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Review

VEGF at the neurovascular interface: Therapeutic implications for motor neuron disease

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Abstract

VEGF was discovered almost 25 years ago, and its angiogenic activity has been extensively studied ever since. Accumulating evidence indicates, however, that VEGF also has direct effects on neuronal cells. VEGF exerts neuroprotective effects on various cultured neurons of the central nervous system. In vivo, VEGF controls the correct migration of facial branchiomotor neurons in the developing hindbrain and stimulates the proliferation of neural stem cells in enriched environments and after cerebral ischemia. Transgenic mice expressing reduced levels of VEGF develop late-onset motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (ALS), whereas reduced levels of VEGF have been implicated in a polyglutamine-induced model of motor neuron degeneration. Recent data further reveal that intracerebroventricular delivery of recombinant VEGF protein delays disease onset and prolongs survival of ALS rats, whereas intramuscular administration of a VEGF-expressing lentiviral vector increases the life expectancy of ALS mice by as much as 30%. Deciphering the precise role of VEGF at the neurovascular interface promises to uncover new insights into the development and pathology of the nervous system, helpful to design novel strategies to treat (motor) neurodegenerative disorders.

Keywords: VEGF; ALS; Neurodegenerative; Blood vessel; Motor neuron; Therapeutic

Half a millennium ago, Vesalius documented that blood vessels and nerve fibers are highly branched in stereotyped patterns and often course along each other throughout the body. Today, evidence emerges that vessels, which arose later in evolution than nerves, appear to have co-opted the same genetic pathways to develop their functionally distinct, but architecturally similar network [1,2]. Another exciting discovery is that vessels and nerves crosstalk. In some cases, vessels produce signals that attract axons to track alongside the pioneer vessel, in other cases, nerves produce signals to guide blood vessels [1,2]. Perhaps the best illustration of how such crosstalk affects vessels and neurons in health and disease is provided by the activities of vascular endothelial growth factor (VEGF) and its receptors.

1. VEGF, a key angiogenic player

Originally purified as a growth factor, capable of increasing vascular permeability and endothelial cell proliferation, VEGF distinguishes itself from other angiogenic factors by a unique combination of properties. Indeed, VEGF is produced and released by cells in close vicinity to endothelial cells [3,4] and, after binding VEGF receptor-1 (VEGFR-1, Flt1), VEGFR-2 (Flk1) and neuropilins (Nrp1; Nrp2), VEGF induces endothelial cells to proliferate, migrate, survive, and assemble into an interconnected vessel network (Fig. 1) [5]. VEGF is transcribed from a single gene into various isoforms (among which $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$). Because of their different affinities for extracellular matrix components and receptor subtypes, these isoforms provide a spatial gradient, critical for vessel patterning [6]. The predominant role of VEGF is illustrated by the fact that loss of even a single allele in the mouse results in haploinsufficiency with early embryonic lethality due to severe vascular defects [7,8]. Additional tissue-specific and conditional inactivation studies of VEGF revealed that VEGF is absolutely required for the formation of the heart and the large vessels that connect with the heart [9] but also for vascular expansion of various organs in the body during postnatal growth (e.g., kidney [10], bone [11], and retina [6]). The adult vasculature seems to be less critically dependent on VEGF, although it still requires low maintenance levels of VEGF.

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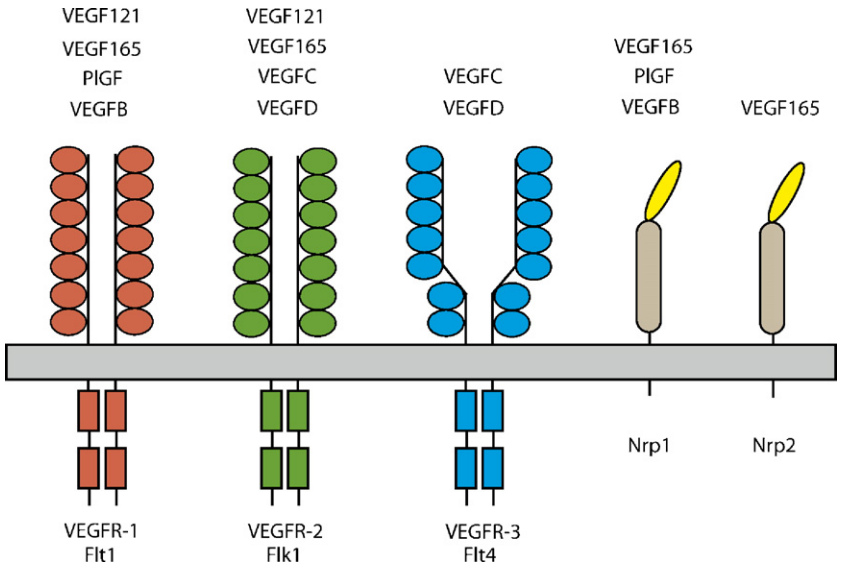

Fig. 1. Ligands and receptors of the VEGF family. Over the last few years, several molecules structurally related to VEGF have been identified, including placenta growth factor (PlGF), VEGFB, VEGFC and VEGFD. All these ligands bind and activate the same receptors, although with different affinities. VEGF is transcribed from a single gene into various isoforms (VEGF121, VEGF164, and VEGF189), exhibiting distinct receptor specificities. Three VEGF tyrosine kinase receptors have been identified: VEGFR-1 (or Flt1), VEGFR-2 (or Flk1), and VEGFR-3 (or Flt4). Neuropilins 1 and 2 (Nrp1 and Nrp2) are another class of high-affinity non-tyrosine kinase receptors for VEGF.

