Upregulation of Pleiotrophin Gene Expression in Developing Microvasculature, Macrophages, and Astrocytes after Acute Ischemic Brain Injury

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Pleiotrophin (PTN) is a heparin-binding, 18 kDa secretory protein that functions to induce mitogenesis, angiogenesis, differentiation, and transformation in vitro. PTN gene (Ptn) expression is highly regulated during development and is highest at sites in which mitogenesis, angiogenesis, and differentiation are active. In striking contrast, with the exception of the neuron, the Ptn gene is only minimally expressed in adults. We now demonstrate that Ptn gene expression is strikingly upregulated within 3 d in OX42-positive macrophages, astrocytes, and endothelial cells in areas of developing neovascularization after focal cerebral ischemia in adult rat. Ptn gene expression remains upregulated in these same cells and sites 7 and 14 d after ischemic injury. However, expression of the Ptn gene is significantly decreased in cortical neurons 6 and 24 hr after injury and is undetectable in degenerating neurons at day 3. Neurons in contralateral cortex continue to express Ptn in levels equal to control, uninjured brain. It is suggested that PTN may have a vital role in neovascular formation in postischemic brain and that postischemic brain is an important model in which to analyze sequential gene expression in developing neovascularization. In contrast, Ptn gene expression in injured neurons destined not to recover is strikingly reduced, and potentially its absence may contribute to the failure of the neuron to survive.

Key words: pleiotrophin gene expression; ischemia; neovascularization; macrophage; astrocytes

Trophic factors are required for growth, differentiation, and maintenance of viability during development and after injury. Pleiotrophin (PTN) is a member of a newly identified family of developmentally regulated, secreted heparin-binding proteins (Milner et al., 1989; Rauvala, 1989; Li et al., 1990); it is an 18 kDa protein that stimulates mitogenesis, angiogenesis, and neurite and glial process outgrowth guidance activities in vitro. In vivo, Ptn gene expression peaks during late embryogenesis and in perinatal growth. Because these are times of active proliferation and differentiation in both mesenchyme and the nervous system, it has been suggested that PTN signals these functions during development as well (Li et al., 1990; Raulo et al., 1992; Wanaka et al., 1993; Matsumoto et al., 1994; Rauvala et al., 1994; Silos-Santiago et al., 1996). In contrast, with the exception of subpopulations of neurons, levels of Ptn gene expression are very much lower in adult animals (Li et al., 1990; Garver et al., 1994; Kurtz et al., 1995; Nakagawara et al., 1995; Silos-Santiago et al., 1996), suggesting that activation of the Ptn gene may occur and activate PTN signaling in responsive cells important in new tissue formation during recovery from injury.

Cerebral ischemia and infarction lead to death of both neurons and glial elements. However, because recovery of brain function is frequently noted in patients with stroke even in the absence of neuronal regeneration, the postischemic expression of trophic factors has been analyzed to identify which factors may be upregulated and to correlate the expression of these factors with tissue recovery. Different neurotrophins (Lindvall et al., 1992; Hsu et al., 1993) and basic fibroblast growth factor (Speliotes et al., 1996; Lin et al., 1997) are expressed after global or focal cerebral ischemia. Previously, the expression pattern of the Ptn gene was analyzed and found to be similar to a number of the neurotrophin genes (Li et al., 1990; Silos-Santiago et al., 1996). Remarkably, its expression is also significantly increased in neurons of the hippocampus, piriform cortex, and parietal cortex after chemically induced seizures (Wanaka et al., 1993), indicating its potential for activation in cells of the CNS. However, the significance of its increased expression levels in cortex after chemically induced seizures is unclear, and its expression after other forms of brain injury has not been studied. Because PTN stimulates proliferation of endothelial cells (Courty et al., 1991; Fang et al., 1992) and has been implicated in tumor angiogenesis (Chauhan et al., 1993; Czubayko et al., 1996; Choudhuri et al., 1997; Relf et al., 1997), the formation of new blood vessels with the increased vascular density frequently noted after cerebral ischemia (Lin et al., 1998) offered the potential to seek contribution of PTN to recovery of brain function after injury through its angiogenic properties. To seek evidence that PTN may contribute to tissue recovery, we have now examined its expression pattern after focal cerebral ischemia in rats.

