VEGF and VEGF Receptor Expression after Experimental Brain Contusion in Rat

MATTIAS K. SKÖLD,1,3 CHRISTINA VON GERTTEN,2 ANN-CHRISTIN SANDBERG-NORDQVIST,2 TIIT MATHIESEN,2 and STAFFAN HOLMIN2

ABSTRACT

Angiogenesis following traumatic brain injury (TBI) may be of importance not only for post-traumatic reparative processes but also for the development of secondary injuries. Vascular endothelial growth factor (VEGF) is a major regulator of endothelial cell proliferation, angiogenesis, and vascular permeability, though its possible involvement in secondary injuries after TBI is largely unknown. This study was undertaken to analyze the expression of VEGF and the VEGF receptors in experimental brain contusion in rat. Twenty-three adult female Sprague-Dawley rats were subjected to a focal cerebral contusion injury by use of a weight-drop model. Four additional rats underwent craniotomy only. The animals were sacrificed 6 h, or 1, 2, 4, 6, 8, or 16 days post-injury. Expression of VEGF and the VEGF receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1) were studied by in situ hybridization and immunohistochemistry. VEGF messenger (m)RNA and protein expression were detected in astrocytes, neutrophils, and macrophages in or adjacent to the injury from 1 day after injury, with a peak expression after 4–6 days. Flt-1 and Flk-1 mRNA and protein were detected in vessels adjacent to the lesion from 1 day after injury throughout day 6 after injury. It was also noted that Flt-1/Flk-1 and VEGF-positive vessels often were negative for SMI-71, a marker for vessels in areas with blood–brain barrier (BBB). In conclusion, we have demonstrated that TBI leads to an upregulation of VEGF, Flt-1, and Flk-1 mRNA and protein in and around the lesion. The data provide a foundation for future pharmacological intervention studies focusing on posttraumatic angiogenesis and possible injury repair effects of the VEGF system in TBI.

Key words: angiogenesis; blood–brain barrier (BBB); brain; contusion; vascular endothelial growth factor (VEGF); Flt-1; Flk-1

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a secreted mitogen with fundamental importance in regulation of angiogenesis and vascular permeability (Ferrara, 2000; Leung et al., 1989; Neufeld et al., 1999; Robinson et al., 2001; Senger et al., 1983). VEGF exerts its function through the phosphotyrosine kinase receptors Flt-1 and Flk-1 (VEGF receptor 1 and 2) (Matsumoto, et al., 2001), where Flk-1 is purported to be the most im-

1Department of Neuroscience, Retzius Laboratory, Karolinska Institutet, Stockholm, Sweden.
2Department of Clinical Neuroscience, Section of Neurosurgery, Karolinska Hospital, Stockholm, Sweden.
3Department of Defence Medicine, Swedish National Defence Research Agency (FOI), Karolinska Institutet, Stockholm, Sweden.
portant receptor for changes in vascular permeability. Earlier studies have demonstrated involvement of VEGF in angiogenesis and breakdown of the blood–brain barrier (BBB) after ischemic CNS injuries (Abumiya et al., 1999; Paul et al., 2001; Proescholdt et al., 1999; Schoch et al., 2002; Van Bruggen et al., 1999; Zhang et al., 2000) but also in CNS inflammation (Croll et al., 2004; Proescholdt et al., 1999) as well as in brain edema in meningoencephalitis (Goldman et al., 1997). VEGF is also a possible direct neurotrophic/neuroprotective factor (Jin et al., 2002, 2000; Oosthuysen et al., 2001; Sondell et al., 1999; Sun et al., 2003). Even though the role of VEGF and its receptors has been studied extensively in ischemia over the last several years, less has been done to evaluate the VEGF expression and its role after traumatic CNS injury. In ischemic CNS injuries, VEGF-stimulated angiogenesis and increases in posttraumatic edema formation and the inflammatory response have been described (Abumiya et al., 1999; Croll et al., 2004; Paul et al., 2001; Proescholdt et al., 1999; Schoch et al., 2002; Sun et al., 2003; Van Bruggen et al., 1999; Zhang et al., 2000). Also, possible neurotrophic/neuroprotective effects of VEGF, either directly or due to VEGF-stimulated vessel sprouting, has been discussed (Hayashi et al., 1998; Jin et al., 2000; Ogunshola et al., 2002; Sondell et al., 1999, 2000; Sun et al., 2003; Zhang et al., 2000). To date, studies on VEGF expression after traumatic CNS injury or traumatic injury–like experiments have been performed by only a few groups. Nag et al. (1997) used a cold injury model that simulates vasogenic edema and showed an upregulation of VEGF protein that correlated to vessel sprouting, although, it was suggested that VEGF was not involved in BBB breakdown at early timepoints after injury. In a cerebral stab and freeze injury model, induction of VEGF protein was demonstrated with immunohistochemical methods (Papavassiliou et al., 1997), and in spinal cord injury, expression of VEGF (Bartholdi et al., 1997) and the VEGF receptors (Sköld et al., 2000) have been found. Posttraumatic treatment with VEGF after spinal cord injury also has potential neuroprotective and tissue sparing effects (Facchiano et al., 2002; Widenfalk et al., 2003).

