Expression of vascular endothelial growth factor by reactive astrocytes and associated neoangiogenesis

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Abstract

Injury to the central nervous system (CNS) invokes a reparative response known as astrogliosis, characterized largely by hypertrophy, proliferation and increased expression of glial fibrillary acidic protein (GFAP), resulting in reactive astrocytosis. Based on our prior observation that peritumoral reactive astrocytes express Vascular Endothelial Growth Factor (VEGF), a highly potent and specific angiogenic growth factor, we have hypothesized that reactive astrocytosis also contributes to the neovascularization associated with astrogliosis. To evaluate this hypothesis we evaluated human surgical/autopsy specimens from a variety of CNS disorders that induce astrogliosis and an experimental CNS needle injury model in wild type and GFAP:Green Fluorescent Protein (GFP) transgenic mice. Using computer image semi-quantitative analysis we evaluated the number of GFAP-positive reactive astrocytes, degree of VEGF expression by these astrocytes, associated Factor VIII-positive microvascular density (MVD) and Ki-67 proliferating endothelial cells. The degree of reactive astrocytosis correlated to levels of VEGF immunoreactivity and MVD in the neuropathological specimens. The mouse–needle–stick brain injury model demonstrated this correlation was temporally and spatially related and maximal after 1 week. These results, involving both human pathology specimens augmented by experimental animal data, supports our hypothesis that the neoangiogenesis associated with reactive astrogliosis is correlated to increased reactive astrocytosis and associated VEGF expression.

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1. Introduction

Reactive astrogliosis is a normal reparative mechanism induced in many CNS disease processes such as ischemia, infection, trauma, degenerative diseases, epilepsy and brain tumors [29,31,49,50,63]. Amongst the various cellular responses of reactive gliosis, there is a prominent astrocytic response [31,58] comprised of normally quiescent astrocytes proliferating, undergoing hypertrophy of their cell processes and bodies with increased expression of the astrocyte specific cytoskeletal intermediate filament Glial Fibrillary Acidic Protein(GFAP) [7,11,14,35,41,50,64,65]. This reactive astrocytic response has been noted to occur within 2 days after experimental brain injury in rodents and approximately 1 week in humans and may persist for several weeks with progressive decline over months to years [43,47,50,63]. The function of this florid reactive astrocytosis is not fully deciphered, but is postulated to play a role in the healing phase after insult to the CNS by monitoring and controlling the molecular and ionic contents of the CNS extracellular space. This is likely achieved via multiple membrane receptors for neurotransmitter peptides, growth factors and specific ion channels that are known to be expressed by normal and reactive
astrocytes [31,50,63]. However, it is hypothesized that even moderate reactive astrocytosis may interfere with eventual reconnection of functional neuronal circuitry by inhibiting axonal regeneration, preventing remyelination or promoting abnormal neuronal connections with increased seizure foci [57].

There is currently little understanding of the molecular mechanisms regulating reactive astrocytosis. Several observations suggest that reactive astrocytosis is a regulated process involving cytokines, inflammatory mediators, growth factors and physiological stimuli such as hypoxia [45,50,69]. For example, axonal growth and regeneration after trauma and cerebral infarction are retarded by inhibitory proteins associated with the intense astrogliotic scar [6,45,50]. Second, reactive astrocytosis although most intense immediately surrounding the area of injury, is found at much removed distances suggestive of soluble trophic factors acting in a paracrine fashion [50]. While a variety of these trophic factors have been studied in astrocyte cell culture experiments, their role and interactions in the astrogliotic response in vivo is poorly understood. Specifically, polypeptide growth factors such as basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF-α), Platelet Derived Growth Factor (PDGF), Ciliary Neurotrophic Factor (CNTF) and cytokines such as Interleukin-1 (IL1) and Tumor Necrosis Factor (TNF-α), have all been implicated in the astrogliotic response [3,13,32,37,49,50,59,68,69].

