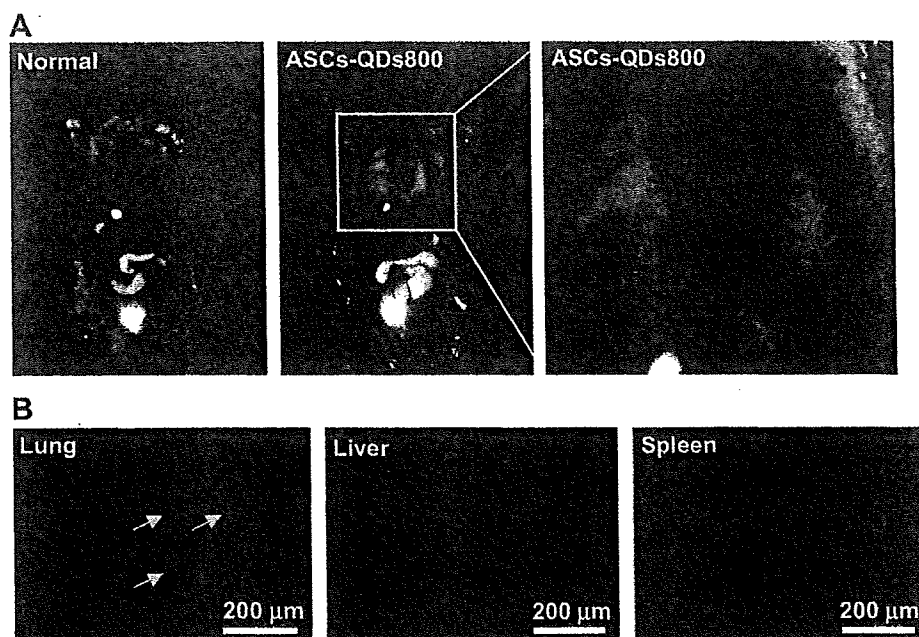


**Fig. 6.** Detection and multiplex imaging capability of QDs in subcutaneous transplantation. **A:** ASCs ( $0.5 \times 10^5$ ,  $1.0 \times 10^5$  and  $3.0 \times 10^5$  cells) labeled with QDs655 (0.8 nm) using R8 were subcutaneously transplanted into the backs of the mice. These images were taken 1 h, 1, 2, 5 and 7 days past injection (excitation filter 575–605 nm, emission filter 645 nm long pass). **B:** The bar graph shows the change of fluorescence intensity of QDs655 for 7 days at the number of ASCs labeled with QDs655, 705 and 800 were subcutaneously injected on the back of the mice. The image was taken 10 min after transplantation with a single excitation light source. **C:** ASCs ( $1.0 \times 10^5$  cells) labeled with QDs655, 705 and 800 were subcutaneously injected on the back of the mice. The image was taken 10 min after transplantation with a single excitation light source. **D:** The graph was the fluorescent spectra of ASCs labeled with QDs655, QDs705 and QDs800.

than the others, thus the cost of synthesis is lower than the others. The cytotoxicity was greatly reduced by using R8. No cytotoxicity was observed in ASCs transduced with less than 16 nM of QDs655, and no drastic decrease in the number of cells was observed with more than 8.0 nM of QDs655. These results suggested that the effect of internalized QDs on the cell cytotoxicity was dependent on surface coating rather than on the total amount of internalized QDs. Furthermore, as observed in a previous report, the current results indicate that ASCs labeled with QDs using R8 had no adverse effects on cell proliferation, measured by the CCK-8 assay [7]. On the other hand, the QDs transduction mechanism using R8 into ASCs was investigated by incubation at 4 °C, with inhibitors of endocytosis, such as sodium azide, and macropinocytosis, such as amiloride

(Fig. 4). The QDs internalization was suppressed by these inhibitors, thus suggesting that the transduction of QDs into ASCs occurred through macropinocytosis.

The transduction time and maintenance of stem cell potency after transduction of QDs are very important, when QDs are employed clinically for regenerative medicine. In this study, the fluorescence intensity of internalized QDs reached almost its peak by 1 h. The transduction time of QDs using R8 was markedly decreased in comparison to Lipofectamine [17]. On the other hand, the CPPs delivery of QDs rescued the cells from the negative effects caused by the internalized QDs to osteogenic and chondrogenic-associated lineage markers [7]. ASCs transduced with QDs655 using R8 cultured with adipogenic and osteogenic



**Fig. 7.** *In vivo* imaging of ASCs labeled with QDs after intravenous injection. A: ASCs ( $5.0 \times 10^5$  cells) labeled with QDs800 (0.8 nm) using R8 were transplanted through the tail vein into mouse. The images were taken 10 min after injection (excitation filter 575–605 nm, emission filter 645 nm long pass). B: The red fluorescence of QDs655 was detected in the lung only, with little or no QDs655 accumulation in the liver or spleen.

medium for 2 weeks differentiated into adipogenic and osteogenic cells, respectively, in the current study. ALP activity of ASCs transduced with QDs655 using R8 following osteogenic differentiation was exhibited at the same level as that for the non-transduced ASCs. Moreover, Jui-Chih Chang et al. checked the influence to the cell surface of ASCs with QDs using cell-penetrating peptide (CPP). In mesenchymal stem cells (MSCs), cell surface receptors such as CD29, CD44, CD73, CD90, CD105, etc. are accredited the prototypical markers. According to the reference, it was found that CPP delivery preserved CD29, CD44, CD73, CD90 and CD105 expressions [7]. These data suggested that ASCs transduced with QDs using R8 maintained their stem cell potency. However, Jui-Chih Chang et al. reported that Pep-1, a CPP, coated QDs, affected the gap junctions of human mesenchymal stem cell [13]. It will be necessary to ascertain the influence of R8 on gap junctions of ASCs in future studies. The fluorescence intensity in differentiated ASCs transduced with QDs655 was sustained at least for 2 weeks. On the other hand, undifferentiated ASCs transduced with QDs655 in the culture medium, showed markedly reduced fluorescence intensity after 2 weeks of culture. The differences may be due to their proliferative activity [5]. However, the QDs655 signal was confirmed, thus suggesting that QDs could be available for labeling of ASCs for 2 weeks.

