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PDGF-α Receptor Expression Following Hypoxic-Ischemic Injury in the Neonatal Rat Brain

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Hypoxia-ischemia (HI) causes injury to oligodendrocytes (OLs), cells which create the myelin sheath in the developing brain. OLs pass successively through progenitor and immature stages during differentiation into mature OLs. Only the OLs in the progenitors stage can express the platelet-derived growth factor-α receptor (PDGF-Rα) so that its expression is one of the cellular markers of OL progenitors. Activation of PDGF-Rα results in OL proliferation, but not OL differentiation. To study the response of OL progenitors after neonatal HI brain injury, we investigated the expression of PDGF-Rα in a neonatal rat stroke model (combination of left common carotid artery ligation and exposure to 8% O_2 for 2 h).

In the injured cerebral cortex, PDGF-Rα mRNA levels increased significantly (p<0.01) with a peak at 0.5 h after HI insult, and returned to baseline levels within 48 h post-injury. PDGF-Rα protein levels increased significantly at 72-96 h (p<0.05) and then returned to basal levels. Immunohistochemistry showed clear staining of PDGF-Rα only in the injured cerebral cortex at 72 h after HI insult. In contrast, no staining was observed in the cortex of sham-operated controls.

These results indicate that the expression of PDGF-Rα increases rapidly and transiently only in the injured cerebral cortex after HI insult and may play a protective role through modulating the glial differentiation under the condition of cellular damage in the developing brain.

Hypoxia-ischemia (HI) causes injury to oligodendrocytes (OLs), cells which create myelin sheaths in the developing brain, resulting in cerebral white matter lesions. Neonates who suffer from severe HI in the perinatal period subsequently develop cerebral palsy and neuro-developmental impairment (33). OLs, astrocytes, and neurons are important components of the central nervous system. They all arise from neural stem cells (31). OLs pass successively through progenitor and immature stages from the neural stem cells and finally differentiate into mature OLs expressing major myelin sheath-forming proteins such as proteolipid protein (PLP) and myelin basic protein (MBP) for rapping axons with myelin membrane (18,21,33). Some experimental results have suggested that OLs have maturation-dependent susceptibility to HI (2,3,6). Immature OLs are more vulnerable than mature OLs to oxidative stress (2) and glutamate receptor-mediated ischemic stress (6). The mechanism to control OL differentiation in the course of HI brain injury has attracted much interest.

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Platelet-derived growth factor (PDGF) is a polypeptide that regulates proliferation and differentiation in many cell types (26). PDGF consists of two homodimers (AA and BB) and one heterodimer (AB), and each of them binds to α or β receptor on the cell membrane. PDGF-AA (PDGF-A chain) binds only to the PDGF-α receptor (PDGF-Rα), whereas PDGF-BB binds to the PDGF-α and -β receptors (11,12,14,28). Binding of PDGF to its receptor results in the activation of intracellular signal pathways (14) and plays important roles in the developing brain.

PDGF-Rα is detected both in neurons and OLs, however, in the immature brain the developing neurons faintly express PDGF-Rα (20). Most of cells that express PDGF-Rα in this stage are the OL progenitor cells, including oligodendrocyte-type 2 astrocyte (O-2A) progenitors (8,10,18,22), and PDGF-Rα is considered as an established marker for OL progenitors (24,29). Expression of this receptor enables OL progenitors to respond to PDGF-A chain and proliferate by themselves (19,21,23,29). Knockout mice of PDGF-A chain have severe hypo-myelination (8), and PDGF is thought to be one of the survival factors for newly formed OL progenitor cells (4). Activation of PDGF-Rα by PDGF-A chain regulates OL differentiation, not to proceed toward the mature OLs.

HI causes injury to OLs more easily in the developing brain (5,15,30). However, little is known about the response and role of PDGF-Rα expressing OL progenitor cells in HI brain injury. We speculate that the PDGF system plays an important role in OL protection through inhibitory regulation of OL differentiation. We investigated the expression of PDGF-Rα in the widely accepted neonatal rat stroke model, and found that the expression of PDGF-Rα in the developing rat cerebral cortex increased rapidly and transiently after HI insult, consistent with a relative increase in OL progenitor population. This phenomenon may reflect repair or survival process for OLs through inhibitory regulation of OL differentiation in the developing brain.

