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Developmental and injury-induced expression of $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins in the rat spinal cord

K. Adam Baker^{a,b,#} and Theo Hagg^{a,*}

a Kentucky Spinal Cord Injury Research Center, Departments of Neurological Surgery, MDR Building, Room 609, University of Louisville, Louisville, Kentucky, USA.

b Anatomical Sciences and Neurobiology, MDR Building, Room 609, University of Louisville, Louisville, Kentucky, USA.

Abstract

Loss and damage to blood vessels are thought to contribute to secondary tissue loss after spinal cord injury. Integrins might be therapeutic targets to protect the vasculature and/or promote angiogenesis, as their activation can promote tubule formation and survival of endothelial cells in vitro. Here, we show that immunostaining with an antibody against the $\alpha 1\beta 1$ integrin heterodimer is present only in blood vessels from postnatal day 1 (P1) through adulthood in Sprague-Dawley rats. After a spinal cord contusion at T9 in adults, the area of $\alpha 1\beta 1$ integrin positive blood vessels increases within 11 mm from the injury site at 3 days post-injury and remains prominent within the injured core only at 7 days. Staining for the $\alpha 6\beta 1$ integrin heterodimer increases in blood vessels between P10 and adulthood and is present in preganglionic neurons of the intermediolateral cell column (IML) at all ages. The $\alpha 6\beta 1$ integrin is also expressed by motor neurons postnatally, and oligodendrocyte precursors (OPCs), as previously reported. After the contusion, the area of $\alpha 6\beta 1$ stained blood vessels was increased at 3 days and most prominently, 1 mm from the injury site, followed by a significant reduction at 7 days, when $\alpha 6\beta 1$ integrin staining is most prominent around the injured core. Staining is also present in a subset of microglia and/or macrophages. These results raise the possibility that $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins in blood vessels might be targeted to reduce blood vessel loss and promote angiogenesis, which may promote tissue sparing after spinal cord injury.

Keywords

angiogenesis, blood vessels; oligodendrocyte precursor; preganglionic neuron; spinal cord injury

1. Introduction

After a contusive spinal cord injury, blood vessels are lost over the first 3 days in adult rats, followed by an angiogenic response between day 3 and 7, and the subsequent regression of the new blood vessels by 14 days post-injury, the time when secondary tissue loss begins (Imperato-Kalmar et al., 1997; Casella et al., 2002, 2006; Loy et al., 2002; Whetstone et al., 2003). The loss of the original and new blood vessels is thought to contribute to secondary

*Corresponding Author: Kentucky Spinal Cord Injury Research Center, MDR Building, Room 609, University of Louisville, Louisville, Kentucky, USA. Tel: (502)852-8058; Fax: (502)852-5148; Email: theo.hagg@louisville.edu

#Current Address: Department of Neurology and Neurosurgery, Montréal Neurological Institute, McGill University, Montréal, Québec, Canada.

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spinal cord damage through a loss of essential nutrients and molecules for metabolism and removal of metabolic by-products. One potential factor contributing to the loss of blood vessels is the disruption of the endothelial cells from their laminin-containing basement membrane, which can induce apoptosis through intracellular signaling cascades by unbound integrin receptors (Frisch and Screaton, 2001; Zhan et al., 2004). Furthermore, integrin activation can promote endothelial cell survival *in vitro* (Scatena and Giachelli, 2002; Stupack and Cheresch, 2004). Therefore, it will be important to identify specific integrins that are associated with the vasculature during development and after spinal cord injury, when endothelial cells need to grow, mature and survive to promote tissue integrity.

Integrins are a family of 24 known heterodimers consisting of one of 18 α and one of 8 β subunits which bind to extracellular matrix molecules and regulate proliferation, growth, migration, differentiation and survival of a variety of cell types (Milner and Campbell, 2002b; Clegg et al., 2003; Stupack and Cheresch, 2004). Two predominant laminin-binding integrins are $\alpha 1\beta 1$ (Ignatius et al., 1990; Tawil et al., 1990) and $\alpha 6\beta 1$ (Sonnenberg et al., 1990). The $\alpha 1\beta 1$ integrin can also bind collagen (preferentially collagen-IV; Ignatius et al., 1990; Tawil et al., 1990). The $\alpha 1$, $\alpha 6$ and $\beta 1$ integrin subunits are expressed by the vasculature in rodent brain (Kloss et al., 1999; Milner and Campbell, 2002a). Integrins containing the $\alpha 1$, $\alpha 6$ and $\beta 1$ subunits have been shown to promote angiogenesis *in vitro* and *in vivo* (Davis and Camarillo, 1995; Senger et al., 1997; 2002; Babic et al., 1998; Pozzi et al., 2000; Gonzalez et al., 2002; Grzeszkiewicz et al., 2002; Leu et al., 2002; 2003). A switch from predominant expression of $\alpha 4$ and $\alpha 5$ by blood vessels in postnatal day 1 (P1) mouse brain to $\alpha 1$, $\alpha 6$ and $\beta 1$ integrin subunits from P7 into adulthood has previously been reported (Milner and Campbell, 2002a). However, their presence in the postnatally developing spinal cord has yet to be fully described. Additionally, no studies have investigated changes in the expression of integrins by the vasculature following spinal cord injury. Moreover, little is known about the distribution of the $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin heterodimers, i.e., the functional forms of integrins. Previous studies have primarily used antibodies directed against individual integrin subunits and relied on co-localization to suggest the possible existence of functional heterodimers.