QDs exhibit several advantages in comparison to conventional organic labels, such as a high luminance, resistance to photobleaching, a range of excitation wavelengths and narrow emission bandwidths. Therefore, QDs have a potential application for *in vivo* imaging. ASCs labeled with QDs655 using R8 were subcutaneously injected with saline into the backs of the mice. ASCs labeled with QDs655 could be detected up to 7 days following subcutaneous transplantation, but the fluorescence intensities were drastically decreased. This reduction might be due to the rapid division of ASCs as suggested in a previous report [22]. When the ASCs labeled with QDs655 were incubated with DMEM/F12 medium containing 2% FBS, the fluorescence of QDs was maintained for 2 weeks at almost the same label. This result was suggested that internalized QDs were diluted by cell proliferation in the culture medium and

incorporated into the cytoplasm of ASCs after escape from the lysosome fraction system [23]. Another advantage of QDs is their ability to for multiplex imaging of different QDs at the same time [22]. When ASCs transduced with QDs655, 705 and 800 respectively were transplanted into the back of mouse subcutaneously at the different points, their fluorescence of all three species could be observed at the same time 1 h after transplantation.

On the other hand, QDs fluorescence below the wavelength range 650 nm was not used often for sensitive imaging of deeper tissues in recent papers [22,24,25]. Deep tissue imaging (millimeters to centimeters) requires the use of far red and near infrared light in the spectral range 650–900 nm [26], because these regions eliminated overlap with major absorption peaks of blood and water *in vivo*. When ASCs labeled with QDs800 were transplanted through the tail vein into a mouse, the fluorescence could be detected in the lung. ASCs labeled with QDs655 and 705 could not be observed *in vivo* because of the strong autofluorescence of liver tissue. Besides, it was reported that QDs fluorescence could be detected in other organs such as the liver and spleen in other papers [12]. In this study, mice were killed 10 min after ASCs transplantation and this suggested that almost all ASCs stayed in the lung. Therefore, *i.v.* ASCs transplantation may be useful treatment modality for lung disease in the future [24].

As for the fate of QDs using R8 *in vivo*, Neera V. Gopee et al. showed that when QDs were injected intradermally (ID) on the right dorsal flank of the female SFH-1 hairless mice, the QDs could be observed moving from the injection sites apparently through the lymphatic duct system to regional lymph nodes within minutes. Residual QDs remained at the site of injection until necropsy at 24 h. Moreover, using inductivity coupled plasma mass spectrometry (ICP-MS), showed accumulation in the liver, regional draining lymph nodes, kidney, spleen, and hepatic lymph node [27].

On the other hand, Hak Soo Choi et al. showed that when QDs leaked into the vascular in the intravenously injection, the QDs were accumulated in the liver. And then, the QDs were transported to the kidney and cleared by urinary excretion [28].

## 5. Conclusion

ASCs possess the ability of self-renewing and differentiating into various mesenchymal cell types. Because of their potential clinical application, it has therefore become important to label the cells for tracing as transplanted cells. The concentration ratio of QDs:R8 of  $1:1 \times 10^4$  was optimal for delivery into ASCs and the cells could be labeled within an 8.0 nm concentration of QDs using R8. In addition, ASCs could be labeled within about 1 h and the fluorescence intensity level of QDs was during 14 days at least. The differentiation ability of ASCs was not inhibited by QDs transduction using R8. Moreover, ASCs labeled with QDs could be detected following subcutaneous transplantation and injection through tail vein *in vivo*. This study suggested that QDs labeling using R8 could be utilized for the imaging of ASCs.

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## Appendix

Figures with essential color discrimination. All figures of this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.01.134.

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## Neuroprotective and Neurotoxic Phenotypes of Activated Microglia in Neonatal Mice with Respective MPTP- and Ethanol-Induced Brain Injury

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### Key Words

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

### Abstract

Activated microglia play important roles in the inflammatory process and progression in Parkinson's disease. These cells produce various cytokines, nitric oxide, and neurotrophins, which are pleiotropic in their action, i.e., neuroprotective or neurotoxic. In an *in vivo* study on a mouse model with nigrostriatal lesions produced by the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), microglia activated by systemic lipopolysaccharide (LPS) were neurotoxic toward dopamine neurons in aged mice, but unexpectedly, neuroprotective in neonatal mice. In contrast to microglia in the MPTP model, LPS-activated microglia in neonatal mice in a model made by the stereotaxic injection of ethanol into the striatum were neurotoxic, and systemic LPS administration in the ethanol-injury model caused marked increases both in the volume of necrotic lesions and in the number of degenerative neurons in the striatum. Thus, activated microglia in the neonatal mouse brain play either a neuroprotective or neurotoxic role depending on the type of brain injury.

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### Introduction

Microglia play important roles in inflammatory processes in various neurodegenerative diseases including Parkinson's disease (PD). Activated microglia play either a neuroprotective or neurotoxic role by producing pro-inflammatory cytokines [e.g., tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), nitric oxide (NO), reactive oxygen species (ROS)], or neurotrophic factors [e.g., nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF)]. Sawada et al. [1] separated two subsets of microglia from mouse brain based on their capacity for intracellular ROS production stimulated by phorbol-12-myristate-13-acetate (PMA): one cell line was neurotoxic when cocultured with neurons and had a high capacity for ROS production, whereas the other cell line was neuroprotective and showed no ROS production. On the other hand, it is well known that neonatal microglia are very proliferative and easily activated under normal circumstances compared with adult microglia [2, 3]. Thus, a question arises as to whether or not activated neonatal microglia are different from those in adult or aged cells in terms of neuroprotective or neurotoxic phenotypes.

