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NEUROTROPHINS: NOVEL MEDIATORS OF ANGIOGENESIS

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1. ABSTRACT

Angiogenesis is a highly coordinated physiological process in which new blood vessels are formed to meet the oxygenation demands of local tissues. Several classes of growth factors, including members of the vascular endothelial growth factor family, angiopoietins, platelet derived growth factor, fibroblast growth factors and ephrins have been implicated in regulating specific aspects of angiogenesis, both during embryonic development and in response to injury. This review focuses on a distinct family of growth factors, the neurotrophins, and their receptors as newly identified angiogenic molecules. The expression of neurotrophins and their receptors are regulated both temporally and spatially by the vasculature of the embryo and adult, and dynamic changes occur following vascular injury. Recent studies that identify the vascular cells responsive to neurotrophins, that genetically dissect neurotrophin actions in vessel development and remodeling, and that uncover neurotrophin effects in models of tissue ischemia are discussed.

2. INTRODUCTION

Angiogenesis, the sprouting and formation of new blood vessels, is a highly regulated process that requires the assembly and differentiation of numerous cell types to form the arteries, capillaries and veins from a pre-existing vascular bed. The primitive vasculature, composed of an endothelial plexus, is patterned into arteries and veins by the recruitment of pericytes and vascular smooth muscle cells by soluble growth factors secreted by endothelial cells (1-3). As the vessels mature, endothelial cells are stabilized by interactions with the extracellular matrix and by the ensheathment with pericytes and eventually, smooth muscle cells. In the adult, pathological angiogenesis occurs under a variety of conditions including ischemia, rheumatoid arthritis, proliferative retinopathy, psoriasis, atherosclerosis and cancer (4, 5).

Several angiogenic factors have been identified that regulate the growth of new vessels under pathological conditions. Among these are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), the fibroblast growth factors and placental derived growth factor (PlGF). The role of these growth factors in angiogenesis has been the focus of several recent reviews (6-10). The focus of this review is on a novel class of growth factors, the neurotrophins, and their potential role in angiogenesis and the mechanisms that regulate their angiogenic activity.

3. NEUROTROPHINS AND THEIR RECEPTORS

3.1. Trk Family of Receptor Tyrosine Kinases

The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4/5 (NT-3 and NT-4), are a family of highly conserved proteins best characterized by their critical role in the differentiation and survival of neurons (11). They mediate their actions by binding to two classes of receptors: the Trk family of receptor tyrosine kinases and $p75^{NTR}$, a member of the TNF receptor family (12) (Figure 1). The trk receptors, trk A, trk B and trk C, exhibit ligand specificity with NGF binding to Trk A, BDNF and NT 4/5 to Trk B, and NT-3 to Trk C. In addition to kinase active isoforms, *Trk B* and *Trk C* genes encode truncated isoforms, generated by alternative splicing (13, 14). The truncated isoforms inhibit intracellular signaling when co-expressed with full-length trk isoforms (15, 16). The dominant negative action of truncated trk receptors has

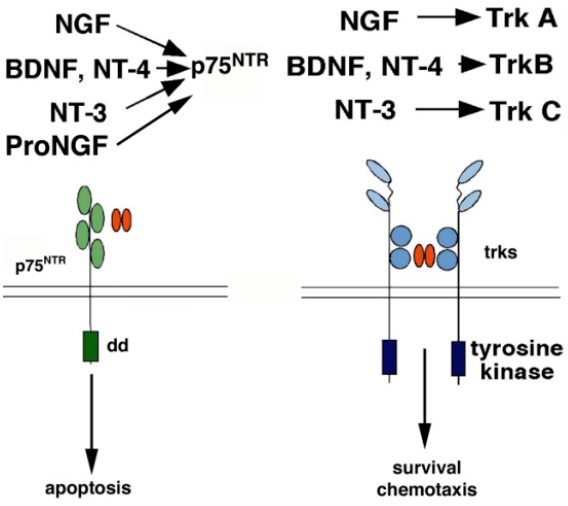

Figure 1. Schematic representation of neurotrophin interactions and biological activities with both Trk receptors and $p75^{NTR}$. dd=death domain.

been extended to *in vivo* studies, as transgenic mice overexpressing the truncated receptor exhibit a phenotype similar to that observed with targeted deletion of kinase active Trk C or NT-3 (17, 18).