MATERIALS AND METHODS

Focal ischemia model. Long-Evans male rats (body weight, 300–350 gm) were used in this study. Housing and anesthesia concurred with guidelines established by the Institutional Animal Welfare Committee, in accordance with the Public Health Services Guide for the Care and Use of Laboratory Animals, of the United States Department of Agriculture regulations, and the Guidelines of Panel on Euthanasia of the American
Veterinary Association. Rats were allowed free access to water and rat chow until surgery. Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.m.). The left femoral artery was cannulated for monitoring arterial blood pressure and heart rate and for arterial blood gas analysis. Mean arterial pressure was maintained at >80, and blood gas was maintained at pH 7.4 ± 0.1, PaO2 >80, and PaCO2 7 ± 1. The rectal temperature was monitored and maintained at 37.0 ± 0.5°C via an electronic temperature controller linked to a heating lamp. The right middle cerebral artery (MCA) was exposed as described previously using microsurgical techniques (Liu et al., 1989; He at al., 1993). Briefly, after a 2 cm vertical skin incision midway between the right eye and ear and splitting of the temporalis muscle, a 2 mm burr hole was drilled at the junction of the zygomatic arch and the squamous bone. The right MCA was ligated with a 10–0 suture under an operating microscope. Complete interruption of blood flow at the MCA occlusion site was confirmed using microsurgical techniques (Liu et al., 1989; He at al., 1993). Briefly, after a 2 cm vertical skin incision midway between the right eye and ear and splitting of the temporalis muscle, a 2 mm burr hole was drilled at the junction of the zygomatic arch and the squamous bone. The right MCA was ligated with a 10–0 suture under an operating microscope. Complete interruption of blood flow at the MCA occlusion site was confirmed using microsurgical techniques (Liu et al., 1989; He at al., 1993). Results of the infusion of Ptn antisense RNA probe were treated as follows: (1) hybridization with a 35S-labeled Ptn sense RNA probe, and (2) digestion with RNase A (50 ng/ml) for 1 hr at 37°C before hybridization with a 35S-labeled Ptn antisense RNA probe to confirm the specificity of the 35S-labeled Ptn antisense RNA probe (data not shown).

**RESULTS**

The pleiotrophin (Ptn) gene was predominantly expressed in cortical neurons in adult rat and mouse brain (Li et al., 1990; Wanaka et al., 1993; Silos-Santiago et al., 1996). In control sections from sham-operated brain and the left (contralateral to the ischemic lesion) hemisphere, the pattern of PTN expression was similar to that described previously (Wanaka et al., 1993; Silos-Santiago et al., 1996). Ptn mRNA and immunoreactivity of the PTN protein are readily seen in cortical neurons, but little or no expression of Ptn was observed in glia or the endothelial cells of the blood vessels in the normal brain or uninjured hemisphere (Fig. 1A–C). However, remarkable changes were demonstrated in the intensity of PTN expression in the right (injured) hemisphere. At 6 hr and 1 and 7 d after reperfusion, expression of the Ptn gene in cortical neurons in the ischemic brain was drastically reduced. The decrease in Ptn gene expression was more rapid and to a greater degree in the ischemic core than in the peri-infarct region. Ptn mRNA signals were low in degenerating neurons in the outer layers of the infarct but not detectable in the ischemic core 6 hr after induction of the lesion (Fig. 1D).

In contrast, transcripts of Ptn and PTN immunoreactivity were readily detected in glial cells in ischemic cortex, especially in the periphery of the infarct and the regions surrounding the injured area when examined 1–3 d after induction of the ischemic lesion (Figs. 2, 3A–C). Using an adjacent section immunostained with the anti-GFAP antibody, it was established that expression of the Ptn gene was localized to GFAP(+) astrocytes (Fig. 3D). The astrocytes were hypertrophic in appearance and exhibited strong GFAP(+) staining in the cell bodies and in the thick processes of these cells in the areas immediately adjacent to the border of injury area (Fig. 3D). At 1 and 3 d after ischemia, the astrocytes were increased in number in the white matter adjacent to the infarct (data not shown). In contrast, the contralateral side had little GFAP(+) staining in cortical astrocytes (data not shown). The morphology and distribution of the anti-Ptn immunostained cells bordering the injured area and peri-infarct region corresponded to that of GFAP(+) astrocytes in the same area, suggesting that the Ptn and GFAP(+) gene-expressing cells in sites immediately surrounding the ischemic lesion are astrocytes.