To confirm the importance of the $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins for the spinal cord vasculature, we investigated their expression during postnatal development in rats using heterodimer-specific antibodies. Moreover, to determine whether these integrins might be modulated after spinal cord injury, we documented their changes after contusive injury in adult rats.

2. Results

2.1. $\alpha 1\beta 1$ integrin is present only in endothelial cells of normal developing and contused adult rat spinal cord

Within the spinal cord, immunostaining for the $\alpha 1\beta 1$ integrin heterodimer appeared to be associated exclusively with blood vessels as early as the first day after birth (P1) and throughout adulthood (Fig. 1). Immunostaining was also evident in the meninges from P15 to adulthood, likely representing meningeal fibroblasts as previously reported (Milner and French-Constant, 1994). The number and complexity of blood vessels increased throughout development, most notably between P10 and P15 to reach a level similar to that seen in adults. Larger-diameter vessels appeared predominant up to P15 (Fig. 1A-D) and smaller-diameter vessels appeared to increase at P20 (Fig. 1E). Up to P10, vessels appeared to be equally distributed throughout the entire spinal cord section. From P15 to adulthood, the blood vessels appeared to gradually become more prominent within gray than white matter. The adult distribution of the blood vessels is consistent with previous reports (Blight, 1991; Tator, 1995; Loy et al., 2002). The exclusive co-localization of $\alpha 1\beta 1$ integrin with blood vessels of naïve adult rats was shown in sections co-stained for SMI-71 (Fig. 2A-C), which recognizes endothelial barrier antigen (Sternberger and Sternberger, 1987).

We also determined whether $\alpha 1\beta 1$ integrin would remain present after a spinal cord contusion in adult rats, and thus be available for therapeutic intervention. After a moderate T9 spinal contusion, more $\alpha 1\beta 1$ -positive blood vessels appeared to be present within 11 mm of the injury epicenter (the farthest distance analyzed) starting as early as 1 day (Fig. 3A-L). At 7 days, large-diameter vessels were seen within the center of the spinal cord, where tissue damage is evident (Fig. 3D). To further illustrate the extent of increased $\alpha 1\beta 1$ integrin expression, horizontal sections from an animal with a moderate contusion processed 7 days post-injury from a previous study (Baker and Hagg, 2005) were stained (Fig. 3N). A dramatic upregulation of $\alpha 1\beta 1$ immunoreactivity in blood vessels can readily be seen and extends throughout the entire lesion and perilesional areas. A large proportion of these $\alpha 1\beta 1$ -positive vessels extend longitudinally from rostral to caudal gray matter with processes emanating radially into surrounding white matter. Within the most severely damaged region of the epicenter, $\alpha 1\beta 1$ staining was also seen in thin fibrous structures that originated from the blood vessels (arrows; Fig. 3O). However, these structures did not co-express SMI-71 (Fig. 2D-F) and could represent migrating endothelial cells or growing capillaries as the SMI-71 antibody recognizes only mature blood vessels.

Quantification of the percentage of tissue area occupied by $\alpha 1\beta 1$ -positive blood vessels showed an increase from 1.6 % in sham-operated animals to ~4-5% from day 1 to 7 days post-injury at 1 mm from the injury epicenter (Fig. 4A). These increases also appeared to occur at 1 and 3 days, at the 6 and 11 mm distances. Significant differences were observed for distance and time separately using a 2-way ANOVA, and thus 1-way ANOVAs were performed, followed by Tukey's post-hoc tests to determine differences between groups. This revealed a significant increase in the mean ($\pm$ standard error of the mean) blood vessel area at 3 days post-injury ($4.35 \pm 0.86\%$; $p < 0.02$) and a trend towards an increased blood vessel area at 1 day ($4.07 \pm 0.63\%$; $p = 0.057$) compared to sham-operated animals ($1.63 \pm 0.10\%$). Also, a significant drop in blood vessel area was observed from 3 to 7 days ($1.84 \pm 0.63\%$) after injury ($p < 0.05$). No significant difference was observed between the distances from the injury epicenter.

2.2. $\alpha 6\beta 1$ integrin is present in spinal cord blood vessels, IML neurons and OPCs during development and adulthood

The association of $\alpha 6$ and $\beta 1$ integrin subunits with blood vessels has been documented in the murine brain and starts at P7 (Milner and Campbell, 2002a). In the current study, faint $\alpha 6\beta 1$ integrin heterodimer immunostaining of a small number of blood vessels in the spinal cord was seen beginning at P10 (Fig. 5). At P15 and P20, a modest number of small and large blood vessels in both white and gray matter were positive for $\alpha 6\beta 1$ integrin. Cross-sections of vessels were primarily seen within gray matter, indicative of their rostral-caudal orientation, in contrast to vessels within the white matter which were primarily seen extending radially to the cord surface. This pattern is similar to that seen with $\alpha 1\beta 1$ integrin staining. Immunostaining for $\alpha 6\beta 1$ integrin was largely unchanged into adulthood. Co-expression of $\alpha 6\beta 1$ and SMI-71, a marker for mature blood vessels, was clearly detectable in the adult (Fig. 7A-C).