3.2. Trk Receptor Signaling Pathways

Although neurotrophin binding induces Trk receptor autophosphorylation of seven cytoplasmic tyrosine residues (19), mutational analysis has defined two of these to be critical for Trk receptor signaling. Phosphorylation of Tyr-785 is required for the binding and activation of phospholipase Cγ (PLCγ) (19, 20), while phosphorylation of Tyr-490 induces binding of the adapter protein Shc, through its PTB phosphotyrosine binding domain (21). The binding of Shc recruits the Grb-2 adapter protein, resulting in the activation of two signaling pathways: the Ras/MAP kinase pathways, utilizing SOS, and the phosphatidyl inositol 3 kinase (PI-3 kinase) pathway, through Grb-2-associated binder-1 (GAB-1) (21). Activation of all three pathways participates in NGF-induced neuronal differentiation and survival (21).

3.3. The p75 Neurotrophin Receptor

In contrast to the well known biological responses and signaling pathways identified for the Trk receptor family, far less is known about biological activities and signaling pathways downstream of $p75^{NTR}$. Although $p75^{NTR}$ binds all neurotrophins, the receptor can mediate distinct cellular responses. First, when co-expressed with Trk receptors, $p75^{NTR}$ expression enhances the affinity of neurotrophin binding, generating a high affinity site (Kd of 10^{-11} M) consisting of Trk: $p75^{NTR}$ receptor complexes. This contrasts with the low affinity interactions (Kd of 10^{-9} M) exhibited when Trk or $p75^{NTR}$ receptors are expressed independently (12, 22). In addition, $p75^{NTR}$ can alter the ligand specificity of Trk receptors, restricting ligand binding in the case of Trk A, or relaxing specificity with Trk C (23, 24). Second, neurotrophin-induced activation of $p75^{NTR}$ can initiate apoptosis when $p75^{NTR}$ is expressed independently of Trk (25-27).

3.4. Neurotrophins and Their Receptors in the Vasculature

Although the neurotrophins were initially characterized as critical regulators of neuronal differentiation and survival, it has become increasingly apparent that the neurotrophins and their receptors are also expressed in nonneuronal tissues, where their functions are less well defined. Recently, the neurotrophins have been identified as critical regulators of vascular development and the vascular response to injury (25, 28-33). Unlike the best characterized angiogenic factor, VEGF, that acts via its receptor tyrosine kinases Flt-1 and Flk on most endothelial populations from mid-gestation onwards, neurotrophins display spatially and temporally restricted expression in the vasculature. For example, BDNF and Trk B are expressed at the highest levels by endothelial cells lining arteries and capillaries of the heart and skeletal muscle (29). Unlike VEGF and its receptors that are expressed at highest levels in early to mid-gestation, and with waning expression during adulthood, Trk B and BDNF are expressed in mid to late gestation and expression increases from perinatal to adult life (29). Vascular smooth muscle cells of large muscular arteries express low levels of both NGF and BDNF and their cognate receptors, Trk A and Trk B, from late gestation onward. Moreover, the expression of both ligand and receptor is markedly upregulated in the neointima following acute vascular injury and in human atherosclerotic lesions (28). In adult animals, $p75^{NTR}$ is not expressed in the uninjured vascular wall, but is upregulated in vascular smooth muscle cells in regions of chronic vascular injury. Taken together, these results suggest that the expression of neurotrophins and Trk/$p75^{NTR}$ receptors are dynamically regulated in the normal and injured vasculature.

3.5. Biological Activity of the Neurotrophins in the Vasculature

The biological actions of the neurotrophins in the vasculature appear to be both cell-type and receptor-dependent (Table 1). In cardiac microvascular endothelial cells, Trk B and its ligand, BDNF are essential for survival (29). BDNF gene-targeted mice exhibit impaired survival of endothelial cells in intramyocardial arteries and capillaries in the early postnatal period, although the embryonic vasculature can remodel into arteries, capillaries and veins in the absence of BDNF. BDNF deficiency results in a reduction in endothelial focal adherens junction formation and in endothelial cell apoptosis, leading to intraventricular wall hemorrhage, depressed cardiac contractility and early postnatal death. In culture, BDNF promotes the survival of cardiac microvascular endothelial cells from wild type animals that are subjected to serum deprivation, supporting the observation of the *in vivo* phenotype. Gross vascular hemorrhage in BDNF null animals is restricted to the cardiac microvasculature, reflecting the localized expression of BDNF and trk B by capillaries and arterioles in this vascular bed. These results establish an essential role for BDNF in maintaining vessel

Table 1. Neurotrophin actions in the vasculature

Cells	Neurotrophin:receptor	Biological action	References
Cardiac endothelium	BDNF; trk B	Survival	29
Neointimal SMC	NGF: trkA	Chemotaxis	28, 34
	BDNF:trkB	MMP expression	41
Neointimal SMC	NGF, proNGF:p75	Apoptosis	29, 35, 43
Ischemic Hindlimb	NGF:? Receptor	Arteriogenesis	30,31

stability in the heart through direct angiogenic action endothelial cells.