MATERIALS AND METHODS

Animals and Materials

Sprague-Dawley (SD) rat pups with their dams were purchased from Charles River Laboratories (Yokohama, Japan). The pups were unsexed, weighed 14-19 g on postnatal day (P) 7. All animal experiments were performed in compliance with the standards of animal care and housing according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine.

cDNAs for rat PDGF-A chain, PDGF-Rα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were produced via RT-PCR and subcloned into pGEM-T vectors (Promega, Madison, WI). cDNA fragments were first isolated from the plasmids using appropriate restriction enzymes, and then used as probes for Northern blot analysis. Rabbit polyclonal anti-PDGF-Rα (sc-338) and polyclonal anti-β-actin (sc-7210) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Hypoxic-Ischemic Brain Damage Model

The HI model we used was originally described by Rice et al. (25) with minor modifications. Each P7 rat pup was lightly anesthetized by diethyl-ether inhalation. The left carotid artery was isolated surgically, double-ligated, and severed. After closure of the neck incision, pups were allowed to recover for 30 min in a plastic chamber placed in a 37.5˚C water bath, and then returned to their dam for at least 1.5 h. The pups were then distributed randomly into the HI or sham-operated (control) group. Pups in the HI group were placed into a plastic chamber in a 37.5˚C water bath in order to maintain appropriate rectal temperature and then exposed to 8% O_2 hypoxia for 2 h. Rectal temperature was monitored

(Digital Thermometer, Unique Medical, Tokyo, Japan) and kept at 37.0 ± 0.5˚C during the procedure. After the hypoxic exposure, pups were allowed to recover in the same chamber under room-air for up to 1 h before being returned to their dams. No hypoxic condition was imposed on the sham-operated control group. At designated times after HI insult, the pups were decapitated under deep diethyl-ether inhalation anesthesia, and brains were removed immediately. Cerebral cortex including hippocampus was dissected out from the striatum, weighed wet, and stored at –80˚C until use.

Northern Blot Analysis

Left cerebral cortex was homogenized in GIT buffer [4 M guanidine thiocyanate, 25 mM sodium acetate (pH 6.0), 0.8% 2-mercaptoethanol, 0.5% N-lauroylsalcocile] using a tissue homogenizer. The homogenate was then ultracentrifuged with a 5.7 M CsCl cushion at 20˚C for at least 16 h. Total RNA fraction was collected, dissolved in diethylpyrocarbonate–treated water, and stored at –80˚C until use.

Twenty micrograms of total RNA was size-fractionated via electrophoresis on a 1% agarose gel containing 1.8% formaldehyde and 0.1 µg/ml ethidium bromide. Equal loading of RNA was confirmed by visual inspection of the stained ribosomal RNA under ultraviolet illumination. Total RNA on the gel was transferred to a nylon membrane in 10XSSC (1XSSC: 0.15 M NaCl and 0.015 M sodium citrate) by capillary action. Prehybridization and hybridization were performed using the ExpressHyb™ hybridization solution (Clontech, Palo Alto, CA) according to the manufacturer's instructions. cDNAs were labeled with [α-^{32}P]dCTP by means of the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). The hybridized membranes were washed twice in 2XSSC with 0.05% SDS at room temperature for 10 min and then twice in 0.1XSSC with 0.1% SDS at 50˚C for 20 min before exposure to an X-ray film with an intensifying screen at –80˚C. GAPDH, a housekeeping gene, served as a RNA loading control. Quantification of the corresponding bands on the autoradiograph was performed by means of scanning densitometry.

Western Blot Analysis

Left cerebral cortex was lysed in 10Xvolume of the lysis buffer [0.02 M Tris-HCl (pH 7.5), 0.04 M EDTA, 1% Triton X-100, 1 mM PMSF] and homogenized by shearing using a 23-guage needle. The homogenate was then centrifuged at 12,000x *g* for 30 min at 4˚C, and the supernatant was stored at –20˚C until use. Protein concentrations were determined using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA).

