Transcriptional activation of endothelial cells by TGFβ coincides with acute microvascular plasticity following focal spinal cord ischaemia/reperfusion injury

Richard L Benton*,†, Melissa A Maddie‡, Toros A Dincman*,‡, Theo Hagg†,§ and Scott R Whittemore*†

ABSTRACT

Microvascular dysfunction, loss of vascular support, ischaemia and sub-acute vascular instability in surviving blood vessels contribute to secondary injury following SCI (spinal cord injury). Neither the precise temporal profile of the cellular dynamics of spinal microvasculature nor the potential molecular effectors regulating this plasticity are well understood. TGFβ (transforming growth factor β) isoforms have been shown to be rapidly increased in response to SCI and CNS (central nervous system) ischaemia, but no data exist regarding their contribution to microvascular dysfunction following SCI. To examine these issues, in the present study we used a model of focal spinal cord ischaemia/reperfusion SCI to examine the cellular response(s) of affected microvessels from 30 min to 14 days post-ischaemia. Spinal endothelial cells were isolated from affected tissue and subjected to focused microarray analysis of TGFβ-responsive/realted mRNAs 6 and 24 h post-SCI. Immunohistochemical analyses of histopathology show neuronal disruption/loss and astroglial regression from spinal microvessels by 3 h post-ischaemia, with complete dissolution of functional endfeet (loss of aquaporin-4) by 12 h post-ischaemia. Coincident with this microvascular plasticity, results from microarray analyses show 9 out of 22 TGFβ-responsive mRNAs significantly up-regulated by 6 h post-ischaemia. Of these, serpine 1/PAI-1 (plasminogen-activator inhibitor 1) demonstrated the greatest increase (>40-fold). Furthermore, uPA (urokinase-type plasminogen activator), another member of the PAS (plasminogen activator system), was also significantly increased (>7.5-fold). These results, along with other select up-regulated mRNAs, were confirmed biochemically or immunohistochemically. Taken together, these results implicate TGFβ as a potential molecular effector of the anatomical and functional plasticity of microvessels following SCI.

Key words: endothelin, insulin-like growth factor binding protein 3 (IGFBP-3), interleukin-6 (IL-6), matrix metalloproteinase 9 (MMP-9), plasminogen-activator inhibitor 1 (PAI-1), urokinase-type plasminogen activator (uPA).

INTRODUCTION

Following traumatic SCI (spinal cord injury), significant vascular disruption occurs at the site(s) of injury. This interruption of vascular support is thought to be a key mediator of multiple secondary injury cascades, all of which contribute to loss of functional tissue (Nelson et al., 1977). In
the intact CNS (central nervous system), the microvasculature is composed of an integrated unit consisting of ECs (endothelial cells), pericytes, astrocytes and neurons. Any perturbation of the normal functional and/or anatomical integration of the microvasculature results in neural pathology (Hawkins and Davis, 2005). Ultrastructural studies have documented vascular pathology minutes after SCI (Goodman et al., 1979; Koyanagi et al., 1993) and this persists throughout the acute injury phase (Whetstone et al., 2003; Benton et al., 2008a). In fact, ECs appear to be the first cells to die following contusive SCI (Griffiths et al., 1978; Casella et al., 2006). These immediate vascular events, including increased permeability of the BSCB (blood-spinal cord-barrier), induce oedema and contribute to detrimental inflammation (Amar and Levy, 1999; Mautes et al., 2000). In the subacute phase of injury, the penumbral microvasculature is also pathologically transformed by loss of astrocytic investment (Whetstone et al., 2003), regression of pericytes (Benton et al., 2008a) and the perivascular localization of infiltrating inflammatory cells (Popovich and Jones, 2003).

This second and more prolonged phase of microvascular instability has been hypothesized to be a primary event leading to chronic histopathology after SCI (Casella et al., 2002; Loy et al., 2002). Cellular protection/stabilization of microvascular elements within penumbral microvasculature remains a largely unexplored therapeutic avenue due to a relative lack of understanding of key molecular pathways pathologically induced in smvECs (spinal microvascular ECs). This is a critical issue as preservation of metabolic support of spinal tissue spared by the primary injury event should result in enhanced substrate for chronic recovery.

A number of effectors influence BSCB function following traumatic SCI, including the critical vasoactive molecules ephins/ephrins, VEGF (vascular endothelial growth factor), and functionally related co-factor(s), the angiopoietins (Sharma, 2005). The neurotrophins BDNF (brain-derived neurotrophic factor), NGF (nerve growth factor) and NT3 (neurophin 3) also modulate EC survival and proliferation in vivo (Ward and LaManna, 2004), but their role in SCI-induced microvascular plasticity is unknown. Several secreted cytokines, including TNFα (tumour necrosis factor α) and TGFβ1 (transforming growth factor β) isoforms are increased following SCI and are thought to be potent regulators of EC survival, proliferation and function, as well as BSCB integrity (O’Brien et al., 1994; McTigue et al., 2000; Han and Suk, 2005), acting, in part, via the induction of VEGF expression (ten Dijke and Arthur, 2007). Previous in vitro evidence suggests that TGFβ1 can act in concert with VEGF to induce EC apoptosis (Ferrari et al., 2006), a surprising finding with potentially important implications for microvascular stabilization in the anterior spinal cord. Furthermore, MMPs (matrix metalloproteinases) are established regulators of vascular destabilization and EC dysfunction following SCI (Noble et al., 2002) and cortical ischaemia (Cunningham et al., 2005). A pathological connection may exist between TGFβ1 and MMPs, as TGFβ1 signalling increases MMP expression (ten Dijke and Arthur, 2007).

Furthermore, MMPs are activators of latent TGFβ1 in various in vivo contexts (ten Dijke and Arthur, 2007), suggesting the potential for a potent, reciprocal, feed-forward pathological loop in the microvasculature following SCI.

Thus the principal goal of the present study was to determine whether ECs are induced by TGFβ1 in the early phases of SCI utilizing multiple novel approaches. Using a previously described model of focal spinal ischaemia (Benton et al., 2008), we sought to replicate the non-traumatic stress/ischaemia present in penumbral spinal tissue (i.e. immediately surrounding the traumatic injury site). Furthermore, we utilized new methodology to isolate spinal cord microvascular ECs (Benton et al., 2008b) from this ischaemic spinal cord. These approaches lead to the identification of multiple TGFβ/BMP (bone morphogenetic protein)-responsive/released mRNAs expressed in the EC compartment concomitant to the onset of significant histopathology following spinal ischaemia. Taken together, the present results indeed implicate TGFβ1, known to be pathologically altered after SCI, in the acute pathological transformation of the spinal microvasculature.