Part of any reparative tissue process is neoangiogenesis, vividly demonstrated in the collagen fibrovascular response associated with wound healing and one that can be speculated to be an important component of reactive astrocytosis in the CNS [10]. Amongst the regulators of neoangiogenesis, Vascular Endothelial Growth Factor (VEGF) is highly potent and specific by activating its cognate receptors on the endothelial cells resulting in their proliferation, migration and sprouting [17,27,61]. VEGF has been implicated in most physiological and pathological processes involving neovascularization including embryogenesis, wound healing, tumor growth, myocardial ischemia, ocular neovascular diseases and chronic diseases such as rheumatoid arthritis [16,21]. In addition to its angiogenic effects, VEGF is also a potent edemogenic agent, resulting in disruption of the blood–brain-barrier (BBB), and hence was also known as Vascular Permeability Factor (VPF). We observed that VEGF/VPF was co-expressed with GFAP-positive reactive astrocytes in the peritumoral edematous brain around meningiomas, where its expression was correlated to the vascularity and associated edema induced by these CNS tumors [56]. This suggested that reactive astrocytes may express VEGF, modulating neoangiogenesis and also the edema and breakdown of the blood brain barrier associated with reactive astrogliosis. To address this hypothesis, immunohistochemical techniques were used to quantify reactive astrocytosis, neoangiogenesis and VEGF expression from a spectrum of conditions including Alzheimer’s disease, brain abscess, peritumoral brain from metastatic carcinoma, head injury and cerebral infarcts. Further, to evaluate the temporal relationship of reactive astrocytosis and neoangiogenesis, a stereotactic brain injury model was utilized. In order to obtain the proliferative index (Ki67 positive cells/1000 endothelial cells×100) of endothelial cells, surgical specimens from brain abscess and metastatic carcinoma were stained for Ki-67. In addition, a CNS injury model was utilized in transgenic mice which express Green Fluorescent Protein (GFP) under the control of the astrocyte specific GFAP promoter [5], to co-localize VEGF expression in reactive astrocytes.

2. Methods

2.1. Evaluation of human neuropathological diseases

In order to evaluate neoangiogenesis and VEGF expression in a variety of reactive astrogliotic specimens, 23 cases of neurosurgical and autopsy specimens obtained from our neuropathology department were examined. These cases represented a spectrum of benign and malignant diseases that are known to induce reactive astrocytosis in the surrounding brain and included: four Alzheimer’s Disease (AD), three bacterial abscesses, five carcinomas, three head injuries and seven infarcts (two acute: <7 days, two subacute: 7–30 days, three old: >30 days). Normal autopsy brain tissue was used as a control. Standard hematoxylin and eosin (H&E) staining was performed on 5-μm tissue sections from paraffin embedded tissue blocks, which were independently reviewed by a neuropathologist (PS). Sequential sections were incubated overnight at 4°C with a primary rabbit antibody against GFAP (1:3000, Dako Corp, CA) to mark reactive astrocytes, a human anti-Factor VIII antibody (1:2000, Dako Corp) to determine the microvascular density (MVD) or a polyclonal rabbit anti-VEGF antibody (1:50, Santa Cruz Biotechnology Inc., CA) that recognizes all four VEGF isoforms. Detection was through the ABC (Vector)-DAB (VectaStain Elite, CA) system. The sections were analyzed using the MicroComputer Image Device (MCID; Imaging Research Inc., Canada), linked to a color CCD camera (Sony DXC 970 MD) mounted on a transmitted-light microscope (Zeiss Axioskop) fitted with a Ludl Biopoint motorized stage. Microscope fields (400×magnification) were acquired and digitized for each of the sequential sections stained for GFAP, Factor VIII and VEGF, with 4–6 high-powered fields (HPF) analyzed in each tissue section. These HPFs were chosen to be adjacent to the site of tissue necrosis by the underlying pathology (i.e. adjacent to the abscess wall, carcinoma, infarct, contusion) or in the mesial temporal lobes of the AD specimens. All raw values were reported as a mean±standard error of the means.
(S.E.M.) and also represented as a percentage of the maximum value obtained. The extent of astrocytosis was assessed by averaging the number of GFAP-positive reactive astrocytes/4–6 high-powered fields (HPF). MVD counts were derived by averaging the number of Factor VIII-positive vessels/4–6 HPF and VEGF staining was quantified by averaging the percentage VEGF immunoreactivity/4–6 HPF.

