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Roles of the endogenous VEGF receptors *flt-1* and *flk-1* in astroglial and vascular remodeling after brain injury

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Abstract

Following trauma to the brain significant changes occur in both the astroglial and vascular components of the neuropil. Angiogenesis is required to re-establish metabolic support and astrocyte activation encompasses several functions including scar formation and the production of growth factors. VEGF has seminal involvement in the process of brain repair and is upregulated during many pathological events. VEGF signaling is regulated mainly through its two primary receptors: *flk-1* (KDR/VEGF-R2) is expressed on vascular endothelium and some neurons and *flt-1* (VEGF-R1) in the CNS, is expressed predominantly by activated astrocytes. Using an injury model of chronic minipump infusion of neutralizing antibodies (NA) to block VEGF receptor signaling, this study takes advantage of these differences in VEGF receptor distribution in order to understand the role the cytokine plays after brain injury. Infusion of NA to *flk-1* caused a significant decrease in vascular proliferation and increased endothelial cell degeneration compared to control IgG infusions but had no effect on astrogliosis. By contrast infusion of NA to *flt-1* significantly decreased astroglial mitogenicity and scar formation and caused some increase in endothelial degeneration. Neutralization of the *flt-1* receptor function, but not *flk-1*, caused significant reduction in the astroglial expression of the growth factors, CNTF and FGF by seven days. These data suggest that after CNS injury, endogenous VEGF upregulation (by astrocytes) induces angiogenesis and, by autocrine signaling, increases both astrocyte proliferation and facilitates expression of growth factors. It is likely that VEGF plays an important role in aspects of astroglial scar formation.

Keywords

Astrocytes; gliosis; angiogenesis; VEGF; CNTF; bFGF; nestin

Introduction

The CNS responds immediately to trauma by triggering a series of cellular events that are designed to repair the vasculature and wall off the injured area (Fitch and Silver 1997; Fawcett and Asher 1999; McGraw et al. 2001). Revascularization and repair of the blood-brain barrier

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reestablish metabolic and trophic support to the injured tissue (de Paermentier et al. 1986; Krum and Rosenstein 1988; Orita et al. 1989). Activated astrocytes form a gliotic scar to provide structural support, and isolate the injury site by reestablishing the glia limitans, thus restricting the migration of inflammatory cells into healthy tissue (Stichel and Müller 1998; Sofroniew 2005). Such reactive gliosis is characterized by astroglial proliferation, hypertrophy, process extension, and increased synthesis of intermediate filaments, including GFAP and vimentin, as well as production of many bioactive molecules (Eddleston and Mucke 1993; Silver and Miller 2004). Astroglial reactivity is stimulated by a number of factors, including thrombin, bFGF, CNTF, TGF α , TGF β 1, TGF β 2, TNF α , and IL-1 (Fawcett 1997; Campbell 2001; Swartz et al. 2001), several of which are microglial-derived cytokines (Liberto et al. 2004), and by trauma-induced release of purines such as ATP (Neary and Kang 2005).

One of the cytokines with seminal involvement in the process of brain repair is vascular endothelial growth factor (VEGF), which is upregulated during many pathological events in the CNS, including ischemia (Kovacs et al. 1996; Cobbs et al. 1998; Lennmyr et al. 1998; Issa et al. 1999; Lee et al. 1999; Pichiule 1999; Plate et al. 1999; Jin et al. 2000), cold lesions (Nag et al. 1997; Papavassiliou et al. 1997), spinal cord injuries (Bartholdi et al. 1997; Tsao et al. 1999; Vaquero et al. 1999), brain contusion (Skold et al. 2005) and direct wounding (Krum and Rosenstein 1998, 1999). VEGF is a secreted dimeric protein that is fundamentally important for angiogenesis during development, tissue regeneration/repair and tumor growth throughout the body (Leung et al. 1989; Klagsbrun and D'Amore 1996; Thomas 1996; Ferrara et al. 2003). The major isoform present in most mammalian tissues is VEGF₁₆₅, a 45 kDa heparin binding, secreted glycoprotein (Ferrara et al. 2003). VEGF binds to the tyrosine kinase receptors *flt-1* (VEGF-R1) and (predominantly) *flk-1* (KDR/VEGF-R2) that are expressed by vascular endothelial cells and triggers the mitotic and migratory processes necessary for angiogenesis in the periphery (Shibuya and Claesson-Welsh 2006). VEGF is also a vascular maintenance factor that promotes endothelial cell survival by acting through the *flk-1* receptor (Yang, W. and de Bono 1997; Darland et al. 2003; Huang et al. 2003). In the intact adult CNS, VEGF protein expression is limited to the choroid plexus, area postrema, and cerebellar granule cells (Monacci et al. 1993), and VEGF receptors are normally expressed at very low levels (Peters et al. 1993; Kremer et al. 1997; Soker et al. 1998).