Beginning at P1, faint $\alpha 6\beta 1$ immunostaining was seen in cell bodies and processes of motor neurons in the ventral horn and continued through P20 (Fig. 6A,D,G,J,M,P), confirming a previous report of $\alpha 6$ mRNA in motor neurons during early postnatal development (Hammarberg et al., 2000). Immunostaining for $\alpha 6\beta 1$ integrin by motor neurons appeared to decrease throughout development and was faint in adults. Robust staining for $\alpha 6\beta 1$ integrin was present in preganglionic neurons of the IML at thoracic (Fig. 6B,E,H,K,N,Q) and lumbar levels (not shown) of the spinal cord from P1 through adulthood. In horizontal sections, staining was seen in the processes that extended medially to the central autonomic nucleus dorsal to the central canal, giving a ladder-like appearance (Fig 6C,F,I,L,O,R). These ladders consist of the IML dendrites as has been previously described (Vera et al., 1986; Bernstein-Goral and

Bohn, 1988; Markham et al., 1991; Ezerman et al., 1996). In the adult rat spinal cord, large, round preganglionic neuronal cell bodies in the IML gave rise to thick or fasciculated processes that were not positive for SMI-71, excluding the possibility that the staining represented blood vessels (Fig. 7A-C).

A few other cell types in the adult spinal cord also contained $\alpha 6 \beta 1$ immunostaining. OPCs have been shown to express $\alpha 6$ and $\beta 1$ integrin subunits (Milner and ffrench-Constant, 1994). Here, cells stained for the OPC marker NG2 exhibited faint staining for $\alpha 6 \beta 1$ (Fig. 7D-F). These cells appeared morphologically like OPCs, as they had small cell bodies, small nuclei and were unipolar, bipolar or multipolar with fine processes extending radially into the surrounding parenchyma (Horner et al., 2002). Glial fibrillary acidic protein (GFAP)-positive astrocytes did not express $\alpha 6 \beta 1$ integrin (Fig. 7G-I). However, $\alpha 6 \beta 1$ -positive processes from the IML neurons were intimately associated and appeared to be intertwined with GFAP-positive processes within the white matter. These GFAP-positive fibers extended from the gray matter towards the surface of the spinal cord and were much larger in diameter than the fine astrocytic processes seen elsewhere. Resting microglia, identified by Iba1-staining and characterized by small cell bodies and many ramified processes, did not express $\alpha 6 \beta 1$ integrin in the normal spinal cord (Fig. 7J-L). In the dorsal and ventral roots that contain Schwann cells, $\alpha 6 \beta 1$ immunostaining was only seen in blood vessels (not shown).

2.3. Transient upregulation of $\alpha 6 \beta 1$ in blood vessels of the contused adult spinal cord

Following spinal contusion, $\alpha 6 \beta 1$ immunostaining appeared to increase within 1 mm of the injury site up to 7 days post-injury, with little change in expression being seen at 6 and 11 mm (Fig. 8). At 7 days, a rim of $\alpha 6 \beta 1$ positive blood vessels was present around the damaged injury core tissue (Fig. 8D, but was sparser than the $\alpha 1 \beta 1$ staining (Fig. 3 D) within the core. The $\alpha 6 \beta 1$ -positive blood vessels seen within the damaged tissue did not have fibrous processes (Fig. 7D-F) as was seen for $\alpha 1 \beta 1$ -positive blood vessels at this time and in this damaged tissue (Fig 3O). Quantification of the percentage of $\alpha 6 \beta 1$ -positive blood vessel area showed increases from about 1% in sham-operated rats to ~1-2% at 1 and 3 days, within the 11 mm distance analyzed (Fig. 4B). Significant differences were observed for distance and time separately using a 2-way ANOVA, and thus 1-way ANOVAs were performed, followed by Tukey's post-hoc tests to determine differences between groups. This revealed a significantly greater percent blood vessel area at 1 mm ($1.39 \pm 0.20\%$) than at 11 mm ($0.75 \pm 0.16\%$; $p < 0.05$) and in animals 3 days post-injury ($1.65 \pm 0.23\%$) than either sham ($0.69 \pm 0.10\%$) or 7 day animals ($0.67 \pm 0.12\%$) ($p < 0.01$ and $p = 0.002$, respectively).

The $\alpha 6 \beta 1$ staining in the spinal motor neurons and preganglionic IML neurons and their associated processes was not changed within 11 mm of the lesion site (data not shown). More OPC cell bodies were $\alpha 6 \beta 1$ integrin-positive around the injury site compared to those in regions farther away from the injury or in sham-operated rats (not shown). The $\alpha 6 \beta 1$ immunostaining in NG2-positive cells, morphologically similar to OPCs was unchanged at 1 day after injury compared to naïve animals. However an increased number of $\alpha 6 \beta 1$ /NG2+ cells appeared to be present at 3 days and remained stable through 7 days post-injury. Due to the intense expression of NG2 after injury, possibly representing perivascular cells of arterioles (Murfee et al., 2005) and endothelial cells (Jones et al., 2003), it is difficult to accurately identify OPCs so the number of precursors expressing $\alpha 6 \beta 1$ was not determined. As in the normal spinal cord, astrocytes did not express $\alpha 6 \beta 1$ after spinal contusions (data not shown). At 1 day post-injury, little change in Iba1-positive microglia/macrophages was seen compared to naïve animals and none of these cells had $\alpha 6 \beta 1$ integrin immunostaining (not shown). At 3 and 7 days following the spinal cord contusion many microglia/macrophages were seen near the injury site. Some of these microglia/macrophages were positive for $\alpha 6 \beta 1$ integrin (Fig. 6M-O). These Iba1-, $\alpha 6 \beta 1$ -positive microglia/macrophages had small swelled cell bodies with 1 or 2 visible short

processes. Previous studies have shown that facial nerve transection and lipopolysaccharide-activated microglia express $\alpha 6$ and $\beta 1$ subunits (Kloss et al., 1999; 2001). Here, only a small subset of Iba1-positive microglia/macrophages exhibited staining for the $\alpha 6\beta 1$ integrin heterodimer, likely representing activated microglia.