In three cases of brain abscess and two cases of metastatic carcinoma, sections were stained for Ki-67 (MIB-1, 1:50, Immunotech) after microwave antigen retrieval and the proliferative index of the endothelium was obtained. The proliferative index was determined by counting 1000 endothelial cell nuclei and determining the percentage staining positive for Ki-67.

### 2.2. Wild type and transgenic GFAP:GFP mouse stereotactic needle brain injury

To determine the temporal association between reactive astrocytosis, neoangiogenesis and VEGF expression, a reproducible stereotactic needle stick injury model to the frontal cerebral cortex of mice was used [42,47,50]. A total of 27 CD1 mice were anesthetized with 0.5 ml of 2.5% Avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol) (Sigma-Aldrich Chemical Company Inc., WI) by intraperitoneal (i.p.) injection. The head was stabilized in a stereotactic frame and a burr hole drilled 2 mm anterior to the coronal suture and 2 mm lateral to the sagittal suture. The needle of a 5-μl Hamilton syringe was then inserted through the burr hole to a depth of 2.5 mm for a duration of 1 min, then withdrawn. The contra-lateral frontal lobe was used as the non-injured control side in all evaluations. The track was made in cortical gray matter, not penetrating sub-cortical areas that normally contain higher numbers of GFAP-positive cells [8]. All mice tolerated the procedure well. Three mice were sacrificed on each of days 1–9 post-injury by cervical dislocation and the brains fixed in formalin for 24 h and embedded in paraffin for histological processing. Axial, 6-μm thick paraffin sections at right angles to the needle tract were cut, stained with anti-GFAP, anti-Factor VIII and anti-VEGF antibodies and analyzed with the computer assisted image analysis system as above.

Three FVB/N background GFAP:GFP transgenic mice (a gift from Dr. A. Messing, Madison, WI, USA) underwent a similar needle tract injury and were evaluated 7 days after injury with immunofluorescence microscopy. Mice were sacrificed by cervical dislocation. Brains were removed within 5 min of sacrifice, embedded in OCT (Tissue Tek) embedding medium on dry ice and stored at −70°C until further processing. Axial cryostat sections were fixed in ice cold acetone for 3 min at −20°C, washed with PBS for 10 min and blocked in a solution of PBS containing 1% BSA and 0.9% NaCl for 30 min at room temperature. Double immunofluorescence for VEGF and endogenous GFAP was performed by incubating with a rabbit anti-GFAP antibody (1:10, Dako) or mouse monoclonal anti-VEGF antibody (1:10, UBI) for 1 h at room temperature. After three washes in PBS containing 1% BSA and 0.9% NaCl, the sections were incubated with either Cy5-conjugated goat anti-mouse IgG (1:50, Jackson ImmunoResearch Laboratories Inc.) or Cy3-conjugated goat anti-rabbit IgG (1:20, Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature. Sections were washed in PBS and mounted for immunofluorescent microscopy. Sections were evaluated with the green immunofluorescence filter to detect expression of the GFP transgene under the GFAP promoter and for either endogenous GFAP (Cy3) or VEGF (Cy5).

