

# Regulation of nitric oxide generation by up-regulated arginase I in rat spinal cord injury

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Recently, arginase is suggested to regulate nitric oxide production by competing with nitric oxide synthase for the same substrate, L-arginine, in experimental asthma. We investigated the role of arginase and its relationship to nitric oxide production after spinal cord injury. Rats were subjected to laminectomy and complete transection of their spinal cords (injury group) or laminectomy only (sham group). In the injury group, arginase I was increased in the macrophages at the transection edge, and the peak was observed 48 h after spinal cord injury. However, nitric oxide production decreased significantly in the injury group despite increased nitric oxide synthase2 mRNA expression compared with the sham group. We also demonstrated the reduction in L-arginine concentrations, which was inversely associated with changes in arginase activity. Therefore, arginase appeared to regulate nitric oxide production by consuming L-arginine. The regulation of arginase activity and L-arginine levels may improve nitroxidative stress and reduce tissue damage in spinal cord injury.

**Key Words:** nitric oxide, arginase, L-arginine, nitric oxide synthase, spinal cord injury

Neuronal regeneration is extremely limited in a variety of neurological disorders of the central nervous system (CNS). Spinal cord injury generally causes tissue damage through both primary and secondary mechanisms.<sup>(1)</sup> The primary injury, such as contusion to the spinal cord, results in the direct damage of neurons, astrocytes, oligodendrocytes and endothelial cells. The secondary injury leads to biochemical and pathological changes in the spinal cord. Neurological dysfunction is influenced by the degree of secondary damage including glutamate excitotoxicity,<sup>(2)</sup> edema,<sup>(3)</sup> ischemia,<sup>(4)</sup> Ca<sup>2+</sup> overload,<sup>(5,6)</sup> reactive oxygen species (ROS)<sup>(7-9)</sup> and nitric oxide (NO).<sup>(10-12)</sup>

NO has been shown to play important but contradictory physiological and pathological roles in CNS. Generally, NO acts as a neurotransmitter and a vasodilator at physiologically moderate levels.<sup>(13)</sup> However, a relatively large amount of NO which is mainly produced by nitric oxide synthase I (NOS1) and NOS2 is neurotoxic.<sup>(14,15)</sup> Moreover, the superoxide anion radical (O<sub>2</sub><sup>-</sup>), which is released from the infiltrated mononuclear cells and following intracellular generation by Nox or Duox easily reacts with NO by induced NOS, and then reactive nitrogen species (RNS) such as peroxynitrite is produced.<sup>(16,17)</sup> In turn, they oxidize sulfhydryl groups, DNA, lipids and proteins causing severe tissue damage.<sup>(18,19)</sup> Therefore, reducing the production of ROS and RNS is prerequisite for the prevention or therapy of spinal cord injury.

Arginase I and II are major metabolic enzymes in the liver and

kidney, and participate in the urea cycle to convert L-arginine to urea and L-citrulline.<sup>(20)</sup> In spinal cord neurons of mice, arginase I and II are expressed as key enzymes for probable pathway source from L-arginine to ornithine, glutamate and gamma-aminobutyric acid.<sup>(21)</sup> High levels of arginase I expression have been detected in neurons and macrophages after hemi-transected spinal cord injury.<sup>(22)</sup> Recently, many researchers have investigated the roles of arginase I and L-arginine homeostasis in airway inflammation and hyperresponsiveness in experimental asthma models and asthmatic patients.<sup>(23-27)</sup> These studies have shown that highly expressed arginase deprives L-arginine of NOS, and results in loss of NO and relaxation of bronchial smooth muscle in experimental asthma. However, in spinal cord injury, the relationship between NO and arginase is unclear.

Recently, the transplantation of biomaterials, such as various extracellular matrices, has been reported for the treatment of spinal cord injury. We previously reported that, after damaged spinal cord tissue was completely resected, reactive astrocytes invaded the collagen filament of amputation stump and released neurotrophic factors such as brain-derived neurotrophic factor and neurotrophin 3.<sup>(28)</sup> Precise elucidation of the tissue environment for axonal growth, including the presence of nitroxidative stress, at the site of the transection injury is of great importance.

Therefore, the aim of this study was to provide insight into the tissue environment by investigating the regulation of NO generation by arginase following spinal cord transected injury in rats.

## Materials and Methods

**Animals and groups.** Adult female Wistar rats (Charles River Japan, Inc., Yokohama, Japan) weighing 200–220 g were used. Animals were randomly assigned to two groups: the sham group (laminectomy only, *n* = 9) and the injury group (laminectomy + spinal cord injury, *n* = 9).

**Surgical procedures.** For all procedures, the rats were anesthetized with an intramuscular injection of a mixture of ketamine (64 mg/kg) and xylazine (4.5 mg/kg). The surgical instruments were sterilized, and the animals were kept on a heating pad during surgery. The rectal temperature was monitored and maintained at a normal temperature throughout the surgery. The laminectomy was performed at the Th9 vertebral level, and the dorsal aspect of the dura was exposed. In the injury group, the spinal cord was then completely transected with a fine microcutter and the wound was

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closed. In the sham group, rats underwent laminectomy without transection injury. After surgery, the animals were caged in a warm environment and given sufficient water and feed. Manual compression was performed twice daily to empty the bladder until preparation. All animal care and procedures were performed according to the NIH guidelines; this study was approved by Okayama University School of Medicine Animal Committee.