3. Discussion

In this study, we show in the rat spinal cord that 1) during development, $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin expression increases in blood vessels between P10-15, suggesting that they have a role in the formation and/or maintenance of the blood-spinal barrier, although the earlier and more extensive expression of $\alpha 1\beta 1$ integrin suggests different functions between the two, 2) after spinal cord contusion in the adult, $\alpha 1\beta 1$ integrin is upregulated in blood vessels over a greater distance from the injury site and more prominently in the injured core than $\alpha 6\beta 1$ integrin, 3) there is a transient increase in blood vessel area up to 3 days after injury for both integrins, and 4) whereas $\alpha 1\beta 1$ integrin is specific for endothelial cells and blood vessels, $\alpha 6\beta 1$ integrin is also present in preganglionic neurons of the IML, and as reported by others for the mRNA of the integrin subunits in spinal motor neurons and a subset of microglia/macrophages and for the protein heterodimer in OPCs.

3.1. $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins in developing spinal cord blood vessels might play roles in blood-spinal cord barrier formation and maintenance

Blood vessels in the brain have been shown to express $\alpha 1$, $\alpha 6$ and $\beta 1$ integrin subunits (Kloss et al., 1999; Milner and Campbell, 2002a) and our results with antibodies specific for the $\alpha 1\beta 1$ and $\alpha 6\beta 1$ heterodimers show that they exist in the spinal cord. We also show that $\alpha 1\beta 1$ and particularly $\alpha 6\beta 1$ expression is low at early postnatal times and increases dramatically from P10 to P15 in the rat, when the complexity of the vasculature greatly increases. This is consistent with the observed switch from a predominant expression of $\alpha 4$ and $\alpha 5$ integrin subunits in P1 mouse brain to expression of $\alpha 1$ and $\alpha 6$ integrin subunits from P7 into adulthood (Milner and Campbell, 2002a). The ligand laminin appears around P7-9 in blood vessels of the brain (Cassella et al., 1996) and spinal cord (Rafalowska et al., 2000) when the blood-brain barrier and blood-spinal cord barriers are known to mature (Hunter et al., 1992; Cassella et al., 1996; Milner and Campbell, 2002a). The increased expression of $\alpha 1\beta 1$ and $\alpha 6\beta 1$ in conjunction with or just after formation of the blood-spinal barrier during development suggests that they may be important for maturation of the barrier. The continued presence of these integrins in blood vessels during adulthood raises the possibility of a role for those integrins in maintaining the barrier, perhaps by promoting endothelial cell survival, as has been shown for $\alpha v\beta 3$ (reviewed in Scatena and Giachelli, 2002). This proposed role in the blood-spinal cord barrier would be in apparent contrast to the reported role of $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins in angiogenesis *in vitro* and in other systems (Davis and Camarillo, 1995; Senger et al., 1997; 2002; Babic et al., 1998; Pozzi et al., 2000; Gonzalez et al., 2002; Grzeszkiewicz et al., 2002; Leu et al., 2002; 2003). However, it would be consistent with the fact that the barrier is unique to CNS blood vessels in which a different function of $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins may exist.

The $\alpha 1\beta 1$ integrin was already present in many blood vessels at P1 whereas the $\alpha 6\beta 1$ integrin was only clearly noticeable in a small number of vessels after P10. This suggests that $\alpha 1\beta 1$ integrin has a different role than the $\alpha 6\beta 1$ integrin. One possibility is that $\alpha 1\beta 1$ integrin is involved in angiogenesis during development in addition to the proposed role in blood-spinal barrier formation. The main difference between $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrin is the binding of $\alpha 1\beta 1$ integrin to collagen as well as laminin (Ignatius et al., 1990; Tawil et al., 1990). Collagen is expressed by developing blood vessels at P15 (Rafalowska et al., 2000). In the adult vasculature, expression of $\alpha 1\beta 1$ integrin appeared higher than $\alpha 6\beta 1$ integrin, again suggesting overlapping as well as different functions.

3.2. $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins as potential vascular targets following spinal cord injury

Following spinal cord injury, endothelial cell death and compromised blood vessel integrity leads to ischemia, vessel leakiness, edema followed by inflammation and later occurring secondary tissue loss (Kwon et al., 2004). Previous studies have reported that by 3 days after spinal cord contusion, many blood vessels in and around the epicenter reportedly have died and a transient angiogenic response is seen between 3 and 7 days post-injury in the center of the damaged spinal cord region (Casella et al., 2002, 2006; Loy et al., 2002). In contrast to those results, here we observe an unexpected increase in the integrin-positive blood vessel area at 3 days post-injury followed by a subsequent reduction in area at 7 days. Our results showed that all SMI71-positive vessels were also $\alpha 1\beta 1$ -positive (Fig. 2A-C), which was used as a blood vessel marker. This suggests that the increase in $\alpha 1\beta 1$ integrin staining represents an earlier angiogenic response and not an injury-induced expression of integrins in blood vessels which normally do not express them. The main difference between those previous studies (Casella et al., 2002, 2006; Loy et al., 2002) and the present are the type of impactor device (NYU vs. IH) and the strain of rats used (Fisher 344 vs. Sprague-Dawley), suggesting that the post-injury angiogenic response may vary between those impactors and/or strains.