In smooth muscle cells, receptor expression dictates the biological activities of the neurotrophins. In smooth muscle cells expressing Trk receptors, neurotrophins are potent chemotactic agents, but they do not induce proliferation (28, 34). In contrast, neurotrophins induce apoptosis of smooth muscle cells expressing p75NTR. This paradox of neurotrophin action, in promoting both chemotactic and apoptosis activities in smooth muscle cells, was recently clarified by the discovery of novel ligands for p75NTR (35). Neurotrophins are synthesized as proneurotrophins of 30 kD, which can be cleaved intracellularly to a mature, 13.5 kD C-terminal product, the isoform that had previously been considered biologically active. However, cells can secrete both proneurotrophins and mature neurotrophins and subsequent studies demonstrated that the proNGF selectively binds p75NTR, but not Trk. Moreover, proNGF is 10-20 times more effective in inducing p75NTR-mediated apoptosis (35). These observations suggest that proNGF is a selective and effective ligand for the pro-apoptotic p75NTR receptor, whereas cleaved, mature NGF selectively activates the pro-chemotactic and pro-survival activities of Trk.

4. ROLE OF MATRIX METALLOPROTEINASES IN ANGIOGENESIS

4.1. Overview of the MMPs

The migratory activity of both endothelial and smooth muscle cells requires highly coordinated responses, including severance of cell-cell and cell matrix contacts, reorganization of the cytoskeleton and remodeling of the surrounding matrix (36). The proteolytic activity of the matrix metalloproteinases (MMPs) is thought to be a principle mechanism regulating matrix remodeling and cellular migration in angiogenesis. Based upon their substrate specificity, the MMPs are grouped into three subfamilies: the collagenases (MMP-1, MMP-8 and MMP-13), the gelatinases (MMP-2 and MMP-9), and the stromolysins (MMP-3 and MMP-10) (37). The activity of the MMPs is tightly regulated by two distinct mechanisms. First, MMPs, released as zymogens, must be cleaved to an active form by proteases, primarily plasmin. Second, tissue inhibitors of metalloproteinases, or TIMPS are present in high levels in the vasculature and regulate MMP activation and subsequent enzymatic activity. Thus, the differential regulation of MMP and TIMP synthesis regulates matrix remodeling and endothelial and smooth muscle cell migration during angiogenesis.

4.2. MMPs and angiogenesis

MMPs play multiple roles in the angiogenic process(5, 38). The remodeling of extracellular matrix allows for the release and migration of endothelial and smooth muscle cells into new tissue, as well as the release of extracellular matrix bound angiogenic factors. Conversely, MMP degradation of specific matrix proteins may generate anti-angiogenic proteins, such as endostatin (5). The ability to generate both pro and anti-angiogenic agents suggests that the expression of MMPs may serve a role as an angiogenic switch in tumor progression, favoring the formation of blood vessels within tumors (5) and in wound healing.

4.3. Neurotrophins and MMP expression

Neurotrophin-induced activation of Trk receptors can increase the expression and activation of selective MMPs, in both vascular and neuronal cells. For example, NGF potently induces MMP-3 expression in neuronal cells (39, 40), leading to enhanced neurite outgrowth. In the vasculature, NGF-treatment of Trk A-expressing smooth muscle cells increases the expression and activation of MMP-9, but not MMP-2 or MMP-3 (41). The increase in MMP-9 expression may be especially important in response to vascular injury, as MMP-9 can degrade the extracellular matrix surrounding medial smooth muscle cells to promote their migration into the intima, thus contributing to lesion development (42). NGF-induced activation of the MAP kinases Erk-1 and Erk-2 is required for the increase in MMP-9 expression in smooth muscle cells, as inhibitors of this signaling cascade abrogate NGF-induced MMP-9 expression. Thus, increased MMP expression and activation is one potential mechanism by which neurotrophins participate in angiogenesis in response to injury.

5. NEUROTROPHIN/RECEPTOR EXPRESSION IN RESPONSE TO INJURY

While low levels of the neurotrophins and their receptors are expressed in the vasculature of selective adult tissues, expression is markedly upregulated in response to injury. For example, in response to vascular injury, increased expression of both NGF and BDNF and their cognate receptors, Trk A and Trk B is observed in the neointima which develops following balloon deendothelialization of the rat thoracic aorta (28). Similarly, Trk B, BDNF and NGF are expressed at increased levels in human atherosclerotic and restenotic lesions as compared to the uninjured adult vasculature (28). Although $p75^{NTR}$ is expressed in embryonic vasculature, expression in adult vessels is not detectable. However, $p75^{NTR}$ is rapidly upregulated by endothelial and vascular smooth muscle cells in regions of acute and chronic vascular injury (25). To assess the biological action of $p75^{NTR}$ in such settings, the $p75^{NTR}$ (-/-) mouse was utilized in a model of vascular injury consisting of the flow

restricted carotid artery (43). In $p75^{NTR}$ (-/-) animals, increased lesion formation and decreased apoptosis was observed in flow restricted carotid arteries, as compared to $p75^{NTR}$ (+/+) littermates. These studies suggest that $p75^{NTR}$ may promote neointimal remodeling in this model system.