At day 3, transcripts of the Ptn gene were not seen in the area of the infarct. However, both transcripts and PTN protein were
Figure 1. Top. Sections of a brain from a sham-operated rat hybridized with $^{35}$S-Ptn antisense cRNA probe (A, dark field; B, bright field) or $^{35}$S-Ptn sense cRNA probe (C, bright field). Ptn hybridization signal was highly expressed in neurons of cortex (large arrow). A little signal was found in glial cells (small arrow) and microvascular endothelium (medium arrow). D (bright field). Section hybridized with $^{35}$S-PTN antisense cRNA shows that PTN mRNA decreases to a greater degree in degenerating neurons in the ischemic core (C) then in the periphery (P) of the infarct compared with normal neurons (N) 6 hr after reperfusions. Magnification: A–C, 200×; D, 100×.

Figure 2. Coronal sections of a brain 3 d after ischemia hybridized with $^{35}$S-Ptn antisense cRNA probe (dark field). A strong hybridization signal is shown at the border of the infarct (arrows) and microvasculature in the infarct (B, arrows). Magnification: A, 10×; B, 40×.
expressed at striking levels in the microvasculature and macrophages (Figs. 4, 5, 6B). Virtually all of the cells that expressed detectable levels of Ptn transcripts were immunoreactive with anti-PTN antibodies as well. Of particular importance, a large number of macrophages were found near the vessels (large arrow) at the border of the infarct (C), which corresponded to the hypertrophic anti-GFAP(+) astrocytes in the similar area on day 3 (D, large arrow); the small arrow denotes neurons under degeneration. Magnification, 200×.

Seven days after ischemia, the injured area consisted of large numbers of PTN-positive macrophages and hyperplastic blood vessels (Fig. 6B). The macrophages exhibited a wide range of morphological appearances, with round, oval, triangular, or square shapes and with the majority lacking identifiable processes. PTN immunoreactivity was readily identified in the cytoplasm of these macrophages and in the endothelial cells of the hyperplastic blood vessels (Fig. 6B).
Fourteen days later, the infarct contained numerous macrophages with various morphological features (Fig. 6D), and residual necrotic tissue was usually surrounded by macrophages (Fig. 6C). GFAP(+) astrocytes were no longer seen. There was very little PTN immunoreactivity in the microvascular endothelium or the cells surrounding the vessels.

Figure 4. Sections of an ischemic brain on day 3 hybridized with $^{35}$S-Ptn antisense cRNA (A, C, dark field; B, D, bright field). PTN transcripts were found in the endothelium of blood vessels (large arrow) and glial cells (small arrow). Endothelial sprouts were seen in A and B (large arrow). Magnification, 200×.
Figure 5. Sections of an ischemic brain on day 3. 

A, B, PTN immunoreactivity was found in endothelial cells (small arrow) and macrophages (large arrow) in the infarcted region after staining with anti-PTN antibody. 

C, D, Macrophages surrounding blood vessel (large arrow) were identified by immunostaining with anti-OX42 antibody (C, arrow, frozen section) and histochemistry staining with GSA-1B4 (D, arrow). Magnification: A, B, 400×; C, D, 200×.
DISCUSSION

In this work, it is demonstrated that levels of the Ptn gene are differentially expressed in different cell types in ischemic rat brain. A very striking increase in the levels of expression of the Ptn gene was found in microglia and macrophages within areas of the exuberant neovasculature that formed at the margins of the infarct and in the endothelial cells of the newly formed vessels themselves. As described previously, a remarkable angiogenic response is seen after severe focal cerebral ischemia in this rat model (Lin et al., 1998). In the present study, it was observed that both the endothelial cells in neovasculature and the cells identified by the different specific macrophage and microglial markers that associate with the sites of angiogenesis exhibit intense Ptn mRNA signals, initially at 3 d and continuously through day 14. Because PTN is a potent angiogenic agent in vitro, and tumors that derive from Ptn-transformed cells have striking new vessel formation (Chauhan et al., 1993), it is highly likely that PTN signaling is a very important contributor to the neovascularization in postischemic brain. It is also highly likely that the differential regulation of the Ptn gene in recovery from ischemic injury results from a specific set of “angiogenic” signals that are responsible for coordination of gene expression needed for the development of new blood vessels characteristic of ischemic injury. PDGF may be a candidate to initiate Ptn gene activation locally.

Ptn gene expression is increased by PDGF (Li et al., 1992a,b). PDGF is released by platelets, and the expression of PDGF-A is upregulated within 24 hr in different cells at sites of injury. The neuron fails to express the PDGF-α receptor (Yeh et al., 1993). For this reason, it is possible that the neuron cannot respond to the same PDGF-A signal potentially responsible for upregulation of the Ptn gene in the context of ischemic brain injury.