**Table 1.** Effects of LPS-induced activated microglia in mouse models with MPTP- or ethanol-induced neurodegeneration

Model	Toxin administration	Toxin + LPS administration
Neonatal mice (P7) treated with MPTP	Decreased DA neuron numbers in SN Decreased DA and DOPAC levels in midbrain Normal IL-1 $\beta$ and IL-6 levels in midbrain	Normal DA neuron numbers in SN Increased TH activity, DA and DOPAC levels in midbrain Increased IL-1 $\beta$ and IL-6 levels in midbrain
Neonatal mice (P7) treated with ethanol	Moderate decrease in number of striatal neurons in striatum	Severe decrease in number of striatal neurons in striatum
Aged mice (60 weeks) treated with MPTP	Moderate decrease in number of DA neurons in SN	Severe decrease in number of DA neurons in SN

SN = Substantia nigra; TH = tyrosine hydroxylase.

### Neuroprotective Phenotype of Microglia Activated by Lipopolysaccharide in Neonatal Mice with MPTP-Induced Injury

Many reports on postmortem PD brains and PD animal models indicate that activated microglia have neurotoxic effects and may play a significant role in progression of the disease. Also aging is thought to be an important factor in idiopathic PD [4]. Aging may promote the toxic change from the protective to the toxic phenotype of activated microglia, as hypothesized by Sawada et al. [1]. This hypothesis of a toxic change in activated microglia had its origin in the *in vitro* experiments conducted by Vilhardt et al. [5]. Sawada et al. [6] showed in neonatal and aged mice different phenotypes of microglia activated by systemic lipopolysaccharide (LPS) treatment on dopamine (DA) neurons under 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced injury (table 1). MPTP is a chemical that causes PD-like symptoms in humans and various animals. Although the number of DA neurons in the substantia nigra in aged mice treated with MPTP was significantly decreased, an increase in the number of LPS-activated microglia produced a further decrease in the number of these neurons. By contrast, neonatal (postnatal day 7) mice treated with MPTP showed that the initial decrease in the levels of DA neurons, tyrosine hydroxylase activity, DA, and the metabolite 3,4-dihydroxyphenylacetic acid in the midbrain changed to an increase, along with marked LPS-induced activation of microglia and increased levels of pro-inflammatory cytokines of IL-1 $\beta$  and IL-6. These results suggest that LPS-activated microglia in neonatal mice may have neurotrophic poten-

tial. Imai et al. [7] also showed the neuroprotective effects of exogenously administered microglia isolated from neonatal gerbils in an ischemic gerbil model; the exogenous microglia migrated to ischemic hippocampal regions in the host, and enhanced neuroprotection accompanied by the expression of neurotrophic factors BDNF and GDNF.

### Neurotoxic Phenotype of Microglia Activated by LPS in Neonatal Mice with Local Ethanol Injury

Sawada et al. [manuscript in preparation] examined whether or not LPS-activated neonatal microglia also show the neuroprotective phenotype in an ethanol-induced brain injury model [8–10] produced by the stereotaxic injection of 100% ethanol (2  $\mu$ l) into the mouse striatum. This local injection of ethanol produced more severe neuronal damage than seen in the MPTP-induced PD model. This ethanol-induced model produced brain injury without hemorrhage, infection or other unexpected effects that might affect the cytokine networks in the brain. After the ethanol-induced damage, activated microglia accumulated in the boundary area of the necrosis and eliminated damaged neurons by causing delayed neuronal death. A large lesion with a necrotic core was observed in the ethanol-injected striatum, and activated microglia had migrated to the outside of this necrotic mass, where Fluoro-Jade B (FJB)-positive degenerative neurons were observed. By previous treatment with systemic LPS or saline as a control, the volumes of necrotic and degenerative areas in the striatum were further increased along with an in-

crease in the number of activated microglia (table 1). The number of inducible NO-synthesizing enzyme (iNOS)-positive microglia also tended to be increased by the LPS treatment, suggesting NO as a toxic agent. These results suggest that LPS-activated microglia in the neonatal mouse striatum play a neurotoxic role in the ethanol-induced brain injury.

### The Mechanisms of Neuroprotective and Neurotoxic Phenotypes of Neonatal Microglia

The mechanisms responsible for the difference in the phenotypes of activated neonatal microglia between the MPTP- and ethanol-injury models remains to be further examined. Neonatal microglia are activated by macrophage colony-stimulating factor (M-CSF) from late gestation to 2 weeks, and are very proliferative and easily activated under normal circumstances. As described above, LPS-activated microglia in the neonatal MPTP model showed a neuroprotective phenotype in the substantia nigra, but a neurotoxic phenotype was observed in neonatal mice that received ethanol-induced striatal damage. In the MPTP model, elevated levels of IL-1 $\beta$  and IL-6 in the midbrain were observed, which suggests that these pleiotropic cytokines produced by activated neonatal microglia may have neuroprotective consequences. As a hypothesis, LPS-activated microglia in the ethanol-injury mouse model might be only transiently neuroprotective, rapidly converting to the neurotoxic phenotype that secretes toxic molecules such as NO. Inoue [11] reviewed microglial activation by ATP and ADP released from injured neurons. At low concentrations of ATP, microglia

release neurotrophic substances such as plasminogen, TNF- $\alpha$ , and IL-6. Stronger or longer stimulation with ATP induced iNOS synthesis in microglia with high concentrations of NO to cause neuronal death. Thus, a high concentration or long-time exposure to extracellular ATP may change the phenotype from protective to toxic.

Since ethanol itself has been shown to influence microglial activation [12–16], the influence of ethanol on microglial activation by local administration might be different depending on the dose, duration, and timing of exposure. Thus the combined effects of LPS with ethanol on microglial activation may also augment the neurotoxic phenotype. Chronic exposure to ethanol was reported to cause increased production of superoxide anion in cultured neonatal hamster microglia; a similar increase was not seen during acute ethanol exposure [13].