Substantial experimental evidence further implicates VEGF in angiogenesis in pathological conditions: VEGF mRNA is markedly upregulated in the vast majority of human tumors, while occlusion of the left anterior descending coronary artery results in a dramatic increase in VEGF levels in the pig myocardium [12]. Presently, both therapeutic angiogenesis using recombinant VEGF and VEGF gene transfer (for the treatment of coronary or limb ischemia) and inhibition of VEGF-mediated tumor vascularization are actively being pursued clinically [12]. Two years ago, an anti-VEGF antibody was approved as the first successful angiogenesis inhibitor for the treatment of colorectal cancer, underlining the overall feasibility of angiogenesis inhibition strategies [13].

2. Neural-derived VEGF guides the neurovasculature

The vasculature of the central nervous system (CNS) undergoes considerable angiogenesis in both the fetus and premature infant to support blood supply to the rapidly developing cortex until reaching its normal density in adulthood. This process is controlled by a strict temporal and spatial-regulated expression of VEGF by neurons and glia cells in the brain [4,14–16]. Initially, VEGF is expressed by neurons of the outer cortex layers, but as development proceeds, VEGF expression progresses to the deeper layers of the cortex. Cortical vascularization proceeds in a similar fashion with vessels starting from meningeal layers early in development but then penetrating to deeper (VEGF-expressing) cortical layers to form the dense vascular network seen at later stages. Interestingly, glial expression of VEGF coincides with the time at which glial end feet begin to invest blood vessels. Glial cells may thus produce the VEGF signals required to maintain the vasculature.

The importance of neural-derived VEGF is illustrated by findings that neuronal inactivation of VEGF in the early embryonic mouse brain impairs angiogenesis and causes neuronal degeneration of cerebral structures such as the hippocampus [17,18]. Neuronal inactivation of the main VEGF receptor, Flk1, did not result in an overt phenotype during neural development, suggesting that in these mice, VEGF affected neural development primarily by affecting vascular development [17]. Mice overexpressing VEGF in neurons exhibited increased densities of vessels in the cerebral cortex, thereby confirming that neuron-derived VEGF is a critical parameter determining the density of the vasculature in the developing CNS [19]. VEGF also provides positional cues directing the growth of capillaries towards the ventricular zone, since loss of the normal VEGF gradient in mice expressing only the $VEGF_{121}$ isoform leads to impaired growth of blood vessels towards the midline of the developing hindbrain [20]. The role of VEGF within the central nervous system is however not restricted to its effect on the vasculature as VEGF is also involved in other aspects of neurodevelopment (see further below).

During normal development, the specification of endothelial cells in the arteries in some peripheral tissues is co-determined by the presence of nerves. This leads to an intimate neurovascular association, such as for instance in the skin of the embryonic limb, where arteries follow the branching pattern of nerves, while veins do not [21]. In mutant embryos lacking sensory nerves (neurogenin 1 and 2 homozygous mutants are characterized by a virtually complete absence of peripheral nerves), arteries fail to properly differentiate, while in embryos with a disorganized patterning of nerves (in mice lacking semaphorin 3A, the pattern of peripheral nerve growth is completely disorganized, axons appear more fasciculated and are less finely branched), the trajectory of blood vessel branching is also altered and, remarkably, follows that of the misrouted nerves [21]. In vitro, VEGF expressed by sensory neurons and Schwann cells induces arterial marker expression in isolated endothelial cells, suggesting that peripheral nerves

determine the pattern of blood vessel branching and arterial differentiation in the skin via local secretion of VEGF [21]. Consistently, VEGF administration or inhibition of VEGF signaling using soluble Flt1 perturbed vascularization of developing forelimbs in quail embryos, but had little effect on the pattern of innervation [22]. Likewise, inhibition of Flk1 or VEGF sequestration using soluble Flt1 had no effect on axonal outgrowth in embryonic DRG explants, while connectivity, branching and structural integrity of the capillary network were severely disrupted [23]. However, when VEGF signaling from nerves to vessels is disrupted – as accomplished by intercrossing floxed VEGF mice with Wnt1-Cre mice, which express the Cre recombinase in both sensory nerves and peripheral glia – the expression of arterial markers was strongly reduced, although nerve-vessel alignment itself was apparently unperturbed [24]. Although the latter observation suggests that additional factors are necessary for the correct alignment between arteries and nerves, nerve-derived VEGF appears to be essential for the specification of arteries in the skin of the embryonic limb [24].

3. The duality of neuropilins implicates VEGF in neural patterning

Neuropilins are 130- to 140-kDa cell-surface receptors with short intracellular domains insufficient for the independent transduction of biological signals [25]. They have been implicated in vessel patterning because they are receptors of specific VEGF isoforms (Fig. 1) [26]. Neuropilins co-assemble with the VEGF receptor Flk1 and thereby enhance the proliferation and migration of endothelial cells in response to VEGF. Concomitantly, zebrafish or mice lacking Nrp1 exhibit branching and remodeling defects of vessels [27,28]. Neuropilins are also receptors for the semaphorins, which are secreted repellent factors involved in neuronal guidance [29–31]. They serve as co-receptors for receptors belonging to the plexin family and as such plexin/neuropilin complexes are able to transduce semaphorin-mediated repulsion [32,33]. Consequently, mice lacking Nrp1 suffer also from a severely disorganized neural development [34]. Mice expressing a variant Nrp1, only capable of binding VEGF but not semaphorins, do not exhibit vascular defects, suggesting that the vascular role of Nrp1 is mediated by the binding of VEGF to Nrp1 [35].