**Spinal cord tissue collection.** Animals were sacrificed for collecting spinal cord tissue at the indicated times after surgery. Rats were anesthetized and perfused through the left ventricle with 100 ml of normal ice-cold saline. In the injury group, a 1-cm-long spinal cord segment (0.5 cm rostral and 0.5 cm caudal from the injury site) containing the injury epicenter was removed from each rat. In the sham group, a corresponding 1-cm-long segment of the spinal cord was removed.

**NOx measurement.** The generation of NO in the spinal cord was expressed as NOx, nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>). Homogenates (25% w/v) of the spinal cord tissue were prepared using a Teflon homogenizer in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA at 4°C. The homogenates were then centrifuged at 12,000 × g for 20 min at 4°C. Nitrate reductase (0.66 U/ml) from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO) and NADPH (6.6 μM) were added to the supernatant to convert NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup>. After incubation at room temperature for 30 min, the protein was removed by adding acetonitrile followed by centrifugation. NO<sub>2</sub><sup>-</sup> was further reduced to NO in a PurgeVessel containing the reducing agent potassium iodide in acetic acid, and NO was subsequently detected by the ozone-chemiluminescence method using an NO analyzer 280i (Sievers, Boulder, CO).<sup>(29)</sup>

**Western blot analysis.** The western blot analysis was performed as previously reported.<sup>(30)</sup> Homogenates were prepared in 20 mM of Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and a protease inhibitor cocktail (Complete Mini Tablets from Roche, Basel, Schweiz) at 4°C with a Teflon homogenizer. The samples were dissolved in 2% SDS and 6% β-mercaptoethanol solution by boiling for 5 min followed by centrifugation at 12,000 × g for 20 min. A total of 50 μg of protein from the supernatant of each sample was separated using SDS/PAGE in a 10% polyacrylamide gel and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.7) for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the following primary antibodies: polyclonal rabbit anti-arginase I and arginase II (1:500; Santa Cruz Biochemistry, Santa Cruz, CA), and monoclonal mouse anti-nitrotyrosine (1:500; Upstate, New York, NY). The membranes were washed 3 times with TBS-T for 5 min each and then incubated for 1 h at room temperature with the following secondary antibodies conjugated with horseradish peroxidase (DAKO, Tokyo, Japan): anti-rabbit for arginase I and II (1:2000 dilution), and anti-mouse for nitrotyrosine (1:1500). After washing 3 times for 10 min each with TBS-T, a chemiluminescent marker (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer Life Sciences Inc., Boston, MA) was used for detection. The results were compared as relative values using β-actin as an internal reference. Autoradiographs were analyzed densitometrically using image analysis software (Scion Image program, Scion Corp., Frederick, MD).

**Arginase activity assay.** Fifty microliters of 10 mM MnCl<sub>2</sub> in 50 mM Tris-HCl (pH 7.5) was incubated with 50 μl of tissue homogenate at 55°C for 10 min. Twenty-five microliters was then transferred into Eppendorf tubes and incubated in the presence of 25 μl of 0.5 M arginine (pH 9.7) at 37°C for 1 h. The reaction was stopped by the addition of 400 μl of an acid mixture containing H<sub>2</sub>SO<sub>4</sub>, H<sub>3</sub>PO<sub>4</sub> and H<sub>2</sub>O (H<sub>2</sub>SO<sub>4</sub>:H<sub>3</sub>PO<sub>4</sub>:H<sub>2</sub>O = 1:3:7 volumes). Isonitrosopropiophenone at a concentration of 9% (dissolved in 100% ethanol) was added, and the mixture was heated at 100°C

for 45 min. The amount of urea formed was determined spectrophotometrically at 540 nm. The activity of arginase was expressed as UV units per min per mg protein.<sup>(31)</sup>

**Measurement of L-arginine concentration by high performance liquid chromatography (HPLC).** The concentration of L-arginine in the spinal cord was quantified by fluorescence HPLC system (HITACHI, Tokyo, Japan), as previously described.<sup>(32)</sup> Briefly, L-arginine was extracted from homogenates using Oasis MCX solid phase-extraction cartridges (Waters, Milford, MA). First, the column was conditioned with 2 ml of methanol/water/ammonia solution (50:45:5, vol/vol/vol) and phosphate buffer saline (PBS). The tissue sample was then passed through the SPE column, and the column was washed with 2 ml of 0.1 M HCl and methanol. The fraction containing arginine was collected with 1 ml of methanol/water/ammonia solution (50:45:5, vol/vol/vol) and dried in a vacuum centrifuge. After the drying process, the residue was reconstituted with water and mixed with an equal amount of derivatization solution (5 mg/ml ortho-phthalaldehyde, 10% methanol, 0.5% 3-mercaptopropionic acid in 200 mM borate buffer, pH 8.5), and the reaction was allowed to occur for 30 min at room temperature. The sample was introduced into the fluorescence HPLC system using a TSKgel ODS-100V column (4.6 × 250 mm, 5 μm, Tosoh, Yamaguchi, Japan). The mobile phase consisted of 9% acetonitrile in acetate buffer (pH 6.3) at a flow rate of 1.5 ml/min, and the excitation and emission wavelengths were 340 and 455 nm, respectively. After each measurement, 100% acetonitrile was allowed to flow in to completely eliminate any wastes remaining in the column.