Although the limitations of the local ethanol-injury model should be carefully considered, this model has advantages for examining the protective or toxic mechanisms of activated microglia in a specific brain region at any age. Further studies on the molecules secreted from the LPS-activated microglia around the area injured by ethanol should provide valuable information on mechanisms of neurodegeneration.

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## Delayed neural damage is induced by iNOS-expressing microglia in a brain injury model

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### ABSTRACT

Most CNS diseases begin with inflammation with subsequent neural damage eventually occurring; however, the process leading from the onset of inflammation to neural damage remains obscure. We used an artificial brain injury mouse model and examined how neural damage occurred in the brain parenchyma. The damaged area in each mouse was clearly observed by magnetic resonance imaging (MRI), and the progression of damage was observed to occur in a biphasic manner (acute damage, within 1 week; delayed damage, after 2 weeks). We found that the delayed neural damage was absent in iNOS-deficient mice (iNOS-KO mice). Then, we analyzed brain tissues and determined that delayed neural damage was accompanied by an increase in the levels of NO end products and iNOS expression, with accumulation of iNOS-expressing microglia around the injured area. In addition, the expression of IL-1 $\beta$  mRNA was increased in areas affected by acute damage, but not in those affected by delayed damage. These findings suggest that delayed neural damage might arise from NO production by iNOS-expressing activated microglia and that such activated microglia might become a therapeutic target for many CNS diseases.

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Many diseases of the central nervous system (CNS), such as brain injury, infection, inflammation, ischemia, trauma, brain tumors, and neurodegenerative diseases, are associated with neural damage. CNS diseases are often accompanied by inflammatory responses, including microglial activation leading to increased phagocytic activity, secretion of pro-inflammatory cytokines, and NO and ROS production; these eventually lead to neural damage. Therefore, many brain diseases are triggered by inflammatory responses/microglial activation prior to the occurrence of severe tissue damage; however, the process leading from the onset of inflammation to neural damage remains obscure.

Neural damage due to diseases are often related to nitric oxide (NO) [10] and reactive oxygen species (ROS) [7]. One of the responses to cerebral ischemia is an increase in the production of NO [6], and synergistic neurotoxicity in a Parkinson's disease model was shown to be involved in the production of NO and ROS [4]. NO is produced by many cells in the brain including neurons, astrocytes, microglia, and endothelial cells, and acts as a neurotransmitter under physiological conditions [5]. In contrast, in CNS diseases, NO production is increased and NO becomes a neurotoxic mediator. Therefore, neural damage might arise from NO production in CNS diseases accompanied by inflammation. In brain, although NO is biosynthesized by two types of nitric oxide synthase (NOS), constitutive NOS and inducible NOS, the latter is mainly induced

in microglia, astrocytes, and invasive macrophages around sites of inflammation, and the concentration of NO markedly increases around such sites [2,23]. Moreover, NO production and release via iNOS induction cause neuronal damage around sites of inflammation [1], such that metabolites of NO might be appropriate for use as an index of exacerbation around inflammation.

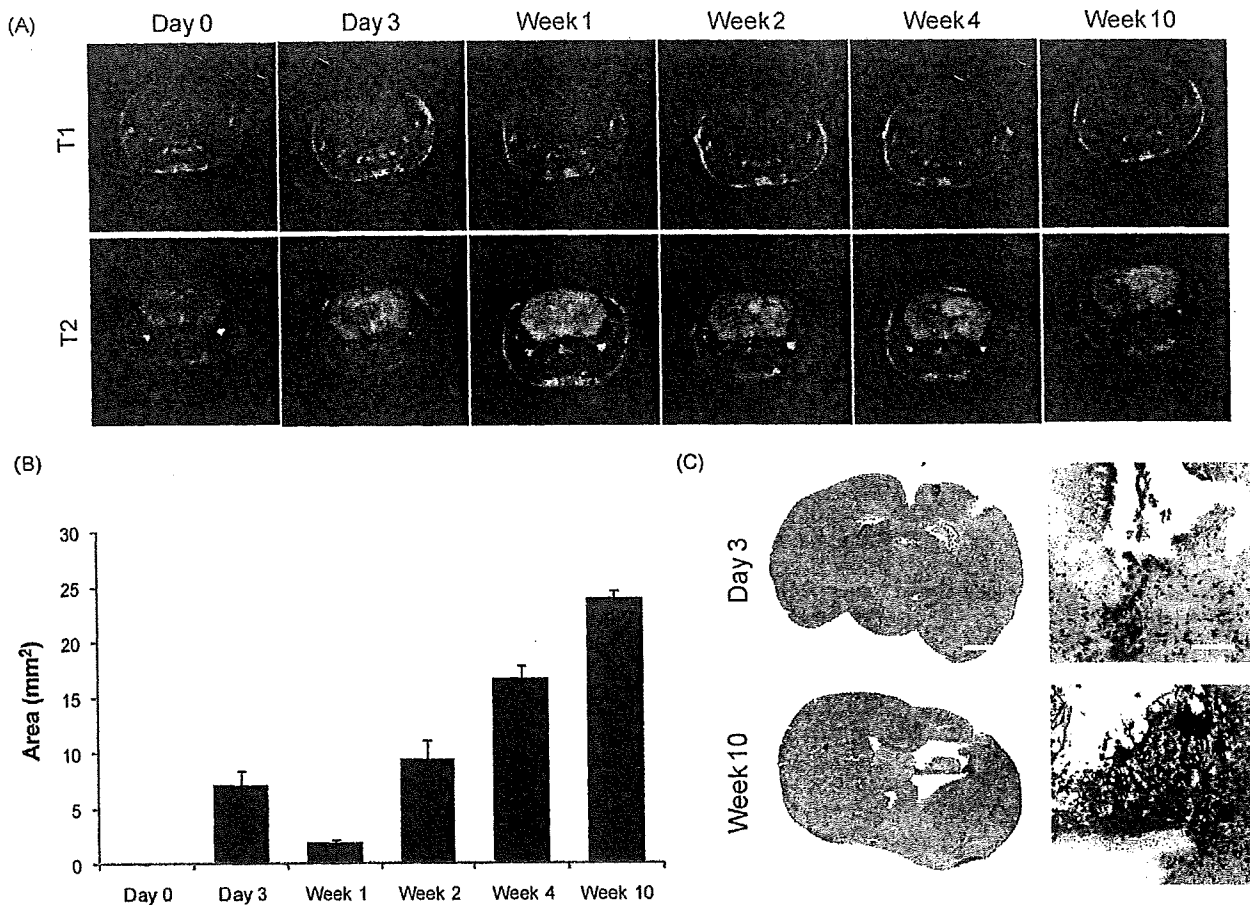
An artificial brain injury model produced by a stereotaxic injection of ethanol into striatum is known to exhibit neural cell damage accompanied by microglial activation but without migration of inflammatory cells from the bloodstream [21,22]. As a feature of this model, activated endogenous microglia accumulate around the injured area and express iNOS, which results in an increase in the level of NO at the injured site [21]. To clarify the processes behind the damage caused by brain injury, imaging techniques for use on living animals, such as MRI, can provide useful information regarding the severity of neural damage at an injured area. In MRI, T1-weighted images are useful in investigating anatomical structure and T2-weighted images are effective in finding lesion sites in brains by increasing signal intensities [13].