In the injured CNS, VEGF is implicated in post-traumatic angiogenesis, which is dependent upon the upregulation of endothelial *flk-1* (Krum and Rosenstein 1998; Rosenstein et al. 1998; Issa et al. 1999; Krum and Rosenstein 1999; Plate et al. 1999; Proescholdt et al. 1999; Silverman et al. 1999; Jin et al. 2000; Beck et al. 2002; Harrigan et al. 2002; Krum et al. 2002; Mani et al. 2003; Croll et al. 2004; Skold et al. 2005). Concomitantly, VEGF protein is also strongly upregulated in astroglia and inflammatory cells near the damaged area (Bartholdi et al. 1997; Nag et al. 1997; Papavassiliou et al. 1997; Krum and Rosenstein 1998, 1999; Tsao et al. 1999; Vaquero et al. 1999; Salhia et al. 2000; Chodobski et al. 2003; Skold et al. 2005); in ischemia models, neurons have also been reported to express VEGF (Kovacs et al. 1996; Cobbs et al. 1998; Lennmyr et al. 1998; Issa et al. 1999; Lee et al. 1999; Pichiule 1999; Jin et al. 2000).

After traumatic insults, the primary VEGF receptors present a particularly interesting dichotomy in their cellular distribution. The *flk-1* receptor is upregulated in neurons, while *flt-1* is upregulated almost exclusively in reactive astrocytes (Krum and Rosenstein 1998, 1999; Lennmyr et al. 1998; Krum and Khaibullina 2003; Krum and Rosenstein 2004). Although some studies, using different experimental paradigms, have indicated endothelial cells upregulate *flt-1* as well (Widenfalk et al. 2003; Skold et al. 2000), we have not observed unequivocal *flt-1* expression in vascular endothelial cells adjacent to the wound site in our model; however, we find strong *flt-1* expression in the astroglial endfeet that are closely applied to the endothelium (Krum and Rosenstein 1999; Krum et al. 2002). Several recent studies have

demonstrated VEGF's trophic and protective effects on neurons both *in vivo* and *in vitro* (Silverman et al. 1999; Jin et al. 2000, 2001; Matsuzaki et al. 2001; Lambrechts et al. 2003; Rosenstein et al. 2003; Azzouz et al. 2004; Khaibullina et al. 2004). It is now apparent, however, that astrocytes also respond to applied VEGF *in vivo* and *in vitro* (Krum et al. 2002; Khaibullina et al. 2004), and it has been recently demonstrated that endogenous VEGF is an important factor for stimulation of astroglial mitosis after brain injury (Krum and Khaibullina 2003).

Using our injury model of osmotic minipump infusion (Krum and Khaibullina 2003), the present study addresses specifically whether endogenous VEGF, acting via the *flt-1* receptor, regulates astroglial proliferation and survival. Since the *flt-1* receptor is strongly up-regulated in reactive astroglia and is negligible in blood vessels after a penetrating injury to the cerebrum (Krum and Rosenstein 1998, 1999; Krum et al. 2002; Krum and Khaibullina 2003), would its blockade by a specific neutralizing antibody abrogate astrocytic activation, survival and mitogenic responses without decreasing the angiogenic response? Conversely, would inhibition of endothelial *flk-1* receptor activity using the same methodology cause a specific decrease in angiogenesis and endothelial survival, while sparing the astroglial response? The experimental mitigation of such responses in these experiments may provide important clues in understanding the relative contributions of reactive gliosis and revascularization to the overall process of CNS wound repair. This investigation takes advantage of the difference in VEGF receptor expression between astroglia and vascular endothelial cells in order to understand how VEGF regulates wound repair with regard to the relative mitotic activities and survival of these cell types.

The results show that inhibition of the *flk-1* receptor caused significant decreases in vascular endothelial proliferation around the wound site with concomitant increases in vascular degeneration compared to IgG control infusions, yet there were no significant effects on astroglial maintenance or growth factor expression. In contrast, inhibition of the *flt-1* signaling decreased astroglial participation at the injury site with respect to mitogenicity and scar formation. These results confirm that the differential VEGF receptor expression observed between astroglia and endothelial cells has functional importance. These data strongly suggest that, following a penetrating injury, endogenous VEGF acts through *flt-1* receptors on reactive astrocytes to increase their proliferation, by autocrine means and serves to facilitate expression of the trophic factors bFGF and CNTF. Endogenous VEGF may thus play an important role in the formation and maintenance of the astroglial scar.