The role of VEGF and the VEGF receptors in both traumatic spinal cord and cerebral injuries is still largely unknown, and its expression after cerebral contusion has not been studied. The present article tested the hypothesis that VEGF and the VEGF receptors are upregulated after TBI. We focused on VEGF and its receptors in a model simulating brain contusion, with delayed inflammatory response and biphasic edema via the use of a weight-drop model (Feeney et al., 1981; Holmin et al., 1995a, 1997). Since the later posttraumatic phase is often followed by neuronal destruction (Povlishock et al., 1992), edema (Bullock et al., 1990; Holmin et al., 1995b; Stein et al., 1993), and clinical deterioration (Mathiesen et al., 1995; Statham et al., 1989), we felt it appropriate to study VEGF expression over a prolonged time frame. Our findings suggested that VEGF mRNA and protein were upregulated in astrocytes and inflammatory cells in the vicinity of the lesion, and that this upregulation took place during the first 1–6 days after injury with a peak expression around 4 days. We also found that vessels in this region were capable of expressing the VEGF receptor Flk-1 and Flt-1. Further, vessels in this border-zone binding VEGF protein and expressing Flk-1 and Flt-1 were often not immunopositive for SMI-71, a marker for vessels in areas with an intact BBB. Taken together, these findings indicate that VEGF could be one factor involved in angiogenesis after TBI as well as BBB changes, edema formation, and inflammation.

**MATERIALS AND METHODS**

**Tissue Preparation**

Twenty-seven adult female Sprague-Dawley rats, weighing 270–280 g each, were deeply anaesthetized by intramuscular injection of 0.15 mL of Hypnorm (10 mg/mL fluanisonum and 0.2 mg/mL fentanylium). In addition, 0.1 mL of Xylocain-Adrenalin (5 mg/mL lidocaine and 5 μg/mL adrenaline) was injected subcutaneously in the sagittal midline of the skull before the skin incision was made. The rats were placed in a stereotactic frame, and under microscopic guidance, a 2-mm craniotomy was drilled 3 mm posterior and 2.5 mm lateral to the bregma. A standardized parietal contusion was produced in 23 rats by letting a steel rod weighing 24 g with a flat end diameter of 1.8 mm fall onto the intact dura from a height of 7 cm (modified after method of Feeney et al., 1981). The rod was allowed to compress the tissue with a maximum of 3 mm, resulting in a contusion injury of the brain (Holmin et al., 1997). Four rats underwent sham operation (craniotomy without contusion). The animals showed no deficits in motor, balance, or sensory function during the posttraumatic phase. The surgically treated rats were anesthetized by intramuscular injection of 0.15 mL of Hypnorm and killed by decapitation at 6 h (n = 3), or 1 day (n = 4), 2 days (n = 3), 4 days (n = 4), 6 days (n = 3), 8 days (n = 3), or 16 days (n = 3) after the contusion. The sham-operated animals were killed 4 days (n = 4) after craniotomy. After decapitation, the brains were quickly dissected out, and the contused and the non-injured contralateral sides of the brain were cut out in an approximately 0.5-cm slice, frozen on dry ice, and thereafter stored in sealed boxes in −70°C.
VEGF EXPRESSION FOLLOWING BRAIN CONTUSION