The neuronal role of Nrp1, on the other hand, seems to be mediated both by semaphorins and VEGF. Recent evidence indicates that VEGF and its co-receptor Nrp1 are required for the migration of facial branchiomotor neurons [36]. Facial branchiomotor neurons are born in a ventral position of the hindbrain segment termed rhombomere 4 and then move their somata caudally to travel through rhombomere 5 and into rhombomere 6. This migration spans several days, covers many cell diameters, and culminates in the formation of the paired motor nuclei of the VIIth cranial (facial) nerve, which control the movement of the facial musculature. In mice lacking Nrp1, the migration of facial branchiomotor neuron somata was compromised, resulting in the formation of misshapen and mal-positioned facial motor nuclei [36]. VEGF controlled the migration of soma of facial branchiomotor neurons by interacting with Nrp1 but was not required for their axon guidance [36]. Instead, semaphorine 3A was essential for axonal guidance, but not for the pathfinding of the somata of the branchiofacial motor neurons [36]. These observations demonstrate, for the first time, that VEGF contributes to neuronal patterning in vivo, and that different compartments of one cell can be co-ordinately patterned by structurally distinct ligands for a shared receptor.

In zebrafish, the segmental outgrowth of axons and somata from primary motor neurons in the trunk is also mediated by Nrp1 [37]. Indeed, knockdown of Nrp1 by morpholino oligonucleotides induces aberrant branching of motor axons, additional exit points of motor axons from the trunk and migration of motor neurons out of the spinal cord [37]. Co-injection of semaphorin3A or VEGF-directed morpholinos with Nrp1 morpholinos at a concentration not inducing motor axon or motor neuron outgrowth defects when injected alone did induce significant defects [37]. Nrp1 in primary motor neurons may thus integrate signals from several ligands, including semaphorin3A and VEGF, and are needed for proper segmental growth of primary motor neuron cell bodies and nerves in zebrafish. In conclusion, VEGF seems to play an important role as a guidance molecule, not only on the developing vasculature, but also during neurodevelopment.

4. The VEGF system in invertebrates

VEGF and its receptors appeared first in evolution in the central nervous system (CNS) of species, such as the worm and fruitfly, which lack a well-developed vascular system. In the *Caenorhabditis elegans* genome, there is a family of four tyrosine kinase receptors, which are structurally related to VEGF receptors [38]. The common expression sites of these receptors (*V*ascular *E*ndothelial growth factor *R*eceptor or ver genes) are specialized cells of neural origin: ver-1 is expressed in the support (glial) cells of amphid and phasmid neurons, ver-2 in chemosensorial neurons, and ver-3 in the ALA neuron of the dorsal ganglia. In the fruit fly *Drosophila melanogaster*, the hemocytes (or primitive blood cells) express a receptor tyrosine kinase, related to mammalian *P*DGF and *V*EGF *R*eceptors (PVR) [39,40]. This receptor is required for hemocytes to migrate in response to three VEGF orthologs, which are expressed along hemocyte migration routes. Loss of PVR function also causes defects in axon tract morphology and in positioning of glial cells. VEGF and its receptors thus seem to have originated from the central nervous system.

5. Neuroprotective activities of VEGF

Recent in vitro findings revealed that VEGF has direct effects on a variety of neuronal cell types. For instance, VEGF increases the survival of cultured hippocampal, cortical, cerebellar, granule, dopaminergic, autonomic and sensory neurons under various conditions of stress (reviewed in [41–43]). In general, the survival activity of VEGF relies on the same tyrosine kinase receptors and downstream protein kinases described previously in non-neural tissues, including activation of mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K)/Akt, and Rho/Rac, and suppression of the pro-apoptotic caspase-3 [44]. When applied to primary cortical neurons, VEGF increases the expression of the neuronal microtubule markers TUJ1 and MAP-2,

suggesting that VEGF also regulates the growth, development and structural stability of neurons [45]. VEGF protects NSC34 motor neuron-like cells against hypoxia and oxidative-stress-induced apoptosis — an effect, blocked by a combination of anti-Nrp1 and anti-Flk1 antibodies [46], as well as primary motor neuron cultures under basal conditions and after hypoxia/hypoglycemia-induced cell death [47]. Notably, intrathecal infusion of Flk1 antisense oligonucleotides in rats, followed by a hypoxic challenge for 7 days, results in approximately 50% loss of motor neurons [48].

6. Effects of VEGF on other neural cell types

VEGF also affects other neural cell types. For instance, VEGF has mitogenic effects on astrocytes, in mesencephalic explant cultures [49] and following intracerebral VEGF delivery in vivo [50]. Most astrocytes express the VEGF receptor Flt1, while immunoexpression for the Flk1 receptor is absent [51]. VEGF administration to fetal and postnatal cortical organotypic explant cultures cause a significant increase in astroglial proliferation and a dose-dependent increase in GFAP and nestin immunoreactivity, whereas co-treatment with antisense oligonucleotides to Flt1 results in a dramatic decrease in GFAP and nestin immunoreactivity, confirming the role of Flt1 in mediating VEGF's gliotrophic effects [51]. VEGF also induces proliferation of microglia through Flt1, both in the murine BV2 cell line as in primary microglial cells, as well as chemotaxis in Boyden chamber assays [52]. VEGF also prolongs the survival and stimulates the proliferation of Schwann cells in explant cultures of superior cervical and dorsal root ganglia [53]. Schwann cells express Flt1, Flk1 and Nrp1, but the effect of VEGF on Schwann cell migration appears to be mediated by Flk1, as addition of a neutralizing antibody against Flk1 completely blocks VEGF-induced migration [54]. Thus, the direct effects of VEGF on neurons and Schwann cells are mediated predominantly by Flk1 (and Nrp1), whereas the effects of VEGF on astrocytes and microglia are directed by Flt1.

Besides its effects on differentiated neurons, VEGF also stimulates neurogenesis in vitro and in vivo. As a competitive antagonist of Sema3A for Nrp1 binding, VEGF also promotes the survival, migration, and proliferation of neural stem cells [55]. After infusion of VEGF into the lateral ventricle of adult rats, the number of BrdU-positive neurons increases, not by stimulating the proliferation of neural stem cell progenitors, but by preventing them to undergo apoptosis [56]. Enriched environments, exercise and hippocampus-dependent learning tasks stimulate neurogenesis [57]. VEGF, by acting through Flk1, seems to mediate some of the enriched environment effects on neurogenesis and cognition [57].