Materials and Methods

Minipump Implantation

Osmotic minipumps (Model 2001, Alza Corp, Palo Alto, CA; delivery rate 1 μ l/hr) were filled with either 1) 20 μ g/ml (delivery of 0.48 μ g/day) goat anti-mouse *flt-1* neutralizing antibody (*flt-1*-NA; R & D Systems) in artificial CSF (aCSF; Harvard Apparatus); 2) 1 μ g/ml goat anti-mouse *flk-1* neutralizing antibody (*flk-1*-NA; R & D Systems) in aCSF (delivery of 0.024 μ g/day); or 3) with 20 μ g/ml normal goat IgG (R & D Systems) in aCSF to provide isotopic control infusions. The antibodies are directed toward the extracellular domain of each receptor. Although there is no specific information about which of the seven immunoglobulin-like extracellular domains are targeted, the antibodies are specific in that they have very limited cross-reactivity with other VEGF receptors (e.g., *flt-1*-NA has 0.2% cross-reactivity with *flk-1*; *flk-1*-NA has <2% cross-reactivity with *flt-1*). The dosages employed for the present study are over twice what the Neutralization Dose₅₀ (ND₅₀) is for each neutralizing antibody, according to the manufacturer's specifications. Neutralizing antibodies to VEGF receptors have been used previously to successfully block endogenous rodent *flt-1* and *flk-1* (Witte et al. 1998; Dias et al. 2001; Kunkel et al. 2001). Young adult Wistar rats were anaesthetized with ketamine/ xylazine (60 mg/kg/5 mg/kg) and each received a single infusion cannula that was placed in

the right striatum, as described previously (Rosenstein et al. 1998; Krum et al. 2002; Krum and Khaibullina 2003) Briefly, after a skin incision over the right side of the skull, a pocket was formed over the neck and shoulder blades to hold the minipump. A 2 mm hole was drilled in the skull 1 mm posterior to the coronal suture and 5 mm lateral to the sagittal suture. Using a stereotaxic apparatus, the cannula was inserted through the cortex and into the striatum to a final depth of 3 mm. The cannula was cemented in place and the incision sutured. Survival times were 3 and 7 days, with 6–7 animals/group used for each time period. After the appropriate time interval the animals were anaesthetized as described above and were perfused intracardially with 4% paraformaldehyde in 0.1 M cacodylate buffer with 3% sucrose. The cannulae were removed and the brains blocked for vibratome sectioning followed by processing for routine paraffin embedment. All surgical procedures were performed within the guidelines provided by and with the approval of the GWUMC Institutional Animal Care and Use Committee.

Immunohistochemistry

Paraffin or vibratome sections were immunostained for the following antigens: cell proliferation marker, proliferating cell nuclear antigen (PCNA; monoclonal, 1:500, SIGMA); an endothelial basement membrane marker, laminin (polyclonal, 1:200, SIGMA); an astrocytic marker, glial fibrillary acidic protein (GFAP; monoclonal, 1:500, SIGMA); nestin (1:200); basic fibroblast growth factor (bFGF; 1:100; Santa Cruz); ciliary neurotrophic factor (CNTF; 1:500); TUNEL (Promega, as per kit instructions). Some of the antibodies were used to co-localize antigens to a particular cell phenotype, such as PCNA/laminin or PCNA/GFAP for proliferating endothelial cells and astroglia respectively. TUNEL was co-localized with either GFAP or laminin. TUNEL and PCNA were visualized using nickel enhancement. Controls included omission of primary antibodies and replacement of the primary antibody with normal serum from the appropriate species. None of the immunohistochemical controls exhibited specific immunoreactivity.

While most of the immunoreactions were carried out using 7 mm paraffin sections, free-floating vibratome sections (60 µm) were used for nestin, and VEGF receptor (*flt-1* and *flk-1*) immunostaining. Paraffin sections were deparaffinized and dehydrated prior to incubation with antisera. All sections were incubated overnight at 4°C with the primary antibodies, which were diluted in 0.05 M TBS containing 1% normal serum. After washing in TBS, the sections were incubated in the appropriate dilutions of secondary antibody for 30 min, followed by exposure to peroxidase-anti-peroxidase. Visualization of the reaction product was done by using either diaminobenzidine (DAB) alone or by nickel intensification of the DAB reaction product if the sections were to be double-labeled.

For confocal fluorescence microscopy, sections incubated with primary antibodies were exposed to a solution of the appropriate fluorochrome(s) for 1 hr. at room temperature and viewed with a Bio-Rad 1024 laser confocal microscope.