until use. The tissue was cut in 14-μm-thin coronal sections, thawed onto Probe-On slides, and stored in −70°C until use.

The use of animals for all experiments was approved by the Swedish ethical committee (Stockholms Norra Försöksdjursetiska Nämnd, approval nos. N144A and 144/89).

In Situ Hybridization (ISH)

Fresh-frozen tissue from rats with survival times of 6 h, or 1, 2, 4, 6, 8, or 16 days plus tissue from sham-operated animals was cut in an RNase free environment on a cryostat (Microm HM 500M, Heidelberg, Germany) in 14-μm-thin coronal sections thawed onto Probe-On object-slides (Fisher Scientific, Pittsburgh, PA) and stored in sealed boxes at −70°C until used. Synthetic oligonucleotides were synthesized (CyberGene AB, Huddinge, Sweden). The sequence of the probes was checked in a GeneBank database search to exclude significant homology with unrelated genes. The synthesized oligonucleotides were complementary to the following Rattus norvegicus mRNAs: VEGF (nt 365–414, GeneBank accession no. AF062644), glioma-derived VEGF (nt 25–72, GeneBank accession no. M32167), Flk-1 (nt 1978–2027, GeneBank accession no. U93306), Flt-1 (nt 4516–4565, GeneBank accession no. M32167), D28498), and Neuropilin-1 (nt 2580–2629, GeneBank accession no. M32167). The probes was labelled at the 3'-end with deoxyadenosine-alpha-(thio)triphosphate [35S] (NEN, Boston, MA) by using terminal deoxynucleotidyl-transferase (Amersham-Pharmacia, Uppsala, Sweden) to a specific activity of 1.5–6 × 10^5 cpm/μL and hybridized to the sections, without pre-treatment, for 16–18 h at 42°C. The hybridization mixture contained 50% formamide (Fluka/H9262, Sigma-Aldrich, Sweden), 4 × SSC (1 × SSC, 0.15 M NaCl and 0.015 M sodium citrate), 1 × Denhardt’s solution (0.02% each of polyvinyl-pyrrolidone, bovine serum albumin and Ficoll), 1% sarcosyl (N-lauroylsarcosine; Sigma-Aldrich, Sweden), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate (Amersham-Pharmacia, Sweden), 500 μg/mL sheared and heat denatured salmon sperm DNA (Sigma-Aldrich, Sweden), and 200 mM dithiothreitol (DTT; Sigma-Aldrich, Sweden). Following hybridization, the sections were washed several times in 1 × SSC for 15 min at 60°C, rinsed in distilled water, and dehydrated in ascending concentrations of ethanol. The sections were then coated with NTB2 nuclear track emulsion (Kodak, Rochester, NY). After 3–5 weeks, the sections were developed in D-19 developer (Kodak) for 4 min at room temperature and fixed in AL-4 fixative (Kodak) for 5 min and coverslipped. Some of the slides were counterstained with cresyl violet (Sigma C5042, USA) and then dehydrated in ascending concentrations of ethanol and mounted in Entellan (Histolab products AB, Göteborg, Sweden). Sense probes for each of the employed antisense probes were used as negative controls, and embryonic tissue with known mRNA expression patterns were used as positive controls.

