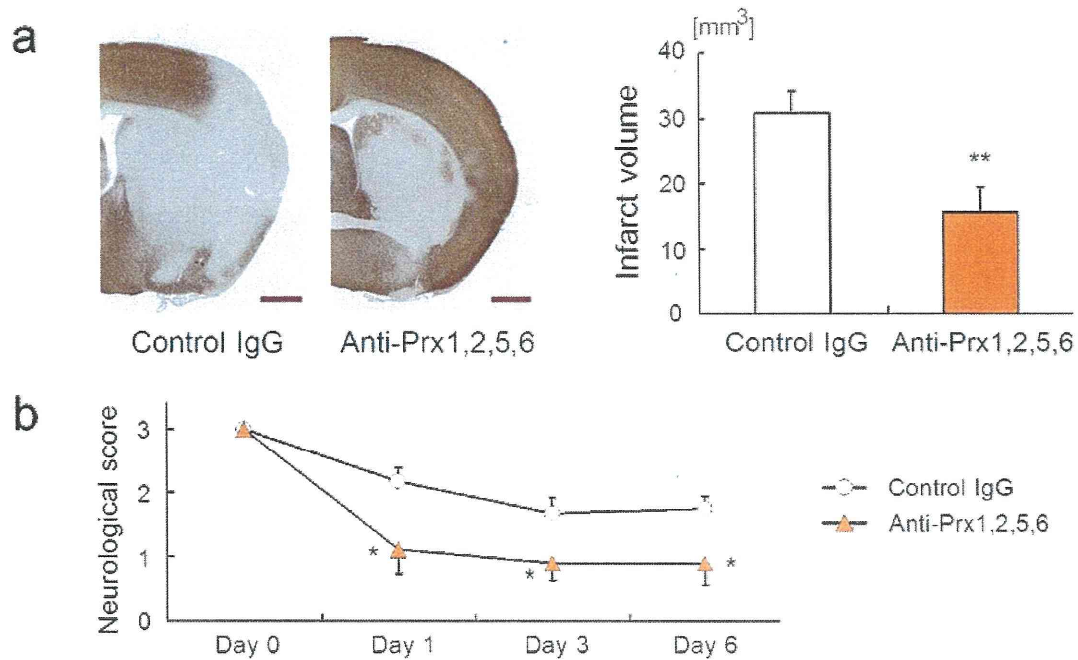
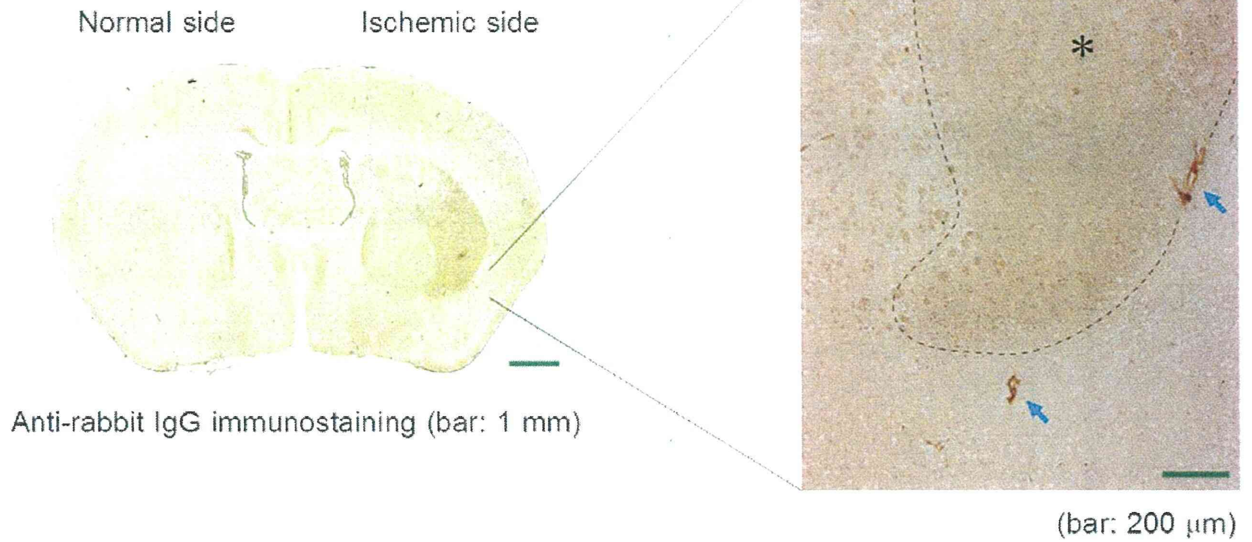
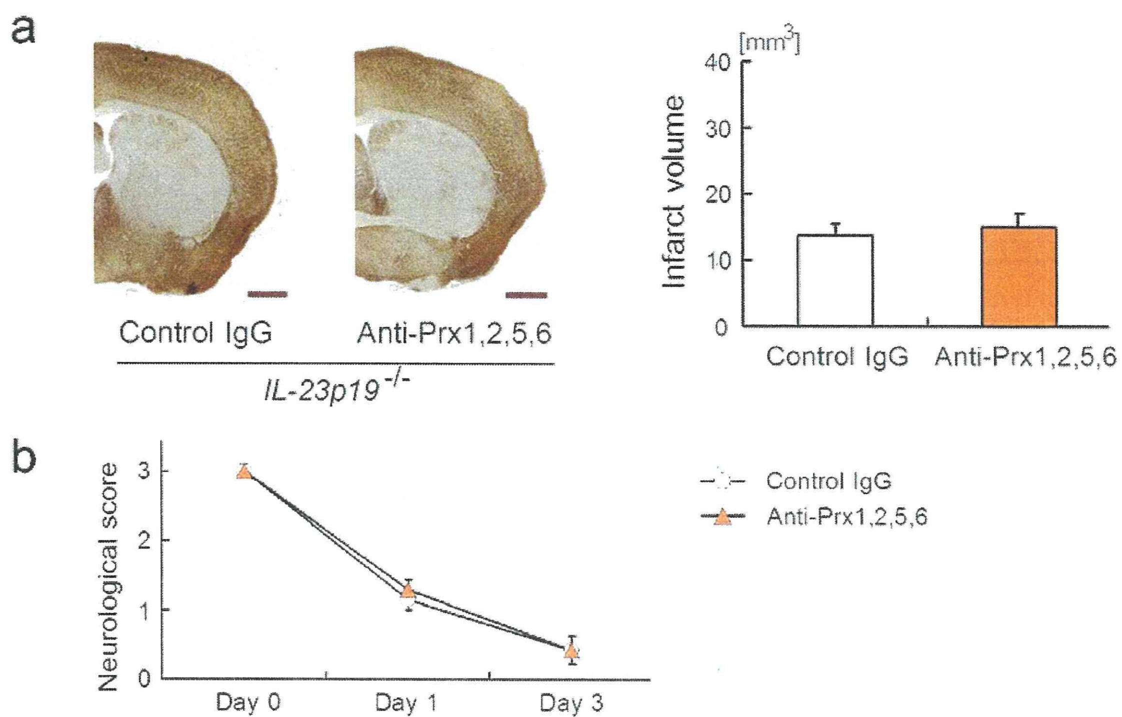
peri-infarct area on day 4 (bar: 50 μm). The number of positive cells within three 0.1 mm^2 areas in the peri-ischemic parietal cortical region was expressed as an average value. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT BM-transferred WT mice (one-way ANOVA with Dunnett's correction; the error bars represent s.e.).