Calculation of proliferation indices for vessels and astrocytes

PCNA is a reliable marker for proliferating cells particularly when only the granular, darkly stained reaction product present within nuclei is counted as an indication of proliferative activity. Results obtained with PCNA immunostaining are very similar to those obtained by ³[H]-thymidine autoradiography (Galand and Degraef 1989; Thaete et al. 1989; Hall et al. 1990; Yamada et al. 1992; Amat et al. 1996). Bromodeoxyuridine (BrdU) is also widely used as a proliferation marker, but its use was precluded in our immunohistochemical protocol. Double immunostaining for BrdU with laminin, GFAP or the other markers used was unsatisfactory due to the rigorous processing necessary for BrdU immunolocalization.

Following double-immunolabeling of sections for laminin and PCNA, the proliferation index (P.I.) for vascular profiles was calculated for controls and for each VEGF concentration, as described previously (Krum et al. 2002; Krum and Khaibullina 2003). Because the concentration of infused agents falls off as an exponential function of the distance from the infusion site (Kasamatsu et al. 1981; Lum et al. 1984), the tissue at a distance of 600 μm around the perimeter of the entire infusion track were assessed for the presence of PCNA (in the endothelial nuclei) and laminin-positive vessels. Two observers blinded to the treatments counted darkly stained PCNA(+) nuclei. It was not possible to visualize unlabeled (PCNA-negative) endothelial nuclei with these immunolabeling methods, so a true endothelial proliferation index could not be calculated. Instead, the values were averaged and expressed as P.I.s for vascular profiles ($\text{P.I.} = [\# \text{PCNA}(+) \text{ vessel profiles}] \div [\text{total } \# \text{ vessel profiles}] \times 100$). Astroglia that were double-labeled for PCNA/GFAP, as well as GFAP(+) astrocytes with visible nuclei that were not immunoreactive for PCNA, were counted in six areas within 600 μm of the infusion track. Astroglial proliferation indices were calculated using $\text{P.I.} = [\# \text{PCNA}(+)/\text{GFAP}(+) \text{ cells}] \div [\text{total } \# \text{GFAP}(+) \text{ cells}] \times 100$.

Quantitation of vascular and astroglial TUNEL immunoreactivity

Quantification of TUNEL-positive nuclei was performed using sections that were double-labeled for laminin/TUNEL (endothelial cells) and GFAP/TUNEL (astrocytes), as described previously (Krum and Khaibullina 2003). Similar to the measurement of vascular proliferation index, TUNEL(+) endothelial cell nuclei and laminin(+) vessels were counted at a distance of 600 μm around the perimeter of the entire infusion track by two observers blinded to the treatments. Similar to vascular PCNA counting, it was not possible to visualize unlabeled (TUNEL-negative) endothelial nuclei with these immunolabeling methods to obtain a strict measurement of endothelial degeneration. Instead, the values were averaged and expressed as a percentage ($[\# \text{TUNEL}(+) \text{ vessel profiles}] \div [\text{total } \# \text{ vessel profiles}] \times 100$). GFAP(+) astroglia were also counted within 600 μm of the infusion site. Since it was possible to visualize unlabeled astroglial nuclei as well as TUNEL(+) astroglia, a true measure of astroglial degeneration was obtained and expressed as a percentage.

Quantitation of reactive astroglia

The total number of astrocytes adjacent to the wound site (within 600 μm ; derived from the PCNA/GFAP and TUNEL/GFAP counts) were added together for each animal and averaged in order to determine the relative abundance of reactive astroglia resulting from each treatment.

Quantitation of bFGF(+) and CNTF(+) astrocytes

To determine the percentage of reactive astroglia that express bFGF or CNTF in response to VEGF receptor neutralization, paraffin sections were fluorescently immunostained for bFGF/GFAP or CNTF/GFAP and viewed with a Biorad confocal microscope. Using the confocal images, astroglia that were double-labeled, as well as astrocytes that were immunoreactive for GFAP alone, were counted within 600 – 700 μm of the infusion site. Two sections of the wound area from each animal, taken 100 μm apart, were counted and the percentage of double-labeled astrocytes was determined for each animal. Percentages were then averaged for each experimental and control group ($n = 6$ each).

Measurement of wound areas

For each animal, five sections were taken at approximately 100 μm intervals through the central portion of the infusion site. The length (from cortex to striatum) and width of the wound cavities were measured using a 4X objective in conjunction with a stage micrometer. Length and width measurements were multiplied to obtain the areas of the wounds. Wound cavity areas were averaged separately for each time point in both VEGF receptor-NA infusions and controls.

Statistics

Data were reported as mean $\pm$ standard error of mean (S.E.M.). The Students t-test for paired data and ANOVA tests with Tukey correction were used for statistical analysis. Statistical significance was set at $P < 0.05$.