Image Analysis of ISH

Semiquantitative measurements of the in situ VEGF hybridizations were performed. Briefly, the hybridization signal was recorded in a microscope (Nikon Eclipse E600) equipped with darkfield condenser (Nikon) and digitized by using a digital camera (Nikon CoolPix 4500). The method is based on the assumption that differences in gray-scale vary linearly. The gray-scale of the darkfield image was adjusted and segmented by using the enhance contrast and density slicing features of the NIH Image software (version 1.63, NIH, Bethesda, MD). An area of 2.95 mm × 3.91 mm, covering the lesion area, was analyzed at the injured side and at the corresponding non-injured side in the same section from the same animal. Two consecutive slides were analyzed per animal. The contrast and density slice features were equilibrated and adjusted for gray-scale differences between measurements in the same animal to make comparison of signal from injured and non-injured areas possible. The mean grain density of each lesioned side was divided with the corresponding value of the contralateral non-injured side of the same section. The ratios thus obtained were pooled, and a mean ratio value, designated labeling ratio, was calculated. This procedure reduces the influence of a possible difference in staining intensity between slides or brain sections at different locations on the same slide.

Immunohistochemistry (IHC)

Sections from the same animals described above were used. The tissue was air dried and thereafter soaked in 0.01M PBS for 20 min. After a short fixation in 4% formalin for 1 min and rinsing in 0.01M PBS for 10 min, the sections were incubated in a humid chamber at 4°C for 24 h with either rabbit polyclonal antibody against VEGF amino-terminus (Santa Cruz-152, SDS, Sweden, dilution 1:100), goat polyclonal antibody against recombinant rat VEGF164 (R&D Systems Inc., Minneapolis, MN, dilution 1:50), rabbit polyclonal antibodies against Flt-1 (Santa Cruz-316, SDS, Sweden, dilution 1:100) and Flk-1 (Santa Cruz-315, SDS, Sweden, dilution 1:100), mouse monoclonal antibodies against an endothelial protein found in areas with blood–brain or blood–nerve barriers (SMI-71, Sternberger Monoclonals Inc., Baltimore, MD, dilution 1:1000), goat polyclonal antibodies against
glial fibrillary acidic protein (GFAP; Santa Cruz-6170, SDS, Sweden, dilution 1:100), mouse monoclonal antibodies against rat macrophage/microglia surface protein ED1 (ED1, Serotec Antibodies, Sweden, dilution 1:500), and mouse monoclonal antibodies against rat granulocytes (Clone MOM/3F12/F2, Serotec Antibodies, Sweden, dilution 1:200). Sections were also double-labeled with different sets of combinations of the above mentioned antibodies. All the primary antibodies were diluted in a solution of 0.3% Triton, 5% bovine serum albumin (BSA), and 0.1% sodium azide in 0.01M PBS. Donkey serum (5%) was also added to minimize background.

**FIG. 1.** Autoradiographs of adult rat cerebral sections at 1 and 2 days after weight-drop injury showing *in situ* hybridization signal for VEGF mRNA with use of an oligoprobe. (A) In a thin zone just around the central lesion (indicated with arrows), expression of VEGF mRNA can be seen at day 1 after the injury. (B) Adjacent to the central lesion, expression of VEGF mRNA can be seen (indicated with arrows) 2 days after the injury. Bar = 200 μm (A), 0.5 mm (B). c.l., Central lesion; b.s., brain surface.
staining. The sections were then rinsed in 0.01M PBS and incubated for 45 min at 20°C with 0.01% PBS + 0.1% sodium azide + 0.3% Triton containing either Cy3-conjugated donkey anti-mouse IgG, (Jackson Immu­noResearch, Inc., West Grove, PA, dilution 1:500), Cy2-conjugated donkey anti-rabbit IgG, Cy2-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, Inc., dilution 1:200), or mixtures of them depending on the primary antibodies on the double-labeled sections. After the sections were rinsed in PBS, they were mounted in a mixture of glycerol and PBS (1:3), and coverslipped. The sections were examined in a Nikon Eclipse E600 microscope equipped with epifluorescence and appropriate filter combinations for the fluorophores used here. Photos were captured with a Nikon Coolpix 4500 digital camera. Controls were performed by preabsorption of the antibodies with their specific blocking peptide.