7. VEGF in ALS

Amyotrophic lateral sclerosis (ALS) – also termed Lou Gehrig's disease – is a progressive, adult-onset neurodegenerative disorder characterized by degeneration and loss of large motor neurons in the spinal cord, brainstem, and cerebral cortex, leading to muscle atrophy, paralysis and death usually within 5 years after onset of clinical symptoms (reviewed in [58–62]). ALS occurs sporadically in 90% of cases, the remaining 10% being familial (FALS) [63]. Several types of familial ALS have been assigned to loci of the human genome. ALS1, an autosomal dominant form of adult ALS affects 14 to 23% of individuals with familial ALS and is associated with more than 100 different mutations in the gene encoding Cu/Zn superoxide dismutase (SOD1) on chromosome 21 [64]. Mutant SOD is generally believed to cause motor neuron degeneration by gaining a toxic property [65]. Mice and rats, expressing a mutant SOD1 transgene, develop ALS with clinical and anatomo-pathological features, that are highly reminiscent of those found in human ALS and are by far the most frequently studied animal model for ALS [66,67]. However, the mechanisms by which this toxic function causes ALS are still debated and remain elusive [61]. Only very recently, additional genes associated with familial ALS were discovered: (i) identical missense mutations in the vesicle-trafficking protein VAPB gene have been identified in families with atypical ALS (ALS8) [68]; (ii) homozygous mutations in ALSIN, a putative guanine nucleotide factor for GTPase, have been found in a family with juvenile-onset ALS (ALS2) [69,70] and (iii) missense mutations in senataxin, a gene that encodes a novel DNA/RNA helicase, have been found in autosomal dominant forms of juvenile ALS (ALS4) [71]. The genetic causes for the other forms of ALS are not yet known.

Recently, a novel role for VEGF in ALS was documented. Mice with a subtle deletion of the hypoxia response element (HRE), to which the hypoxia-inducible transcription factors HIF-1α and HIF-2α bind, in the VEGF gene promoter ($VEGF^{\delta/\delta}$ mice), produced insufficient VEGF levels leading to adult-onset motor neuron degeneration, reminiscent of ALS [46] (Fig. 2A, B). Many neuropathological features seen in $VEGF^{\delta/\delta}$ mice, such as specific loss of choline-acetyltransferase-positive motor neurons, the ultrastructural signs of motor neuron degeneration, axonal spheroids, aberrant neurofilament inclusions, Wallerian degeneration in peripheral nerves and selective loss of large myelinated motor axons, are strikingly similar to those found in mutant SOD1 mice and in ALS patients. Therefore, the $VEGF^{\delta/\delta}$ mouse model may be useful to study the mechanisms of adult-onset motor neuron degeneration and to evaluate therapeutic strategies.

These surprising genetic findings in mice seem to be relevant for human ALS as well. Although spontaneous mutations of the hypoxia response element were not detected in ALS individuals, human subjects homozygous for the −2578A/−1154A/−634G or −2578A/−1154G/−634G haplotypes in the VEGF gene, were more common in the population of ALS patients than healthy individuals [72]. These 'at-risk' haplotypes reduced VEGF gene transcription, impaired IRES-dependent translation, and interfered with translational initiation of a novel long VEGF isoform (L-VEGF), resulting in reduced circulating VEGF levels in vivo. To further characterize the role of VEGF in ALS, $VEGF^{\partial/\partial}$ mice were intercrossed with $SOD1^{G93A}$ mice. Normally, $SOD1^{G93A}$ mice develop clinical symptoms at the age of 90 days and die at the age of 130 days. In contrast, $VEGF^{\partial/\partial}$ mice only develop symptoms beyond the age of 6 months and survive for up to 2 years. Notably, double transgenic $VEGF^{\partial/\partial}$/$SOD1^{G93A}$ mice died sooner than single transgenic $SOD1^{G93A}$ mice, indicating that VEGF also modified motor neuron degeneration in the standard mouse model of ALS [72]. By now, our

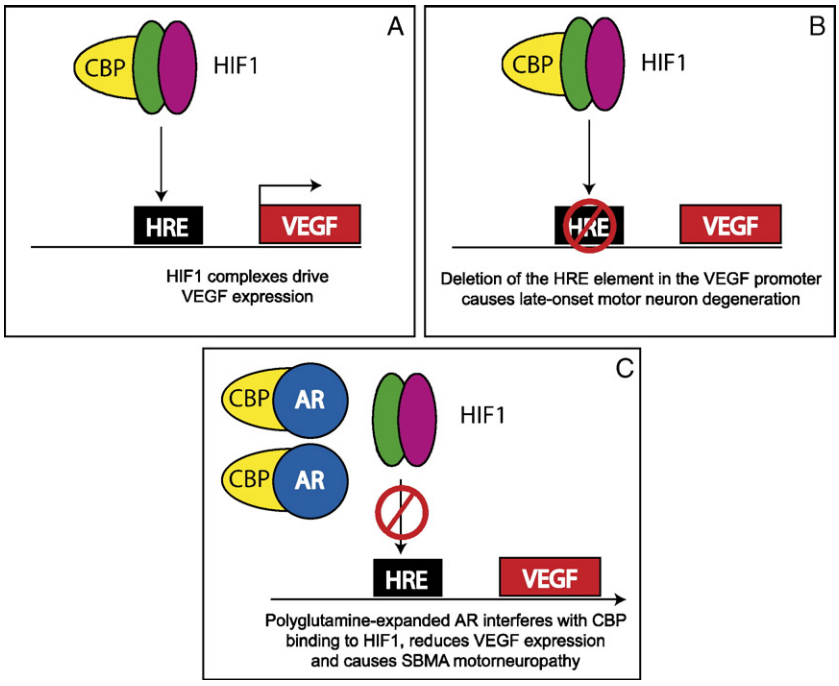

Fig. 2. Deregulation of VEGF expression levels are involved in at least two types of motor neuron degeneration. (A) Under normal conditions, hypoxia-induced factors (HIFs) such as HIF1, co-assemble with transcriptional coactivator CREB-binding proteins (CBP), bind to the hypoxia regulatory element (HRE) in the VEGF promoter and drive expression of the VEGF gene. (B) Deletion of the HRE region in the VEGF promoter causes a late-onset progressive form of motor neuron degeneration, reminiscent to ALS. (C) Expression of polyglutamine-expanded androgen receptor (AR) traps CBP proteins, thereby interferes with CBP binding to HIF and causes SBMA motorneuropathy.