**MATERIALS AND METHODS**

**Focal ischaemic SCIs**

All surgical intervention and subsequent care and treatment of all animals used in the present study were in strict accordance with the PHS Policy on Humane Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996) and approved by the University of Louisville IACUC guidelines. Focal ischaemic grey matter SCI was accomplished by slight modification of methods described previously (Benton et al., 2005). A total of 70 adult (approx. 6–8 week old) female Sprague-Dawley rats (180–200 g; Harlan) were anaesthetized using sodium pentobarbital [Nembutal; 50 mg/kg, i.p. (intraperitoneally)]. Once anaesthesia was achieved, the surgical site was prepared by shaving, and a betadine scrub and laminectomies were performed at the T13 vertebral level, exposing the L1 spinal cord segment. Animals were randomized into groups receiving a 0.75 μl microinjection of vehicle (saline+1 mg/ml rat serum albumin) or rET-1 (rat endothelin-1; 15 pmol; Sigma, E7764). A single midline incision was made in the exposed dura and rET-1 was delivered in one microinjection into the anterior sulcus using bevelled glass micropipettes custom-pulled to an external diameter of 50–60 μm. The incision sites were then sutured in layers and the topical antibiotic Bacitracin was applied to the incision site. Animals received prophylactic injections of gentamicin to prevent infection [1 mg/kg, i.m. (intramuscularly)], and a 10 cc bolus subcutaneous injection of saline was given to prevent peri-operative dehydration. For longer survival times, rats were
housed two per cage and cages were placed on a 37°C heating pad overnight. Immediately following surgery and for 48 h post-operatively, animals also received twice-daily injections of buprenorphine [Bupronex; 0.075 mg/kg, s.c. (subcutaneously), b.i.d. (twice daily)] and their bladders were manually expressed twice daily.

**Isolation of smvECs**

Spinal microvessels in control and injured rat spinal cords were intravascularly labelled using 200 μg of FITC-conjugated LEA (Lycopersicon esculentum agglutinin) lectin (FL-1171, 2 mg/ml; Vector Laboratories) as described previously (Benton et al., 2008b). Following anaesthesia, 100 μl of FITC–LEA was delivered systemically by intravenous injection into the surgically exposed right external jugular vein at a rate of 1.2 ml/h using a syringe pump (model # 780100, KD Scientific). FITC–LEA was allowed to circulate for 15 min prior to perfusion with saline. Spinal cord tissue containing intravascularly labelled microvessels was then processed for microvascular EC isolation. Briefly, 6 and 24 h post-SCI, approx. 1 cm of spinal cord tissue spanning the injection site was isolated, and the ventral intact/lesioned grey matter was dissected and homogenized in ice-cold HBSS (Hanks balanced salt solution). The crude homogenate was triturated using multiple passes through 26 and 30 gauge needles and filtered through a 70 μm mesh. smvECs were purified by FACS using a MoFlo system (DAKO) using identical parameters with those previously described (Benton et al., 2008b); approx. 90% enrichment of microvascular fragments is achieved using this technique. This microvascular fraction contains mainly small-diameter vessels (i.e. <10 μm in diameter) with little evidence of preserved mural cell ensheathment (Supplementary Figure S1 at http://www.asnneuro.org/an/001/an001e015.add.htm). Following FACS isolation, microvascular fragments were collected by centrifugation (16000 g) at 4°C and stored at −80°C.

**TGFβ/BMP-related gene microarray analysis**

Focused microarray analysis of smvEC gene expression was accomplished as previously described (Benton et al., 2008b). Total RNA was extracted from microvascular ECs using the PicoPure™ RNA Isolation Kit (Arcturus Bioscience). Isolated RNA was reverse-transcribed using the Reaction Ready™ First Strand cDNA synthesis Kit (SuperArray Bioscience). Differential gene expression was conducted using the RT2Profiler™ PCR Array (rat, TGFβ signalling PCR array; catalogue number PARN-035, SuperArray Bioscience). qRT-PCR (quantitative real-time-PCR) was performed using an ABI 7900 real-time PCR instrument. Results were analysed using the Microsoft Excel™ analysis template provided by SuperArray Bioscience using the ΔΔCt method. For each gene, the fold-changes were calculated as the difference in gene expression between smvECs from control spinal tissue and that in smvECs from ischaemic spinal tissue.

**Tissue processing and immunohistochemical analysis**

At multiple experimental timepoints post-ischaemia, animals were perfused using 200 ml of 4% (w/v) PFA (paraformaldehyde). Spinal cords were dissected and transversely sectioned at 20 μm on a cryostat. Sections were thaw-mounted on microscope slides and stored at −80°C. On the day of staining, slides were warmed at 37°C for 20 min and the mounting matrix was removed with forceps. Tissue was blocked in 0.1 M TBS (Tris-buffered saline; 100 mM Tris and 150 mM NaCl, pH 7.4), 0.1% Triton X-100, 0.5% BSA and 10% normal donkey serum for 1 h at room temperature (22°C). Primary antibodies were applied in 0.1 M TBS, 0.1% Triton X-100, 0.5% BSA and 5% normal donkey serum overnight in a humidified chamber at 4°C. Table 1 contains all information regarding the primary antibodies used in the present study, including specificity data. Sections were then incubated with TRITC- (tetramethylrhodamine β-isothiocyanate; 1:200), FITC- (1:100) or AMCA- (1:100) conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Negative controls for each epitope consisted of experimental tissue exposed to species-specific pre-immune IgG and did not result in significant staining. All photomicrographs were captured using a Nikon Ti-E automated inverted microscope equipped with a DS-Ri1 digital camera or an LS-500 laser confocal microscope (Olympus) and Nikon Elements BR analysis software or an Eclipse C1 laser confocal microscope (Nikon Instruments). To eliminate the possibility of ‘cross-over’ signal, sequential excitation and capture was performed with tissue containing multiple fluorophores analysed by laser confocal microscopy.

**uPA [urokinase-type PA (plasminogen activator)] zymography**

uPA enzymatic activity was determined as described previously (Benton et al., 2008b), which has been adapted with slight modification of an established protocol (Heussen and Dowdle, 1980). Briefly, 5 mm of spinal tissue, centered on the ET-1 injection site, was rapidly removed from the spinal column. Tissue was then bisected into ventral and dorsal portions. Protein was extracted from the ventral tissue sample containing ischaemic grey matter. Total protein (10 μg) from spinal cord tissues was resolved under non-reducing conditions on SDS/PAGE (10% gels) containing 0.01 unit/ml plasminogen and 1.5 mg/ml gelatin (Sigma). For standardization, 10 μg of rat uPA (American Diagnostica) was loaded on to each gel. After electrophoresis, the gels were washed three times in 2.5% Triton X-100 solution and then incubated in 100 mmol/l Tris/HCl (pH 8.2) buffer for 18 h at 37°C. The gels were stained with 0.1% Amido Black solution and zymograms were quantified by densitometric analysis using ImageQuant software (Molecular Devices). Specifically, gels were scanned and the images converted into greyscale and inverted so areas of enzymatic activity appeared as dark staining. A rectangular box was then placed over the largest
band and the total area of dark pixels was counted. The same box was used to quantify each band for individual experiments. Background densities for each lane were then subtracted from enzymatic values obtained from density measurements of each band. This method has been used routinely for the detection of uPA activity in diseased brain (Burk et al., 2008) and spinal cord (Glas et al., 2007).