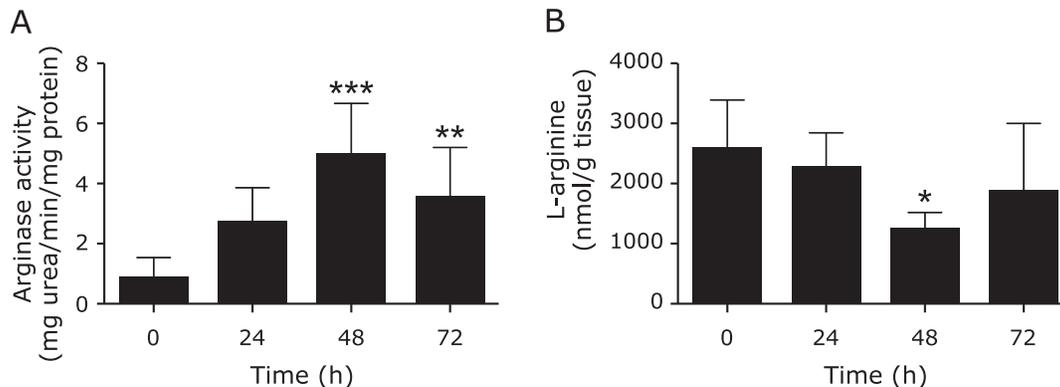
**Immunohistochemistry.** Rats were anesthetized and perfused intracardially with 100 ml of 0.01 M PBS (pH 7.4) followed by 200 ml of 4% paraformaldehyde. The spinal cord was carefully extracted, and a 1 cm segment containing the injury epicenter was post-fixed in 4% paraformaldehyde for 6 h before cryo-protection in 20% sucrose in 0.01 M PBS overnight. The spinal cord segment was embedded in tissue freezing medium and cut into 8 μm sagittal sections using a cryostat. The sections were then collected on silanized slides (Dako). For immunostaining, frozen sections were incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min and washed with 0.01 M PBS three times for 5 min each. The sections were incubated in 5% normal goat serum. The specimens were incubated overnight at 4°C with polyclonal antibodies against arginase I (1:100) (Santa Cruz Biochemistry). Then, specimens were treated with goat anti-rabbit immunoglobulin conjugated with peroxidase labeled-dextran polymer (Dako) for 1 h at room temperature. Visualization was performed with 3,3-diaminobenzidine tetrahydrochloride (Dako) as a substrate. As a negative control, rabbit non-immune immunoglobulin (Dako) was used. Finally, the sections were counterstained with hematoxylin.

Also, to study the possible cellular colocalization of arginase, a double immunofluorescence assay for arginase and ED-1 was performed. ED-1 is widely used as a marker for macrophages. Frozen section were incubated overnight at 4°C with polyclonal rabbit anti-arginase (1:50) and monoclonal mouse anti-ED-1 (1:200) (Santa Cruz Biochemistry). The secondary antibodies were Texas red-conjugated anti-rabbit (1:200) (TAKARA BIO, Shiga, Japan) and FITC-conjugated anti-mouse (1:200) (Santa Cruz Biochemistry) antibodies. Images were obtained using a confocal laser-scanning microscope.

**Reverse transcription-PCR (RT-PCR) for NOS isoforms and cytokines.** Total RNA from spinal cord tissue was isolated by ISOGEN with modifications based on the manufacturer's instructions for the strict collection of RNA. Briefly, the RNA pellet was dissolved again in ISOGEN then washed twice in chloroform to remove contaminating DNA and proteins thoroughly. The RNA samples were stored at -80°C until further analysis. Reverse transcription and PCR was performed using the TaKaRa RNA PCR Kit (AMV) ver. 3.0 (TAKARA BIO) with oligo-dT primers according to the manufacturer's instructions. The primer sets and

**Table 1.** List of primers and RT-PCR conditions

Target gene	Sense	Antisense	Denaturation	Amplification	Cycle	Elongation
NOS1	AGAACGGGGAGAAATTCG	CGCAGAACACATCACAG	94°C, 3 min	94°C, 60 s 56°C, 60 s 72°C, 60 s	32	72°C, 5 min
NOS2	ATGGCTTGCCCTGGAAGTTTCTC	CCTCTGATGGTGCATCGGGCATC	94°C, 3 min	94°C, 45 s 60°C, 45 s 72°C, 90 s	31	72°C, 5 min
NOS3	GGGCTCCCTCTCCGGCTGCCACC	GGATCCCTGAAAAGCGGTGAGG	94°C, 3 min	94°C, 45 s 64°C, 45 s 72°C, 90 s	33	72°C, 5 min
IL-4	CTGCTTTCATATGTACCGGG	TTCAGTGTGTGAGCGTGG	94°C, 3 min	94°C, 60 s 61°C, 45 s 72°C, 75 s	40	72°C, 7 min
IL-13	CATCACACAAGACCAGAAGACTTCC	GGATGGCATTGCAACTGGAG	94°C, 3 min	94°C, 60 s 61°C, 45 s 72°C, 75 s	40	72°C, 7 min
TGF- $\beta$	CTTCAGCTCCACAGAGAAGAAGTGC	CACGATCATGTTGGACAAGTCTCC	94°C, 3 min	94°C, 60 s 54°C, 60 s 72°C, 45 s	35	72°C, 5 min
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA	94°C, 3 min	94°C, 45 s 60°C, 45 s 72°C, 60 s	30	72°C, 5 min