observations have been confirmed by additional studies. Indeed, in SOD1^{G93A} rats, VEGF levels are reduced before disease onset and become progressively lower with disease progression [73]. In humans, low levels of VEGF in the cerebrospinal fluid (CSF) are also a hallmark of early ALS [74]. In a much smaller association study by Terry et al., individuals homozygous for the 'at-risk' haplotypes exhibited a 3-fold increased risk for ALS [75], whereas studies performed within the Dutch and UK population reported that association between the 'at-risk' haplotypes and ALS was lacking [76,77]. Although the precise reason for the lack of association in this population remains unknown, heterogeneity between study populations is not entirely uncommon for association studies and should not, a priori, devaluate a positive association in other populations. On the other hand, independent replicative studies should still be encouraged as meta-analysis of all available published association studies is expected to give a more robust estimate of the genetic effect [78]. VEGF levels in the CSF from hypoxaemic patients with ALS are also lower than VEGF levels from normoxaemic patients with ALS, whereas hypoxaemic neurological controls display higher VEGF levels than normoxaemic neurological controls [79]. In ALS patients, fewer anterior horn cells seem to be capable of expressing normal expression levels of VEGF and VEGFR2 [80]. All together, these observations highlight that deregulation of VEGF expression is implicated in the pathogenesis of ALS in both mice and humans.

To provide a better understanding of the molecular basis of the VEGF system in motor neuron degeneration, we generated transgenic mice overexpressing Flk1 in neurons of the adult nervous system by using the Thy1.2 promoter [81]. The Thy-Flk1 transgenic mice were fertile, appeared healthy, and their motor performance under basal conditions was normal. Intercrossing of the Thy-Flk1 transgenic mice with SOD1^{G93A} mice revealed that the motor performance of the double-transgenic Thy-Flk1/SOD1^{G93A} transgenic mice deteriorated at a later age as compared to the single transgenic SOD1^{G93A} mice. Thy-Flk1/SOD1^{G93A} transgenic mice also lived longer than SOD1^{G93A} single transgenic mice, revealing that overexpression of Flk1 in neurons delays the degeneration of spinal motor neurons in SOD1^{G93A} mice by transmitting survival signals of endogenous VEGF [81].

8. Role of VEGF in SBMA motor neuronopathy

VEGF has also been implicated in another form of motor neuron disease, X-linked spinal and bulbar muscular atrophy or Kennedy's disease, which is caused by a polyglutamine expansion in the androgen receptor. Ellerby, La Spada and colleagues produced transgenic mice expressing the expanded human androgen receptor, which caused a progressive disorder comprising muscle weakness, atrophy, weight loss and early death, and manifested histologically by loss of motor neurons from the anterior horns of the spinal cord and neurogenic (grouped) muscle fiber atrophy [82]. Motor neurons from these mice showed reduced viability in cell culture, which was rescued by treatment with VEGF. In this form of muscular atrophy, the overexpressed levels of the androgen receptor seem to interfere with transcription of the VEGF gene by squelching the transcriptional coactivator CREB-binding protein (CBP), which is necessary to allow HIF to induce VEGF gene transcription [82] (Fig. 2A, C). Thus, a disturbance in the neuronal

effects of VEGF is observed in at least two genetically and phenotypically distinct forms of motor neuron disease.

9. Angiogenin, another angiogenic factor linked with ALS

The discovery of the link between VEGF and ALS has also stimulated interest in evaluating the role that other so-called angiogenic factors could play in modifying the risk of developing ALS. Greenway and colleagues focused their attention on angiogenin (ANG), predicting that, similar to VEGF, functional abnormalities of ANG would enhance the risk of ALS [83]. To test their hypothesis, Greenway et al. led a Euro-American collaborative effort, in which the scientists sequenced the coding and flanking regions of the ANG gene [84]. At least seven different heterozygous missense mutations were identified in the ANG gene from patients with ALS, six of which were in evolutionary conserved regions of the ANG gene. The functional consequences of these ANG mutations were not studied, and therefore, a direct causative link between ANG mutations and ALS is still lacking.

Unfortunately, we still have a poor understanding of the biological properties of ANG, and this imposes limitations in our ability to identify potential molecular mechanisms by which ANG mutations are involved in ALS. Several studies have described ANG as a potent angiogenic factor [85]. Particularly, the angiogenic effects of VEGF are dependent to some extent, upon the activity of ANG, i.e., ANG appears to be a downstream effector of VEGF, at least in endothelial cells [86]. It is therefore reasonable to speculate that ANG and VEGF might be neuroprotective in motor neurons via similar or overlapping pathways. Alternatively, ANG may also have distinctly VEGF-independent modes of action that result in increased susceptibility to ALS, e.g., by regulating innate immunity [87] or ribosomal RNA synthesis [88].