Results

Wound area

All animals survived without any apparent complications from the infusions. Cavities were present around the site of cannula implantation, as described previously (Rosenstein et al. 1998; Krum et al. 2002; Krum and Khaibullina 2003). In general, the inflammatory cell influx into the wound areas appeared similar in all types of infusions, and by seven days a typical connective tissue down-growth from the pial surface had occurred (Logan and Berry 2002). A glia limitans, demarcated by GFAP/nestin(+) astrocytes at the edge of the cavity, was present in all infusion paradigms. Analysis of cavity sizes revealed no significant differences between control IgG infusions and either of the receptor antibody infusions (Fig. 1).

Vascular proliferation and degeneration

Vascular proliferation, as defined by PCNA labeling indices, was significantly decreased in both three and seven day anti-*flk-1* infusions by 65% and 50%, respectively, compared to the IgG controls (Fig. 2A). In contrast, although infusion of *flt-1*-NA produced a trend toward diminished vascular proliferation compared to control infusions (particularly at 3 days postoperative), neutralization of *flt-1* did not have a statistically significant effect on vascular PCNA labeling indices.

TUNEL/GFAP and TUNEL/laminin immunohistochemistry was used to determine the index of vascular and astroglial degeneration. TUNEL immunoreactivity is indicative of DNA fragmentation that occurs in both apoptosis and necrosis (Grasl-Kraupp et al. 1995; Newcomb et al. 1999; Fujikawa et al. 2000). Following *flk-1*-NA infusion, the TUNEL/laminin index was significantly increased by approximately 5-fold at both time points (Fig. 2B). Although control infusions produced an increased vascular density at the wound site (Fig. 2C), laminin immunostaining revealed a generally decreased density of vessels adjacent to the wounds in *flk-1*-NA infusions (Fig. 2D), presumably because of the combined effects of reduced endothelial proliferation and increased vascular degeneration. The majority of the capillaries having TUNEL(+) endothelial cell nuclei appeared to be shrunken, collapsed, and somewhat fragmented. Interestingly, *flt-1*-NA infusion also produced a significant (almost two-fold) increase in TUNEL(+) vessels at the three day time point, but had no statistically significant effect on the vascular TUNEL labeling index at seven days.

Astroglial proliferation and degeneration

Infusion of neutralizing antibody to *flt-1* significantly reduced astroglial proliferation by approximately 55% compared to controls at 3 days and by >80% at 7 days (Fig. 3A). In contrast, infusion of neutralizing antibody to *flk-1* had no significant effect on astroglial proliferation, which was comparable to controls. Notably, the astroglial TUNEL index, indicative of degenerating cells, was not significantly different from controls in either the *flt-1* or the *flk-1* antibody infusions, although it trended higher for *flt-1*-NA at three days (Fig. 3B).

Immunostaining for GFAP revealed both decreased density of reactive astrocytes adjacent to the wounds and decreased hypertrophy in the majority of astrocytes at both 3 and 7 days in the anti-*flt-1* infusions compared to controls (Fig. 4A – D). Consistent with the decreased proliferation index observed in the *flt-1*-NA infusions, the overall number of astrocytes within

the 600 μ m region adjacent to the wounds trended lower at 3 days and was significantly decreased at 7 days compared to both *flk-1*-NA and control infusions (Fig. 4E).

Nestin expression

There were no qualitative or quantitative differences in nestin(+) astroglial distribution between IgG and *flk-1*-NA infusions at either time point, but the *flt-1*-NA infusions had fewer astrocytes, and therefore, fewer nestin(+) astroglia (Fig. 5 A–C). Nestin(+) astrocytes were confined to an area that extended approximately 1 mm from the wound edges in every case. Nestin was consistently co-localized with GFAP, such that portions of the astroglial processes were nestin(+), although a few GFAP(+) cells did not express nestin protein (not shown). Astrocytes in the contralateral cortex and striatum did not express nestin in any of the experimental paradigms.

CNTF and bFGF immunoreactivity

Labeling indices were derived by counting astrocytes that were fluorescently double labeled with bFGF/GFAP or CNTF/GFAP to determine if inhibition of endogenous VEGF-astrocyte interactions would affect astroglial expression of either trophic factor. There were no significant differences between control and receptor antibody infusions at 3 days, but by 7 days, only *flt-1*-NA administration had significantly decreased the percentage of astrocytes that were immunoreactive for bFGF or CNTF (Fig. 6 A – H). *Flt-1* blockade produced astrocytic bFGF and CNTF labeling indices that were only 50% of control infusions, while *flk-1* blockade had no effect on astrocytic expression of these growth factors.