**Fig. 1.** Time-course of changes in arginase activity and L-arginine. In the injury group, arginase activity increased gradually, and was significantly higher at 48 and 72 h than at 0 h (A). L-arginine was extracted from the spinal cord homogenate using Oasis MCX SPE columns, and derivatized with OPA for the fluorescence HPLC system. Reduction in L-arginine concentration was observed at 48 h (B). Data are expressed as mean  $\pm$  SD ( $n = 9$  animals per group). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs 0 h.

PCR conditions in this study are detailed in Table 1. PCR was performed using a Thermal Cycler MP (TAKARA BIO) under optimal thermal conditions specific for each primer set. Electrophoresis was performed using a 2% agarose gel containing 0.5 mg/ml of ethidium bromide. The PCR bands were visualized using an UV transilluminator. The expression of each mRNA was analyzed using the Scion Image program (Scion Corp., Frederick, MD). The results were compared as the relative values using GAPDH as an internal reference.

**Statistical analysis.** Data were expressed as the mean  $\pm$  SD. Differences were analyzed using un-paired  $t$  test or ANOVA with post hoc test.  $p$  values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 5.0c for Mac (GraphPad Software, Inc., San Diego, CA).

## Results

**Arginase activity.** The time-course changes in arginase activity following spinal cord transection injury were investigated. The arginase activity increased gradually after the spinal cord injury. This increase was significant at 48 and 72 h after spinal cord injury compared with that at 0 h ( $p < 0.001$  at 48 h,  $p < 0.01$  at 72 h) (Fig. 1A). Therefore, our investigation was mainly conducted at 48 h after spinal cord injury.

**Concentration of L-arginine in the tissue.** Contrary to arginase activity, the content of L-arginine was gradually reduced in the injury group, and the reduction reached statistical significance at 48 h ( $p < 0.05$ ) (Fig. 1B).

**Western blot analysis for arginase.** The expressions of arginase I and II protein were determined by Western blot analysis. Significant increase of arginase I was observed in the injury group at 48 h compared with low expression in the sham group ( $p < 0.05$ ).

In contrast, arginase II was constitutively expressed in the sham group. At 48 h, expression of arginase II was down-regulated in the injury group ( $p < 0.01$ ) (Fig. 2).

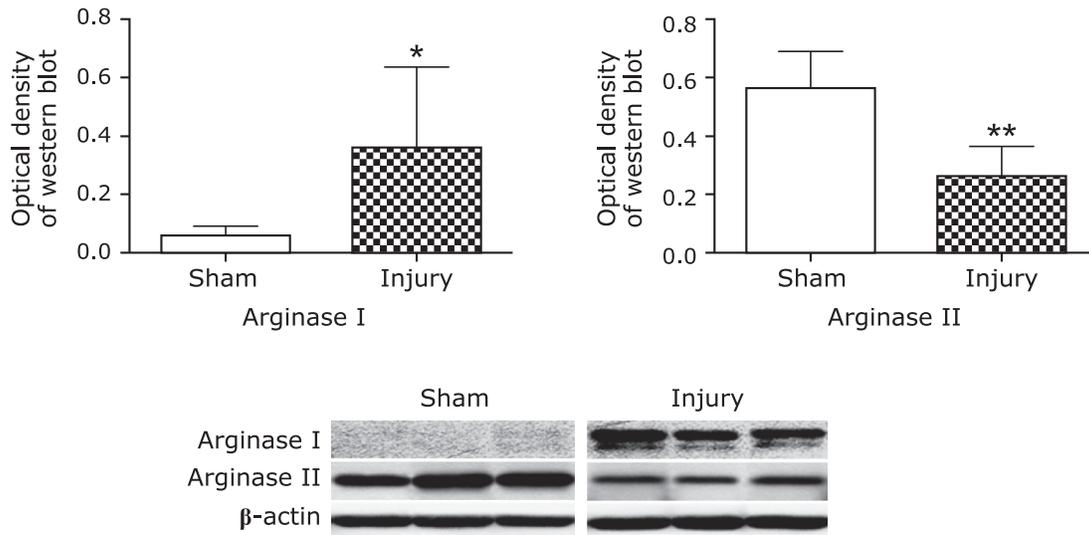
**Expression of mRNA for NOS isoforms.** The mRNA expression of NOS isoforms at 48 h compared with that in the sham group, and the time course of changes in the expression of NOS2 mRNA in the injury group were analyzed by RT-PCR. NOS2 mRNA expression was significantly increased at 48 h compared with that in the sham group ( $p < 0.001$ ). Whereas, no increase in NOS1 and NOS3 mRNA expression level was observed at 48 h (Fig. 3A). Significant increase in the time course of NOS2 mRNA was observed at 24 and 48 h after spinal cord injury ( $p < 0.001$ ) (Fig. 3B).