10. Finding a therapy for ALS

Despite intensive efforts, ALS remains an incurable disorder. Riluzole, a glutamate antagonist, is currently the only drug that has been approved for the treatment of ALS, but it provides only a marginal therapeutic benefit by retarding the decline in muscle strength and prolonging survival by only a few months [89]. Neurotrophic factors have been considered for therapy of motor neuron degeneration, but previous clinical trials have all failed. Indeed, intrathecal infusion of brain-derived growth factor (BDNF) and ciliary neurotrophic factor (CNTF), intracerebroventricular (ICV) delivery of glial cell line-derived neurotrophic factor (GDNF) or systemic administration of BDNF or CNTF did not result in clinical improvement in ALS patients (reviewed by [90–92]). When delivered systemically, insulin-like growth factor (IGF1) marginally reduced disease progression in one but not in another trial [93,94]. In $SOD1^{G93A}$ mice, leukemia inhibitory factor (LIF) improved motor neuron survival without prolonging lifespan in one study but had no effect in another study [95,96], whereas delivery of BDNF, GDNF, CNTF and IGF1 recombinant factors has never been evaluated in $SOD1^{G93A}$ mice. Some of these clinical trials with neurotrophic agents may have failed because of inappropriate delivery of these neurotrophic factors. Indeed, when delivered systemically, neurotrophic factors often fail to cross the blood–brain barrier and thus never reach the motor neurons [97]. In addition, these polypeptides are usually rapidly cleared from the circulation and may evoke a neutralizing immune reaction after repetitive injections [97]. When delivered into the CSF, neurotrophins may also be rapidly cleared into the venous or lymphatic system, or be unable to penetrate into the surrounding parenchyma because they are sequestered by cellular receptors on the ependymal lining — all preventing these neurotrophic factors to reach the degenerating motor neuron [97]. Optimizing the appropriate delivery route is thus critical for therapeutic success.

11. Gene therapy approaches for ALS

Treatment of motor neuron degeneration with neurotrophins requires chronic delivery. This can be achieved by gene therapy or repetitive protein delivery. An advantage of viral gene transfer is that the transduced cells produce a ‘natural’ neurotrophic protein with a mammalian-type glycosylation pattern, which is not recognized as a foreign antigen. In the past, only viral vectors were available which were capable of infecting the motor neuron cell bodies when the viral vector was injected directly into the anterior horn of the spinal cord. Such invasive surgery is obviously not a therapeutic option in humans. More recently, improved vectors have been developed, which facilitate the delivery of neurotrophic factors to the motor neuron soma [98,99]. These vectors take advantage of the intrinsic property of the motor neuron to retrogradely transport cargo from the nerve terminal via its long axon to the cell body. The polio, herpes and rabies viruses are capable of infecting the nerve terminals at the neuromuscular junction. Thereafter, these neurotropic viruses highjack the axonal transport system and are thereby capable of reaching the motor neuron cell body [98,100,101]. Adeno-associated adenoviral vectors (AAV) and lentiviral vectors pseudotyped with rabies-G envelopes have been constructed, which are retrogradelly transported via the axons upon intramuscular injection (as reviewed by Azzouz [102]). These vectors are particularly relevant for the treatment of motor neuron disorders, as they offer the opportunity to administer these vectors via peripheral intramuscular injection.

12. VEGF delivery with the retrogradely transported EIAV lentiviral vector

To evaluate the therapeutic potential of VEGF for ALS, a rabies-G pseudotyped EIAV lentiviral vector encoding the human VEGF gene, EIAV-VEGF, was constructed [99]. After confirming that this vector was able to produce active VEGF, EIAV vectors were injected bilaterally into the hindlimb gastrocnemius, diaphragm, intercostal, facial and tongue muscles of $SOD1^{G93A}$ mice. VEGF treatment significantly extended the lifespan of these mice by an average of 38 days. Thereby, VEGF treatment achieved one of the most effective therapies reported in the field so far, prolonging survival by as much as 30% [99]. The size of the observed effect was comparable to that reported for IGF1 when delivered with AAV2 vectors [98]. Notably, administration of

VEGF therapy at the time of disease onset was also effective, prolonging survival by as much as 19 days [99]. When injecting a similar amount of a rabies-G pseudotyped EIAV vector expressing GDNF, a negligible increase in survival of 6 days was observed [99]. These findings indicate a superior specificity and potency of VEGF over GDNF for ALS. EIAV-VEGF gene transfer was also well tolerated and did not cause vascular side effects.

13. Recombinant VEGF protein therapy

Though clinical trials with viral vectors are being considered, the clinical applicability, feasibility, efficacy and safety of gene therapy for ALS remain to be established. Delivery of recombinant neurotrophic growth factors, on the other hand, is clinically very attractive, as it offers flexible control of the dose and duration of the administered protein. Previous clinical trials with delivery of a neurotrophic factor failed, however, to show a therapeutic effect, presumably because the factors were insufficiently active, immunogenic, rapidly cleared or inappropriately delivered. By using osmotic mini-pumps implanted subcutaneously on the back of SOD1^{G93A} rats, another rodent model of ALS, we were capable of delivering the recombinant protein continuously via a catheter stereotactically implanted in the left lateral ventricles of rats for more than 100 days [81]. Distribution studies revealed that ^{125}I-VEGF, after bolus injection into the left lateral ventricle, diffused from the CSF into the parenchyme of the brain and spinal cord and reached the motor neurons, where it remained intact for several hours [81].