**Statistical analyses**

All quantitative data are expressed as means± S.D. Differences between two groups (SCI microarray results) were compared using a Student’s t test. One-way ANOVA followed by Tukey’s HSD (Honestly Significant Difference) post-hoc analysis was used to compare results for uPA enzymatic activity. Quantitative results from fold-changes in mRNA levels after SCI in smvECs were compared using a two-tailed unpaired Student’s t test, assuming unequal variances. For all analyses, statistical significance was defined at \( P \leq 0.05 \).

**RESULTS**

Neuronal integrity/survival is compromised as early as 3 h following onset of ET-1-mediated focal ischaemia

Although anatomically isolated from the microvasculature, neurons are linked metabolically with ECs and mural cells (i.e.
astrocytes, pericytes and smooth muscle cells) in the CNS. Thus neuronal death/dysfunction may precipitate pathological transformation of the CNS vasculature (Zlokovic, 2008). The results of the present study show that neuronal pathology occurs very early following focal spinal grey matter ischaemia. Significant grey matter pathology is observed by 3 h post-ET-1 injection, as demonstrated by the overt loss of Map2 (microtubule-associated protein 2)- and NeuN-immunoreactivity (Figures 1B, 1E and 1F). Large spinal motor neurons (Figure 1D; arrowheads) were notably absent in the acute phase of insult in affected grey matter by 3 h following onset of ET-1-mediated ischaemia (Figure 1F). At early timepoints following focal ischaemia, only grey matter perfused by target vessels emanating from the anterior spinal artery demonstrate significant pathology (Figure 1B). This decreased Map2- and NeuN-immunoreactivity in affected spinal grey matter is the result of neuronal death and not down-regulation of epitopes as evidenced by loss of Nissl-stained neurons in adjacent sections (Figures 1G–1J).

Temporal course of astrocyte loss from microvessels affected by ischaemic SCI

Following experimental stroke, loss of perivascular astrocytic endfeet has been documented by 6 h post-ischaemia, resulting in substantial permeability and oedema in and around affected microvessels (Polavarapu et al., 2007). In the present study, we examined the temporal loss of functional astrocytic contribution to microvessels following spinal cord ischaemia by Aqp-4 (aquaporin-4) immunostaining (Figures 2A–2D; arrowheads). Aqp-4 is a water channel associated with perivascular astrocytic endfeet (Abbott et al., 2006) and is characteristic of an intact BSCB. At 3 h post-ischaemia, when overt neuronal pathology is initially observed, affected microvessels begin to exhibit partial loss of astroglial investment, with most microvessels devoid of Aqp-4 immunoreactivity (Figures 2E–2H). By 6 h post-ischaemia, no GFAP (glial fibrillary acidic protein)- or Aqp-4-immunoreactive elements are observed in affected grey matter (Figures 2I–2L). This astroglial loss becomes more pronounced by 3 days post-ischaemia, with astrocytes becoming limited to the perimeter of spinal white matter and completely absent from the ischaemic core as determined by GFAP-immunoreactivity (results not shown). Importantly, this temporal course of astrocyte and anatomical/functional decoupling from the EC compartment correlates with the appearance of abnormal EC tight-junctional phenotypes (results not shown), consistent with what is observed after contusive SCI in the adult mouse (Benton et al., 2008a).

Spinal endothelial expression of TGFβ-related/responsive mRNAs is induced concomitantly with microvascular plasticity

Table 2 summarizes the greatest magnitude changes in TGFβ/BMP signalling-related mRNA expression acutely after SCI (see Supplementary Figure S2 at http://www.asnneuro.org/an/001/an001e015.add.htm for the complete mRNA data set). Of the 83 represented genes, approx. 10% were induced at 6 and/or 24 h post-ischaemia. Of these, nearly all were transcripts known to be TGFβ1-responsive. More specifically, several within this class were significantly up-regulated at both 6 and 24 h post-ischaemia including serpine 1/PAI-1 (PA inhibitor-1) (41- and 24-fold), c-fos (34- and 8-fold), plau/uPA (8- and 3-fold) and Tgf (7- and 3-fold). A smaller subset of mRNAs, including IL (interleukin)-6 (27-fold), JunB (5-fold), p27Cip1/Waf1 (4-fold) and Jun (2-fold), had significantly higher expression at 6 h, but not 24 h, post-ischaemia. Interestingly, a number of the genes regulating TGFβ superfamily signalling in the EC compartment responded to ET-1-induced focal ischaemia by significantly down-regulating transcript levels. For example, cystatin C
expression was significantly decreased at both 6 and 24 h after SCI (2- and 5-fold respectively), whereas decreased expression of Fkbp1b and Nbl1 (3-fold) and noggin (4-fold) mRNAs were seen only at 24 h post-ischaemia. A mixed response of TGFβ superfamily receptor genes was observed, with IGFBP-3 [IGF (insulin-like growth factor)-binding protein 3]/TGFβRV mRNA levels exhibiting a 12-fold increase at 24 h post-ischaemia, whereas significant reductions were observed in Itgb5 (6-fold), Bmpr1b (3-fold) and Acvr1 (2-fold) mRNA levels only at 24 h.

**Tissue levels of uPA are increased acutely following ischaemic SCI**

Previous results have shown uPA mRNA expression and bioactivity to be acutely induced in smvECs by traumatic SCI (Benton et al., 2008b). Consistent with these findings, we show that uPA mRNA levels are significantly increased as early as 6 h following ischaemic SCI (Table 2). To determine whether this induction may have biological consequences in the ischaemic spinal cord, we examined uPA enzymatic

---

**Figure 2  Acute astroglial loss from ischaemic microvessels**

In intact spinal grey matter, Aqp-4- and GFAP-immunoreactive astrocytic endfeet are associated with perfused microvessels (A–D; arrowheads). By 3 h post-ischaemia, significant loss of GFAP-immunoreactivity is observed in affected tissue (E), with no detectable Aqp-4 staining associated (F) with microvascular elements (G). By 6 h post-ischaemia, all GFAP- (I) and Aqp-4- (J) immunoreactivity is lost in affected grey matter, despite a preservation of intact microvessels (K and L). Scale bar = 50 μm (C–F).
activity in affected spinal tissue. As early as 3 h post-ET-1 microinjection, uPA proteolytic activity was significantly increased, with levels further increasing by 6 h post-ischaemia (Figures 3A and 3B). The temporal course of this increased enzymatic activity parallels both the loss of astrocytic endfeet in affected microvessels (Figure 2), as well the induction of TGFβ activation of smvECs as demonstrated by microarray results. This result is important in that it implicates uPA in the activation of TGFβ within the ischaemic grey matter and in ECs within affected tissue soon after the onset of ischaemic/reperfusion insult.

**MMP-9, a potent modulator of TGFβ bioactivity, is expressed in affected microvessels**

In most tissues, including the CNS, pools of TGFβ exist in inactive forms within the pericellular space and may be rendered bioactive by one or more activators, which include proteases, integrins and thrombospondins (ten Dijke and Arthur, 2007). Of these potential activators, expression and/or activity of MMP-9 is induced in traumatic SCI (Noble et al., 2002). In control ventral grey matter, no MMP-9 expression was observed in smvECs (Figures 4A and 4B). The earliest timepoint post-ischaemia where MMP-9 immunoreactivity was observed was 12 h, where a marked increase was seen in affected microvessels (Figures 4C and 4D), although some affected microvascular elements did not exhibit detectable immunoreactivity (Figure 4D; arrowheads). By 24 h post-ischaemia, levels of MMP-9 expression remained elevated in all affected microvessels (Figures 4E and 4F). This result suggests a role for MMP-9 in regulation of TGFβ activity in/at affected microvessels in the subacute phases, but not in the immediate phase, of smvECs activation following ischaemic SCI.