Discussion

Endogenous VEGF is induced by various types of CNS injuries and plays a role in neuronal survival and brain repair (Widenfalk et al. 2003; Krum and Rosenstein 2004; Yasuhara et al. 2005; Skold et al. 2006). It is also now apparent that a reduction in VEGF levels in mice can cause neurodegeneration, while increased VEGF levels delay neurodegeneration in models of amyotrophic lateral sclerosis (Lambrechts et al. 2003; Wang et al. 2007). Exogenous VEGF delivered to a wound site results in increased angiogenesis and astroglial proliferation and smaller wound areas when compared to controls (Krum et al. 2002) and can afford neuroprotection in stroke models (Manoonkitiwongsa et al 2004; Kaya et al. 2005). We have previously investigated the role of endogenously-derived VEGF in wound healing by inhibiting it with a specific neutralizing antibody using the same infusion model. The permanent insertion of the infusion cannula creates an injury site similar to a stab wound, and the injured tissue is directly and continuously exposed to the infusate (Krum et al. 2002; Krum and Khaibullina 2003). It was clear in that study that VEGF is an important component of the healing response, since its inhibition significantly decreases both astroglial and vascular proliferation, with a concomitant increase in wound size. In the present study, we took advantage of the differential expression of VEGF receptors by CNS cells, where *flk-1* is expressed primarily by endothelial cells and neurons and reactive astroglia predominantly express *flt-1* in order to determine if the reduction in astroglial proliferation was the result of VEGF blockade alone, or if it was because of the decrement in vascular growth. The specific blockade of each receptor *in situ* over periods of three to seven days allowed us to conclude that VEGF directly stimulates astroglia to proliferate and upregulate growth factor production via their expression of the *flt-1* receptor, since blockade of the *flk-1* receptor resulted only in decreased vascular proliferation and survival and had no effect on the measured astroglial parameters.

Using either antibody, we could not detect any differences in wound size when compared to IgG controls, suggesting that the simultaneous inhibition of endogenously VEGF-induced astroglial and vascular proliferation is necessary to augment wound size during the repair

process (Krum and Khaibullina 2003). The relative loss of vasculature at the wound site due to *flt-1*-NA infusion would, logically, promote hypoxic degeneration of adjacent cells. It is possible, however, that tissue damage could be abrogated to a degree by the presence of unchecked astroglial activation and subsequent scarring, which may have many beneficial functions that include clearance of glutamate and potassium ions or production of cytokines and growth factors, among other considerations (Eddleston and Mucke 1993; Stichel and Müller 1998; Krum et al. 2002; Faulkner et al. 2004; Sofroniew 2005). After infusion of *flt-1*-NA, on the other hand, the relative loss of reactive astroglia could be compensated for by a relatively intact and modestly angiogenic vasculature that might offset the production of a larger wound area.

Vascular effects of VEGF receptor blockade

Infusion of *flt-1*-NA caused a significant decrease in vascular proliferation at three and seven days, and also produced a significant increase in vascular TUNEL staining at both time points, indicative of vascular degeneration adjacent to the infusion/wound site. The decreased density of vessels, and their shrunken, fragmented appearance (compared to control infusions) were also indicative of ongoing vascular degeneration. We have observed similar effects with blockade of VEGF itself in our previous study (Krum and Khaibullina 2003). Controls exhibited an increased vascular density at the wound sites compared to adjacent uninjured brain areas, which presumably was induced by secreted endogenous VEGF (Krum and Rosenstein 1998, 1999).

In contrast, infusion of *flt-1*-NA had no statistically significant effect on vascular proliferation, although there was a marked trend toward decreased vascular proliferation after *flt-1* blockade, and there was only a partial effect on vessel degeneration. Consistent with the results of the present study, others have shown that *flt-1* is the primary receptor that mediates VEGF-induced endothelial proliferation and survival in peripheral blood vessels (Gerber et al. 1998; Zachary and Glick 2001). It is thought that *flt-1* only weakly transduces signals for these functions (Zachary and Glick 2001), but at three days there was a significant increase in vascular TUNEL labeling in our model. Notably, *flt-1* signaling is involved in the prevention of oxygen-induced vessel loss in the retina (Shih et al. 2003a, b). It appears that CNS endothelial cells share proliferative (*flt-1*-dependent) VEGF receptor characteristics with peripheral vascular endothelium, but may be more closely related to retinal endothelium in terms of their survival mechanisms.