The presence of $\alpha 1\beta 1$ integrin in large blood vessels in the core of the spinal cord injury site correlates with the reported angiogenic response seen by day 7 after contusion (Loy et al., 2002). The $\alpha 1\beta 1$ integrin was seen in cells and processes that appeared to derive from the blood vessels. These cells did not express SMI-71, a marker for mature endothelial cells. This suggests that $\alpha 1\beta 1$ integrin is also present on endothelial cells that are migrating or are in the process of early tubule formation after spinal cord injury. In fact, blocking antibodies to the $\alpha 1$ subunit have been shown to prevent attachment and spreading of endothelial cells on collagen-I, -IV and laminin-I (Senger et al., 1997; 2002). The $\alpha 6\beta 1$ integrin was barely present in this damaged region. This suggests that $\alpha 1\beta 1$ but not $\alpha 6\beta 1$ integrin plays a role in angiogenesis in the injured spinal cord. This role of $\alpha 1\beta 1$ integrin is consistent with its role in VEGF-induced angiogenesis in other systems (Senger et al., 1997; 2002) and the increase in VEGF that occurs after spinal cord injury (Bartholdi et al., 1997; Vaquero et al., 1999; Sköld et al., 2000).

The $\alpha 1\beta 1$ integrin is expressed by activated effector memory T cells and is involved in the inflammatory response by enabling transmigration of lymphocytes into tissues (Andreasen et al., 2003; Ben-Horin and Bank, 2004). However, we did not see evidence for $\alpha 1\beta 1$ -positive infiltrating cells, even though T-cells invade the rat spinal cord 3-7 days after spinal cord contusion (Popovich et al., 1997; Sroga et al., 2003). Therefore, the exclusive presence of $\alpha 1\beta 1$ integrin in endothelial cells and blood vessels suggests that it might be a good therapeutic target for enhancing endothelial survival and angiogenesis after spinal cord injury. The $\alpha 6\beta 1$ integrin might be targeted during the first three days when endothelial cells die. However, it would not be selective for blood vessels as other cells also express the $\alpha 6\beta 1$ integrin.

3.3. Potential additional roles for $\alpha 6\beta 1$ integrin

We found $\alpha 6\beta 1$ integrin in the preganglionic neurons of the IML throughout development from P1 into adulthood. This early expression corresponds to the time-frame of dendrite outgrowth (Markham et al., 1991; Ezerman and Forehand, 1996) as well as to supraspinal innervation and synaptic reorganization on IML neurons (Bernstein-Goral and Bohn, 1988; Rajaofetra et al., 1989; 1992). Integrins have been shown to be important for synaptogenesis (Einheber et al., 1996; Nikonenko et al., 2003) and consolidation of long-term potentiation (Chan et al., 2003; Kramar et al., 2006). The synaptic inputs onto the IML neurons are thought to be plastic as they express growth-associated protein-43 (Nacimientos et al., 1993; Ching et al., 1994; Weaver et al., 1997). This raises the possibility that $\alpha 6\beta 1$ integrin on these neurons might be involved in developmental, adult and spinal cord injury-induced plasticity. These neurons are critically

involved in the development of autonomic dysreflexia (Llewellyn-Smith et al., 2006). Furthermore, $\alpha 6 \beta 1$ integrin is a novel immunohistochemical marker for this cell population.

We also observed $\alpha 6 \beta 1$ -positive/NG2-positive OPCs in the non-injured spinal cord. Others have demonstrated by co-immunoprecipitation that cultured OPCs (Milner and ffrench-Constant, 1994), oligodendrocytes (Milner and ffrench-Constant, 1994; Shaw et al., 1996) and astrocytes (Shaw et al., 1996) may have $\alpha 6 \beta 1$ integrins. We did not observe $\alpha 6 \beta 1$ immunostaining in mature oligodendrocytes or astrocytes *in vivo*, suggesting that it is upregulated in cultured cells or that we are observing different isoforms or only the functional heterodimers *in vivo*. Following spinal cord injury there appeared to be an increase in the number of $\alpha 6 \beta 1$ -expressing OPCs. However, the dramatic elevation of NG2 expression made it difficult to identify and quantify the number of $\alpha 6 \beta 1$ -positive OPCs. OPCs have been shown to increase in number as early as 1-2 days after a cerebellar puncture injury (Levine, 1994) as well as after chemical demyelinating lesions of the spinal cord (Talbot et al., 2005). Therefore, the observed increase in $\alpha 6 \beta 1$ -positive cells most likely includes an increased number of OPCs at the injury site. The function of $\alpha 6 \beta 1$ integrin in OPCs is currently unknown.