**Detection of multiple TGFβ-responsive proteins in spinal microvessels in ischaemic SCI**

The rationale for the selection of mRNAs to validate at the protein level was based upon (i) the magnitude of the transcriptional changes revealed by microarray analysis and (ii) the novelty of their role in acute microvascular dysfunction in the context of SCI. Based on these criteria, we chose to define the temporal dynamic of protein expression levels of PAI-1, IL-6 and IGFBP-3, which represent the three most highly up-regulated mRNAs examined in smvECs acutely following ischaemic SCI. Surprisingly for IL-6, we observed a low level of immunoreactivity in grey matter microvessels in control tissue (Figures 5A and 5B; arrowheads). By 6 h post-ischaemia, levels of IL-6 were qualitatively increased in activated microvessels (Figures 5E and 5F; arrowheads). Unexpectedly, little IL-6 expression was detected in affected microvessels in the subacute phases, but not in the immediate phase, of smvECs activation following ischaemic SCI.

### Table 2 Selected microarray results

| GenBank® accession number | Symbol and gene name                                                                 | 6 h post-ET-1 fold-change (P value) | 24 h post-ET-1 fold-change (P value) |
|--------------------------|--------------------------------------------------------------------------------------|------------------------------------|------------------------------------|
| NM_012620                | Serpine 1, serine peptidase inhibitor, clade E, member 1 (PAI-1A/Pai1)               | +40.67 (0.001)                     | +24.28 (0.001)                     |
| NM_022197                | c-fos, FBJ murine osteosarcoma viral oncogene homologue                              | +34.46 (<0.001)                   | +7.66 (<0.001)                    |
| NM_012589                | Il6, IL-6 (IL-6/Ifnb2)                                                              | +27.07 (<0.001)                   | +2.27 (0.085)                     |
| NM_013085                | Plau, plasminogen activator, urokinase (UPAM)                                       | +7.93 (0.042)                     | +3.05 (0.032)                     |
| NM_001015020             | Tgf, transforming growth factor interacting factor                                   | +6.71 (0.01)                      | +3.30 (0.004)                     |
| NM_021836                | Junb, jun-B oncogene                                                                | +5.49 (0.008)                     | +2.04 (0.142)                     |
| NM_012603                | Myc, myelocytomatosis viral oncogene homologue (RNCMYC/c-myc)                       | +3.93 (0.85)                      | +3.52 (0.018)                     |
| NM_080782                | Cdkn, cyclin-dependent kinase inhibitor 1A (p27Cip1/Waf1)                           | +3.92 (0.014)                     | +1.19 (0.520)                     |
| NM_021835                | Jun, Jun oncogene                                                                  | +2.07 (0.029)                     | +1.86 (0.072)                     |

| Genes regulating TGFβ superfamily signalling                            |                                                                 |                                                                 |                                                                 |
|--------------------------|-------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|
| NM_012837                | Cyt3, cystatin C (CYS3)                                            | −2.10 (0.017)                                                   | −5.19 (0.001)                                                   |
| NM_022675                | Fkbp1b, FK506 binding protein 1b                                   | +1.12 (0.84)                                                    | −2.69 (0.02)                                                   |
| NM_031609                | Nbl1, neuroblastoma, suppression of tumorigenicity 1               | −1.64 (0.38)                                                    | −3.10 (0.005)                                                   |
| XM_349354                | Nog, noggin                                                       | +1.70 (0.56)                                                    | −3.98 (<0.001)                                                  |

| TGFβ superfamily receptor genes                                         |                                                                 |                                                                 |                                                                 |
|--------------------------|-------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|
| NM_012588                | Igfbp3, IGFBP-3 (IGF-R-V)                                         | +1.32 (0.76)                                                    | +11.72 (0.003)                                                  |
| NM_147139                | Itgb5, integrin, Jβ5 (RGD1563276)                                 | +1.22 (0.83)                                                    | −5.82 (0.003)                                                   |
| NM_001024259             | Bmpr1b, BMP receptor, type 1 (CFK-43A)                            | +2.11 (0.43)                                                    | −2.89 (<0.001)                                                  |
| NM_024486                | Acvr1, activin A receptor, type 1                                 | +1.17 (0.83)                                                    | −2.48 (0.004)                                                   |

© 2009 The Author(s) This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited. 187
detectable in spinal microvessels 24 h post-ischaemia (Figures 5I and 5J), but was again observed in vessels 3 days following ischaemia (Figures 5M and 5N; arrowheads). This phasic response is consistent with that quantitatively determined for mRNA levels (Table 2). Microvascular expression of IGFBP-3, another TGFβ-responsive molecule involved in cytokine function, was also complex. Low levels of immunoreactivity were observed in control ventral grey matter (Figures 5C and 5D; arrows), with an apparent increase of smvEC expression by 6 h post-ischaemia (Figures 5G and 5H; arrows). Expression levels appear to decrease in affected microvessels by 2 days post-ischaemia (Figures 5K and 5L), with little detectable IGFBP-3 immunoreactivity apparent in perfused microvessels 5 days post-ischaemia (Figures 5O and 5P). This result suggests a transient role for IGFBP-3 in the activation of the smvECs following ischaemic SCI.

The most highly up-regulated mRNA observed in the present study (40- and 24-fold at 6 and 24 h post-ischaemia; Table 1) was that encoding the endogenous uPA inhibitor PAI-1. In sham-control spinal grey matter, no detectable PAI-1 expression was observed in spinal microvessels (Figure 6B). As early as 3 h post-ET-1 microinjection, PAI-1 immunoreactivity was associated with perfused microvessels in affected tissue (Figures 6C and 6D; arrowheads). This increase in PAI-1 expression was maintained throughout the subacute period post-ischaemia, with detectable levels of PAI-1 expression associated with activated smvECs at 2 and 3 days (Figures 6E–6H; arrowheads). Interestingly, 5 days post-ischaemia, little PAI-1 immunoreactivity was observed in perfused microvessels in affected ventral grey matter (Figures 6I and 6J). These results implicate PAI-1 in contributing to the early pathological activation of the microvasculature following focal spinal ischaemia. Moreover, these immunohistochemical data showing correlative protein expression and mRNA levels in ECs support the conclusion that results obtained from FACS-isolated ECs reflect their in situ transcriptional state. This is an important issue considering the stress induced by the isolation protocol.
DISCUSSION

In the present study, we show that induction of focal spinal cord ischaemia results in rapid dissolution of NVU (neurovascular unit) integrity. The use of ET-1 to induce vasospastic spinal cord ischaemia is based upon previous experimental models of ET-1-mediated stroke (O’Neill and Clemens, 2001). In mammals, ET exerts its bioactivity via three structurally related peptides (ET-1, ET-2 and ET-3), which bind two subtypes of G-protein-coupled receptors (ETA and ETB), which are highly conserved across multiple species (Schinelli, 2006).

