NERVE GROWTH FACTOR - ENDOTHELIAL CELL INTERACTION LEADS TO ANGIOGENESIS IN VITRO AND IN VIVO

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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 objective of the present work was addressed to 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.
Subconfluent cultures of HUVECs were assessed for the expression of the two NGF receptors $trkA^{NGFR}$ and $p75^{NTR}$. Both $trkA^{NGFR}$ and $p75^{NTR}$ transcripts were detected in HUVECs by RT-PCR analysis. Accordingly, immunoprecipitation of the cell extracts followed by western blotting showed that $trkA^{NGFR}$ and $p75^{NTR}$ proteins are present in HUVECs, a result confirmed by immunocytochemistry experiments performed on the same batch of HUVEC cultures.
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 the absence of the sustaining factor ECGS. NGF induced a rapid increase of $trkA^{NGFR}$ phosphorylation in HUVECs within 10 min after the addition of the growth factor to the culture medium without significant modification of $trkA^{NGFR}$ protein level. Preincubation of HUVECs with the specific $trkA^{NGFR}$ inhibitor K252a prevented NGF-induced receptor phosphorylation. Furthermore, K252a completely suppressed the basal level of $trkA^{NGFR}$ phosphorylation observed in NGF-untreated cultures. NGF-induced $trkA^{NGFR}$ phosphorylation was associated to a significant increase of $ERK_{1/2}$ phosphorylation. The $trkA^{NGFR}$ inhibitor K252a completely prevented NGF-induced $ERK_{1/2}$ activation. As observed for $trkA^{NGFR}$ phosphorylation, K252a suppressed also the basal levels of $ERK_{1/2}$ phosphorylation in NGF-untreated cultures. Preincubation of HUVEC cultures with the MAPKK inhibitor PD98059 resulted in a complete inhibition of NGF-induced $ERK_{1/2}$ phosphorylation, that was associated with parallel suppression of the mitogenic response to the growth factor.
The capacity of the K252a inhibitor to suppress the basal levels of $trkA^{NGFR}$ and $ERK_{1/2}$ 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. Furthermore, NGF protein expression was dramatically enhanced when cells were maintained under serum-free conditions in a time-dependent manner (24 h and 48 h starvation). It must be pointed out that no viable cells were observed after 60 h of serum deprivation, suggesting that NGF upregulation 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. In addition, a VEGF-neutralizing antibody did not influence NGF-induced HUVEC proliferation.
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 8 day chick embryo CAM via a gelatin sponge implant. CAMs treated with NGF showed the presence of allantoic vessels spreading radially towards 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 previously using $VEGF_{165}$. 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 $VEGF_{165}$. The angiogenic activity of NGF was fully suppressed by neutralizing anti-NGF antibodies, marginally affected by a VEGF neutralizing antiserum, and completely unaffected by a FGF2 neutralizing antibody.

In conclusion, we have shown for the first time that NGF exerts a direct mitogenic effect on endothelium. NGF could thus represent a candidate proangiogenic factor in the developing CNS, inflammation, and tumor growth.

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