**NOx measurement.** At 48 h, significant decrease in NOx contents was observed in the injury group compared with that in the sham group ( $p < 0.05$ ) (Fig. 3C).

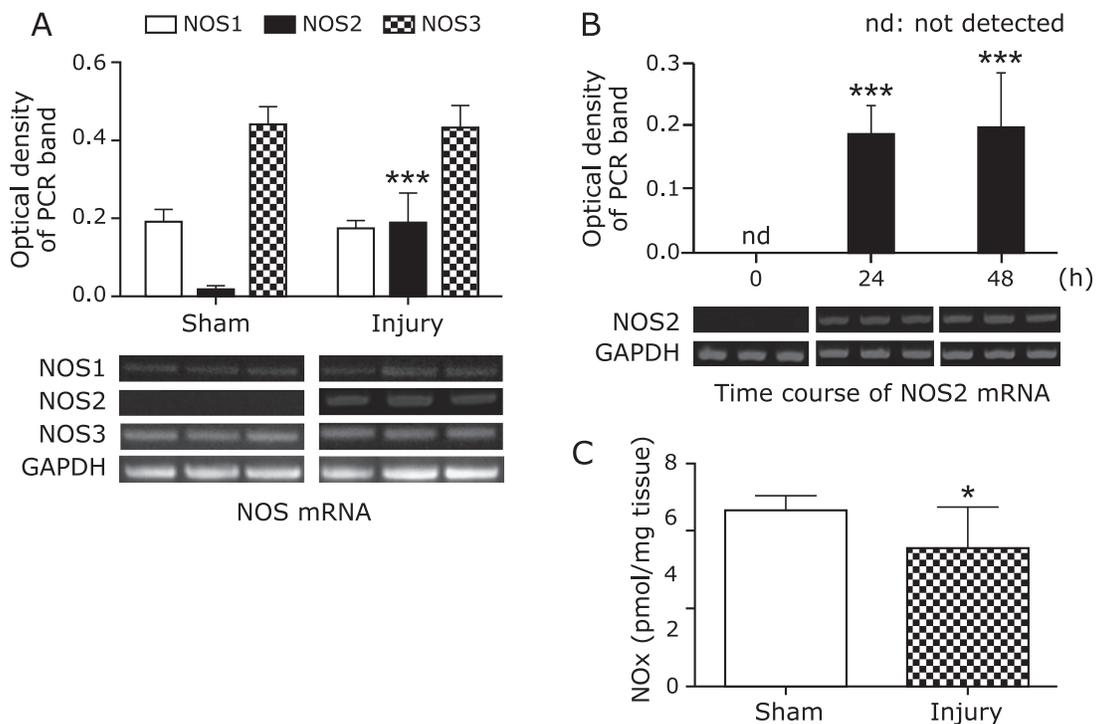
**Western blot analysis for nitrotyrosine expression.** Tyrosine nitrated proteins were detected in the injury group by Western blotting. The bands of low molecular weight range were clearer than that of high molecular weight range. In contrast, in the sham group, nitrotyrosine expression was not detected. Marked increase was observed in the injury group ( $p < 0.05$ ) (Fig. 4).

**Expression of cytokine mRNA.** To investigate the mechanism of arginase induction, mRNA expressions of several cytokines reported to regulate arginase activity were analyzed using RT-PCR for IL-4, IL-13 and TGF- $\beta$ . At 48 h, no significant increase in mRNA expression level of IL-4 or IL-13 was observed. In contrast, mRNA expression of TGF- $\beta$  was markedly elevated at 48 h ( $p < 0.01$ ) (Fig. 5).

**Localization of arginase by immunohistochemistry.** The localization of arginase was investigated by immunohistochemistry. A marked increase in the density of arginase I positive



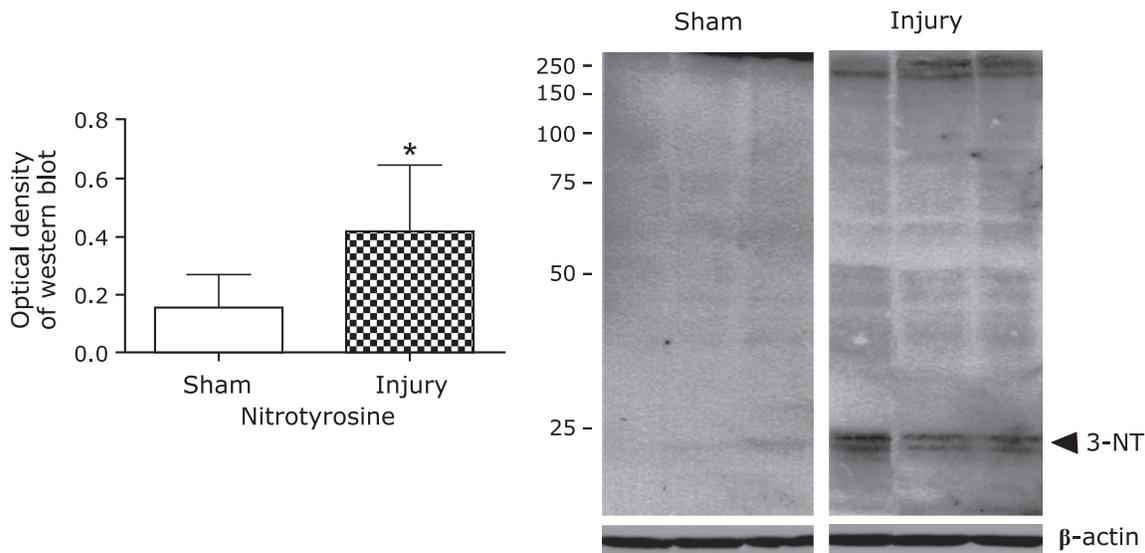
**Fig. 2.** Western blot analysis for arginase expression. Arginase expression in each group was measured by Western blot analysis at 48 h after surgery. Densitometric analysis of arginase expression is shown, and the results are normalized to the expression level of  $\beta$ -actin. Expression of arginase I was significantly increased in the injury group compared with the sham group. Conversely, arginase II was decreased in the injury group. Data are expressed as mean  $\pm$  SD ( $n = 9$  animals per group). \* $p < 0.05$ , \*\* $p < 0.01$  vs sham group.