### 3. Results

#### 3.1. Reactive astrocytosis, VEGF immunoreactivity and neoangiogenesis in human CNS diseases

The mean age of patients with the different diseases were 79 years — AD; 38 years — Abscess; 65 years — Peri-metastatic carcinoma brain; 52 years — Head Injury; 71 years — Cerebral infarcts. Reactive astrocytosis with increased numbers of large and darkly stained GFAP-positive reactive astrocytes were a prominent feature of all these disease processes compared to normal brain, although the degree of reactive astrocytosis varied with the type of pathology, as demonstrated in Table 1, Fig. 1. The most exuberant reactive astrocytosis was induced in the brain surrounding bacterial abscesses (Table 1, Fig. 1-Raw A), with an average raw value of 82±28 GFAP-positive astrocytes/HPF. In comparison, diffuse head injury (Fig. 1-Raw C), induced the lowest amount of reactive astrocytosis, with a raw value 20±7 GFAP-positive astrocytes/HPF, though this result should be tempered due to our lack of knowledge of severity or the time of the autopsy after the head injury. The amount of VEGF immunoreactivity paralleled the degree of induced reactive astrocytosis, except for cerebral infarcts and peri-tumoral brain, where VEGF immunoreactivity was disproportionately higher (Table 1). The number of Factor VIII-positive blood vessels (MVD), as a measure of neoangiogenesis, was related to the degree of reactive astrocytosis and VEGF immunoreactivity. This is best exemplified by MVD, raw counts of 20±3 vessels/HPF, associated with the florid reactive astrocytosis in bacterial abscess, compared to only 6±1 vessels/HPF in brains with prior head injury, AD or infarcts. In these latter conditions, the degree of reactive astrocytosis and associated neoangiogenesis although relatively low compared to brain around bacterial abscess, was still significantly high relative to normal brain (Table 1). In all cases, astrocytosis, VEGF immunoreactivity and MVD was increased from normal brain (Fig. 1-Raw F). In addition, blood vessels associated with brain abscess
Table 1
Semi-quantitative analysis of immunohistochemical staining in a variety of human neuropathologies

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Reactive Astrocytosis (No. GFAP-Positive Cells/HPF)</th>
<th>VEGF Expression (VEGF immunoreactivity/HPF)</th>
<th>Vascularity (No. Factor VIII-stained vessels/HPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1</td>
<td>2±1 (2)</td>
<td>4±1 (9)</td>
<td>2±1 (10)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>4</td>
<td>39±2 (48)</td>
<td>25±4 (54)</td>
<td>6±1 (30)</td>
</tr>
<tr>
<td>Bacterial abscess</td>
<td>3</td>
<td>82±28 (100)</td>
<td>6±1 (100)</td>
<td>20±3 (100)</td>
</tr>
<tr>
<td>Head injury</td>
<td>3</td>
<td>20±7 (24)</td>
<td>13±4 (28)</td>
<td>6±1 (30)</td>
</tr>
<tr>
<td>Infarct</td>
<td>6</td>
<td>22±3 (27)</td>
<td>23±3 (50)</td>
<td>6±1 (30)</td>
</tr>
<tr>
<td>Peritumoral brain</td>
<td>4</td>
<td>28±1 (34)</td>
<td>30±1 (65)</td>
<td>11±3 (55)</td>
</tr>
</tbody>
</table>

* Reactive astrocytosis, VEGF expression and vascularity in a spectrum of human neuropathological specimens. Raw mean values±S.E.M. are shown and values are reported as a percentage of the maximum in brackets. All slides were quantified at 400× magnification. VEGF, vascular endothelial growth factor; S.E.M., standard error of the mean; GFAP, glial fibrillary acidic protein; HPF, High Power Field.

3.2. Temporal and spatial expression of reactive astrocytosis, VEGF immunoreactivity and neoangiogenesis

The mouse stereotactic needle stick injury resulted in a distinct tract that could be consistently visualized on the injured side with a surrounding reactive astrogliotic response (Fig. 2A). The injury induced GFAP-positive astrocytes (Fig. 2B) to express VEGF (Fig. 2C) with accompanying neoangiogenesis, as marked by Factor VIII-positive endothelial cells (Fig. 2D). The number of GFAP-positive reactive astrocytes reached a maximum on day-7 post-injury, which was taken as 100% for comparison with the other time points analyzed (Figs. 2, 3). VEGF immunoreactivity increased to its maximum level (100%) on day 6 post-injury, when the reactive astrocytic response had reached 47±14% of maximal day-7 levels (Fig. 3). The number of Factor VIII-positive blood vessels (MVD) reached a maximal level (100%) on day 5 post-injury, when the degree of reactive astrocytosis was 31±14% and VEGF immunoreactivity was 59±7% of maximal levels on day 7 and day 6 respectively (Figs. 2, 3). Therefore, there was a progressive increase in reactive astrocytosis, VEGF immunoreactivity and neoangiogenesis after injury, with maximal levels clustering around days 5–7. All three parameters subsequently decreased towards normal levels by day 9. While the peak days for each parameter analyzed do not occur on the same day in this experimental paradigm, the overall temporal correlation between them is clearly demonstrated. The GFAP:GFP transgenic mice were used for co-localization experiments in a needle stick injury model to confirm that the reactive astrocytes induced by the needle tract injury (* in Fig. 4) were expressing VEGF. Green immunofluorescence denoted the expression of the GFP transgene under regulation of the GFAP promoter (Fig. 4A). These same GFP expressing cells were reactive astrocytes, as they also expressed endogenous GFAP as detected by double immunofluorescence, where the cells are yellow due to the combination of green and the Cy3 secondary antibody used to detect endogenous GFAP (Fig. 4B). On adjacent sections, the GFP reactive astrocytes are the same cells that are expressing VEGF, where they appear red due to combination of green and Cy5 immunofluorescence used to detect VEGF (Fig. 4C). There were no significant reactive GFP-positive astrocytes in the non-injured contra-lateral hemisphere (Fig. 4D).

