Interleukin-1 receptor antagonist attenuates the severity of spinal cord ischemic injury in rabbits

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Objective: **Thoracic and thoracoabdominal aortic surgery is sometimes complicated by subacute or delayed paraplegia. Pro-inflammatory cytokine interleukin-1 (IL-1) β has been implicated in extensive inflammation and progressive neurodegeneration after ischemia. Using a rabbit model, we investigated the neuroprotective effects of IL-1 receptor antagonist (IL-1ra) in a temporal fashion.**

Methods: **Spinal cord ischemia was induced by aortic cross-clamping in New Zealand White rabbits. The animals were assigned to three groups. Group C (n = 20) received saline (0.2-mL) and Group I (n = 20) received IL-1ra (200-μg/0.2-mL) intrathecally just after reperfusion. Group S (n = 3) underwent sham operation without aortic occlusion. We assessed the neuroprotective effects of IL-1ra by evaluating neurological function, histopathological changes, and in-situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL staining). We also measured the levels of Nitric Oxide (NO) and S100β in cerebrospinal fluid (CSF). Each evaluation was performed sequentially within 120 hours after reperfusion.**

Results: **Group C showed progressive deterioration of motor function which became statistically significant from 48 hours after the onset of reperfusion ($P < .05$, $P < .01$, $P < .001$, $P < .001$ at 48, 72, 96, and 120 hours, respectively). Compared to Group C, a higher number of viable neurons was observed with less severe spinal cord injury in Group I ($P < .01$, .05 and .05 at 24, 72, and 120 hours, respectively). TUNEL-positive neurons were also significantly reduced by the administration of IL-1ra ($P < .01$ and .05 at 24, and 120 hours, respectively). The difference between Group C and Group I with regard to NO was significant at 72 and 120 hours ($P < .05$), while that in terms of S100β was significant only at 24 hours ($P < .05$).**

Conclusions: **Administration of IL-1ra attenuates spinal cord ischemic-reperfusion injury as evidenced by reducing both neuronal necrosis and apoptosis. (J Vasc Surg 2008;48:694-700.)**

Clinical Relevance: **The present study explores the possibility of reducing subacute or delayed paraplegia after thoracoabdominal aortic surgery using a pharmacological agent that is already available for clinical use. IL-1 mediated inflammatory reaction after ischemic insult can be thought as one of the mechanisms responsible for delayed infarct expansion leading to subacute or delayed paraplegia. IL-1ra has known anti-inflammatory properties and was therefore expected to suppress such post-ischemic inflammatory reaction, if given at an appropriate time. The results of the present study indicate that IL-1-targetted anti-cytokine therapy can be a potentially useful strategy for the attenuation of neurological injury after spinal cord ischemia.**

Paraplegia or paraparesis is a serious complication resulting from spinal cord ischemia during descending or thoracoabdominal aortic surgery. Various methods have been suggested for the prevention of this complication such as topical cooling, partial bypass, distal aortic perfusion, intercostal artery reimplantation, and cerebrospinal fluid drainage.[1-3] However, despite the use of these adjuncts, paraplegia still remains a disastrous and unpredictable complication. With a view to further reducing the incidence of paraplegia, we undertook a number of experimental studies in the previous years and evaluated the potential merit of various pharmacologic agents for spinal cord ischemia.[4-6] These studies revealed the promises of novel therapeutics in the prevention of spinal cord ischemic injuries.

Pro-inflammatory cytokine interleukin-1 (IL-1) β has been implicated in extensive inflammation and progressive neurodegeneration after ischemia.[7,8] It is also reported that administration of recombinant human IL-1 receptor antagonist (IL-1ra) helps in the attenuation of neural damage in cerebral ischemia.[9] IL-1ra is already in clinical use indicated for severe rheumatoid arthritis. However, the possible effect of this agent on spinal cord ischemia has not yet been investigated. In the clinical setting, paraplegia sometimes occurs in sub-acute or delayed phase after thoracic or

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thoracoabdominal aortic surgery.[10] We postulated that suppression of inflammatory reaction after ischemia with IL-1ra has the potential of attenuating the severity of the spinal cord injury. We undertook this animal study to demonstrate the neuroprotective effects of IL-1ra after spinal cord ischemia in a temporal fashion.