Seventy micrograms of supernatant protein was mixed with an equal volume of sample buffer [2% SDS, 2% 2-mercaptoethanol, 20% glycerol, 20 mM Tris-HCl (pH 6.8), 0.02% bromophenol blue]. After heat treatment at 95˚C for 2 min, samples were separated electrophoretically on a 7.5 or 15% polyacrylamide gel, and then transferred to polyvinylidine difluoride membranes (Immobilon™, Millipore, Bedford, MA) with a semidry transfer device (ATTO, Tokyo, Japan). Membranes were exposed to either a dilution of 1:7,000 rabbit polyclonal anti-human PDGF-Rα antibody or to a dilution of 1:2,500 rabbit polyclonal anti-human β-actin antibody in TBST [0.05 M Tris-HCl (pH 7.6), 0.3 M NaCl, 0.1% Tween 20] for 1 h at room temperature. After washing, the membranes were incubated with a dilution of 1:5,000 peroxidase-conjugated, swine anti-rabbit immunoglobulins (DAKO A/S, Denmark) in TBST for 1 h at room temperature. Immuno-complexes on the membranes were visualized using an ECL chemiluminescence system (Amersham Pharmacia Biotech) and quantified by means of scanning densitometry.

Immunohistochemistry

At designated times after HI insult, each rat pup was perfusion-fixed with 4% paraformaldehyde in PBS. Coronal sections at the level of the hippocampus were prepared

from the paraffin-embedded samples.

Immunohistochemical staining for the PDGF-Rα was performed using the Avidin-Biotin-Peroxidase Complex Method (Vectastatin® ABC kit, Vector Laboratories, Burlingame, CA). Tissue sections on slides, treated according to the manufacturer's instructions, were incubated with primary antibody: 1:200 dilution of rabbit polyclonal anti-PDGF-Rα antibody in TBST for 30 min at room temperature. After careful washing, the sections were incubated with biotinylated goat anti-rabbit IgG for 30 min. Finally, the sections were incubated with streptoavidin-biotin peroxidase and the peroxidase activity was visualized by application of diaminobenzidine solution (Vector Laboratories, Burlingame, CA). The sections were counterstained with Carrazzi's hematoxylin solution (WAKO, Osaka, Japan).

Statistical Analysis

All data were presented as mean ± SD values for each group. The autoradiograph data of Northern and Western analyses were compared between the HI and the sham-operated control group. Statistical analysis was performed with the *Mann-Whitney* nonparametric rank test, and statistical significance was accepted at $p<0.05$.

RESULTS

Ontogeny of the PDGF-Rα Gene in Developing Rat Cerebral Cortex

Northern blot analysis for PDGF-Rα, observed as a single band at 6.8 kb, in cerebral cortices from rats ranging in age from newborn to adult is shown in Fig. 1. PDGF-Rα mRNA was highly expressed at P0, peaked at P10, and decreased gradually to adult levels (approximately 60% of P10 levels).

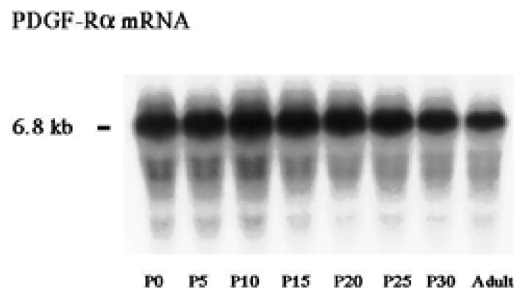

Fig. 1. Ontogeny of the PDGF-Rα gene in the developing rat cerebral cortex. Total RNA (20 μg) extracted from the cerebral cortex of rats on the indicted postnatal day was subjected to Northern blot analysis using PDGF-Rα probes. The number below each lane indicates the postnatal age of rats in days. P0, postnatal day 0.