While it has been shown that *flt-1* is a decoy receptor for VEGF that acts as a sink to regulate the amount of VEGF available to bind to endothelial *flt-1*, thus influencing angiogenic activity, there is also circumstantial evidence that *flt-1* can be phosphorylated by VEGF which stimulates direct effects on growth and migration of non-endothelial cells (see Meyer and Rahimi 2003). If blockade of *flt-1* in our experiments thwarts such a decoy effect, we would expect that the vascular proliferation index would be increased during *flt-1* blockade, since theoretically more VEGF would be available to bind with endothelial *flt-1* receptors, but this was not observed. Since we have not detected *flt-1* expression in vascular endothelial cells in our model, the relative decrease in activated astrocytes that occurs after *flt-1* blockade (see below) may result in decreased amounts of endogenous VEGF (since astrocytes are a primary source of secreted VEGF), which would likely lead to decreased angiogenic activity.

Astroglial effects of VEGF receptor blockade

Flk-1-NA infusion had no significant effect on astrocyte proliferation, degeneration, number, or the expression of nestin, VEGF, CNTF, or bFGF at the wound sites at either time point. All of these parameters were comparable to those of the IgG control infusions, strongly suggesting

that *flk-1* is not a primary functional VEGF receptor for activated astrocytes, at least within the experimental parameters of the present study.

In sharp contrast to the results from *flk-1* blockade, infusion with *flt-1*-NA produced significant decrements in astroglial proliferation at both time points, and a significant decrease in astrocyte number was observed after seven days of infusion. Likewise, seven days of *flt-1*-NA administration decreased astrocytic expression of both CNTF and bFGF by 50% compared to control infusions. These growth factors are produced by activated astroglia. bFGF is trophic for glia, blood vessels and neurons (Finklestein et al. 1988; Gomez-Pinilla et al. 1992; Yang and Cui 1998), while CNTF is neurotrophic (Sleeman et al. 2000). The results suggest that VEGF may have a regulatory influence over astroglial production of these trophic factors, which could positively influence wound repair and neurite growth. There appears to be a delayed effect of *flt-1* receptor blockade since both the decrease in number of astrocytes and the decrease in percentage of them that are labeled for both FGF and CNTF are only significant after 7 days of *flt-1*-NA infusion, and not at 3 days. Thus, VEGF, via its interaction with *flt-1*, appears to be involved in several aspects of the astroglial activation cascade, including mitotic activity and upregulation of bFGF and CNTF. This observation fits with the recent finding that *flt-1* signaling is also involved in inflammatory cytokine/chemokine production by monocytes (Selvaraj et al. 2003).

In our previous work using VEGF-NA infusions (Krum and Khaibullina 2003), there was a very large increase in TUNEL(+) astrocytes at each time point. Interestingly, blockade of *flt-1* activity trended toward an increase in the number of TUNEL(+) astrocytes but the change was not statistically significant. Thus, although *flt-1* signaling is necessary for astroglial mitogenesis, it remains unclear if VEGF/*flt-1* interactions have a major role in astroglial survival after injury. The role of *flt-1* in promoting VEGF's survival effects on various cell types is only now beginning to be defined. *Flt-1* signaling is responsible for preventing oxygen-induced vessel loss in the retina (Shih et al. 2003a, b) and for promoting the survival of chronic lymphocytic leukemia B cells (Lee, Y. K. et al. 2005). It is unknown if there are survival factors for astrocytes after injury; it is possible that there may be only limited astroglial survival, and the prevention of mitogenesis brings about the observation of fewer astrocytes at the wound site.

Nestin is strongly expressed in mature reactive astroglia, and may be a part of the activation cascade that represents a less mature state that is responsive to trophic factors and is capable of migratory activity (Krum and Rosenstein 1999). It is possible that nestin(+) cells are newly arrived to the infusion site from the subventricular zone (SVZ), since evidence suggests that progenitor cells migrate to a wound site and stroke penumbra (Arvidsson et al. 2002; Jin et al. 2003; Zhang et al. 2004). There were no differences in nestin(+) astroglial distribution between *flk-1*-NA infusions and the controls at either time point, but there was an observable reduction in nestin expression after *flt-1*-NA. This reduction was not as pronounced as what we observed previously, in which nestin immunoreactivity was almost completely abrogated ipsilateral to the wound/infusion site after VEGF-NA application (Krum and Khaibullina 2003). The profound loss of nestin expression around the wound site after VEGF-NA infusion may reflect a combined sensitivity of astroglia to the loss of both VEGF-supported vascularity and a lack of direct astroglial stimulation via the *flt-1* receptor. In the present study, nestin immunoreactivity (upregulation) was not observed in the contralateral cortex/striatum in either controls or in antibody-infused brains.