METHODS

Experimental animals and materials. We used male New Zealand White rabbits weighing between 2.5 and 3.0-kg in this study (Japan SLC, Inc. Hamamatsu, Japan). Recombinant human IL-1ra, Anakinra was supplied by Amgen Inc (Thousand Oaks, Calif), at a concentration of 100mg/mL (in: 10-mM citrate, 140-mM NaCl, 0.5-mM EDTA, pH 6.5), which we diluted with sterile 0.9% NaCl prior to injection.

Animal care and surgical procedure. The animal protocol was approved by the Ethics Review Committee for Animal Experimentation of Hamamatsu University School of Medicine and all animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

The rabbits were anesthetized with intravenous sodium pentobarbital (25-mg/kg) and allowed to breathe spontaneously. First, the catheter was inserted intrathecally for drug delivery (described below). The animal was then placed in the supine position and the infrarenal abdominal aorta was exposed through a transperitoneal approach. After intravenous administration of heparin (100-U/kg), spinal cord ischemia was induced by cross-clamping the aorta just below the renal arteries and just above the aortic bifurcation for 21 minutes. The clamps were then released and the flank was closed in two layers. At the end of the protocol, the animal was euthanized with a lethal dose of pentobarbital (200-mg/kg) and the spinal cord (L4 to L6) was quickly harvested. Rectal temperature was continuously monitored throughout the procedure with a flexible probe (Terumo Medical Co, Tokyo, Japan) and maintained at about 38.5°C with heating lamp and electric blanket (Sharp Co, Osaka, Japan).

Intrathecal injection. Intrathecal injection technique was conducted according to the method described by our department previously.[11] After inducing the general anesthesia, the intervertebral space between L6 and L7 was punctured with a 16-gauge needle and a polyethylene tube was inserted through it into the subarachnoid space. The desired position of the catheter was confirmed by cautious aspiration of cerebrospinal fluid. The catheter was removed after intrathecal injection of either IL-1ra or vehicle alone.

Experimental protocol and drug delivery. A total of 43 animals were assigned to three groups in a random fashion. Group S (n = 3) underwent sham operation without aortic occlusion. Intervertebral puncture with catheter insertion was done in this group without drug or saline injection. Group C (n = 20) received saline (0.2-mL) and Group I (n = 20) received IL-1ra (200-μg/0.2-mL) intrathecally after an equal volume of CSF (0.2-mL) was withdrawn. All intrathecal injection was performed at the onset of reperfusion. In this study, we applied a 21-minute obstruction of the abdominal aorta. Three rabbits from Group S and eight each from Group C and Group I were euthanized at 120 hours after the beginning of reperfusion. Six rabbits each from Group C and Group I were euthanized at 24 and 72 hours respectively for histopathological and biochemical examinations. CSF was withdrawn for biochemical assessment after the final evaluation of motor function.

Table 1. Semiquantitative score for histopathologic assessment of ischemic spinal cord injury

Histopathologic assessment	*Score*
Healthy	0
Perineural edema or scattered single-cell necrosis	1
Unilateral necrosis of the central medial portion of anterior horn	2
Bilateral necrosis of the central medial portion of anterior horn	3
Unilateral necrosis of the entire anterior horn	4
Bilateral necrosis of the entire anterior horn	5

Neurological evaluation. Hind-limb motor function was scored at every 24 hours after reperfusion using the modified Tarlov scale as follows; 0 = no movement; 1 = slight movement; 2 = sit with assistance; 3 = sit alone; 4 = weak hop; 5 = normal hop.[12] The neurological assessment was performed by an observer without knowledge of the protocol.