In this study, we produced an artificial brain injury model via ethanol injection with normal and iNOS-knockout mice and examined how neural damage occurred in the brain parenchyma after brain injury by MRI, and histochemical and biochemical analyses.

Each C57BL/6 mouse or iNOS-KO mouse ([B6.129P2-Nos2<sup>tm1Lau</sup>/J], The Jackson Laboratories, ME) was anesthetized and placed in a stereotaxic frame, and the skull was exposed. 1 mm burr holes were drilled and the following coordinates were used to position a 10  $\mu$ l Hamilton syringe: the needle was placed

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**Fig. 1.** Progression of neural damage after brain injury in normal mice (A) T1-weighted (T1) and T2-weighted (T2) images of each mouse before and during 10 weeks after brain injury were obtained by MRI. (B) This graph indicates the injured area observed in T2-weighted images obtained before and after brain injury. Values represent the mean  $\pm$  SD. (C) These photographs show brain sections after HE staining at 3 days and 10 weeks after brain injury. The photographs on the right show magnified images of the injured area shown to the left. Each scale bar in left and right photographs indicates 2 mm and 100  $\mu$ m, respectively.

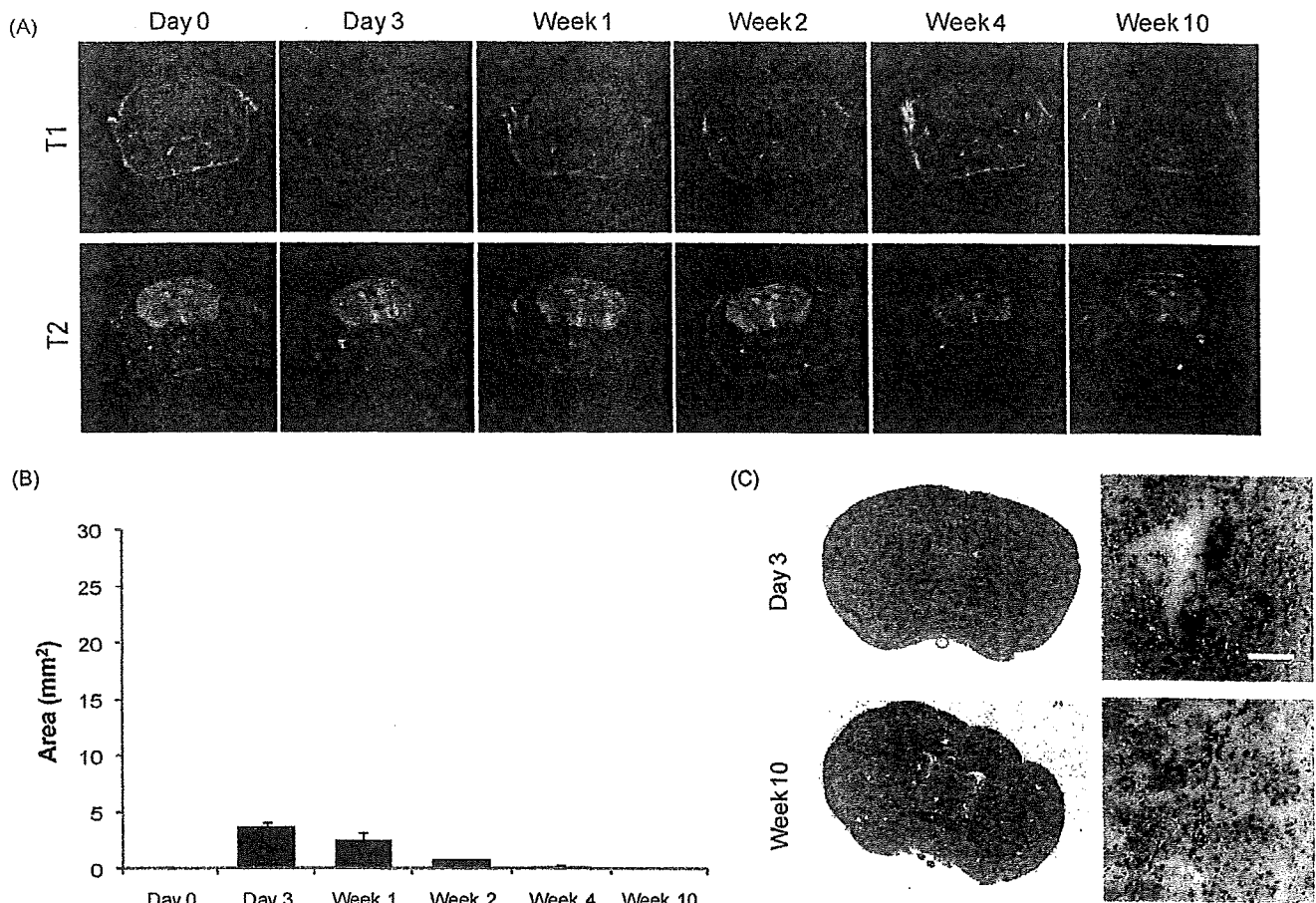
4.0 mm anterior to the bregma, 4.0 mm lateral to the midline, and 3.5 mm ventral to the cortical surface to deliver cells to the striatum. At each site, an injection volume of 5  $\mu$ l of ethanol (EtOH) was delivered at a rate of 1  $\mu$ l/min, and the needle was withdrawn after an additional 5 min. At 3 days, 1, 2, 4, and 10 weeks, the hearts of anesthetized mice were perfused with about 100 ml of isotonic saline, and then each brain was isolated, frozen in liquid nitrogen, and embedded in OCT compound (Tissue Tek; Miles, Elkhart, IN).