Image Analysis of IHC

Evaluation of VEGF protein expression (Santa Cruz-152, SDS, Sweden, dilution 1:100) in GFAP-positive astrocytes, ED1-positive macrophages/microglia, granulocytes, and SMI-71-positive vessels was performed on double immuno-labelled tissue by use of a fluorescence microscope with appropriate filter combinations. Positive cells for respective antibody were counted in six randomly chosen areas of one optical field (240 μm × 180 μm) located along the border of the lesion. Two tissue sections from each animal were counted in random order, and the average constituted one observation.

Statistical Analysis

GraphPad Prism software was used for statistical analysis, and a two-tailed Kruskal-Wallis nonparametric test and Dunnet’s multi-comparison test were used to calculate significant differences (p < 0.05) between control and injured animals.

RESULTS

Upregulation of VEGF mRNA and Protein in the Lesion Area after Cerebral Contusions

Analysis of in situ hybridization. With use of two different VEGF probes, both complimentary to the 5’ end expressed in all known rat VEGF spliceforms, we were able to detect expression of VEGF mRNA from day 1 after injury throughout day 6 after injury. The expression pattern over time was similar with both probes used. At all time points after injury, the expression of VEGF mRNA detected was only in the lesion and the perilesional area. No expression was detected at the very early

FIG. 2. Autoradiographs of adult rat cerebral sections 4 days after weight-drop injury showing in situ hybridization signal for VEGF mRNA with use of an oligoprobe. (A) At the injured side (indicated by arrow), a strong expression of VEGF mRNA can be detected around the lesion area. (B) Higher magnification of the lesion central lesion in A. A distinct VEGF mRNA expression can be detected around the central lesion. (C) Larger magnification of B. Single cells expressing VEGF mRNA can be detected at the central lesion border. Bars = 1 mm (A), 0.5mm (B), and 100 μm (C), c.l., central lesion.
Posttraumatic phase at 6 h after injury (results not shown), but starting at 1 day post-injury expression of VEGF mRNA was detected (Fig. 1A). The expression peaked at day 4 and was almost abolished by day 8 after the injury (Figs. 1–4). VEGF mRNA was expressed in small cells—at day 1 in a thin border zone between the central contusional lesion and the surrounding brain tissue (Fig. 1A), and during later time points both in and around the lesion (Figs. 1B, 2A–C, and 3A,B). The corresponding sense probes for the different mRNAs were negative at all time points after contusion (results not shown), and no activity similar to that found in the injured animals could be found in sham-operated animals (results not shown).

Analysis of immunohistochemistry. With immunohistochemical staining, colocalization of VEGF and a granulocyte-specific antibody (Fig. 5A), a macrophage/microglia antibody ED-1 (Fig. 5B), and the astrocytic marker GFAP (Fig. 5C) could be detected around the lesion from day 1 after injury. Estimation of number of cells expressing VEGF at different time points revealed that both astrocytes and granulocytes had a peak expression of VEGF around 4 days after injury (Fig. 7A,C) though VEGF-expressing granulocytes seemed to be more numerous at earlier time points compared to astrocytes (Fig. 7A,C). ED1-expressing macrophages/microglia did express VEGF (7B); although compared with the astrocytes and granulocytes, the number of VEGF-positive macrophages/microglia were fewer of the total number of cells counted. VEGF immunolabeling could also be detected in vessel-like structures around the lesion from day 1 after injury, with a maximum expression occurring approximately 6–8 days after injury (Figs. 6A,B and 8). Double immunolabeling with SMI-71 antibody (specific for vessels in CNS areas with intact BBB)
and VEGF showed that colocalization of VEGF and SMI-71 could sometimes be found (Fig. 6B); however, vessels positively stained for VEGF were more often negative for SMI-71 (Figs. 6A,B and 8).
VEGF expression in GFAP positive astrocytes after cerebral contusion

A

![Graph showing VEGF expression in GFAP positive astrocytes](image)