Histopathological examination. The harvested lumbar spinal cord was fixed overnight in 10% buffered formalin. Paraffin-embedded spinal cord sections were processed for hematoxylin and eosin staining, and histopathological evaluation was performed under a light microscope. The examination included a count of the viable neurons per section in the ventral gray matter and a graded histological damage scoring (Table I).[13] Slides were examined by a pathologist blinded to the experimental protocol. Readings from 3 sections were taken and values were averaged at 24, 72, and 120 hours after reperfusion.

TUNEL staining. Paraffin-embedded sections were used for in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) with Apoptosis in situ Detection Kit Wako (Wako Co, Osaka, Japan). The sections were counterstained with hematoxylin. Slides were examined by a blinded observer. The ratio of TUNEL–positive neurons relative to the total neural cells in the gray matter was calculated in 3 sections, and the values were averaged at 24, 72, and 120 hours after reperfusion.

Biochemical examination of CSF. CSF was stored at -80°C and thawed at room temperature just before examinations. The levels of Nitric Oxide (NO) and S100β protein were measured with NO (NO_2^-/NO_3^-) Assay Kit (Assay Designs Inc, Ann Arbor, Mich) and Sangtec 100

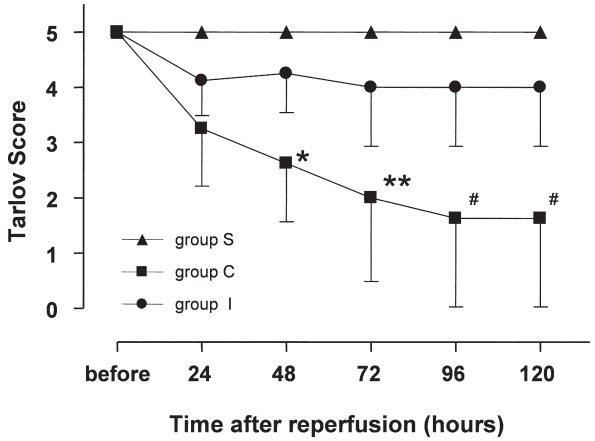

Fig 1. Neurological function scaled by modified Tarlov score at 24, 48, 72, 96, 120 hours after the beginning of reperfusion. Triangles, squares, and circles represent average score of each group while bars show standard deviation (SD). Group I had significantly higher scores in comparison with Group C after 48 hours from the beginning of reperfusion ($*P < .05$, $**P < .01$, $^{\#}P < .001$).

ELISA kit (DiaSorin Inc, Stillwater, Minn), respectively. NO was measured from the values of total Nitrite (NO_2^-) and Nitrate (NO_3^-) detected by the colorimetric Griess reaction that absorbs visible light at 540nm after the enzymatic conversion. S100β protein level was measured by a two-site, one-step, enzyme linked immunosorbent assay with capture antibodies, a detector antibody with horseradish peroxidase and tetramethylbemzidine chromogen. Samples were diluted 50 times with diluting solution. The absorbance was measured at 450-nm.

Statistical analysis. The data are presented as mean ± standard deviation (SD). Statistical analysis of physiologic parameters was performed with one-way analysis of variance (ANOVA). Comparative analysis of neurological recovery score between Group C and Group I was performed by two-way repeated measure ANOVA with Bonferonni's post-hoc test. The non-parametric Mann-Whitney U test was used for comparison between Group C and Group I, with respect to histologic score, the number of the viable neurons, the ratio of TUNEL-positive neurons and the levels of NO and S100β in CSF. Statistical significance was defined as a *P*-value of less than .05.

RESULTS

Physiologic parameters. Mean body weight along with the baseline rectal temperature of each animal was measured before the induction of anesthesia, while the lowest rectal temperature was recorded during the experiment. There were no significant differences in these parameters among the sham, control, and IL-1ra treated groups.