PDGF-A Chain and PDGF-Rα mRNA Levels in the Cerebral Cortex after HI Injury

Northern blot analyses for PDGF-A chain (Fig. 2) and PDGF-Rα (Fig. 3) in the cerebral cortex after HI insult and in sham-operated controls are presented. No change was observed in PDGF-A chain mRNA levels after HI injury (Fig. 2). A statistically significant increase in PDGF-Rα mRNA levels was observed 0.5 h after HI insult as compared to levels of sham-operated controls ($p<0.01$, $n=5$ for each group). PDGF-Rα mRNA decreased progressively for 12 h after HI insult ($p<0.01$), and then increased gradually to the control levels at 24-48 h (Fig. 3).

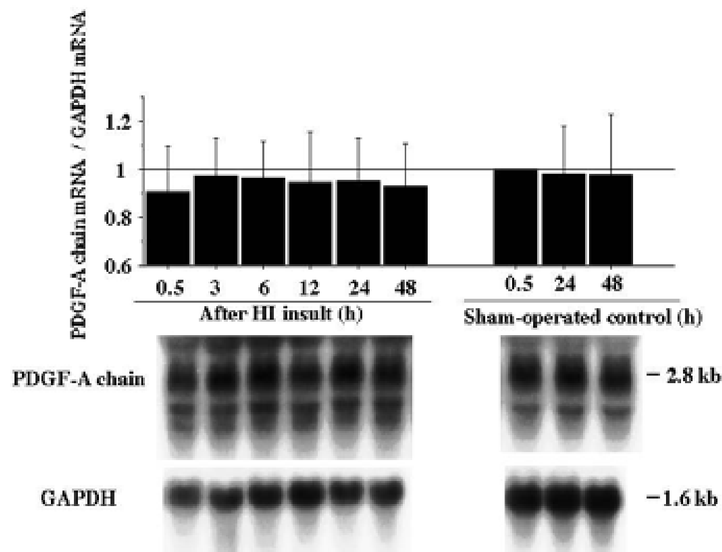

Fig. 2. PDGF-A chain mRNA levels after HI injury.
Total RNA (20 µg) was extracted from cerebral cortex of rat pups 0.5, 3, 6, 12, 24, and 48 h after HI insult (n=5 for each time point), and sham-operated controls 0.5, 24, and 48 h after surgery (n=5 for each time point). The relative abundance of PDGF-A chain mRNA was standardized to GAPDH levels for both groups and then expressed as fold change from sham-operated controls at 0.5 h.

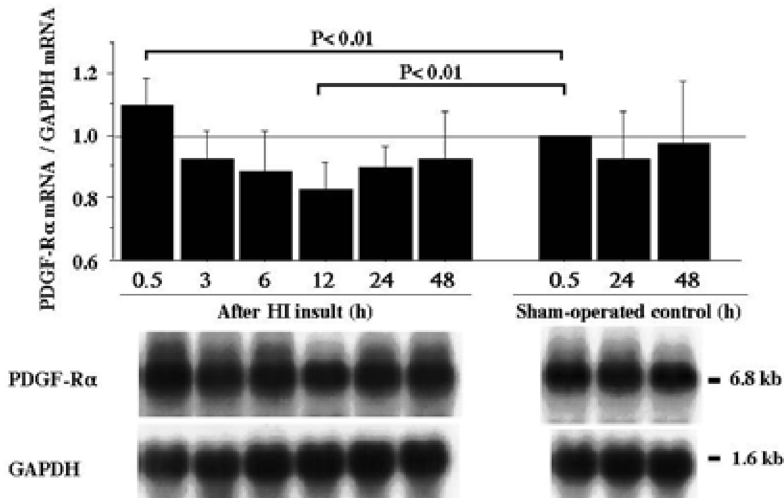

Fig. 3. PDGF-Rα mRNA levels after HI injury.
The identical samples used in Fig. 2 were subjected to Northern blot analysis for PDGF-Rα. The relative abundance of PDGF-Rα mRNA was standardized to GAPDH levels for both groups and then expressed as fold change from sham-operated controls at 0.5 h.

PDGF-Rα Protein Levels in the Cerebral Cortex after HI Injury

Western blot analyses for PDGF-Rα protein, detected as a single band at 175 kD, in the cerebral cortex after HI injury and in sham-operated controls are shown in Fig. 4. Protein levels increased gradually after HI insult to a statistically significant peak levels by 72 to 96 h ($p<0.05$, n=5), thereafter, returned to control levels after 120 h.