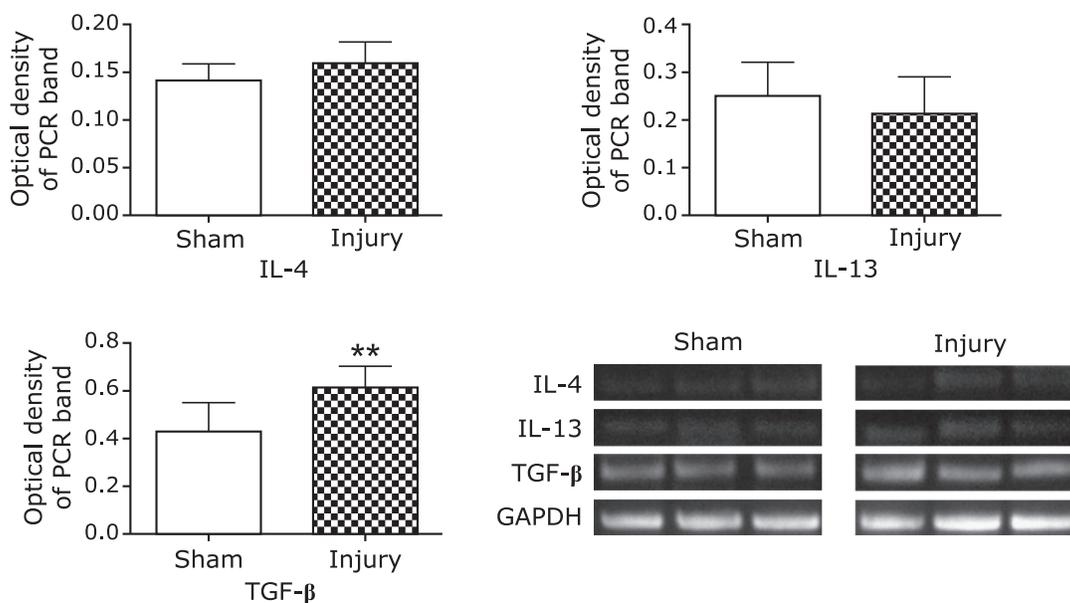
**Fig. 3.** Expression of mRNA for NOS isoforms and NOx concentration. The mRNA expression of NOS was measured by RT-PCR at 48 h in the injury group. Densitometric analysis is shown, and the results are normalized to the expression level of GAPDH. Significant increase of NOS2 mRNA expression was observed at 48 h. However, no increase of mRNA expression of NOS1 or NOS3 was observed compared with that in the sham group (A). Significant increase of NOS2 mRNA expression was observed from the early phase (B). NOx contents were measured in the total homogenate obtained from the sham and injury groups at 48 h. In the injury group, significant decrease was observed (C). Data are expressed as mean  $\pm$  SD ( $n = 9$  animals per group). \* $p < 0.05$ , \*\*\* $p < 0.001$  vs sham group or 0 h.

cells was observed in the vicinity of transection site at 48 h (Fig. 6C), while few cells were visible at 24 h (Fig. 6B). No infiltration of arginase-positive cells to the injury site was observed in the sham group (Fig. 6A) or in the negative control

group (Fig. 6D). Double immunofluorescence staining for arginase I (Fig. 7A) and ED-1 (Fig. 7B), a marker of macrophages also revealed that most of the ED-1 positive cells were co-localized with cells expressing arginase I (Fig. 7C).



**Fig. 4.** Western blot analysis for nitrotyrosine expression. Nitrotyrosine expression was measured by Western blot analysis at 48 h. Arrowhead indicate the protein bands that intensified significantly in the injury group. Densitometric analysis of nitrotyrosine expression is shown, and the results are normalized to the expression levels of  $\beta$ -actin. Nitrotyrosine expression was significantly higher in the injury group than in the sham group. Data are expressed as mean  $\pm$  SD ( $n = 9$  animals per group). \* $p < 0.05$  vs sham group.