4. Discussion

Astrogliosis and the formation of the glial scar, with a predominant reactive astrocytosis component, is an important and widespread neuropathological response to diverse neurological insults, a feature that is well conserved across different species. This prominent, rapid and evolutionarily conserved reparative response, suggests that reactive astrocytosis fulfills an important function in the CNS. However, the exact role of reactive astrocytosis and whether it is beneficial or ultimately detrimental to CNS recovery after injury remains unclear and controversial. Reactive astrogliosis is a dynamic process that leads to a densely interwoven glial scar composed of many cells including reactive astrocytes, microglia, macrophages and endothelial cells [12,24,26,31,53]. Reactive astrocytes
Fig. 1. Astrocytosis and neoangiogenesis in human CNS disease. Photomicrographs (scale bar depicts 50 μm in length) illustrating immunohistochemical staining of a spectrum of human neuropathological disease states with: Row A — Brain Abscess; Row B — Alzheimer’s Disease; Row C — Old Infarct; Row D — Peritumoral Brain; Row E — Head Injury; Row F — Normal Brain; Column 1 — anti-glial fibrillary acidic protein (GFAP) antibody; Column 2 — anti-vascular endothelial growth factor (VEGF) antibody; Column 3 — anti-Factor VIII antibody. When compared to the staining observed in normal brain, GFAP reactive astrocytes and neoangiogenesis as indicated by VEGF immunoreactivity and Factor VIII-stained vessels, is common and increased in all of the human pathologies (reddish/brown staining). GFAP positivity is noted in the reactive astrocytes and astrocytic foot processes around blood vessels. The most intense staining for all three parameters is observed in the tissue taken from the brain surrounding the abscess.
Fig. 2. Visualization and characterization of mouse–needle–stick injury. Immunohistochemical sections of mouse brain stained for GFAP, VEGF and Factor VIII. (A) Coronal section through the injury site (scale bar depicts 2 mm in length) displaying GFAP immunoreactivity (brown staining) in reactive astrocytes straddling the tract site (arrow). The contralateral non-injured site (N) is GFAP negative in the same cortical region. Mouse brain sections at higher power magnification at the site of the needle stick injury (day 7 post-injury) (scale bar depicts 50 µm in length) stained with (B) anti-glial fibrillary acidic protein (GFAP) antibody; (C) anti-vascular endothelial growth factor (VEGF) antibody; (D) anti-Factor VIII antibody. Day 7 post-injury is the maximum GFAP response, also shown in Fig. 3. The injury tract (*) can be seen in the upper left corner of each section. Arrows in B and C are pointing to positively stained reactive astrocytes.

form scars by extending their long, thick cytoplasmic processes toward the site of the lesion [29]. It is likely that the glial scar can serve to separate normal brain from the surrounding damaged tissue, but its formation may also be harmful by essentially creating a mechanical barrier [50] which may impede neuronal dendritic growth, remodeling and axonal regeneration [25,29,50,63,71]. In addition, the glial scar may contribute to electrical instability in the region and in turn promote seizure activity [50]. However, more recent evidence suggests that astrogliosis may actually facilitate CNS recovery through neurotrophic factor production around the area of the lesion [2,34,67]. In summary, like the common collagen fibrovascular wound healing process, reactive astrogliosis serves a beneficial CNS reparative function, however, under certain circumstances it may be detrimental much like exuberant scar and keloid formation in other tissues.