Neurological assessment. Eight animals each from Group C and Group I were observed until 120 hours after reperfusion and were subjected to comparative analysis of motor function (Fig 1). The rabbits euthanized earlier (at 24 [n = 6] and 72 [n = 6] hours) for histopathologic and biochemical examinations had no significant differences at similar time points compared with the remaining animals of the same group in terms of neurological outcome. All rabbits in Group S were normal throughout the observation period. Twenty one minutes of aortic occlusion resulted in severe progressive deterioration of hind-limb motor function in Group C. Treatment with IL-1ra remarkably enhanced the preservation of motor function starting from 48 hours after the beginning of reperfusion (Fig 1; Group C vs Group I; $P < .05$, $P < .01$, $P < .001$, $P < .001$ at 48, 72, 96, and 120 hours, respectively).

Histopathological assessment. Representative photographs of the tissue sections stained with hematoxylin and eosin are shown in Fig 2. The count of viable neurons as evidenced by clear nucleoli and Nissl substance in the ventral gray matter and histological injury score are summarized in Fig 3. In the sham operated animals, the spinal cord was intact and many large neurons were present in the anterior horn. Severe neuronal damage was induced in Group C, as evidenced by a reduced number of viable neurons with higher histological injury score. In contrast, there were fewer evidences of neuronal damage in Group I (Fig 2, 3). Significant differences were found between Group C and Group I in terms of the number of viable neurons at 24, 72, and 120 hours (Fig 3, *A*; Group C vs Group I; $P < .01$, .05 and .05 at 24, 72, and 120 hours, respectively). The differences in histological score reached statistical significance at 24 and 120 hours (Fig 3, *B*; Group C vs Group I; $P < .01$ and .05 at 24 and 120 hours, respectively). There was no evidence of significant macro or microscopic injury caused by intervertebral puncture in any of the animals.

TUNEL staining. Neurons with double-strand breaks in DNA — a feature suggestive of apoptosis — were detected in TUNEL staining by their brown and granular nuclei. Representative photographs of the TUNEL-stained tissue sections along with the ratio between TUNEL-positive and total neurons are shown in Fig 4. Group S had very few cells with evidence of TUNEL-positivity, while numerous scattering TUNEL-positive neurons were detected in Group C (Fig 4, *A*). Administration of IL-1ra remarkably reduced the ratio between TUNEL-positive and total neurons compared with that in Group C (Fig 4, *B*; Group C vs Group I; $P < .05$ at 24 and 72 hours and $P < .01$ at 120 hours, respectively).

Biochemical examination of CSF. The levels of NO and S100β in CSF are shown in Fig 5. Higher concentrations of these parameters were seen until 120 hours after reperfusion in Group C. The difference between Group C and Group I with regard to S100β was significant at 24 hours (Fig 5, *A*; $P < .05$), while that in terms of NO was significant at 72 and 120 hours (Fig 5, *B*; $P < .05$ at both 72 and 120 hours).

DISCUSSION

Cerebral infarct expansion is thought to continue for several days subsequent to the initial infarct expansion that occurs within the first 24 hours.[14,15] The expression of