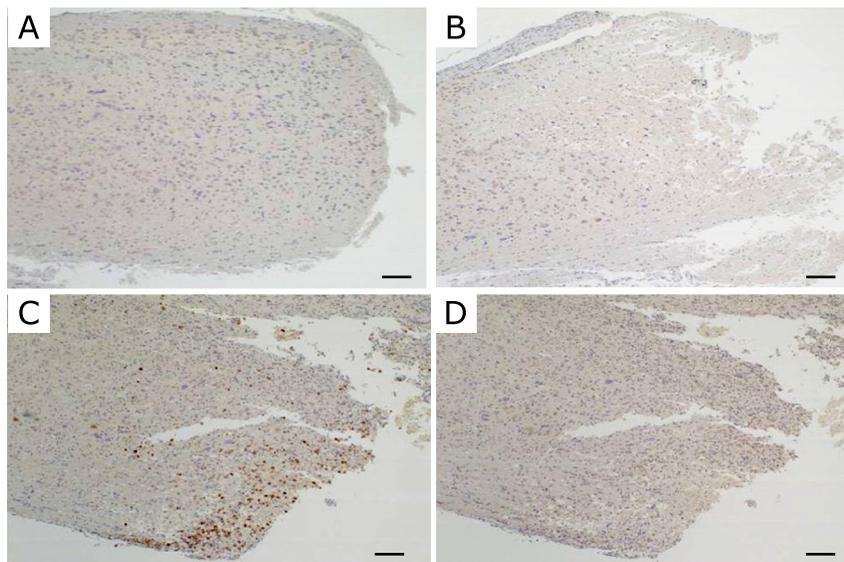


**Fig. 5.** Expressions of cytokine mRNAs for IL-4, IL-13 and TGF- $\beta$ . The mRNA expression level of TGF- $\beta$  significantly increased at 48 h in the injury group compared with the sham group. In contrast, no increase in the expression level of IL-4 or IL-13 was observed. Data are expressed as mean  $\pm$  SD ( $n = 9$  animals per group). \*\* $p < 0.01$  vs sham group.

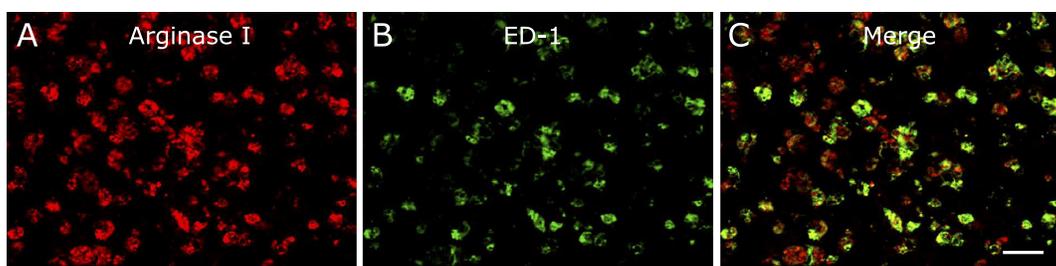
## Discussion

In this study, induction of arginase was demonstrated in transected spinal cord injury. Generally, induction of arginase occurs in two distinct isoforms, arginase I and II, which have different subcellular localization. Arginase I is predominantly expressed in cytosol of hepatic cells as a key enzyme for the urea cycle, while arginase II is expressed in mitochondria of extra-hepatic cells encoded by a different gene.<sup>(20)</sup> Especially, arginase I is highly induced in many tissues following exposure to many

stimulants of Th2 cytokines such as IL-4 and IL-13, growth factors (TGF- $\beta$ ), endotoxin, cAMP-elevating agents, oxygen tension and ROS.<sup>(33,34)</sup> However, the inducer of arginase I in spinal cord injury has not yet been identified. In this study, based on the results of nitrotyrosine generation, ROS might be one of candidates for the inducer of arginase I. Moreover, in our study, up-regulation of TGF- $\beta$  mRNA was demonstrated. Therefore, it seems likely that the TGF- $\beta$  provides some contribution to the induction of arginase I, although the precise mechanisms need to be further investigated.



**Fig. 6.** Immunohistochemistry for arginase positive cells at the injury site. No arginase positive cells were detected at 48 h in the sham group (A) or in the negative control group (D). The number of arginase positive cells in the region of transection at 48 h was significantly increased in the injury group (C), while arginase positive cells were not detectable at 24 h in the injury group (B). Bar = 100  $\mu\text{m}$ .



**Fig. 7.** Double immunostaining for arginase and ED-1, a marker of macrophages. In the injury group, arginase positive cells (A) were co-localized with most of the ED-1 positive macrophages (B) at the edge of transected site at 48 h (C, merge image). Bar = 50  $\mu\text{m}$ .

In mice spinal cord, expression of arginase I and II were localized in the grey matter.<sup>(21)</sup> Although induction of arginase I was demonstrated in neurons and macrophages in hemi-transected spinal cord injury,<sup>(22)</sup> there is little evidence for interactions between arginase I and NO following spinal cord injury. In this study, we detected a remarkable increase in arginase expression in most of the macrophages located at the transection edge at 48 h. In parallel changes in arginase activity, the amount of L-arginine was significantly decreased at 48 h. Moreover, at 72 h, arginase activity had decreased and L-arginine levels had increased. On the other hand, unexpectedly, NO levels were not increased in the injury group compared with the sham group despite up-regulation of NOS2 mRNA. These results indicate that arginase activity and L-arginine levels are linked and that upregulated arginase is associated with low levels of NO for the same substrate, L-arginine. However, it has been reported that the Km for L-arginine is in the 2–20 mM range for arginase,<sup>(35)</sup> but it is in the 2–20  $\mu\text{M}$  range for the NOS.<sup>(36)</sup> From this point of view, it is impossible for arginase to deprive of L-arginine from NOS because the affinity of L-arginine is much higher for NOS than for arginase. However the Vmax of arginase is more than 1,000 times that of the NOS enzymes, indicating similar rates of substrate usage for NO synthesis at low L-arginine concentrations. Sufficient quantities of arginase can limit the availability of L-arginine for NO synthesis