This study demonstrates, using human specimens and experimental models of CNS injury, that reactive astrogliosis is accompanied by a neoangiogenic response, similar to the collagen fibrovascular response elsewhere. Furthermore, we speculate based on our results that a potential source of this angiogenic response lies in increased VEGF secretion by the quiescent reactive astrocytes. This conclusion is based on the overall consistent relationship between GFAP-positive reactive astrocytes, neoangiogenesis (MVD) and VEGF immunoreactivity in a variety of human CNS pathologies (see Table 1, Fig. 1). Reactive astrogliosis, neoangiogenesis and increased VEGF was maximal in response to bacterial brain abscess and probably reflects the acuteness of the illness and the type of response that it induces in the CNS. In an abscess, in response to the released toxins, there is an inflammatory cellular response, components of which themselves release VEGF and other inflammatory cytokines modulators. This leads not only to a vigorous reactive astrogliotic response but it is also one of the few CNS diseases that induces a collagen fibrovascular response.

The amount of VEGF immunoreactivity and MVD exceeded the degree of reactive astrocytosis in the brain around carcinomas (Table 1), perhaps attributable to the additive expression of VEGF from the GFAP positive reactive astrocytes and the carcinoma cells themselves [22]. In cerebral infarcts, irrespective of whether it was acute, subacute or old, VEGF immunoreactivity was higher than the degree of reactive astrocytosis or neoangiogenic response (see Table 1). This may reflect the potent hypoxic induction of VEGF expression by the surviving reactive astrocytes [15], without a concomitant increase in the number of astrocytes or the ability to recruit new blood vessels due to the cerebral ischemia. This data is similar to results in a study by Plate et al., where in a middle cerebral
artery occlusion in rats, VEGF mRNA expression was consistently greater across different time points than the number of CD31 stained vessels [54]. In neurodegenerative CNS diseases like AD, characterized by β-amyloid deposits, neuronal death is accompanied by proliferation of astrocytes, which react in an attempt to detoxify neuronal debris, toxins and produce various trophic substances [33,63]. Furthermore, decreased cerebral perfusion and glucose metabolism found in AD may also induce the reactive astrocytes to up-regulate VEGF in an attempt to increase neoangiogenesis (Fig. 1) [33,63].

The overall results of the mouse-needle-stick injury model are supportive of a temporal correlation between reactive astrocytosis, neoangiogenesis and VEGF expression. All three reached maximal levels between days 5–7, with subsequent return towards basal levels by day 9 (Fig. 3). What is not obvious is why the MVD peaked first (day 5), followed by VEGF (day 6) and then GFAP-positive reactive astrocytes (day 7) (Fig. 3). Perhaps the initial wave of neoangiogenesis on day 5 reflects the response to inflammatory cells induced by the injury in these immunocompetent CD1 mice, with release of other angiogenic stimulants in addition to VEGF [52]. The GFAP:GFP transgenic mice subjected to a similar needle stick injury, clearly demonstrated co-labeling of VEGF to the GFAP positive reactive astrocytes. Our results confirm previous findings that reactive astrocytes after a stab wound injury are VEGF positive [46,52] and also concur with the observation that VEGF mRNA was overexpressed in retinal astrocytes of diabetic rats [46].

Although our study focuses on increased expression of VEGF in reactive astrocytes, it is evident that there are several cell types capable of producing VEGF, that are also prevalent in reactive astrocytosis. These include, platelets, neurons, macrophages/microglia, smooth muscles around vessel walls, megakaryocytes and polymorphonuclear leukocytes [36,44,46,52,54,66,72]. There is some controversy as to which of these cell types are the predominant expressors of VEGF following damage to the CNS. Plate et al. identified cells of the microglial/macrophage lineage to be the major source of VEGF upregulation following a middle cerebral artery occlusion (MCAO) and reported virtually no VEGF production in neurons or reactive astrocytes [54]. Lennmyr et al. demonstrated the most striking changes in VEGF production following MCAO to be in neurons, with only weak-to-moderate VEGF immunoreactivity in monocytes and astrocytes [36]. The reasons for these differences are unclear but may be attributed to differences in methods and use of reagents. Interestingly however, using the same reagents and con-
conditions, we were able to detect increased VEGF immunoreactivity but unable to co-localize VEGF to reactive astrocytes in GFAP:GFP transgenic and CD1 mice with a MCAO (data not shown). This data suggests that there may be differences in VEGF immunolocalization in the stab injury and focal ischemia models [46]. Further evidence indicative of differences in VEGF immunolocalization in cerebral infarct is seen in our human data, where despite increased levels of VEGF immunoreactivity, the correlation to reactive astrogliosis was not as strong as was the correlation in other neuropathologies including head injury and brain abscess. However, this is only speculation and requires further studies with the appropriate controls.