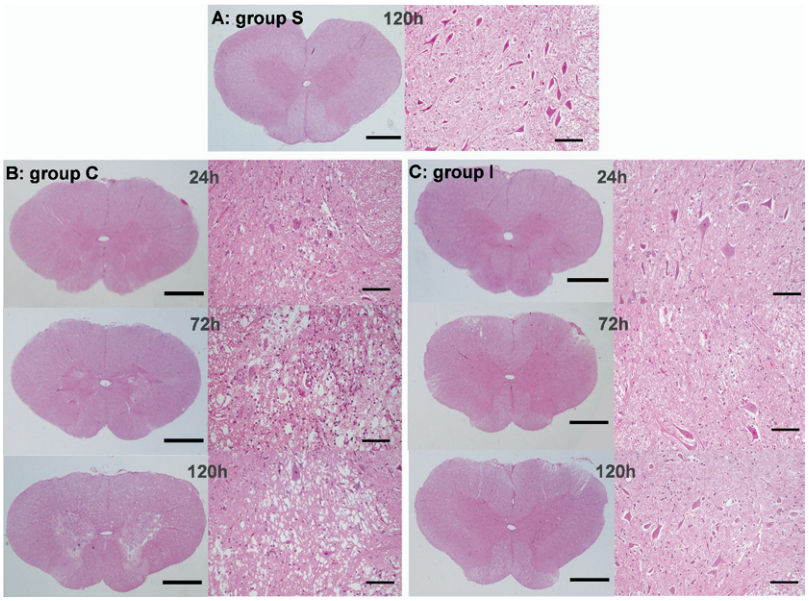

Fig 2. Representative sections of the spinal cord stained with hematoxylin and eosin. Sections from each group were examined with two different magnifications (left panel: 40×, bar = 1-mm, right panel: 200×, bar = 100-μm). The spinal cord of the Group S animals was largely intact (**A**). In Group C, spinal cords showed spread of necrotic changes with vacuolization and gliosis of the gray matter (**B**). In Group I, normal-looking motor neurons were largely preserved with only slight vacuolization (**C**).

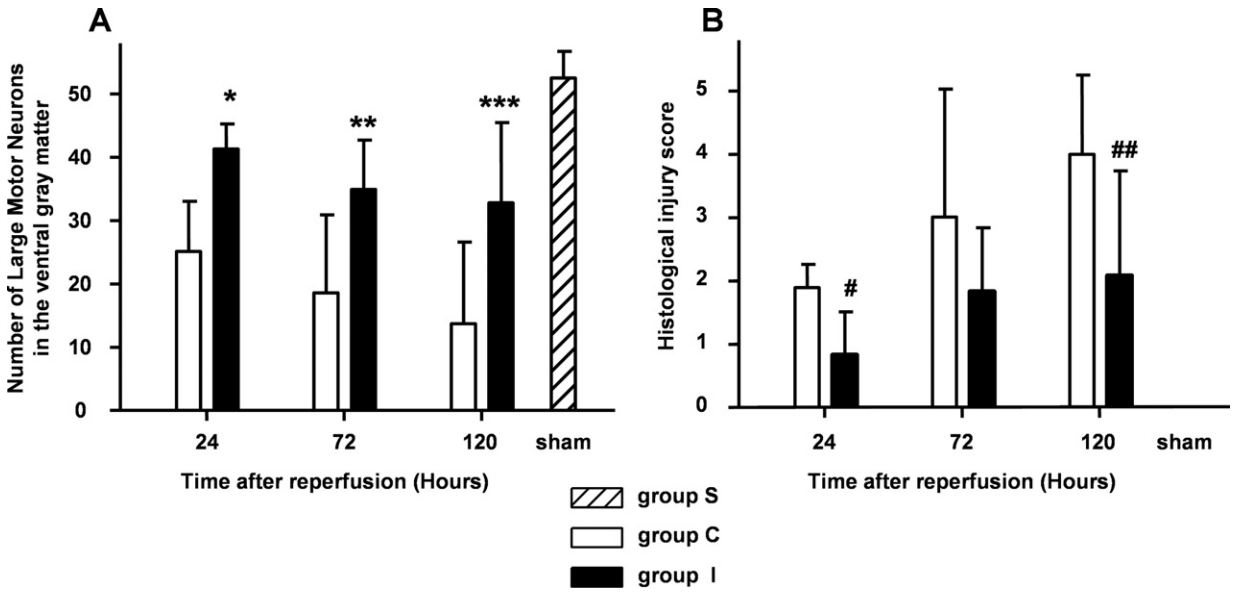

Fig 3. Bar graph showing the number of remaining viable neurons in the ventral gray matter (**A**) and the histological injury score at 24, 72, and 120 hours after the beginning of reperfusion (**B**) in the spinal cord sections. Group C had progressive neurodegeneration, whereas there were fewer evidences of neuronal damages with a higher number of viable neurons and low histological injury score in Group I (*$P < .01$, **$P < .05$, ***$P < .05$, #$P < .01$, ##$P < .05$).