MRI was sequentially performed on the treated mice. Each mouse was anesthetized with 1.0% isoflurane and held in an MRI coil. It was set in MRI equipment (MRTechnology, Inc., Tsukuba, Japan) and serial T1-weighted and T2-weighted images were acquired in accordance with the manufacturer's instructions. The acquired T2-weighted images were analyzed in terms of signal intensity using Photoshop CS2 software and the area ( $\text{mm}^2$ ) of neural damage was calculated.

Brain sections (10  $\mu$ m) were serially cut with a cryostat microtome (Leica Microsystems K.K., Japan), then transferred to MAS-coated slides (Matsunami Glass Ind., Ltd., Japan) and air-dried. The sections were fixed with 4% paraformaldehyde in PBS at 4  $^{\circ}$ C for 15 min and treated with 0.1% triton-X for improvement of permeability. The sections were multiply labeled with monoclonal antibodies against Mac-1 (a murine complement component C3bi receptor marker; BMA, Switzerland) and inducible nitric oxide synthase (iNOS; BD Biosciences, Tokyo, Japan). The reaction was visualized with Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., PA) at a dilution of 1:400 and

Alexa350-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) at a dilution of 1:200. Some sections were also subjected to hematoxylin–eosin (HE) staining for confirmation of morphological changes. Fluorescent images were captured with an imaging system (Apotome; Carl Zeiss, Tokyo, Japan) and HE images were captured with Aperio ScanScope system (Aperio Technology, CA).

The accumulation of nitrite and nitrate in the brain was measured as described previously [11]. In brief, 20 slices of brain sections (10  $\mu$ m) were homogenized in TNE buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.15 M NaCl, 1 mM EDTA), centrifuged at 13,000 rpm for 30 min at 4  $^{\circ}$ C for 15 min, and supernatant fractions were collected. The supernatants were centrifuged at 7000  $\times$  g with a filter (Ultrafree-MC microcentrifuge device, Millipore, Tokyo, Japan) to remove substances larger than 10 kDa. The filtrates were analyzed using a  $\text{NO}_2/\text{NO}_3$  Assay Kit-C II (Dojindo, Kumamoto, Japan). After nitrate was reduced to nitrite by nitrate reductase, the absorbance at 595 nm was measured after reaction with Griess reagent. The obtained value was expressed as the total of NO end products, nitrite and nitrate. Nitrite and nitrate were expressed in micromolar concentrations based on tissue weight. Total RNA was extracted from 40 serial brain sections (10  $\mu$ m/slice) using RNeasy mini kit and RNase-free DNase set (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. RNA (1  $\mu$ g) was reverse-transcribed at 37  $^{\circ}$ C for 90 min in a mixture containing 100 U recombinant M-MLV reverse transcriptase, 0.1  $\mu$ g DNA random hexamers, 40 U RNase inhibitor, and 1.4 mM dNTPs, in a final volume of 50  $\mu$ l. The cDNA was amplified and analyzed