The experiments in this study afforded us the ability to document the segregation of VEGF receptor function between astrocytes and the vasculature *in vivo*. The results shed new light on the function of *flt-1* in astrocytes, indicating that it is the receptor most responsible for binding endogenous VEGF and transmitting the proliferation signal for these cells. Based on

our TUNEL results, *flt-1* activation may not be a strong promoter of astroglial survival, but our results support its role in transmitting survival signals in CNS endothelial cells, similar to what occurs in hypoxic retinal vasculature (Shih et al. 2003a, b). We have also largely confirmed the role of *flt-1* as an important effector of endothelial survival and proliferation in the CNS, as it is in peripheral blood vessels. Thus, inhibition of astrocyte proliferation and growth factor production, without decreasing angiogenesis which is essential to wound repair, can be accomplished by the administration of *flt-1*-NA. Future studies could examine if other astrocyte-derived scar components, e.g. extracellular matrix molecules, could be modified by inhibition of VEGF's specific proliferation and stimulatory effects on astroglia. Such information could be highly useful for strategies designed to promote neurite outgrowth at sites of astroglial scarring (Rhodes and Fawcett 2004; Silver and Miller 2004).

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Fig. 1.

There was more variability in wound size for the experimental infusions vs. IgG controls, particularly at three days post-infusion, but the differences were not significant at either time point. Error bars represent SEM.

Fig. 2.

Vascular effects of VEGF receptor antibody infusions. A. At both time points, the vascular proliferation index quantified within 600 μm of the infusion sites was significantly decreased only after *flk-1*-NA infusions, compared to IgG controls. Vessels were double-labeled with laminin and PCNA. B. *flk-1*-NA administration resulted in significant increases in vascular TUNEL labeling at each time point, while *flt-1*-NA infusion caused significantly increased TUNEL labeling only at three days. * $P < 0.05$ (vs. control). Error bars represent SEM. C. A seven day IgG infusion immunostained for laminin shows profuse vessels filling the wound cavity (W). D. Numerous vessels lie adjacent to the wound cavity of a seven day *flt-1*-NA infusion. E. A seven day *flk-1*-NA infusion shows sparse vasculature around the wound cavity (W). Bar = 75 μm .

Fig. 3.

Astroglial effects of VEGF receptor antibody infusions. A. Quantification of the astroglial proliferation index within 600 μ m of the infusion sites revealed significant decreases only after *flt-1*-NA infusion at both three and seven days, compared to IgG control infusions. B. Astroglial TUNEL expression was not significantly different from IgG control infusions after administration of either receptor antibody. * $P < 0.05$ (vs. control). Error bars represent SEM.

Fig. 4. Quantification of astroglia within 600 μm of the infusion site; GFAP/TUNEL immunolabeling. Dark nuclei are TUNEL (+). A. 3 day IgG infusion and B. 3 day *flk-1*-NA have comparable numbers of astrocytes adjacent to the wound sites (W). C. 3 day *flt-1*-NA infusion shows a slightly decreased astroglial population compared to A and B. D, E. After 7 days, IgG and *flk-1*-NA infusions show large numbers of GFAP(+) reactive astrocytes near the wound area, while in F, *flt-1*-NA administration exhibited markedly reduced numbers of reactive astrocytes. Bar = 100 μm . G. Counts of astrocyte numbers at infusion sites. A statistically significant decrease in astrocytes was detected at 7 days only with *flt-1*-NA infusions compared to IgG controls. * $P < 0.05$ (vs. control). Error bars represent SEM.

Fig. 5. Nestin (red)/GFAP (green) fluorescent immunolabeling reveals a reduced presence of double-labeled astrocytes (yellow) after *flt-1*-NA infusion directly adjacent to the wounds (W) at the 7 day time point. A, IgG infusion. B, *flk-1*-NA infusion. C, *flt-1*-NA infusion. Bar = 75 μ m.

Fig. 6.

A – C, GFAP (red)/bFGF (green) fluorescent immunostaining adjacent to infusion sites (7 day time point). bFGF(+) cells have green nuclei. Numerous astrocytes are double labeled in the IgG infusion (A) and the *flk-1*-NA infusion (B) compared to the few observed in the *flt-1*-NA infusions (C). D – F, GFAP (red)/CNTF (green) fluorescent immunolabeling near infusion area (7 day time point) show a similar pattern of double labeling. IgG (D) and *flk-1*-NA infusions (E) show more CNTF(+)/GFAP(+) astrocytes (yellow) than the *flt-1*-NA infusions (F). Bar = 100 μ m. G and H show that only the 7 day *flt-1*-NA infusions have significantly decreased numbers of astrocytes that are double stained for bFGF (G) and CNTF (H). *P < 0.05 (vs. control). Error bars represent SEM.