IL-1 occurs a couple of hours after the onset of ischemia or reperfusion and continues for several days in the brain and can be detected on microglia, perivascular macrophage, and astrocyte.[16,17] Previous studies have shown that neural inflammation which induces pro-cytokine IL-1β plays a key role in this process involving the glial cells as well as the invading immune cells and has detrimental effect on central nervous system (CNS) injury.[9,17] Lu et al[18] have recently

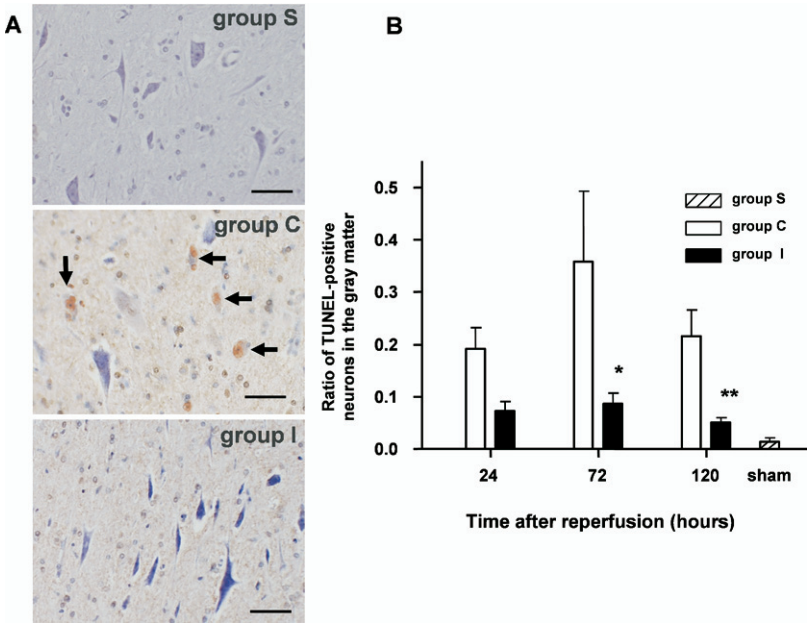

Fig 4. Representative sections of TUNEL staining (**A**: original magnifications; 200×, bar = 20-μm) with ratio of TUNEL-positive neurons relative to the total neurons in the gray matter (**B**). Arrows show the TUNEL-positive neurons. Numerous scattering TUNEL-positive neurons were detected in Group C compared with Group I (*$P < .05$, **$P < .05$).