Figure 5

**smvEC expression of IL-6 and IGFBP-3 after SCI**

Basal levels of expression for both IL-6 (A and B; arrowheads) and IGFBP-3 (C and D; arrows) were detected in the vasculature in control spinal grey matter. Increased IL-6 immunoreactivity was observed in smvECs at 6 h post-ischaemia (E and F; arrowheads). Interestingly, this expression appeared to exhibit a biphasic pattern, with qualitative levels diminished at 1 day (I and J), but reappearing in activated microvascular profiles by 3 days post-ischaemia (M and N; arrowheads). Similar to IL-6 staining, levels of IGFBP-3 immunoreactivity were intensified by 6 h post-ET-1 microinjection (G and H; arrows). However, levels were then found to diminish progressively beginning at 2 days post-ischaemia (K and L; arrows), with little IGFBP-3 immunoreactivity associated with perfused microvessels 5 days post-ischaemia (O and P; arrow). Scale bar=50 μm (A–P).
In the healthy CNS, receptor expression is observed in ependymal/subependymal cells (Tuschick et al., 1997), Bergmann glia of the cerebellum (Tuschick et al., 1997), epithelial cells of the choroid plexus (Tsaur et al., 1997), neurons (Yamada and Kurokawa, 1998) and astrocytes (Schinelli et al., 2001), with the highest levels of expression observed in the vasculature (Yu et al., 1995; Chen et al., 2000; Hansen-Schwartz et al., 2002). The ET system is up-regulated in response to brain ischaemia (Edvinsson, 2009), suggesting that it may play a role in the aetiology of clinical stroke. While less is known about the precise role of ET-1 in CNS trauma, blockade of ET-1 receptors has been shown to ameliorate tissue loss and facilitate functional sparing following experimental SCI in rats (Uesugi et al., 1998; Peters et al., 2003; Ogawa et al., 2008). Furthermore, blood levels of ET-1 are elevated in patients suffering from traumatic SCI (Wang et al., 2007). These data, combined with the results of the present study, support the idea that ET-1 does play a role in secondary injury cascades following SCI.

Although the primary action in nervous tissue is in regulation of intrinsic vascular tone (Schinelli, 2006), it has more recently been implicated in the modulation of nociception and sensory function (Khodorova et al., 2009). Although this implicates direct activation of neurons, little data exist demonstrating receptor-mediated neurotoxic actions of ET-1. Indeed, previous results show that direct application of 1 μM ET-1 to mixed cultures of neonatal neurons and astrocytes results in neither cell death nor potentiation of excitotoxicity, whereas as little as 50 nM is sufficient for cellular activation, primarily via ETB (Benton et al., 2005). In the present study, acute grey matter pathology following ET-1 microinjection is limited to areas corresponding to patterns of intrinsic blood supply of targeted penetrating microvasculature (Mautes et al., 2000). This supports the idea that the mechanism(s) of insult observed is likely to be due to interruption of blood flow to affected grey matter and not a direct cytotoxic action.

Results from the present study demonstrate early and robust transcriptional activation of TGFβ-responsive genes in the EC compartment in response to spinal ischaemia/reperfusion injury. Previous studies have determined that it is primarily the TGFβ1 isoform that is acutely increased following traumatic SCI (Semple-Rowland et al., 1995; Streit et al., 1998; Nakamura et al., 2003; Wang et al., 2009). In clinical cases of SCI, TGFβ1 appears to be primarily associated with acute injury responses, with other isoforms being expressed at more chronic phases of injury (Buss et al., 2008). Among the postulated effects of this early TGFβ1 response in traumatic SCI are included altered microglial chemotaxis (Yao et al., 1990) and astrocyte proliferation (Lindholm et al., 1992). Present results suggest that TGFβ1 activation of the EC compartment in the early phase of spinal insult may play a role in acute microvascular dysfunction/plasticity following SCI. Also, these results suggest conservation in the TGFβ1 response in traumatic and atraumatic SCI.

Figure 6 smveC expression of PAI-1 is rapidly induced by ischaemic SCI
No detectable PAI-1 immunoreactivity was observed in control spinal grey matter (B). As early as 3 h post-ischaemia, robust PAI-1 immunoreactivity is observed in affected smveCs (C and D; arrowheads). This expression level is maintained in smveCs for up to 3 days post-ischaemia (E–H; arrowheads). By 5 days post-ischaemia, PAI-1 immunoreactivity returned to basal levels in perfused spinal microvessels (I and J; arrowheads). Confocal imaging definitively co-localizes PAI-1 to FITC–LEA activated/perfused spinal microvessels in the xz and yz planes (F and H; arrows). Scale bar = 50 μm (A–J).
The results of the present study suggest de novo synthesis of TGFβ isoforms is unchanged in ECs following ischemic SCI (see Supplementary Figure S2), a result confirmed by ELISA analyses (results not shown). These observations support the conclusion that EC stimulation by TGFβ results from EC exposure to extracellular sources of bioactive cytokine. Platelets are a rich source of TGFβ1, containing 40-100 times as much as other cells (Assoian et al., 1983). Early studies of traumatic SCI established platelet aggregation as a precipitating event in secondary vascular responses to injury (Griffiths et al., 1978; Goodman et al., 1979). However, observations of acute platelet aggregation in acute ET-1 (Griffiths et al., 1978; Goodman et al., 1979). However, precipitating event in secondary vascular responses to injury studies of traumatic SCI established platelet aggregation as a times as much as other cells (Assoian et al., 1983). Early exposure to extracellular sources of bioactive cytokine. conclusion that EC stimulation by TGF

Another possibility supported by the present results, is that local inactive pools of TGFβ are rendered bioactive by one of several potential pathways induced by focal spinal ischemia. TGFβ1 exists in a biologically inactive form in complex with the remaining portion of its precursor molecule, LAP (latency-associated peptide), which is bound to a LTBP (latent TGFβ binding protein) (LTBP-1, 3 or 4) forming the LLC (large latent complex). Virtually all of the TGFβ1 released from platelets and other cells is in the LLC (Annes et al., 2003). In the CNS, TSP-1 (thrombospondin-1) is a very potent activator of TGFβ1, as are the proteases plasmin, mast cell chymase, metalloproteases [including MMP-9 and MT1-MMP (membrane-type 1-MMP)] and thrombin (ten Dijke and Arthur, 2007). Interestingly, 24 h after contusive SCI in the mouse, up-regulated expression of both TSP-1 (58-fold) and uPA (21-fold) was observed in purified microvascular ECs (Benton et al., 2008b). uPA cleaves plasminogen to plasmin which directly activates TGFβ (Tkachuk et al., 1996) as well as indirectly by cleaving pro-MMP-9 to active MMP-9 enhancing bioactivation of TGFβ from extracellular stores (Zhang et al., 2005). Thus not only is TGFβ activated acutely after SCI, it is also done so in a positive-feedback loop further enhancing its actions. The results of the present study support the local activation of TGFβ by uPA in the immediate phase of ischemia/reperfusion, with other possible effectors including, but not limited to, MMP-9, resulting in TGFβ bioactivity/ bioavailability in the subacute phase of microvascular plasticity in response to ischemic SCI. Furthermore, these results suggest that the molecular mechanism of NVU dysfunction (i.e. increased expression of uPA, MMP-9 and TSP-1) may be similar in both traumatic and atraumatic (i.e. ischemic and excitotoxic) forms of SCI.