Number of positive cells

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<tr>
<th>Time after injury</th>
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**FIG. 7.** Line graphs showing the number of VEGF positive astrocytes (A), macrophages/microglia (B), and granulocytes (C). (A) An initial decrease in the number of GFAP positive astrocytes around the cerebral contusion is followed by an increase both in number GFAP positive astrocytes and astrocytes expressing both GFAP and VEGF. (B) A delayed maximum level of ED1 positive macrophages/microglia could be shown but only a minor part of these cells seemed to co-express VEGF. (C) A rapid increase in the number of granulocytes was observed in the perilesional area over the first 4 days where after a decline in the number of granulocytes could be observed. A substantial number of the granulocytes where also immunopositive for VEGF. The values are means ± standard deviation.
Flk-1 and Flt-1 but Not Np-1 Is Upregulated in the Perilesional Area after Cerebral Contusion

Analysis of in situ hybridization. Probes against the VEGF receptors Flk-1, Flt-1, and Np-1, previously used in experiments on traumatic spinal cord injuries (Sköld et al., 2000), were hybridized to 14-μm sections of contusion material from all different time points between 6 h and 14 days. From day 1 after the injury, we could visualize vessel-like structures positively marked with Flk-1 (Fig. 9A–C). The expression seemed to be limited to the area adjacent to the lesion cavity and could be observed from day 1 throughout day 6. The VEGF receptor Flt-1 could be detected only at day 2 postoperatively. As with Flk-1, expression seemed limited to the lesion border (Fig. 10). The VEGF receptor Np-1 could not be detected in or around the contusion lesion at any time point after the injury. The corresponding sense probes for the different mRNAs were negative at all analyzed time points after contusion (results not shown).

Analysis of immunohistochemistry. With immunohistochemical methods, we could detect Flk-1 and Flt-1 expression in vessels adjacent to the lesion from day 1 after injury throughout day 4 after injury (Fig. 11A,B). In the same area adjacent to the lesion cavity, SMI-71-immunopositive vessels were few (Fig. 11C), with many vessels expressing Flk-1 while lacking SMI-71 immunoreactivity (Fig. 11D).

DISCUSSION

In this work, we demonstrated that an experimental TBI induced an upregulation of VEGF and the VEGF receptors Flk-1 and Flt-1. The expression of VEGF and the two VEGF receptors was limited to the lesion area or to tissue just around the lesion (Fig. 12). VEGF expression was most prominent approximately 4–6 days after the injury. Double immunolabelling showed expression of VEGF in granulocytes, macrophages/microglia, and astrocytes. We also found expression of VEGF and the VEGF receptors Flk-1 and Flt-1 in vessel-like structures in and around the lesion area. Flk-1 and Flt-1 was expressed at both mRNA and protein level from day 1 until day 6 after injury, and vessels labelled with VEGF, Flk-1, or Flt-1 were often negative for a marker of vessels in areas with BBB.

VEGF and Traumatic Brain Injuries

With a few interesting exceptions (Krum et al., 1998; Nag et al., 1997; Papavassiliou et al., 1997), the expression of VEGF and the VEGF receptors has not been studied thoroughly in traumatic cerebral injury or, to our knowledge, in a contusional model of injury.

In a cold injury model developed to induce vasogenic edema, VEGF immunoreactivity occurred from 6 h to 14 days after injury (Nag et al., 1997). Interestingly, VEGF immunoreactivity did precede angiogenesis occurring
from day 3 after injury. In this study, we also found expression of both VEGF mRNA and protein before the time-point when angiogenesis is reported to take place in response to traumatic CNS injuries (Imperato-Kalmar et al., 1997; Nag et al., 1997). The early finding of VEGF in our model is also interesting from the perspective of the reported early induction of integrin αvβ3, an essential factor in angiogenesis, after ischemic CNS injuries (Okada et al., 1996), which can be induced by VEGF (Abumiya et al., 1999; Senger et al., 1996).