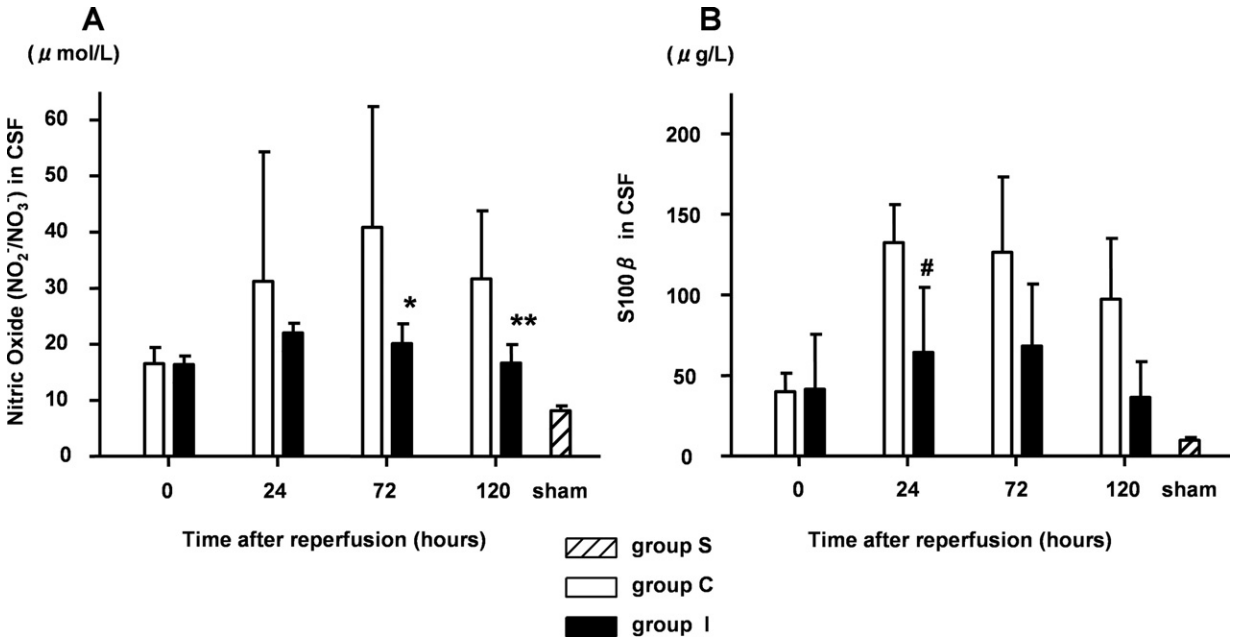

Fig 5. Concentrations of NO ($NO2^-/NO3^-$) and S100β protein in CSF (**A**, **B**). Higher concentrations of these parameters were seen until 120 hours after reperfusion in Group C. Difference between Group C and Group I with regard to NO was significant at 72 and 120 hours (*$P < .05$, **$P < .05$), while that in terms of S100β was significant only at 24 hours (#$P < .05$). NO, nitric oxide; CSF, cerebrospinal fluid.

shown that IL-1 production was elevated after spinal cord ischemia and high concentration was maintained up to 3 days after reperfusion. They have also shown that reduction of IL-1 expression could lead to the attenuation of reperfusion injury. In the present study we noted significant progressive deterioration of motor function with histologically demonstrable neuronal injury in the control rabbits. In contrast, administration of IL-1ra led to a remarkable

attenuation of the neuronal injury with preservation of motor functions. Thus, the results of our study suggest that IL-1 contributes to the severity of ischemia-reperfusion injury which can be attenuated by blocking its signal pathways. Despite the difference in the levels of intervention in the cytokine chain, the findings of these two studies are basically similar in the sense that both achieved attenuation of neuronal injury by minimizing the effect of IL-1.

A key aspect of our study is the route of drug administration. We preferred the intrathecal route for drug delivery as the elimination half-life of IL-1ra is very short when given intravenously.[19] On the other hand, the drug persists in the CNS for a significantly longer duration when given intraventricularly. Konsman et al[20] have shown that IL-1ra injected into the lateral ventricle rapidly diffuses from the CSF into brain parenchyma. Loddick et al[21] have reported that the effects of IL-1ra were still evident in both cortex and striatum over 48-hour after cerebral ischemia using intracerebroventoricular administration of this agent. The intrathecal route also facilitated cerebrospinal fluid (CSF) sampling for biochemical evaluations. The dosage of the drug in the present study was decided on the basis of previous studies in rat cerebral ischemia models.[21,22] Our decision to use a 21-minute aortic occlusion was also based on previous studies from our laboratory with similar spinal cord ischemic time.[4,11] All experimental animals were allowed to breathe spontaneously in this study like most other studies of similar nature using rabbit spinal ischemia model. Although spontaneous breathing may alter the level of pCO2 after the induction of anesthesia which may influence neuroprotection under ischemic condition, we did not anticipate any significant differences with regard to this parameter among the study groups as we employed the same anesthetic protocol for all the animals in this study.