We also found that a subset of Iba1-positive microglia/macrophages in the injured spinal cord is $\alpha 6 \beta 1$ -positive, suggesting that $\alpha 6 \beta 1$ expression may represent activated microglia, as has been previously shown (Kloss et al., 2001). As the role for $\alpha 6 \beta 1$ integrin in microglial function is unknown, it is unclear how modulation of the microglial $\alpha 6 \beta 1$ integrin would affect the pathophysiology of spinal cord injury.

3.4. Conclusion

In conclusion, we have identified the selective expression of the $\alpha 1 \beta 1$ integrin heterodimer in the vasculature of the spinal cord and its potential as a target for improving endothelial survival and angiogenesis after spinal cord injury. The $\alpha 6 \beta 1$ integrin may also play a role in endothelial functions. In addition, it is most likely also involved in the function or plasticity of the IML neurons, OPCs and in a subset of microglia/macrophages after a spinal cord injury.

4. Materials and methods

All experiments were conducted in accordance with the National Institutes of Health's guidelines on the care and use of laboratory animals and were approved by the Animal Care Committee of the University of Louisville. Rat pups were randomly selected at P1 (first day of birth), P5, P10, P15 and P20 from Sprague-Dawley dams housed singly (n=4 for each time-point; Harlan, Indianapolis, IN). Twelve young adult female Sprague-Dawley rats (Harlan; 8-9 weeks and weighing 180-200g) were used to study $\alpha 1 \beta 1$ and $\alpha 6 \beta 1$ expression in the normal spinal cord and at 1, 3 or 7 days (n=3 for each group) following a moderate contusion at spinal cord level T9.

4.1. Spinal cord contusion

Young adult rats were anesthetized with 3.3 ml/kg of an anesthetic mixture consisting of 25 mg/ml of ketamine hydrochloride (Abbott Laboratories, North Chicago, IL), 1.2 mg/ml of acepromazine maleate (The Butler Company, Columbus, OH), and 0.25 mg/ml of xylazine (Butler) in 0.9% saline, injected intramuscularly. Their backs were shaved and cleansed with betadine, lacrilube ophthalmic ointment (Allergan, Irvine, CA) was placed on their eyes to prevent drying and 50 mg/kg gentamicin sulfate antibiotic (Boehringer Ingelheim, St Joseph, MO) was injected intramuscularly to reduce infections. Laminectomies were performed at spinal cord level T9 and a moderate contusion performed through the intact dura of the exposed spinal cord with the Infinite Horizon (IH) impactor set at a force of 150 kdyn (Scheff et al., 2003; Precision Systems Instrumentation, Lexington, KY). Those rats were perfused at 1, 3 or

7 days post-injury. Naïve rats served as normal controls. Afterwards, the layers of muscle and skin were sutured separately and Bacitracin zinc antibiotic ointment (Altana Inc., Melville, NY) applied to the incision area. All animals were injected subcutaneously with 10 cc of lactated ringer's solution with 5% dextrose. Bladders were manually expressed twice daily throughout the experiment.

4.2. Tissue processing

The rat pups were anesthetized with an initial 0.1 ml injection of the anesthetic mixture and received subsequent injections of 0.05 ml until adequately anesthetized. The pups were perfused transcardially with 10 ml of ice-cold 0.1M PBS followed by 20 ml of 4% paraformaldehyde in 0.1M phosphate buffer. The spinal cords were collected and post-fixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose for 48 hours. Approximately 5 mm long sections of spinal cord were taken from cervical (C4-7), thoracic (T6-T9) and lumbar (L2-L5) regions and placed in tissue freezing medium (OCT Compound, Sakura Finetek USA Inc., Torrance, CA) in embedding molds. Blocks of tissue were stored at -80°C prior to cryostat sectioning in either the transverse (n=2) or horizontal (n=2) planes. Every third, 20 μm thick, section was collected on warm electrostatic slides and stored at -80°C before being immunohistochemically processed.

The young adult rats that received sham and contusion surgeries were anaesthetized with 4 ml/kg of the anaesthetic mixture and perfused with 100 ml of 0.1M PBS followed by 200 ml of 4% paraformaldehyde (pH = 7.4 in 0.1M phosphate buffer). The lesion epicenter was identified and 3 cm of the surrounding spinal cord removed and affixed with 30-gauge needles to a strip of dental wax (SPI Supplies, West Chester, PA) to prevent curvatures of the spinal cord, and post-fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer at 4°C . Next, the cord was cryoprotected for 2 days in 30% sucrose. The spinal cord was cut into 5 mm long pieces up to 1.5 cm rostral and caudal to the lesion epicenter and the pieces placed in OCT Compound in embedding molds for cryostat sectioning. Thirty micrometer transverse sections were collected on warm electrostatic slides and stored at -80°C prior to immunohistochemical processing.