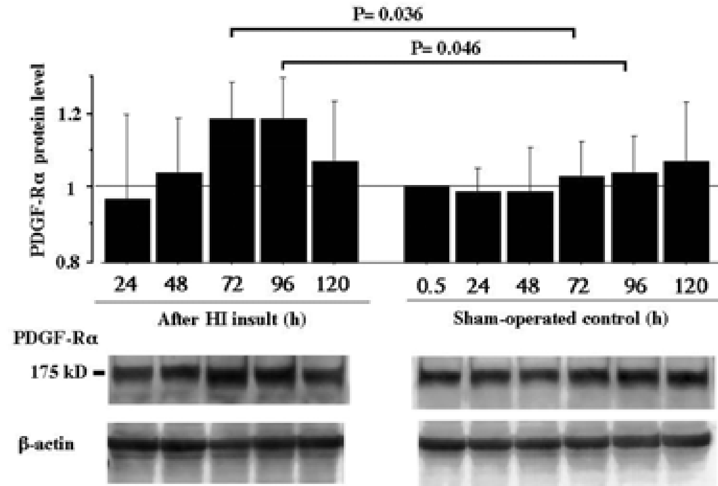

Fig. 4. PDGF-Rα protein levels after HI injury.
Total protein (70 μg) was extracted from cerebral cortex of rat pups 24, 48, 72, 96, and 120 h after HI insult (n=5 for each time point), and at 0.5, 24, 48, 72, 96, and 120 h after surgery from sham-operated controls (n=5 for each time point). Data were expressed as fold change from protein levels of sham-operated controls at 0.5 h. β-actin served as a loading control.

PDGF-Rα Immunostaining after HI Injury

Immunohistochemical staining for PDGF-Rα in the cerebral cortex and striatum 72 h after HI insult are shown in Fig. 5. Immunopositive granular cells were clearly observed in the injured areas of the cerebral cortex (Fig. 5A), but not in the striatum (Fig. 5B). As expected, immunopositive cells were not observed in the cortex or striatum of sham-operated controls (Fig. 5C, D) nor in the right, non-ligated side of the brain (data not shown). In addition, when immunostaining was performed without the PDGF-Rα primary antibody, no immunopositivity was observed (data not shown).

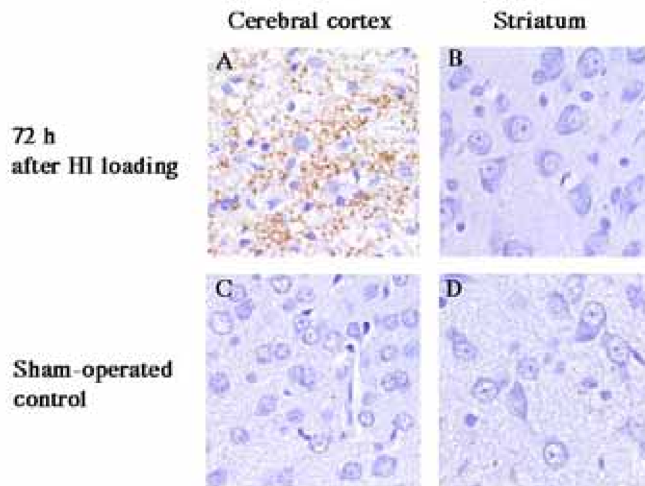

Fig. 5. Immunohistochemical staining for PDGF-Rα.
Coronal sections of the left, ligated side of cerebral cortex and striatum 72 h after HI insult and in sham-operated controls were stained for PDGF-Rα. A: cerebral cortex after HI injury, B: striatum after HI injury, C: cerebral cortex of the sham-operated control, D: striatum of the sham-operated control. Immunopositive granular cells were clearly observed in the damaged areas of the cerebral cortex (A), but not in the striatum (B). Magnification at 400X.