Compared to rats treated with artificial cerebrospinal fluid (aCSF), SOD1^{G93A} rats receiving 0.2 μg VEGF/kg/day exhibited a delayed disease onset, an improved motor performance, a longer spontaneous activity and prolonged survival [81]. When scoring spontaneous activity, for instance, VEGF-treated animals consistently performed better than controls for as long as 35 to 42 days, whereas survival of SOD1^{G93A} rats was prolonged by 22 days after VEGF treatment. SOD1^{G93A} rats tolerated the VEGF treatment well without adverse effects. To evaluate whether ICV delivery of VEGF would be capable of prolonging survival when initiated at the time of disease onset, 0.2 μg VEGF/kg/day was administered from 85 days of age, when the first signs of motor impairment emerged. Despite the very rapidly progressing disease course of this model, treatment of SOD1^{G93A} rats with ICV delivered VEGF significantly improved survival by 10 days [81]. VEGF treatment at 60 days also changed the disease subtype from a severe to a much milder form. Indeed, significantly fewer SOD1^{G93A} rats suffered from the severe forelimb (FL) onset type of disease after VEGF than aCSF delivery. Compared to aCSF-treated rats with hindlimb (HL)- or FL-onset, VEGF-treated rats survived 17 or 27 days longer, respectively [81]. This is a relevant finding, as involvement of brain stem and cervical disease results in a worse prognosis in both rats and humans. The more pronounced therapeutic effect of VEGF on forelimb than hindlimb muscles is likely attributable to the higher VEGF levels in the bulbar/cervical than in the lumbar spinal cord upon ICV delivery.

Recently, systemic delivery of recombinant VEGF was also shown to prolong the survival of SOD1^{G93A} mice. As VEGF is unlikely to cross the blood–brain barrier, these data may possibly suggest that VEGF affects motor neuron survival indirectly via effects on the vasculature [103].

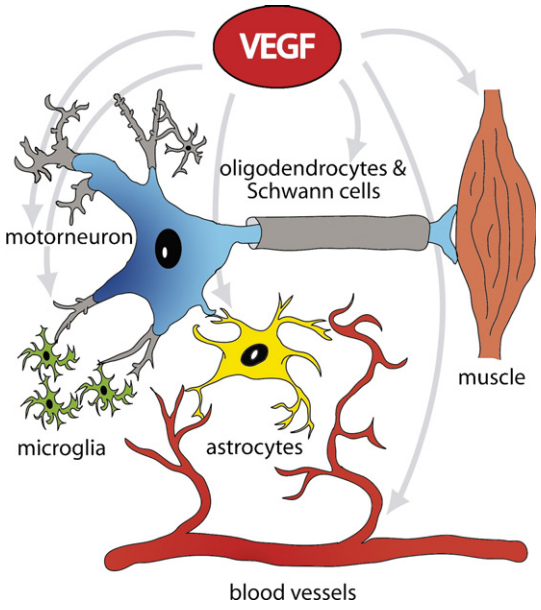

Fig. 3. VEGF is a pleitrophic factor: VEGF affects motor neurons, microglia, astrocytes, muscle fibers, blood vessels, oligodendrocytes and Schwann cells.

14. Therapeutic mechanisms of VEGF

VEGF has distinct biological activities, which differ from those of other neurotrophic factors. Similar to IGF1, VEGF has pleiotropic effects on various different types of neuronal and glial cells, stimulates neurogenesis, is axonally transported in an antero- and retrograde direction and stimulates neural perfusion [41,45,81]—all these effects might have contributed to promote motor neuron survival in ALS models (Fig. 3). In addition, while VEGF and other neurotrophins are capable of affecting motor neurons, their importance in the pathogenesis of ALS may differ substantially. Indeed, even though over- or under-expression of BDNF, GDNF, CNTF or LIF affected motor neurons during development and injured motor neurons after birth, and although adenoviral gene transfer of some of these factors stimulated motor neuron survival in ALS mouse models, loss of these neurotrophins did not cause adult-onset ALS-like motor neuron degeneration and paralysis in mice.

15. Role for VEGF in acute neurological diseases

VEGF has also been implicated in several acute neurological disorders, in which it exerts both positive and negative effects. In addition to its protective effects, VEGF expression has also been implicated in blood–brain barrier breakdown after cerebral ischemia [104,105], and as an inflammatory mediator, it may also contribute to inflammatory responses under certain disorders. All these activities of VEGF make it challenging to determine whether VEGF will aggravate or improve clinical outcome of experimental paradigms, and whether these effects are due to its neuro-, glio- or endotheliotrophic activities.

Following cerebral ischemia, disruption of the blood–brain barrier occurs acutely, followed by leakage of plasma proteins into the brain parenchyma (edema) and infiltration of inflammatory cells into the infarcted region. VEGF may stimulate vascular leakage and vessel permeability and have detrimental effects after cerebral ischemia. Early post-ischemic administration of VEGF resulted in prominent brain edema, hemorrhagic transformation of the infarct, and enlargement of the ischemic lesion [106], whereas intraperitoneal administration of a fusion protein that sequesters VEGF reduced infarct size in another study [107], indicating that the effects of VEGF in cerebral ischaemia can indeed be detrimental. Likewise, acute intraparenchymal injection of VEGF after spinal cord injury also resulted in exacerbation of the spinal cord lesion volume [108]. On the other hand, VEGF expression is strongly induced after acute focal cerebral ischemia, as early as 1–3 h, and peaks at 24–48 h after onset of ischemia [109–112], suggesting that VEGF may have beneficial effects as well. Indeed, administration of VEGF at 48 h after stroke protected neurons and improved neurological outcome [113,114]. From 7 days after the stroke, cerebral microvascular perfusion and vessel architecture in the ischemic penumbra were increased and led to improved vascular perfusion. In a model of severe spinal cord ischemia induced by clamping the thoracic vessels, most of the ventral horn neurons degenerate leading to complete paralysis in mice [115]. VEGF treatment rescued some of the motor neurons from death in this model and resulted in a significantly improved neurological outcome [115]. After spinal cord injury, Widenfalk et al. also observed beneficial effects of VEGF delivery attributable to angio- and neuroprotective effects as revealed by the increased density of blood vessels and the reduced number of apoptotic cells within the lesion [116]. Remarkably, intracerebroventricular administration of VEGF also enhanced neurogenesis in a stroke model as the number of BrdU-positive labeled cells of neuronal lineage was increased after 4 weeks [113,114]. Like VEGF, its homologue VEGF-B (Fig. 1) also seems to exert a neuroprotective effect in cerebral ischaemia [117] as the infarct volume after cerebral ischemia was increased in VEGF-B-knockout compared with wild-type mice, and neurological function was more impaired. Notably, VEGF-B also stimulated neurogenesis [118]. Thus, in animal models of stroke, early delivery of VEGF (within 1 h of onset) worsens outcome by increasing vascular permeability and brain edema, whereas late administration of VEGF (48 h after onset) is beneficial. How exactly VEGF exerts its protective effects in stroke still remains to be further established, but it most likely involves a combination of neuroprotection, vascular perfusion and neurogenesis.