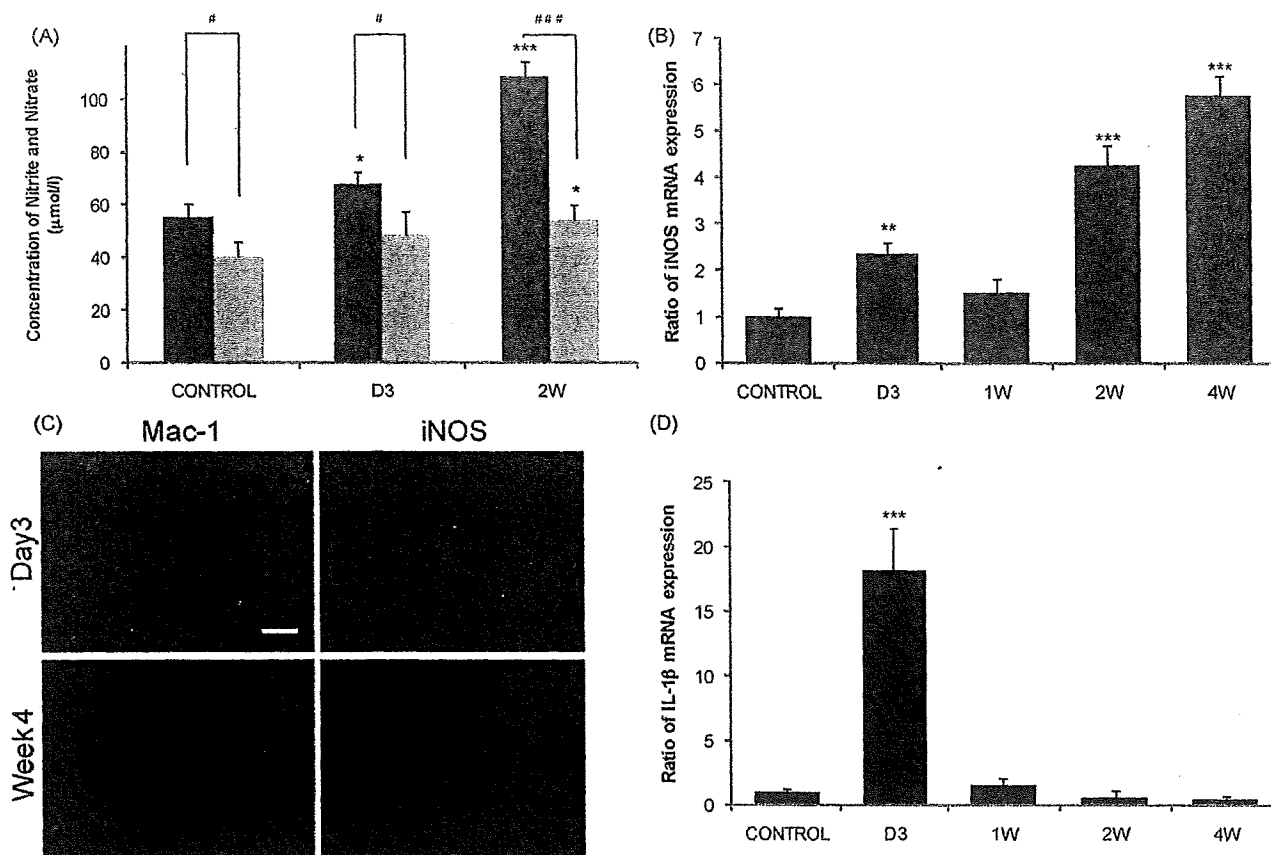


**Fig. 2.** Progression of neural damage in late phase was not observed in iNOS-KO mice (A) T1-weighted (T1) and T2-weighted (T2) images of each iNOS-KO mouse before and during 10 weeks after brain injury were obtained by MRI. (B) This graph indicates the injured area observed in T2-weighted images of iNOS-KO mice obtained before and after brain injury. Values represent the mean  $\pm$  SD. (C) These photographs show brain sections after HE staining at 3 days and 10 weeks after brain injury. The photographs on the right show magnified images of the injured area to the left. Each scale bar in left and right photographs indicates 2 mm and 100  $\mu$ m, respectively.

with Power SYBR Green PCR Master Mix and 7900 Real-Time PCR System (Applied Biosystems, Tokyo, Japan) using primer pairs specific to iNOS (sense primer: GCA GCT ACT GGG TCA AAG ACA A; antisense primer: TCT CTG CCT ATC CGT CTC GTC), IL-1 $\beta$  (sense primer: CGT GGA CCT TCC AGG ATG AG; antisense primer: GGA GCCTGT AGT GCAGCT GTC), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sense primer: TGC ACC ACC AAC TGC TTA G; antisense primer: GAT GCA GGG ATG ATG TTC) for 40 cycles (95  $^{\circ}$ C for 15 s, 60  $^{\circ}$ C for 60 s). Expression of iNOS and IL-1 $\beta$  mRNA was analyzed by the Delta-Delta Ct method [3].

To estimate the severity of injury in the brain of living animals by MRI, T1-weighted and T2-weighted images from each normal C57BL/6 mouse before and after the brain injury were examined (Fig. 1A), since T1-weighted images are generally useful in investigating anatomical structures and T2-weighted images are effective in finding lesion sites in tissues by increasing the signal intensity. The damaged area was clearly observed by MRI and the progression of damage was found to occur in a biphasic manner (Fig. 1B). The first damage occurred within 1 week (acute damage), and subsequent damage occurred more than 2 weeks after the brain injury (delayed damage). To confirm individual neural damage histologically, brain sections were analyzed by hematoxylin-eosin (HE) staining (Fig. 1C). As observed by MRI, HE staining also indicated slight destruction of brain tissue in the acute damage phase and severe destruction in the delayed damage phase. The accumulation of many infiltrating cells was observed around the injured area in the delayed damage phase. Neural damage that occurred

owing to the artificial brain injury was also investigated in iNOS-KO mouse by MRI (Fig. 2A). Similar acute damage to that in normal mice was observed; however, delayed damage was not observed in either T1-weighted or T2-weighted images (Fig. 2A and B). HE staining indicated that in the acute phase a similar extent of tissue destruction occurred to that in the normal mouse; however, no tissue destruction was observed in the delayed phase for the iNOS-KO mouse (Fig. 2C). Then, the amount of NO end products was measured in the brain tissues of normal and iNOS-KO mice by the Griess method (Fig. 3A). NO end products were detected in uninjured brains of both normal and iNOS-KO mice; their levels were significantly lower in iNOS-KO mice than in normal mice. In normal mice, the level of NO end products increased slightly but significantly at 3 days then further increased at 2 weeks after the injury up to about 2-fold that in the uninjured brain. In contrast, the levels of NO end products in brains in iNOS-KO mice at 3 days after the injury were unchanged and their levels hardly increased at 2 weeks after the injury. The expression of iNOS was analyzed by quantitative PCR (Fig. 3B). The expression of iNOS mRNA increased in a biphasic manner in the injured hemisphere of normal mouse brain. The expression of iNOS mRNA increased 2.5-fold in the acute phase and 6-fold in the delayed phase. Immunohistochemical analysis with antibodies against Mac-1 and iNOS indicated that the activated form of microglia expressed iNOS in brain sections of both acute and delayed phases (Fig. 3C). At 3 days after the brain injury, some of the microglia cells around the injured area expressed iNOS. They showed round and amoeboid forms and were dispersed. At 4