The timing of drug delivery constitutes another difficult issue. In the present study, we administered IL-1ra at the beginning of reperfusion. We think that the initial deterioration of neurologic status in Group I was due to the effect of spinal ischemia itself and its resultant excitotoxicity. That the histological injury scores between the two groups did not reveal significant difference at 72 hours could be considered a limitation of the scoring system.

In our study, the ratio between TUNEL-positive and total countable neurons was significantly lower in the IL-1ra treated group as compared with that in the control group suggesting fewer apoptosis in the former group. Apoptosis has been shown to be an important mode of neuronal death in the ischemic spinal cord which is thought to be occurring towards the earlier part of the ischemic stress.[23] However, in the present study, we could detect TUNEL-positive neurons over 72 hours. This indicates that, in addition to the initial ischemic stress, secondary inflammatory stimuli associated with IL-1 signals cause a more severe neurological injury, and that IL-1ra attenuates such injury by reducing apoptotic and necrotic neuronal death. We think that the very small number of TUNEL-positive neurons detected in Group S resulted from spinal cord harvesting procedure.

IL-1ra acts by blocking the IL-1 receptor and its signal pathways and does not directly inhibit its production. To clarify the possible mechanism of ischemic neurological injury caused by IL-1, we aimed at demonstrating the cytotoxic mediators associated with IL-1 signals rather than the production of IL-1 itself. Previous studies on cerebral ischemia have demonstrated that cytotoxic factors are associated with IL-1 signals and that the neurotoxic effect of IL-1 could be mediated by such factors as NO and S100β. NO produced by IL-1 signal-mediated immunostimulation of glial cells exacerbates ischemic neural damage in the CNS through an oxidative process.[24] Combined with superoxide, NO is transformed into reactive nitrogen oxide radical ($ONOO^-$), which is a free radical having strong tissue toxicity.[25,26] S100β is an astrocytic protein generally known as a biomarker of neural cell damage. However, it has recently been suggested that inhibiting the products of S100β can be a potential therapeutic target in acute stroke patients.[27] This indicates that S100β itself might exacerbate ischemic neuronal damage, a possible mechanism of which might be propagation of neuroinflammation through astrocytic gap junction and also enhancement of NO production in the ischemic brain.[15,28] In the present study, significantly lower levels of NO and S100β were detected in CSF in IL-1ra-treated animals in the subacute phase after reperfusion. Although the production of NO and S100β is not regulated by IL-1 signals alone, we think that it is affected by IL-1 signals in a direct fashion and also by an unknown secondary mechanism under the complex cytokine network. We think that the reduction of these parameters resulted in the suppression of the cytokine cycles and attenuation of the continuous oxidative stress.

While the results of the present study are highly encouraging, they should be interpreted bearing in mind its limitations. We performed only a two-dimensional assessment of the histopathological severity of ischemia-induced spinal cord injury that actually occurred in a three-dimensional manner. Moreover, we were able to demonstrate only a few of the possible mechanisms by which IL-1ra attenuates neurological injury. However, to our knowledge, this is the first report to describe the effect of this agent on spinal cord ischemic injury. Further studies using more refined techniques are warranted to elucidate the correlation between neurological function and the extent of IL-1 mediated ischemia-reperfusion injury of the spinal cord.

In conclusion, the administration of IL-1ra resulted in a significant preservation of the motor function after transient spinal cord ischemia and protected spinal cord against ischemia-reperfusion injury by reducing necrosis and apoptosis. This study suggests that IL-1-targetted anti-cytokine therapy possesses therapeutic potential with regard to neurological injury after spinal cord ischemia.

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AUTHOR CONTRIBUTIONS

Conception and design: SA
Analysis and interpretation: SA, ES, KY
Data collection: SA, ES, AB
Writing the article: SA
Critical revision of the article: TK, ES, KY, AB, HT
Final approval of the article: SA, TK, ES, KY, AB, HT
Statistical analysis: HT
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