With immunostaining for SMI-71, a marker for vessels in areas with BBB (Sternberger et al., 1987), and VEGF we found that those markers were not always co-expressed in brain vessels. Since the VEGF expression occurs before angiogenesis is known to take place, it is possible that mature SMI-71 antigen expressing vessels in the vicinity of the lesion can change their status and become leaky upon binding of VEGF. Studies have also shown absence of tight junction proteins in brain vessels after injuries similar to those used herein (Bellander et al., 1996), and it is known from in vitro experiments that hypoxia-induced changes of the ZO-1 expression are mediated by VEGF (Fischer et al., 2001), further illustrating the potential importance of VEGF in changed BBB status after cerebral contusion. The time-frame for VEGF expression following contusion demonstrated here also matched findings from stab and freeze injuries where VEGF immunoreactivity was reported to be maximal on days 3 and 4 after injury (Papavassiliou et al., 1997). The delayed peak of VEGF expression found in this study is especially interesting considering that the model employed is known to be associated with biphasic edema formation (Holmin et al., 1995a), although no direct in-

FIG. 9. Sections of rat brain after contusion showing in situ hybridization signal for Flk-1 mRNA at 1, 2, and 6 days after the injury. At the central lesion border, vessel-like structures marked with Flk-1 oligoprobe can be seen at 1 (A), 2 (B), and 4 (C) days after injury. Insert in B is a magnification of the indicated area (arrow) and shows a single vessel at the lesion border expressing Flk-1 mRNA. Note that, at all different time points observed, the receptor expression is limited to the lesion border. Bar = 200 μm. c.l., central lesion.

FIG. 10. Sections of rat brain after contusion showing in situ hybridization signal for Flt-1 mRNA at day 2 after the injury. Vessel-like structure at the injury border is indicated (arrow). Bar = 100 μm. c.l., central lesion.
FIG. 11.  Fluorescence photomicrographs of sections immunohistochemically stained for Flt-1, Flk-1, and the BBB marker SMI-71. (A) At the border (marked with asterisks) of the contusion area, vessels immunopositive for Flk-1 can be observed at 4 days after injury. (B) Vessels stained with antibodies against Flt-1 can be observed around the contusion area at 4 days after injury (indicated by arrows). (C) SMI-71 immunopositive vessels outside the border (marked with asterisks) between central lesion and intact tissue at 2 days after contusion injury. (D) Double-immunolabeling with antibodies against SMI-71 (green) and Flk-1 (red) at 4 days after injury in the perilesional area. Note that double immunolabeling with SMI-71 and Flk-1 can be seen in some vessels (indicated with arrow), whereas others express Flk-1 only. Bar = 50 μm (A,B), 20 μm (C,D).

FIG. 12.  Schematic drawing showing a coronal section through the rat brain 2.5 mm posterior to bregma. Black area indicates the lesion area after cortical impact. Red spots indicates typical finding place for VEGF mRNA/protein expression, and green spots typical finding place for VEGF receptor mRNA/protein expression after cerebral contusion. lv, lateral ventricle; 3v, third ventricle; hi, hippocampus.
fluence of VEGF on edema development is shown in the present work.

Flk-1 and Flt-1 Expression: Potential Role in Angiogenesis and Vascular Leakage

Normal adult CNS does not express the VEGF receptors (Millauer et al., 1993). Our findings of Flk-1 and Flt-1 mRNA and protein expression from 1 day after injury throughout at least day 6 are novel in cerebral contusion injuries. Thus, both the receptors and the ligand, VEGF, were expressed in the same time, indicating that the contusion itself is the primary site of VEGF actions. Since the knowledge of VEGF receptors in traumatic CNS injury is limited, comparisons can only be made with findings from ischemic injuries. It is known that VEGF and VEGF receptors are expressed after ischemic CNS injury (Abumiya et al., 1999; Issa et al., 1999; Kovacs et al., 1996; Lennmyr et al., 1998; Marti et al., 2000), and there are also indications of a temporal and spatial correlation between BBB leakage and VEGF expression (Zhang et al., 2002).