**Fig. 3.** Microglia produced NO via iNOS expression in delayed neural damage after brain injury. (A) The concentrations of total NO end products at 3 days and 2 weeks after brain injury were analyzed by the Griess method. Dark gray bars indicate normal mice and light gray bars indicate iNOS-KO mice. (B) This graph indicates the expression of iNOS mRNA in the brain during 4 weeks after brain injury. (C) These photographs show brain sections at 3 days and 4 weeks after brain injury. The photographs on the left indicate Mac-1 staining and those on the right indicate iNOS staining. Photographs on the same row indicate the same location. The scale bar indicates 100 μm. (D) This graph indicates the expression of IL-1β mRNA in the brain during 4 weeks after brain injury. CONTROL, mice without brain injury; D3, mice at 3 days after brain injury; 1W, mice at 1 week after brain injury; 2W, mice at 2 weeks after brain injury; 4W, mice at 4 weeks after brain injury. Values represent the mean ± SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. CONTROL and # $P < 0.05$ , ### $P < 0.001$  vs. normal mice before and after brain injury using Student's *t*-test.

weeks after the brain injury, most of the microglia cells around injured areas expressed iNOS. They showed branched forms as well as amoeboid forms and most of them accumulated near the injured site. To determine the state of microglial activation, the expression of IL-1β was analyzed by quantitative PCR (Fig. 3D). The expression of IL-1β mRNA in the injured hemisphere of the brain increased only in the acute phase but not in the delayed phase.

We demonstrated that neural damage occurred in a biphasic manner after artificial brain injury. We successfully observed by MRI and histological staining that the first damage occurred within 1 week (acute phase), and subsequent damage occurred more than 2 weeks after the brain injury (delayed phase). Since delayed neural damage was not observed in iNOS-KO mice, iNOS expression seems to be responsible for tissue destruction in delayed neural damage. We also found that activated microglia cells were the major cells that expressed iNOS in the delayed phase (Fig. 3C). Most of the activated microglia cells were iNOS-positive and did not produce pro-inflammatory cytokine, IL-1β, in the delayed phase, whereas only some of the activated microglia cells were positive for iNOS with IL-1β expression in the acute phase (Fig. 3C and D). In this study, a brain injury model produced by cerebral ethanol injection was used because the migration of invasive hematopoietic cells such as monocytes and macrophages is hardly induced [21,22]. Then, although monocytes and macrophages as well as microglia cells express Mac-1 (CD11b) antigens [14], accumu-

lated Mac-1-expressing cells originate from endogenous microglia. Since the local production of pro-inflammatory cytokines initiates inflammatory responses, the activation of microglia in the acute phase might be involved in the inflammatory process. This suggests that activated microglia might play distinct roles between acute and delayed phases after brain injury. Microglial activation was observed to occur in a sequential manner, therefore, the first inflammatory activation might trigger further activation of microglia cells resulting in their toxic conversion, leading to an attack of neuronal tissues with NO and other toxic substances such as ROS.

Several lines of evidence suggest that there are some functional differences between activated microglia; some of them exhibit cytotoxic properties and produce several toxic materials to eliminate damaged cells that are beyond the point of recovery, while others exhibit protective characteristics, including the release of neurotrophic factors and the protection of cells from damage [17,18]. The former state of activated microglia is sometimes referred to as the 'further activated' or 'fully activated' form. These two different characteristics of activated microglia play an important role in the pathophysiology of common disorders such as Alzheimer's disease and Parkinson's disease. Recently, it was reported that [<sup>11</sup>C]PK11195 PET imaging could successfully distinguish between the two different activated states of microglia [8].

Another possibility is the existence of subtypes of microglia. Microglial cells show a rather uniform distribution in terms of cell numbers throughout the brain with only a minor population in some brain regions. The *in situ* morphologies of these cells, however, may vary markedly from elongated forms observed adjacent to neuronal fibers to spherical cell bodies with sometimes extremely elaborate branching. Furthermore, human fetal microglia cells display heterogeneity in phenotype identified by CD68 [16]. This heterogeneity gave rise to the hypothesis that these cells are differentially conditioned by their microenvironment and, therefore, also display specific patterns of differential gene expression. For example, mRNAs of TNF- $\alpha$ , CD4, and Fc gamma receptor II are differentially expressed in microglia isolated from different areas of the brain [15]. It has been reported that the productions of IL-12 [20] and histamine [9] are different in different subtypes of microglia. Furthermore, the subtypes of microglia show distinct acidification profiles by treatment with pepstatin A [12] and selective clearance of oligomeric beta-amyloid peptide [19]. We observed that morphologies and patterns of iNOS expression were different in the acute and delayed phases. Thus, microglial subtypes may display distinct regulation of the progression of neural damage.

We observed that the total amount of NO end products was lower in injured and uninjured brains of iNOS-KO mice than those of normal mice and that in the delayed phase after injury of iNOS-KO mice the amount slightly increased compared with that in uninjured brain, despite the deficiency in iNOS. Because NO is synthesized by two types of NOS (constitutive and inducible), this increase might have resulted from activation of constitutive NOS.

We have clearly indicated that the progression of neural damage that occurs in the brain of individual living animals can be monitored by MRI. The feasibility of this was confirmed by histochemical analysis; the observed changes in brain damage matched between the two methods (Figs. 1 and 2). In the conventional protocol, neural damage is estimated from brain sections obtained from animals sacrificed at different time points by histochemical analysis, which means that the results do not represent the changes in an individual animal. In this study, MRI was used to continuously provide useful information on the severity of neural damage at the injured area in each living mouse. Thus, it is very important that non-invasive imaging techniques for use on living animals, such as MRI, are developed and adopted in many experiments in place of time-course analysis using tissue sections.

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