Angiogenesis is the sprouting of capillaries from pre-existing blood vessels and during embryonic development is a fundamental process in the formation of the vascular system. In the adult, angiogenesis plays a crucial role in normal and pathological processes such as wound healing, diabetic retinopathy and tumorigenesis [1,10,23]. The angiogenic phenotype is activated when the normal balance that exists between inducers and inhibitors of angiogenesis is disrupted [4,21,51]. This induction results in angiogenesis through chemotactic and mitogenic effects on endothelial cells which line blood vessels [21]. A number of peptides are known to be angiogenic in vivo [23]. However, VEGF, a homodimeric glycoprotein is a mitogen that specifically acts on endothelial cells and is postulated to be the main positive regulator of angiogenesis in vivo [55,60,70]. There are many regulators of VEGF, with hypoxia being the most potent physiological inducer of VEGF [19,20,28,38,62]. The factors responsible for increased VEGF production by astrocytes after damage to the CNS are unknown. However, hypoxia may be the common regulator of VEGF expression by reactive astrocytes in response to a variety of CNS insults that create a relative hypoxic gradient in the surrounding brain. Whether modulation of angiogenic factors such as VEGF would favor repair in various non-neoplastic CNS con-

Fig. 4. Co-localization of VEGF in reactive astrocytes of GFAP:GFP transgenic mice. Photomicrograph (scale bar depicts 20 μm in length) demonstrating the co-localization of reactive astrocytes and VEGF expression in GFAP:GFP transgenic mouse brains post-stereotactic needle stick injury. (*) denotes the area of the tract injury. (A) Endogenous reactive astrocytes (arrows) emit intense green fluorescence. Typical astrocyte morphology with cell bodies and numerous processes can be seen. (B) Double labeling on the same section with anti-GFAP antibody and endogenous GFP immunofluorescence. The reactive astrocytes are stained yellow (arrows) due to the combination of the GFP green fluorescence and the anti-GFAP antibody detected via a Cy3-conjugated secondary antibody. (C) Double labeling on an adjacent section showing the co-localization of GFP-positive reactive astrocytes and VEGF protein expression as demonstrated by the red immunofluorescence staining (arrows). (D) The non-injured contra-lateral hemisphere of the brain in the same area demonstrated no significant GFP-positive green astrocytes.
ditions is uncertain. For example, experiments which modulate VEGF in the zone of cerebral infarction as to rescue the penumbra by neoangiogenesis [33], similar to experiments with VEGF gene therapy of ischemic cardiomyocytes [39], are under current study in our laboratory.

This study has demonstrated using several experimental and neuropathological models that reactive astrocytosis is accompanied by neoangiogenesis, part of which we can postulate is based on VEGF expression by the reactive astrocytes. The process of reactive gliosis involves many different cell types (with reactive astrocytes being a major component), several of which are capable of secreting VEGF. Therefore, the functional importance of reactive astrocytes and VEGF towards the neoangiogenesis accompanying reactive gliosis requires further experimentation. Presently hypomorph (under-expressing) and hypermorph (over-expressing) VEGF transgenic animals are being used to address this issue, since both the homozygous and heterozygous VEGF knockouts are embryonically lethal [9,17]. Another ongoing strategy utilizes small molecule inhibitors of the VEGF receptors expressed by the endothelial cells to inhibit the VEGF mediated neoangiogenic response in reactive astrocytosis. These and other potential strategies will help to further our molecular understanding of reactive astrocytosis and associated neoangiogenesis and may provide therapeutic reagents to modulate this process favorably where indicated in the future.

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