Of the TGFβ-responsive genes examined, the largest change was the 11-fold increase in IGFBP-3 24 h post-ischemia, a result also demonstrated at the protein level by immunohistochemistry. The role of IGFBP-3 on EC function in the injured spinal cord is unknown, but its overexpression may have implications for smvEC plasticity following SCI. IGFBP-3 is normally expressed at high levels by hepatic ECs (Zimmermann et al., 2000) and is the most abundant circulating binding protein (Guler et al., 1989). The main function of IGFBP-3 is to bind IGF, which results in enhanced bioavailability and activity (Guler et al., 1989). In addition, there are a number of critical IGF-independent actions of IGFBP-3, including wound healing, cell adhesion/migration, gene transcription, cytosolic trafficking and neuronal protection (for a review see Yamada and Lee, 2009). Data on EC-specific responses suggest a controversial and context-dependent role in vascular plasticity. IGFBP-3 accelerates EC senescence in vitro (Kim et al., 2007) and inhibits angiogenesis in HUVECs (human umbilical vein endothelial cells) and chick chorioallantoic in vitro models (Oh et al., 2006). By contrast, IGFBP-3 enhances differentiation of EC precursors in vitro and enhances angiogenesis in an oxygen-induced retinopathy mouse model (Chang et al., 2007). IGFBP-3 remains a poorly understood biomarker of CNS pathology. Increased IGFBP-3 expression is associated with amyloid-β plaque formation in affected microvasculature in brains of Alzheimer’s disease patients (Rensink et al., 2002). Furthermore, the IGF system has been implicated in both experimental stroke recovery/susceptibility in patients. Specifically, IGFBP-3 mRNA is acutely up-regulated in the mouse brain in response to transient ischemia (O’Donnell et al., 2002), a response that was associated with the post-ischemic inflammatory response. Interestingly, plasma levels of IGFBP-3 are inversely correlated with both increased risk of (Johnsen et al., 2005) and recovery from (Schwab et al., 1997) clinical stroke. Taken together, the latter findings suggest a protective role for IGFBP-3 in CNS pathology. To date, no results exist regarding the putative role of the IGF/IGFBP-3 system in microvascular function and/or neuroprotection following SCI.

Also of note is the present result demonstrating mRNA levels of IL-6 to be significantly up-regulated in activated ECs following SCI, with protein levels increased acutely, but not sustained beyond, 24 h post-SCI. With respect to IL-6, in addition to its role as a multifunctional pro-inflammatory cytokine involved in the regulation of the acute inflammatory response (Papassotriopoulos et al., 2001; Rose-John et al., 2007), IL-6 protects HUVECs from H2O2-induced cell death (Waxman et al., 2003). On the other hand, IL-6 induces tight junction dysfunction and hyperpermeability in rat heart microvascular ECs in vitro (Tinsley et al., 2008). In the context of traumatic SCI, IL-6 mRNA levels are increased 3–24 h post-injury in astrocytes, neurons and resident microglia (Pineau and LaCroix, 2007). Similarly, IL-6 mRNA is elevated 3–24 h in response to permanent MCAO (middle cerebral artery occlusion) in rats, with expression peaking at 12 h post-ischemia (Wang et al., 1996). More recently, serum levels of IL-6 were shown to significantly increase 24 h following transient MCAO (Martínez-Revelles et al., 2008). Similar results have been observed in patients within hours following stroke onset, with a positive correlation seen between plasma
levels of IL-6 and both infarct size and clinical outcome (Tarkowski et al., 1995; Huang et al., 2006). While no data currently exist regarding whether the action of IL-6 is deleterious in SCI, recent findings have linked IL-6 signalling in the EC compartment with blood–brain barrier failure in EAE (experimental autoimmune encephalomyelitis) (Linker et al., 2008). Additional investigations will be needed to determine the contribution of increased EC expression of IL-6 on inflammation and/or angiogenesis following SCI.

The temporal response of uPA mRNA and activity observed in the present study is consistent with observations made in experimental stroke (Hosomi et al., 2001). Specific inhibition of uPA in experimental stroke decreases infarct size by protecting blood–CNS barrier integrity (Hamann et al., 2004). The acute increase in uPA mRNA in the EC compartment observed in the present study is likely to be of functional consequence as uPA activity is increased in smvECs isolated 72 h post-ischaemia (R. L. Benton, unpublished data). Furthermore, significant inductions of PAI-1 (mRNA and protein) and uPAR (uPA receptor) (R. L. Benton, unpublished data) in ECs after ischaemic SCI suggest a role for the PAS (PA system) in vascular dysfunction and secondary injury progression following SCI, as has been established in stroke. The implications of the response observed here, as well as in other types of neuropathology, are likely to be a fundamental event underlying secondary degeneration. The PAS is best known for its function in thrombolysis, but it also plays substantial roles in many cellular processes outside of the bloodstream, especially in the CNS (Zhang et al., 2005). The central active protease of the PAS is plasmin, which is generated by cleavage of plasminogen by the PAs, tPA (tissue-type PA) and uPA. While both PAs are secreted proteases, uPA is unique in that following secretion it binds its cell-surface receptor (uPAR) where it is then activated, directing proteolysis to the pericellular space (Cubellis et al., 1986). tPA appears to be the primary PA expressed in the intact CNS and is involved in neuronal migration and synaptic plasticity (Seeds et al., 1997, 1999, 2003). By contrast, little uPA expression is observed in the adult CNS in the absence of pathobiology (Tsirka et al., 1997), with a single report demonstrating uPA up-regulation in the injured spinal motor neurons (Minor and Seeds, 2008). The clinical relevance of this observation is substantiated by the observation of increased uPAR expression in ECs in human cases of both TBI (traumatic brain injury) and stroke (Beschorner et al., 2000). Furthermore, several members of the PAS (uPA, uPAR and PAI-1) are expressed in ECs and astrocytes within human stroke foci (Dietzmann et al., 2000).

In conclusion, the increased TGFβ1 signalling demonstrated in the present study might be a double-edged sword. On one hand, TGFβ1 is known to confer neuroprotection against various insults, including stroke (Dhandapani and Brann, 2003). However, increased TGFβ signalling probably has destabilizing actions in pathologically activated vessels, in part via induction of both VEGF and MMP expression (ten Dijke and Arthur, 2007). While uPA and plasmin are likely regulators of the action of TGFβ1 on spinal microvasculature (Sato and Rifkin, 1980; Sato et al., 1990; Rainger and Nash, 2001), the present study suggests a comparable role for regulating spinal microvascular plasticity and/or dysfunction following SCI. Furthermore, it may be possible that the activation of the EC compartment by TGFβ may be downstream of a yet-to-be identified cytokine cascade, involving IL-6 and/or other growth factors. Acutely attenuating the action of TGFβ1 in microvessels following SCI may have therapeutic potential.