Angiogenic actions for $p75^{NTR}$ have also been revealed in the analysis of a newly generated $p75^{NTR}$ gene-targeted mouse (44). The initial mouse deleted in $p75^{NTR}$ results in ablation of the full-length $p75^{NTR}$ protein. However, an alternatively spliced short $p75^{NTR}$ isoform (s-$p75^{NTR}$) that lacks most of the extracellular domain, but retains an intact transmembrane and cytoplasmic domain, is expressed at low levels. Unexpectedly, newly generated $p75^{NTR}$ (-/-) animals lacking expression of both full length and short $p75^{NTR}$ isoforms exhibit partially penetrant late gestational embryonic lethality, with evidence of cerebral hemorrhage and aortic aneurysmal dilation (44). As $p75^{NTR}$ is expressed by several vascular populations during embryogenesis, further studies will be necessary to uncover the mechanisms by which $p75^{NTR}$ promotes vessel stabilization in the embryo.

Increased expression of neurotrophins and Trk receptors has also been demonstrated in ischemic models of injury (45). In a rat model of myocardial ischemia and reperfusion, increased NGF mRNA levels were observed in cells that were in close association with capillaries, venules and arterioles. Increased NGF expression was also observed in the coronary arteries supplying the reperfused myocardium (45). The increase in NGF expression was observed within 2 hours of reperfusion, and remained elevated up to 120h post reperfusion. In contrast, a transient increase in BDNF expression was observed predominantly by myocytes, within 2 hours of reperfusion, mostly in myocytes. These results suggest that the vascular cell expression of neurotrophins and their receptors is carefully regulated in response to injury and further studies should allow dissection of the initiating events.

6. EVIDENCE FOR ANGIOGENIC ACTIVITY OF THE NEUROTROPHINS

Although BDNF is essential for the survival of endothelial cells in the developing vasculature of the myocardium (29), suggesting a critical role in angiogenesis in the developing embryo, the effects of neurotrophins in postnatal angiogenesis has also been evaluated. Recent data suggests that delivery of exogenous NGF can induce vascularization of the chick embryo chorioallantoic membrane through proposed mitogenic actions on endothelial cells (31). In addition, NGF induces endothelial cell hyperplasia and increases vessel density in the superior cervical ganglia of newborn rats, concomitant with an increase in VEGF expression. These results suggest that NGF, via either direct or indirect effects, may stimulate neoangiogenesis in postnatal tissues (32). Recent evidence also demonstrates that neurotrophins may also participate in the angiogenic response to injury. Following induction of hindlimb ischemia, NGF protein levels increase in muscle lysates, suggesting that NGF induction accompanies tissue ischemia (30, 33). Administration of NGF protein to the ischemic hindlimb of rodents increases the microvascular response, with statistically significant augmentation in arteriolar density (30, 33). NGF treatment was also associated with a decrease in the apoptosis of both endothelial cells and myocytes in the ischemic muscle, and an improved perfusion ratio of the ischemic hindlimb as compared to vehicle-treated ischemic limbs (33). However, it is not clear whether NGF is mediating direct effects on $p75^{NTR}$ or Trk A expressing vascular cell populations. Indeed, the angiogenic actions of NGF were abrogated by concomitant delivery of VEGF neutralizing antibodies, or the administration of inhibitors or nitric oxide synthase. Thus, further studies will be needed to mechanistically dissect NGF action in ameliorating blood flow and promoting vascularization in models of limb ischemia.

Few studies have examined potential roles for neurotrophins in tumor angiogenesis, despite the initial identification of NGF from sarcoma tissue (46). Neurotrophins and/or receptor expression has been characterized in numerous tumors, including cancers of pancreatic (47), prostate (48), medullary thyroid (49), and breast origin (50) and leukemia (51), Wilm's tumor (52) and neuroblastoma (53). However, expression patterns in these tumors are most consistent with autocrine actions upon the tumor cells themselves. A recent study, however, documents the upregulation of NGF in hepatocellular carcinoma, with Trk A expression by smooth muscle cells in tumor arterioles, but absent in uninvolved areas (54). Further study will be needed to determine whether tumor derived neurotrophins mediate actions, such as induction of metalloproteinase activity, in tumor vasculature and could thus modulate tumor angiogenesis.

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