^{\text{NGFR}}$ phosphorylation in HUVECs within 10 min after addition of the

growth factor to the culture medium without significant modification of the $\text{trkA}^{\text{NGFR}}$ protein level. Preincubation of HUVECs with the specific $\text{trkA}^{\text{NGFR}}$ inhibitor K252a prevented NGF-induced receptor phosphorylation. Furthermore, K252a completely suppressed the basal level of $\text{trkA}^{\text{NGFR}}$ phosphorylation observed in NGF-untreated cultures. NGF-induced $\text{trkA}^{\text{NGFR}}$ phosphorylation was associated with a significant increase of $\text{ERK}_{1/2}$ phosphorylation. The $\text{trkA}^{\text{NGFR}}$ inhibitor K252a completely prevented NGF-induced $\text{ERK}_{1/2}$ activation. As observed for $\text{trkA}^{\text{NGFR}}$ phosphorylation, K252a suppressed the basal levels of $\text{ERK}_{1/2}$ phosphorylation in NGF-untreated cultures. Preincubation of HUVEC cultures with the MAPKK inhibitor PD98059 resulted in a complete inhibition of NGF-induced $\text{ERK}_{1/2}$ phosphorylation, which was associated with parallel suppression of the mitogenic response to the growth factor. Together, the data indicate that NGF/ $\text{trkA}^{\text{NGFR}}$ triggers a mitogenic response in HUVECs that is mediated by $\text{ERK}_{1/2}$ activation.

3. NGF exerts an autocrine role in HUVECs

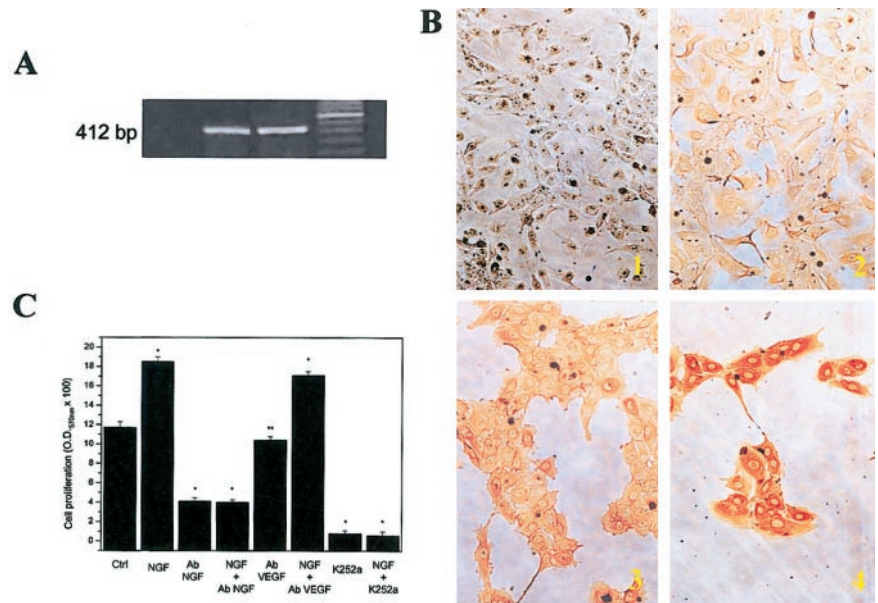
The capacity of the K252a inhibitor to suppress the basal levels of $\text{trkA}^{\text{NGFR}}$ and $\text{ERK}_{1/2}$ phosphorylation in untreated HUVEC culture raises the hypothesis that NGF of endothelial origin may play an autocrine role in HUVECs. RT-PCR analysis and immunocytochemistry revealed that HUVECs express NGF mRNA and protein under standard culture conditions (**Fig. 1A**). NGF protein expression was dramatically enhanced when cells were maintained under serum-free conditions in a time-dependent manner (24 and 48 h starvation) (**Fig.**

¹ To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.01-1000fje>; to cite this article, use *FASEB J.* (June 21, 2002) 10.1096/fj.01-1000fje