4.3. Immunohistochemistry

Staining for integrins was performed on sections from C4-7, T6-T9 and L2-L5 postnatal rat spinal cords and on sections at approximately 1, 6 and 11 mm from the lesion epicenter. The slides containing the sections were first rinsed in 0.1M tris-buffered saline (TBS), quenched in a 10% methanol, 3% hydrogen peroxide solution, and blocked in TBS containing 5% normal horse serum, 0.3% Triton X-100 (Sigma-Aldrich) for 30 min.. Sections were then incubated in primary antibodies against the heterodimers of $\alpha 1\beta 1$ (VLA-1; 1:3000; MAB2513; Chemicon International, Temecula, CA) or $\alpha 6\beta 1$ integrins (VLA-6; 1:1000; MAB1410; Chemicon) in TBS containing serum and 0.3% Triton X-100 overnight at 4°C . To visualize antibody binding, sections were incubated in biotinylated horse anti-mouse IgG (1:500; Vector Laboratories, Burlingame, CA) in TBS containing 5% serum; followed by incubation with an avidin-biotin-complex (ABC) amplification kit (1:600; Elite Vectastain kit; Vector Laboratories) in TBS. Peroxidase activity was visualized by a 3,3'-diaminobenzidine step (Sigma-Aldrich) and the reaction product intensified with 0.06% nickel ammonium sulfate in 0.1M phosphate buffer. Sections were rinsed with 0.1M phosphate buffer before being cover-slipped. The slides were air-dried overnight before being dehydrated in a series of ethanol and xylene solutions and coverslipped in Entellan (Electron Microscopy Sciences, Ft. Washington, PA).

To identify the cell types that express $\alpha 1\beta 1$ and $\alpha 6\beta 1$ integrins in the normal and injured spinal cord, double immunofluorescence staining for $\alpha 6\beta 1$ or $\alpha 1\beta 1$ and various cell-specific markers was performed. Briefly, slides were rinsed in 0.3% Triton in TBS and blocked with 10% normal

donkey serum, 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100 in TBS and incubated overnight in mouse anti- $\alpha 6\beta 1$ IgG (1:300) or mouse anti- $\alpha 1\beta 1$ IgG (1:1000) and another primary antibody (see below) in 5% serum, 0.5% BSA, 0.3% Triton in TBS at 4°C. On the following day, the slides were rinsed in TBS and incubated for 2h in 1:500 donkey anti-mouse IgG (Alexa488; Molecular Probes, Eugene, OR) and 1:500 donkey anti-rabbit IgG (Alexa594; Molecular Probes) or a Texas red-conjugated donkey anti-mouse IgM (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) all in 5% serum, 0.5% BSA in TBS. The slides were rinsed once in phosphate buffer and coverslipped with ProLong anti-fade (Molecular Probes). Cell types analyzed included blood vessels (mouse anti-endothelial barrier antigen IgM; SMI-71; 1:100; Sternberger Monoclonals Inc., Lutherville, MD), microglia/macrophages (rabbit anti-Iba1 IgG; 1:3000; Wako Chemicals USA, Richmond, VA), OPCs (rabbit anti-NG2 IgG; 1:100; AB5320; Chemicon) and astrocytes (rabbit anti-GFAP IgG; 1:300; AB5804; Chemicon).

4.4. Image Processing and Blood Vessel Quantification

All brightfield and fluorescent photomicrographs were taken using a Leica DMIRE2 inverted microscope with an attached Spot RT^{KE} digital camera and associated Spot software for Windows (Version 4.0.8; Diagnostic Instruments Inc., Sterling Heights, MI) and saved in Adobe Photoshop 6.0. If necessary, the brightness and contrast of the images were altered to best represent what was seen through the microscope. To quantify changes in $\alpha 6\beta 1$ - and $\alpha 1\beta 1$ -positive blood vessel area and staining intensity, a variation of the method previously reported by Loy and colleagues (2002) was used. Briefly, photomicrographs of the dorsal white matter (DWM), right dorsal gray matter (DGM), right ventral WM (VWM) and left VGM at 1, 6 and 11 mm rostral and caudal to the injury epicenter were taken at 200X magnification. Areas within the above regions were outlined in ImageJ (Version 1.36b, National Institutes of Health, Bethesda, MD) and thresholding used to select only the positive-stained blood vessels and the total blood vessel area was measured for each section and each rat. The total blood vessel area was recalculated as a percentage of the outlined tissue area. As no difference was seen between rostral and caudal sections, they were combined. To assess whether significant differences existed in the percent blood vessel area with respect to the distance from the injury epicenter or time after injury, ANOVAs were performed and considered significant at $p < 0.05$.

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Abbreviations

DGM, dorsal gray matter; DWM, dorsal white matter; GFAP, glial fibrillary acidic protein; IML, intermediolateral cell column; OPC, oligodendrocyte precursor; PBS, phosphate-buffered saline; TBS, tris-buffered saline; VGM, ventral gray matter; VWM, ventral white matter.

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Figure 1.

Expression of $\alpha 1\beta 1$ integrin in the developing spinal cord. $\alpha 1\beta 1$ -positive blood vessels are readily observed at all developmental ages in transverse sections through the thoracic spinal cord. The number of blood vessels and complexity of the vasculature gradually increases through P1 (A), P5 (B), P10 (C), P15 (D) and P20 (E) spinal cord with vessels appearing to be thickest at P15. $\alpha 1\beta 1$ immunostaining can also be observed on the surface of the spinal cord, likely representing menigeal fibroblasts.

Figure 2.

Expression of $\alpha 1\beta 1$ by blood vessels in the naïve and injured spinal cord. (A-C) $\alpha 1\beta 1$ expression is specific to SMI-71-positive blood vessels in naïve animals. (D-E) Thin fibrous $\alpha 1\beta 1$ -positive, SMI-71-negative 'streamers' (arrows) can be seen within the lesion cavity and may represent small immature vessels not yet possessing a mature blood-spinal cord barrier, and thus not yet expressing SMI-71.

Figure 3.