ACKNOWLEDGEMENTS

We thank Christine Nunn for surgical support, Kim Fentress for post-operative animal care and Christopher Worth for his technical expertise with FACS.

FUNDING

This work was supported by the University of Louisville School of Medicine Foundation [grant numbers RR15576, NS045734 (to R.L.B.); Norton Healthcare; and the Commonwealth of Kentucky Challenge for Excellence (to S.R.W. and T.H.).

REFERENCES

Abbott NJ, Ronnback L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7:41–53.
Allaire E, Hasenstab D, Kenagy RD, Starcher B, Clowes MM, Clowes AW (1998) Prevention of aneurysm development and rupture by local overexpression of plasminogen activator inhibitor-1. Circulation 98:249–255.
Amar AP, Levy ML (1999) Pathogenesis and pharmacological strategies for mitigating secondary damage in acute spinal cord injury. Neurosurgery 44:1027–1039.
Annes JP, Munger JS, Rifkin DB (2003) Making sense of latent TGFβ activation. J Cell Sci 116:217–224.
Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB (1983) Pathogenesis and pharmacological strategies for regulating spinal microvasculature following SCI may be
Casella GT, Marcillo A, Bunge MB, Wood PM (2002) New vascular tissue rapidly replaces neural parenchyma and vessels destroyed by a contusion injury to the rat spinal cord. Exp Neurol 173:63–76.

Casella GT, Bunge MB, Wood PM (2006) Endothelial cell loss is not a major cause of neuronal and glial cell death following contusion injury of the spinal cord. Exp Neurol 202:6–20.

Chang KH, Chang-Ling T, McFarland EL, Afzal A, Pan H, Baxter LC, Shaw LC, Caballero S, Sengupta N, Li CS, Sullivan SM, Grant MB (2007) IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. Proc Natl Acad Sci USA 104:10595–10600.

Chang Y, McCarron RM, Ohara Y, Bembry J, Azzam N, Lenz FA, Shohami E, Mechemoul R, Spatz M (2000) Human brain capillary endothelium: 2-arachidonoylglycerol (endocannabinoid) interacts with endothelin-1. Circ Res 87:323–327.

Corti S, Locatelli F, Papadimitriou D, Donadoni C, Del Bo R, Fortunato F, Strazzer S, Salani S, Bresolin N, Comi GP (2000) Multipotentiality, homo properties, and pyramidal neurogenesis of CNS-derived LeX(ssea-1) prourokinase to the urokinase receptor of human U937 cells. J Biol Chem 276:15819–15822.

Cunningham LA, Wetzel M, Rosenbaum GA (2005) Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia 50:329–339.

Edvinsson L (2009) Cerebrovascular endothelin receptor upregulation in the rat brain: increase after injury and inhibition of astrocyte proliferation. J Cell Biol 117:395–400.

Linker RA, Lüderer F, Kallen K, Lee D, Engelhardt B, Rose-John S, Gold R (2008) IL-6 transsignaling modulates the early effector phase of EAE and targets the blood-brain barrier. J Neuroimmunol 205:64–72.

Linkert S, Cheer DA, Davenport JB, Burke DA, Onifer SM, Whittemore SR (2002) Temporal progression of angiogenesis and basal lamina deposition after contusion spinal cord injury in the adult rat. J Comp Neurol 445:308–324.

Martínez-Revellés S, Jiménez-Altava L, Caracuel L, Pérez-ASENSIO FJ, Planas AM, Vila E (2008) Endothelial dysfunction at rat mesenteric resistance artery after transient middle cerebral artery occlusion. J Pharmacol Exp Therap 325:363–369.

Mautes AE, Weinzierl MR, Donovan F, Noble LJ (2000) Vascular events after spinal cord injury: contribution to secondary pathogenesis. Phys Ther 80:673–687.

McTigue DM, Popovich PG, Morgan TE, Stokes BT (2000) Localization of transforming growth factor-β1 and receptor mRNA after experimental spinal cord injury. Exp Neurol 163:220–230.

Minor KH, Seeds NW (2008) Plasminogen activator induction facilitates recovery of respiratory function following spinal cord injury. Mol Cell Neurosci 37:143–152.

Nakamura M, Houghting RA, MacArthur L, Bayer BM, Bregman BS (2003) Differences in cytokine gene expression profile between acute and secondary injury in adult rat spinal cord. Exp Neurol 184:313–325.

Nelson E, Gertz SD, Rennels ML, Ducker TB, Blaumanis OR (1977) Spinal cord injury. The role of vascular damage in the pathogenesis of central hemorrhagic necrosis. Arch Neurol 34:332–333.

Noble LJ, Donovan F, Igarashi T, Goussév S, Webb Z (2002) Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early events. J Neurosci 22:5762–5765.

O’Brien MF, Lenke LG, Lou J, Bridwell KH, Joyce ME (1994) Astrocyte response and transforming growth factor-β1 localization in acute spinal cord injury. Spine 19:2321–2329.

O'Donnell SL, Frederick TJ, Krady JK, Vannucci SJ, Wood TL (2002) IGF-1 and microglia/macrophage proliferation in the ischemic mouse brain. Glia 38:85–97.

O'Neill MJ, Clemens JA (2001) Rodent models of focal cerebral ischemia. Curr Protoc Neurosci 9.11.

Ogawa T, Sasatomi K, Hiragata S, Seki S, Nishizawa O, Chermansky CJ, Pflug V (1987) Expression of the plasminogen activator system and the inhibitors PAI-1 and PAI-2 in postischemic lesions of the CNS and brain injuries following dramatic circulatory arrests: an immunohistochemical study. Pathol Res Pract 196:15–21.

Protoc Neurosci 9:9.

Ogawa T, Sasatomi K, Hiragata S, Seki S, Nishizawa O, Chermansky CJ, Pflug V (1987) Expression of the plasminogen activator system and the inhibitors PAI-1 and PAI-2 in postischemic lesions of the CNS and brain injuries following dramatic circulatory arrests: an immunohistochemical study. Pathol Res Pract 196:15–21.

Protoc Neurosci 9:9.

Ogawa T, Sasatomi K, Hiragata S, Seki S, Nishizawa O, Chermansky CJ, Pflug V (1987) Expression of the plasminogen activator system and the inhibitors PAI-1 and PAI-2 in postischemic lesions of the CNS and brain injuries following dramatic circulatory arrests: an immunohistochemical study. Pathol Res Pract 196:15–21.

Protoc Neurosci 9:9.
Polavarapu R, Gongora MC, Yi H, Ranganathan S, Lawrence DA, Strickland D, Yerkes M (2007) Tissue-type plasminogen activator-mediated shedding of astrocytic low-density lipoprotein receptor-related protein increases the permeability of the neurovascular unit. Blood 109:3270–3278.

Popovich PG, Jones TB (2003) Manipulating neuroinflammatory reactions in the injured spinal cord: back to basics. Trends Pharmacol Sci 24:13–17.

Quick AM, Czopp MA (2005) Pregnancy-induced up-regulation of aquaporin-4 protein in brain and its role in eclampsia. FASEB J 19:170–175.