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Figure 1. A) RT-PCR analysis of NGF mRNA in HUVECs. Lane M, 100 bp marker DNA ladder, with the 600 bp to provide internal orientation. Negative control: lane 1. Lane 2: positive control (U373 MG cells); lane 3: HUVECs. B) NGF Immunoreactivity in HUVECs incubated with normal rabbit serum (1) or a specific anti-NGF serum in normal conditions (2) or starved for 24 (3) and 48 (4) h. Note the increasing NGF-like immunoreactive staining in HUVECs at each different time of starvation. C) Effects of either specific NGF, VEGF-neutralizing antibodies, or the $trkA^{NGFR}$ inhibitor K252a on basal and NGF-stimulated HUVEC proliferation. Vertical bars are means \pm SE; ** $P < 0.05$ and * $P > 0.01$ vs. respective controls (one-way analysis of variance, followed by Duncan's least significance test).



1B). No viable cells were observed after 60 h of serum deprivation (data not shown), suggesting that NGF up-regulation may be part of the cell response to nutrient depletion. To assess whether endogenous NGF may exert an autocrine role in HUVEC cultures, cells were incubated in the presence of a NGF-neutralizing antibody or the selective $trkA^{NGFR}$ inhibitor K252a. Both substances caused a significant decrease of HUVEC proliferation under standard culture conditions. A VEGF-neutralizing antibody did not influence NGF-induced HUVEC proliferation (Fig. 1C). These data indicate that proliferation induced autocrinally by NGF results from direct interaction with the endothelial cell in a manner similar to other angiogenic factors.

4. NGF is angiogenic in the chick embryo CAM

The capacity of NGF to interact directly with endothelial cells in vitro prompted us to evaluate the angiogenic potential of NGF when delivered in vivo to an 8 day chick embryo CAM via a gelatin sponge implant (Fig. 2). CAMs treated with NGF showed the presence of allantoic vessels spreading radially toward the sponge in a spoked wheel pattern, whereas no vascular reaction occurred in CAMs treated with PBS. Microscopically, the sponges adsorbed with NGF showed a collagenous matrix containing numerous small blood vessels and fibroblasts localized among the sponge trabeculae. Numerous host capillaries piercing the sponge were also detected at the boundary between the sponge and the CAM mesenchyme, unlike samples treated with PBS. Similar findings had been reported using VEGF₁₆₅. Accordingly, morphometric evaluation of the vascular density of the CAM at day 12 of incubation demonstrated that NGF exerted an angiogenic effect in the chick embryo quantitatively similar to that elicited by VEGF₁₆₅. The angiogenic activity of NGF was fully suppressed by neutralizing anti-NGF antibodies, marginally affected by a VEGF-neutralizing antiserum, and

completely unaffected by an FGF2-neutralizing antibody.

CONCLUSIONS

Our results clearly indicate that HUVEC proliferation triggered by NGF is specifically mediated by $trkA^{NGFR}$ via activation of the MAPK pathway. Our data extend previous observations on the capacity of NGF to induce cell proliferation and up-regulation of cell adhesion molecules in human dermal microvascular endothelial cells, suggesting that the responsiveness to NGF represents a widespread property of endothelial cells of different ori-