$\alpha 1\beta 1$ integrin positive blood vessels increase after thoracic spinal cord contusions. (A, E, I) Few blood vessels are detected by $\alpha 1\beta 1$ immunostaining in sham-operated animals. The $\alpha 1\beta 1$ integrin staining appears to increase at 1 (B, F, J) and 3 days (C, G, K) after contusion up to 11 mm from 1 mm of the injury epicenter. At 7 days, increased integrin expression is seen 1 mm from the injury epicenter prominently within the injured core (D) but appears similar to baseline levels at 6 (H) and 11 mm (L). (M) Schematic indicating the location of the panels in Fig. 3A-L and Fig. 8A-L. (N) A horizontal section demonstrating $\alpha 1\beta 1$ integrin staining in the spinal cord 7 days after injury. Many $\alpha 1\beta 1$ -positive blood vessels are seen extending primarily rostrocaudally through the lesion site with vessels extending radially from these vessels into the lateral white matter. (O) A higher magnification of vessels within the lesion cavity demonstrates the thin $\alpha 1\beta 1$ integrin-positive processes often seen attached to blood vessels at this time-point following injury (arrows). CC = central canal.

Figure 4.

Integrin-positive blood vessel increases after spinal cord injury: quantification. (A) The percentage of $\alpha 1\beta 1$ -immunopositive blood vessel area increases up to 3 days after injury within 11 mm of the injury epicenter. However, at 7 days an increase in area is only seen at 1 mm, with decreases below that of sham-operated animals being seen at 6 and 11 mm. (B) An increase in the percentage of $\alpha 6\beta 1$ -positive blood vessel area is seen 1-3 days after injury up to 11 mm from the epicenter, whereas at 7 days the percent blood vessel area is similar to that of sham-operated animals at 1 mm and at other distances the blood vessel area drops below that of sham-operated animals.

Figure 5.

Expression of $\alpha 6 \beta 1$ integrin in the developing spinal cord. From P10 (D) to adulthood (F) staining of blood vessels with $\alpha 6 \beta 1$ was seen (arrows). In P1 (A), P5 (B) and P10 (C) transverse sections of the spinal cord, $\alpha 6 \beta 1$ staining was also seen within preganglionic cell somata within the intermediolateral cell column (IML) and associated processes (arrowheads) motor neurons in the ventral horn and their processes.

Figure 6.

$\alpha 6\beta 1$ expression within the ventral horn and IML of the rat spinal cord. Left and middle columns: higher magnification images of Figure 4. Left column: expression of $\alpha 6\beta 1$ integrin is highest within ventral horn motor neurons at P1 (A) and P5 (D). Also note the $\alpha 6\beta 1$ -positive processes extending from the motor neurons towards the ventrolateral spinal cord (the transition zone to the ventral root) at this age. Expression of $\alpha 6\beta 1$ integrin is reduced but still evident in motor neurons at later ages (arrows). The middle column shows transverse sections and right column horizontal sections (rostral is at the top, midline at the right): $\alpha 6\beta 1$ -positive preganglionic neurons of the intermediolateral nucleus are seen at each developmental age. Furthermore, $\alpha 6\beta 1$ -positive dendrites can be seen as early as P1 (B,C) extending medially with fewer processes projecting laterally. At P10 (E,F) $\alpha 6\beta 1$ -positive processes appear to project also rostrocaudally within the IML cell column and at the midline. The complexity of the $\alpha 6\beta 1$ -positive dendrites greatly increases at P20 (R,S) with intense staining seen within dendrites ventral to the central canal. A fragment of $\alpha 6\beta 1$ -positive meninges can be seen in (R).

Figure 7.

Blood vessels, oligodendrocyte precursors and a subset of activated microglia have $\alpha 6 \beta 1$ integrin immunostaining in the adult rat spinal cord. A-C) In the normal spinal cord, $\alpha 6 \beta 1$ integrin is present in SMI-71-positive blood vessels (arrows) in normal spinal cord. Note that the $\alpha 6 \beta 1$ -positive processes from the intermediolateral (IML) cell column (arrowheads) do not co-express SMI-71, suggesting that they are neuronal processes and not blood vessels. D-F) NG2-positive oligodendrocyte precursors also have $\alpha 6 \beta 1$ integrin immunostaining. G-I) GFAP-positive astrocytes do not have $\alpha 6 \beta 1$ integrin staining. These IML processes (arrowhead) are intimately intertwined with the astrocytic processes within the white matter. J-L) Resting microglia in the normal spinal cord also did not exhibit $\alpha 6 \beta 1$ integrin staining. M-O) A subset of Iba1-positive microglia/macrophages (arrows) express $\alpha 6 \beta 1$ integrin, 7 days after spinal cord contusion around the epicenter.

Figure 8.

$\alpha 6 \beta 1$ integrin-positive blood vessels increase after thoracic spinal cord contusions. (A, E, I) Sham-operated rats demonstrated low levels of $\alpha 6 \beta 1$ in blood vessels and clear staining of IML preganglionic neurons and their processes. After spinal contusions, $\alpha 6 \beta 1$ staining appeared to be increased in blood vessels at 1 and 3 days, 1 mm rostral to the injury site (B and C, respectively) and at 6 (F and G, respectively) and 11 mm (J and K, respectively). (D, H, L) At 7 days, $\alpha 6 \beta 1$ integrin staining patterns approached normal levels. The same labeling pattern was seen in sections caudal to the lesion.