Rainger GE, Nash GB (2001) Cellular pathology of atherosclerosis: smooth muscle cells prime cocultured endothelial cells for enhanced leukocyte adhesion. Circ Res 88:615–622.

Rensink AM, Gellekink H, Otte-Hüller I, ten Konkelaar HJ, de Waal RM, Verbeek MM, Kremers B (2002) Expression of the cytokine leukemia inhibitory factor and pro-apoptotic insulin-like growth factor binding protein-3 in Alzheimer's disease. Acta Neuropathol 104:525–533.

Rose-John S, Waetzig GH, Scheller J, Grotzinger J, Seegert D (2007) The IL-6/sIL-6R complex as a novel target for therapeutic approaches. Expert Opin Ther Targets 11:613–624.

Sato Y, Rifkin DB (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β-like molecule by plasmin during co-culture. J Cell Biol 109:319–329.

Sato Y, Tsuiboi R, Lyons R, Moses H, Rifkin DB (1990) Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J Cell Biol 111:767–763.

Schellini S, Zanassi P, Paolillo M, Wang H, Feliciello A, Gallo V (2001) Stimulation of endothelin B receptors in astrocytes induces CAMP response element-binding protein phosphorylation and c-fos expression via multiple mitogen-activated protein kinase signaling pathways. J Neurosci 21:8842–8853.

Schellini S (2006) Pharmacology and physiopathology of the brain endothelelial system: an overview. Curr Med Chem 13:627–638.

Schwab S, Spranger M, Krempien S, Hacke W, Bettendorf M (1997) Plasma permeability of the neurovascular unit. Blood 109:3270–3278.

Schinelli S, Zanassi P, Paolillo M, Wang H, Feliciello A, Gallo V (2001) Stimulation of endothelin B receptors in astrocytes induces CAMP response element-binding protein phosphorylation and c-fos expression via multiple mitogen-activated protein kinase signaling pathways. J Neurosci 21:8842–8853.

Seeds NW, Siconolfi LB, Haffke SP (1997) Neuronal extracellular proteases facilitate cell migration, axonal growth, and pathfinding. Cell Tissue Res 290:367–370.

Seeds NW, Basham ME, Haffke SP (1999) Neuronal migration is retarded in mice lacking the tissue plasminogen activator gene. Proc Natl Acad Sci USA 96:14118–14123.

Seeds NW, Basham ME, Ferguson JE (2003) Absence of tissue plasminogen activator gene or activity impairs mouse cerebellar motor learning. J Neurosci 23:7366–7375.

Semple-Rowland SL, Mahant K, Popovich PG, Green DA, Hassler Jr G, Stokes BT, Streit WJ, Semple-Rowland SL, Mahant K, Popovich PG, Green DA, Hassler Jr G, Stokes BT (1998) Endothelin ETA receptor expression in the mouse hippocampus. J Neurosci 18:615–622.

Sharma HS (2003) Pathophysiology of blood-spinal cord barrier in traumatic injury and repair. Curr Pharm Des 11:1533–1539.

Stemberger NH, Stemberger LA (1987) Blood-brain barrier protein recognized by monoclonal antibody. Proc Natl Acad Sci USA 84:8169–8173.

Streit WJ, Semple-Rowland SL, Hurley SD, Miller RC, Popovich PG, Stokes BT (1998) Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. J Neurosci Res 59:227–239.

Tuschick S, Kirischuk S, Kirchhoff F, Liefeldt L, Paul M, Verherrings A, Kettenmann H (1997) Bergmann glial cells in situ express endothelinB receptors linked to cytoplasmic calcium signals. Cell Calcium 21:409–419.

Uesugi M, Kasuya Y, Hayashi K, Goto K (1998) SB209670, a potent endothelin receptor antagonist, prevents or delays axonal degeneration after spinal cord injury. Brain Res 786:235–239.

Watson L, Lim G, Zeng Q, Sung B, Ju Y, Goss C, Yang L, Mao J (2004) Expression of central glucocorticoid receptors after peripheral nerve injury contributes to neuropathic pain behaviors in rats. J Neurosci 24:8595–8605.

Wang TD, Wang YH, Huang TS, Su TC, Pan SL, Chen SY (2007) Circulating levels of markers of inflammation and endothelial activation are increased in men with chronic spinal cord injury. J Formos Med Assoc 106:919–928.

Wang X, Yue TL, Young PR, Barone FC, Feuerstein GZ (1995) Expression of interleukin-6, c-fos, and zf268 mRNA in rats ischemic cortex. J Cereb Blood Flow Metab 15:166–171.

Wang J, Chen W, Liu W, Wu J, Shao Y, Zhang X (2009) The role of thrombospondin-1 and transforming growth factor-β after spinal cord injury in the rat. J Clin Neurosci 16:818–821.

Ward NL, LaManna JC (2004) The neurovascular unit and its growth factors: coordinated response in the vascular and nervous systems. Neurol Res 26:870–883.

Waxman AB, Mahboubi K, Knickelbein RG, Mantell LL, Monzo N, Pober JS, Elias JA (2003) Interleukin-11 and interleukin-6 protect cultured human endothelial cells from H2O2-induced cell death. Am J Respir Cell Mol Biol 29:513–522.

Whetstone WD, Hsu JY, Eisenberg M, Werb Z, Noble-Haeusslein LJ (2003) Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. J Neurosci Res 74:227–239.

Watanabe M, Kurokawa K (1998) Histochemical studies on endothelin and the endothelin-A receptor in the hypothalamus. J Cardiovasc Pharmacol 31(Suppl 1):S215–S218.

Watanabe M, Lee KW (2009) Perspectives in mammalian IGF-BP-3 biology: local vs. systemic action. Am J Phys Cell Physiol 296:C954–C976.

Yao J, Harvath L, Gilbert DL, Colton CA (1990) Chemotaxis by a CNS macrophage, the microglia. J Neurosci Res 27:36–42.

Ye P, Price W, Kassiotis G, Kollai G, D’Ercole AJ (2003) Tumour necrosis factor-ca regulation of insulin-like growth factor-I, type 1 IGF receptor, and IGF binding protein expression in cerebellum of transgenic mice. J Neurosci Res 71:721–731.

Yin KJ, Cirrito JR, Yan P, Hu X, Xiao Q, Pan X, Bateman R, Song H, Hsu FF, Turk J, Xu J, Hsu CY, Mills JC, Holtzman DM, Lee JM (2006) Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism. J Neurosci 26:10939–10948.

Yu JC, Pickard JD, Davenport AP (1995) Endothelin ETA receptor expression in human cerebrovascular smooth muscle cells. Br J Pharmacol 116:2441–2446.

Zhang Y, Pothakos K, Tsirka SA (2005) Extracellular proteases: biological and behavioral roles in the mammalian central nervous system. Curr Top Dev Biol 66:161–188.

Zimmermann EM, Lem L, Hoyt EC, Pucilowska JB, Lichtman S, Lund PK (2000) Cell-specific localization of insulin-like growth factor binding protein mRNAs in rat liver. Am J Physiol Gastrointest Liver Physiol 278:G447–G457.

Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 57:178–201.