DISCUSSION

OLs are thought to be very vulnerable in the developing period and their damage easily causes white matter lesions leading to leukomalacia. Recently, repair processes of myelin sheath have been studied in relation with the differentiation of OL lineage. OLs have the capability of cellular repair mechanisms after trauma, virus-induced demyelination, and ischemic damage in adult mouse brain (13,16,17). The appearance of immature OLs derived from OL progenitors, may contribute to myelin repair after demyelination induced by laser irradiation in adult rat spinal cord (27). Myelin repair processes have been reported to arise from a number of bioactive factors. PDGF-A chain leads to myelin sheath repair after demyelination induced by lysophosphatidylcholine *in vitro* (7) and *in vivo* (1). PDGF-A chain knockout mice have defective OL development and show severe hypomyelination (8), and therefore, PDGF-A chain may be one of the important factors for myelin repair. However, in the developing brain, the repair processes for OLs after brain injury has not been fully elucidated. We investigated the expression of PDGF-A chain mRNA and protein level in the injured cerebral cortex, and found no changes in their expression after HI injury (Fig. 2, protein level; data not shown).

PDGF-Rα mRNA was highly expressed abundantly in the early period after birth, the timing at which OLs initiate to differentiate into more mature OLs. The levels of PDGF-Rα mRNA and protein increased significantly after HI injury in the developing rat cortex. Increases in PDGF-Rα message were very rapid following injury; however, all measurements were performed following 2-h exposure to hypoxia after ligation of the carotid

artery. Induction of PDGF-Rα mRNA may have been caused by hypoxia consistent with a transcription time of 2-3 h. Or, the levels decreased rapidly reaching bottom at 12 h after insult, suggesting in part that there exists a post-transcriptional mechanism to increase the PDGF-Rα message level.

The increase of OL progenitors which express PDGF-Rα after brain injury, may play a crucial role in the repair processes of the developing brain (29). Groves et al. (9) showed that demyelinated lesions in the spinal cords of adult rats were repaired after transplantation of O-2A progenitors. Furthermore, Tontsch et al. (32) reported that transplantation of OL progenitors leads to extensive myelination in the brains of normal newborn rats and in the spinal cord of the myelin-deficient newborn rat. It has been shown recently that OLs have a maturation-dependent vulnerability to HI (2,3,6). We propose that OL progenitors, which are less mature than the immature OLs, proliferate after HI injury and then start to differentiate when the repair process has begun.

One of the neuro-pathological patterns of human hypoxic-ischemic encephalopathy, "status marmoratus", results from neuronal necrosis, gliosis, and 'hypermyelination' in basal ganglia, thalamus, and cerebral cortex. In injured areas of cortex, we observed earlier appearance of PLP immunostainings than in non-injured control cortex (in preparation). This finding is never observed in the normal developing brain, and may be a part of "hypermyelination" phenomena in the developing brain after HI injury.

Our results also indicate that not all parts of the developing brain show the same response after HI injury. PDGF-Rα was expressed in the cerebral cortex, but not in the striatum and external capsule 72 h after HI insult, although it has been reported that some restoration of mature OLs occurs in the striatum and external capsule in a neonatal HI brain damage model (hypoxia for 1.5 h) following a 2-week recovery period (15). This difference would reflect the degree of HI and the recovery time after HI. The myelin repair can occur after mild to moderate HI, but not after more severe HI.

Whether all OL progenitor cells that express PDGF-Rα differentiate only to mature OLs is still of concern. It has been demonstrated that all PDGF-Rα-positive cells differentiate to mature OLs when cultured under appropriate conditions *in vitro* (10). However, OL progenitors can differentiate into not only OLs, but also type 2 astrocytes. Astrocytosis does occur after HI brain injury and may also be reflected by an increase in OL progenitor cells. The mechanism of which OL progenitors expressing PDGF-Rα form mature OLs or type 2 astrocytes needs to be further studied.

In conclusion, we demonstrated a relative increase in PDGF-Rα expression in the developing rat cortex following HI injury. This finding suggests that the survival and repair processes for OLs are regulated through the differentiation of OL progenitor cells after HI injury in the developing rat brain. There is a possibility that more mature OLs have capability to return to the more immature stages of OLs (to the OL progenitors) to raise the threshold against cellular damage through the PDGF-Rα system, which is one of the crucial factors to control OL differentiation under brain damage. The exact mechanism of this regulation needs to be further elucidated.

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