In addition to this, administration of VEGF to ischemic rats has been shown to enhance angiogenesis and neurological recovery when given late (48 h) after middle cerebral artery occlusion (MCAO), but increased BBB permeability and ischemic injury when given early (1 h) after MCAO (Zhang et al., 2000). Experimental antagonism to VEGF after cortical ischemia by adding of Flt-1–IgG before ischemia, after reperfusion and at 1 day after injury, resulted in reduction of edema and sparing of cortical tissue (Van Bruggen et al., 1999). This further supports the suggestion that early VEGF expression might be important for development of vasogenic edema and that similar strategies may be effective for treatment of traumatic CNS injuries. In the current study we found both early and late expression of VEGF receptors and VEGF itself, indicating probable roles in permeability changes as well as in early angiogenesis after contusion injury. While VEGF expression had a delayed peak, no obvious changes in intensity of VEGF receptor expression were observed. The VEGF receptors are of profound importance for angiogenesis, and knockouts are lethal at embryonic stage (Fong et al., 1996; Shalaby et al., 1995). It is therefore likely that the VEGF receptor expression shown here is a part of post-traumatic angiogenesis. Since the early expression described here does precede the time typically associated with angiogenesis it is likely that presence of VEGF receptors and VEGF indicate the presence of signaling associated with vascular proliferation.

VEGF and Inflammation

Increase in adhesion molecules are of importance in inflammation and the diapedesis of leukocytes over ves-

VEGF as Neuroprotective/Neurotrophic Factor

VEGF is a neuroprotective/neurotrophic factor. It can therefore be speculated that the presence of VEGF and its receptors following contusion can have protective roles similar to those shown for other growth factors known to be increased following brain trauma, for example, IGF-1 (Nordqvist et al., 1997; Sandberg-Nordqvist et al., 1996). After spinal cord injury, the addition of VEGF protein (Widenfalk et al., 2003) or protein combined with VEGF165 coding adenovirus (Facchiano et al., 2002) improved functional outcome and decreased secondary degeneration. Also, it is known that VEGF can exert a direct neuroprotective effect on cultured CNS neurons exposed to hypoxia and that Flk-1 is essential for neuroprotection in this CNS-model (Jin et al., 2000). VEGF expression of neurons in response to hypoxia has a paracrine/autocrine role for maintenance of neurons (Ogunshola et al., 2002) and the administration of VEGF in vivo after MCAO occlusion can have beneficial effects (Sun et al., 2003). VEGF can also stimulate neurogenesis via Flk-1 both in vitro in cultured cortical neurons as well as in vivo in adult rats (Jin et al., 2002). In our present study Flk-1 expression was limited to vessels in the vicinity of the injury, probably making a direct Flk-1-mediated influence on neurons less likely in this model. Besides direct neuroprotective/neurotrophic actions of
VEGF, it is also possible that VEGF allows growth factors to reach the injury site by altering BBB permeability. We have previously shown VEGF/VEGF receptor upregulation (Sköld et al., 2000) in spinal cord injury model associated with neuronal regeneration (Risling et al., 1983) and BBB downregulation (Risling et al., 1989). Clearly, however, additional experiments are needed to demonstrate a possible link between VEGF, BBB downregulation, and neuronal regeneration.

CONCLUSION

In conclusion, the present investigation showed that an experimental brain contusion caused increased intracerebral expression of VEGF and the VEGF receptors Flk-1 and Flt-1. The expression of both VEGF and the VEGF receptors was limited to the lesion and/or the perilesional area, with a maximum expression of VEGF at approximately 4 days after injury. These data extend our knowledge on VEGF in traumatic CNS injuries and will hopefully serve as a foundation for future studies on the importance of the VEGF system in TBI.

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Address reprint requests to: Mattias Sköld, M.D., Ph.D.
Department of Neuroscience
Retzius väg 8
B1:5 Karolinska Institutet
S-17177 Stockholm, Sweden

E-mail: Mattias.Skold@neuro.ki.se