by intact cells.<sup>(20)</sup> In fact, previous studies has been indicated that the majority of L-arginine was consumed for the production of urea rather than NO in L-arginine metabolism, and inhibition of arginase or supplementation of culture media with L-arginine promoted NO generation.<sup>(37,38)</sup> Considering this background, many researchers have focused on the induction of arginase I in experimental asthma, because increased expression of arginase would lower the concentration of L-arginine available as a substrate for NOS activity. As a result, the loss of NO inhibits dilatation of the bronchial smooth muscle.<sup>(23,24)</sup> In this study, L-arginine was possibly consumed by the up-regulation of arginase. Furthermore, the decrease of L-arginine might be progressed in spinal cord transection injury because vascular supply was significantly restricted in the injury edge. Therefore, it is probable that the elevated expression of arginase reduces L-arginine levels in the injured spinal cord and contributes to the inhibition of NO production through depriving L-arginine.

Nitroxidative stress is involved in secondary damage following spinal cord injury. Generally, there are two experimental models in spinal cord injury. One is a contusion model and the other is a transected model. In contusion spinal cord injury, there are many evidences to show the contribution of oxidative stress.<sup>(9–11)</sup> Increase in oxidative stress shown by high expression of NADPH oxidase subunits and nitrotyrosine formation is observed at 16 h

after spinal cord injury.<sup>(39)</sup> Superoxide can easily react with NO, which results in the formation of peroxynitrite. In turn, peroxynitrite is readily protonated under physiological conditions, leading to the generation of peroxynitrous acid.<sup>(40)</sup> Peroxynitrous acid may react with tyrosine or undergo peroxide bond homolysis to generate hydroxyl radical and nitrogen dioxide. The reaction of the hydroxyl radical and nitrogen dioxide with tyrosine can also give nitrated tyrosine.<sup>(41)</sup> Nitrotyrosine can also be produced through a heme peroxidase-dependent mechanism. It has been shown that heme proteins with potential peroxidase activity can cause tyrosine nitration reactions using nitrite and hydrogen peroxide as a substrate.<sup>(42)</sup> In this study, augmented nitrotyrosine formation of some proteins was demonstrated in the injury group. Therefore, it is clear that nitroxidative stress was involved in transected spinal cord injury. However, increased NO production was not observed in the injury group.

The formation of peroxynitrite is supported by another theory. Usually, the coupling of NOS monomers is needed to produce NO. However, under specific conditions such as ischemia, loss of cofactor tetrahydrobiopterin (BH4) and low concentrations of L-arginine, uncoupled NOS is formed, and it can generate O<sub>2</sub><sup>-</sup> via the oxidase domain of NOS.<sup>(43,44)</sup> In fact, the generation of O<sub>2</sub><sup>-</sup> by NOS2 has been documented *in vitro*.<sup>(45)</sup> Similarly, *in vivo*, Okazaki *et al.*<sup>(46)</sup> reported that NOS2 derived superoxide generation was reduced by treatment with the NOS-cofactor, BH4 and inhibition of NOS2 uncoupling switched superoxide production toward NO generation in the ischemia-reperfusion injured heart isolated from diabetic rats. O<sub>2</sub><sup>-</sup> generated by the uncoupled oxidase domain of NOS and NO generated by coupled NOS can easily react with each other, possibly resulting in the formation of peroxynitrite.<sup>(47)</sup> Therefore, in transected spinal cord injury, under ischemia and L-arginine depletion, O<sub>2</sub><sup>-</sup> generation by uncoupled NOS may lead peroxynitrite formation, although there is no evidence to show that peroxynitrite was formed by uncoupling NOS in this study.

In this study, marked increase in arginase I expression and concomitant decrease in L-arginine were demonstrated at 48 h after spinal cord injury. Moreover, decrease in NOx contents and increased nitrotyrosine formation were observed. It was suggested that induced arginase might down-regulate NO production in spite

of up-regulation of NOS2 because of L-arginine consumption. Therefore, regulation of arginase activity or L-arginine level may improve nitroxidative stress and attenuate tissue damage. This may provide a novel clinical approach to the treatment of spinal cord injury. However, it should be investigated more precise mechanisms of nitroxidative stress in spinal cord injury in the future.

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## Declaration of Interest

The authors have no conflicts of interest related to this manuscript.

## Abbreviations

BH4	tetrahydrobiopterin
cAMP	cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HPLC	high performance liquid chromatography
IL	interleukin
mRNA	messenger ribonucleic acid
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffer saline
PCR	polymerase chain reaction
ROS	reactive oxygen species
RNS	reactive nitrogen species
SDS	sodium dodecyl sulfate
TGF	transforming growth factor

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