Blood–brain barrier breakdown, edema and inflammation are also observed after seizures, although the magnitude of these events is substantially milder than seen after cerebral ischemia [119]. Because of these similarities, mediators of post-ischemic injury are now also being investigated in seizures. One day after a status epilepticus, VEGF expression is dramatically increased both in neurons and glial cells of the hippocampus and limbic cortex [119]. VEGF also protects cultured hippocampal neurons against glutamate excitotoxicity [120], whereas intra-hippocampal infusion of VEGF appears to protect hippocampal neurons from seizure-induced damage [119]. To examine the effect of VEGF on the intrinsic properties and synaptic responses of hippocampal neurons, VEGF was administered to adult rat hippocampal slices. VEGF reduced the amplitude of excitatory responses elicited after stimulation and was able to suppress epileptiform activity in epileptic rats, but not in normal rat slices [121]. VEGF may thus also play a role to decrease epileptiform activity in the epileptic brain.

16. VEGF in other neurodegenerative disorders

VEGF is also important for diseases of the peripheral nervous system. Intramuscular administration of plasmid DNA encoding VEGF improved nerve function in rats with diabetic (streptozotocin-induced) polyneuropathy [122], and intramuscular delivery of naked VEGF DNA had a similar effect in rabbits with ischemic neuropathy [54]. Recently, Fink and colleagues administered a replication-defective herpes simplex virus (HSV) vector expressing VEGF intramuscularly to mice with streptozotocin neuropathy and found preservation of nerve fibers, increased sensory nerve amplitudes and thermal pain perception, improved autonomic function and greater nerve vascularity [123]. An additional study demonstrated that VEGF application via matrigel in a silicone sciatic nerve chamber enhanced myelin axon counts by 78% and significantly improved motor performance [124]. In these experiments, VEGF increased angiogenesis in the chamber and enhanced Schwann cell proliferation and migration. Though it is attractive to postulate a direct neuroregenerative role of VEGF in these studies, it is difficult in many of these examples to assess whether neural repair or neuroprotection are the results of a direct neurotrophic effect or are secondary to VEGF-driven angiogenesis and hence improved perfusion of the ischemic region.

Alzheimer's disease (AD) is characterized by amyloid plaques, neurofibrillary tangles, cerebral hypoperfusion and amyloid angiopathy. The observation that cerebral hypoperfusion precedes the onset of clinical symptoms in AD suggests that chronic brain hypoperfusion increases the risk for AD [125]. Amyloid-β has direct effects on the cerebral vasculature: it causes vasoconstriction, reduces the blood flow, and even induces endothelial cell damage. Furthermore, VEGF co-localizes with and binds amyloid-β plaques in the brains of AD patients [126]. Such a deposition of VEGF in plaques may reduce the availability of VEGF and thereby causally contribute to cerebral hypoperfusion in AD and further aggravate memory decline. Other studies have also suggested that cerebral microvascular amyloid accumulation is a better correlate with dementia than parenchymal amyloid plaques [127], indicating that AD has also a neurovascular component. Interestingly, the −2578A VEGF allele, which correlates with reduced VEGF expression levels in plasma and serum, also confers an increased risk for patients with Alzheimer disease in one study [128], but not in another [129]. Others reported that expression levels of VEGF in the CSF of AD patients are increased and that VEGF immunoreactivity is also enhanced around perivascular astrocytes and walls of cerebral vessels in the subjects with AD when compared to elderly [130,131]. However, since cerebral hypoperfusion leads to cerebral ischemia, which increases VEGF expression levels, increasing VEGF expression levels may be a compensatory repair mechanism to counteract

insufficient vascularity, reduced perfusion and neuronal apoptosis. Although little is known about the involvement of VEGF in AD, recent studies on VEGF and AD suggest that VEGF may indeed contribute to neurodegeneration and vascular dysfunction in AD.

There is some evidence suggesting that VEGF could have a protective role against the selective degeneration of dopaminergic neurons in Parkinson's disease. Unilateral implantation of encapsulated VEGF-producing cells into the striatum of adult rats, 1 week before administration of 6-OHDA, reduces amphetamine-induced rotational behavior, augments tyrosine-hydroxylase-positive neurons and fibers, and increases vascularization and proliferation of glia [132,133]. Furthermore, a single bolus injection of VEGF into the striatum of unilaterally 6-OHDA lesioned rats before transplantation of solid ventral mesencephalic grafts into the same striatum results in more homogeneous distribution of small blood vessels throughout the graft and accelerated recovery of amphetamine-induced rotational asymmetry [134]; therefore, VEGF may also improve the function of fetal ventral mesencephalic tissue grafts.

All these studies suggest that VEGF might have therapeutic potential for several neurological diseases. However, when considering VEGF therapy, careful optimization of the VEGF dose will be required to avoid hemangioma, edema formation and ventriculomegaly, an increase in tumor angiogenesis and tumor-associated intracranial hemorrhage.

17. Conclusion

Originally discovered in 1983, VEGF has come forth as the key mediator of angiogenesis in health and disease. Only recently have direct effects of VEGF on neuronal cell types been described. It is now evident that VEGF is implicated in ALS, but also in other neurodegenerative disorders. Evidence that VEGF gene transfer or protein delivery improves the outcome of ALS is most promising.

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