During embryogenesis, Ptn mRNA is primarily expressed by progenitor cells in the subependymal layer of the brain in developing neuroepithelium and in the ependymal cells themselves, suggesting roles in cell division of both neural and vascular progenitors. During the perinatal stage, Ptn mRNA is seen in cells of neural as well as glial origins. In the adult brain, Ptn expression is restricted to selective neuronal subpopulations, including cerebral cortex (Li et al., 1990; Wanaka et al., 1993; Silos-Santiago et al., 1996). It is interesting to note that after ischemia, expression of Ptn in glia becomes evident again in the adult brain and is preferentially distributed in the regions surrounding the injured area. The identification of Ptn expression in astrocytes with GFAP immunoreactivity indicated that the injured brain reverts to a perinatal pattern of glial Ptn expression. Because Ptn is also expressed in the ependymal cells in embryogenesis and therefore is a potentially important contributor to early vasculogenesis in developing brain, its expression in neo-

Figure 6. Sections of ischemic brain on days 3, 7, and 14. A. 35S-Ptn cRNA hybridization signals were detected in macrophages (arrow) near the infarcted area (top) on day 3 (bright field). B. A number of macrophages with PTN immunoreactivity (small arrow) with variable morphology in the infarcted area on day 7 (large arrow denotes a blood vessel). C. 35S-Ptn antisense cRNA hybridization signals were detected in numerous macrophages (arrow) surrounding residual necrotic tissue at the infarcted region on day 14 (bright field). D. A number of macrophages stained with GSA-IB4 with various morphological features in the infarct on day 14. Magnification: A, B, 400×; C, D, 100×.
vascular endothelial cells may also reflect a reversion to the perinatal pattern of Ptn gene expression.

Ptn expression in cortical neurons is different; selected populations of cortical neurons continue to exhibit basal levels of Ptn gene expression in normal adult brain (Wanaka et al., 1993; Silos-Santiago et al., 1996). After severe focal ischemia leading to infarction, a striking loss of expression of the Ptn gene was observed in neurons that were irreversibly injured within the ischemic core. A substantial reduction was found in stressed or severely injured neurons at the periphery. Thus, in striking contrast to the glial elements, macrophages, and endothelial cells, Ptn gene expression is not activated in neurons. As noted above, it is likely that an important signal in recovery from ischemia brain injury is PDGF-A; the absence of the PDGF-α receptor in neurons may therefore underlie the failure of neurons to express PTN in ischemic injury. The neurons in which Ptn gene expression is downregulated seem destined for cell death, in contrast to the neurons distant from the site of injury and neurons on the contralateral side that retain the high levels the Ptn mRNA signal intensity that is characteristic of uninjured neurons.

The significance of Ptn expression relative to the fate of neurons under ischemic insult is not clear. However, expression of selected genes may distinguish cell survival from cell death (States et al., 1996). Heat shock protein 70 (HSP70), a stress gene with putative cytoprotective action, is expressed only in neurons that survive ischemic insult but not in those neurons that sustain irreversible injury. Neurons dying of apoptosis can be separated from those that survive and express HSP70 by the absence of expression of this marker gene. Despite the neurotrophic properties of PTN, Ptn gene expression was not enhanced in injured neurons in the periphery of the ischemic core, suggesting that Ptn is not a stress gene for cortical neurons in the context of focal cerebral ischemia. In contrast, this failure of an increase in Ptn gene expression contrasts with increased Ptn expression and enhanced neuronal activity after chemical seizure (Wanaka et al., 1993), suggesting that perhaps Ptn gene expression contributes to selective maintenance of neuronal viability. Because the principle activities directed by PTN in neurons in culture are neurite outgrowth and perhaps axonal guidance (Rauvala et al., 1989, 1994; Li et al., 1990; Nolo et al., 1996), it is likely that the major role of PTN in neurons is related to differentiation and thus to promoting and maintaining the differentiated state of cortical neurons.

The present study demonstrates an altered pattern of Ptn expression affecting neuronal, glial, macrophage, and endothelial cell populations in the brain after focal cerebral ischemia–reperfusion. The fact that PTN protein was expressed in parallel with its mRNA signals supports the view that Ptn expression probably plays important pathophysiological roles in the restorative processes of the brain in response to ischemic injury and that neovascularization may be the principle role of PTN in this context.

REFERENCES