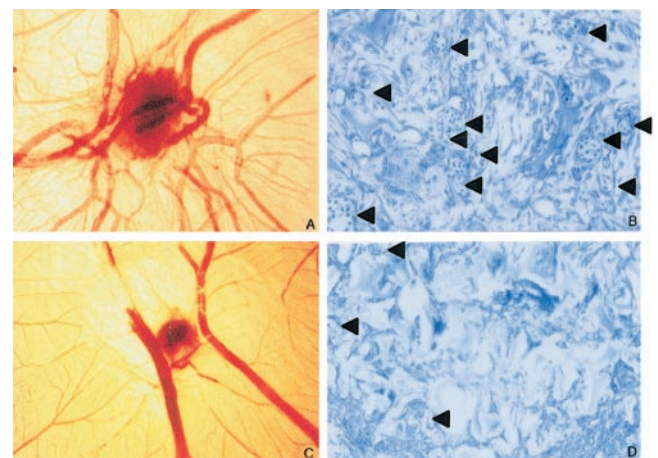


Figure 2. Effect of NGF on CAM neovascularization. A) Gelatin sponge adsorbed with NGF surrounded by allantoic vessels that develop radially toward the implant in a 'spoked wheel' pattern. B) Newly formed blood vessels and infiltrating fibroblasts within an abundant network of collagen fibers. Absence of vascular reaction around the vehicle-treated sponge at macroscopic (C) and microscopic levels (D). Original magnifications: A, C: 50 \times ; B, D: 250 \times .

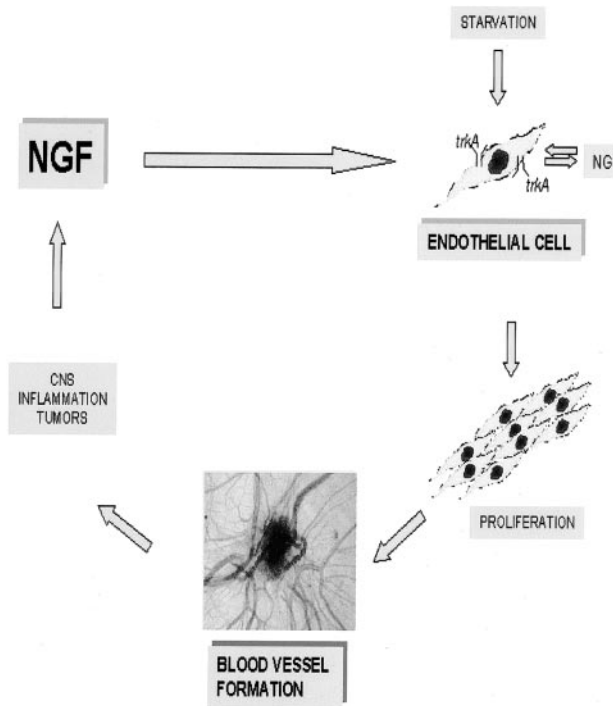


Figure 3. Schematic representation of NGF-dependent angiogenesis and its pathophysiological role.

gin. We have shown that NGF induces a potent angiogenic response in the CAM of the developing chick embryo. Data showing indirect angiogenic effects of NGF,

together with NGF's ability to exert a direct mitogenic effect on endothelial cells in vitro suggest redundancy of NGF-mediated angiogenesis in vivo. Therefore, NGF released by different cell types may be paracrinally active on endothelial cells, stimulating neovascularization. This may be of high relevance in various pathological conditions, including inflammation and tumor growth, processes in which a pivotal role is played by new blood vessel formation and where NGF could promote angiogenesis as well as activation of other cell types (Fig. 3). However, our work demonstrates that NGF also plays an autocrine role in endothelium. Indeed, we have shown for the first time that HUVECs express NGF mRNA and protein, causing partial activation of the $trkA^{NGFR}$ pathway observed under basal conditions and prevented by NGF-neutralizing antibodies or by the $trkA^{NGFR}$ inhibitor K252a. NGF expression in HUVECs was increased by serum deprivation, suggesting that NGF up-regulation may be part of an endothelial cell response to nutrient/trophic depletion (Fig. 3). It is therefore possible to hypothesize that nutrient/trophic shortage, together with hypoxic conditions and the cytokine milieu, may activate epigenetic autocrine (e.g., NGF production by endothelium) and paracrine (e.g., VEGF production by tumor or stromal cells) signals leading to endothelial cell proliferation/survival. In conclusion, we have shown for the first time that NGF exerts a direct mitogenic effect on endothelium. NGF thus could represent a candidate proangiogenic factor in the developing CNS, inflammation, and tumor growth (Fig. 3). [F]