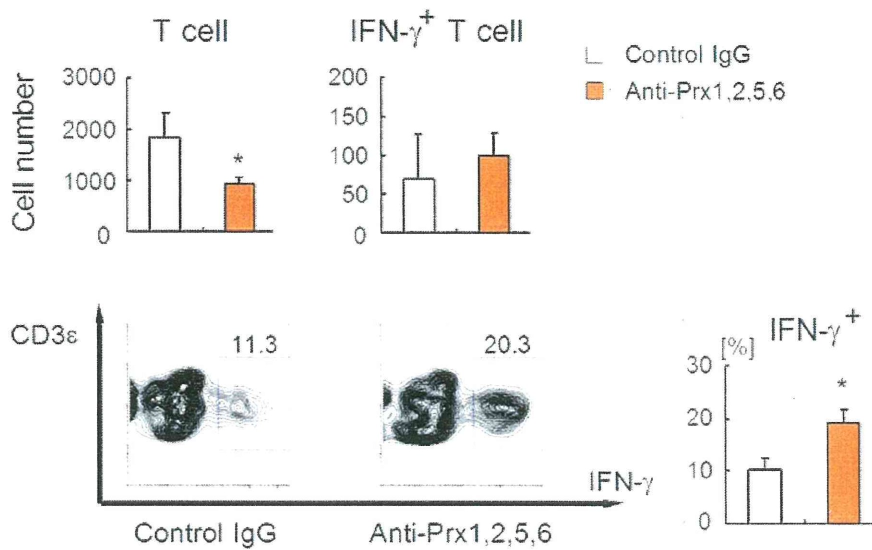
Supplementary Figure 13. (**a**) Infarct volume on day 7 (bar: 1 mm) and (**b**) neurological scores on days 1, 3, and 6 of mice treated with control IgG or anti-Prx antibody mixture just after the induction of brain ischemia (500 μg/mouse). $^*p < 0.05$, $^{**}p < 0.01$ vs. control IgG-administered mice (two-sided Student's *t*-test; the error bars represents s.e.).



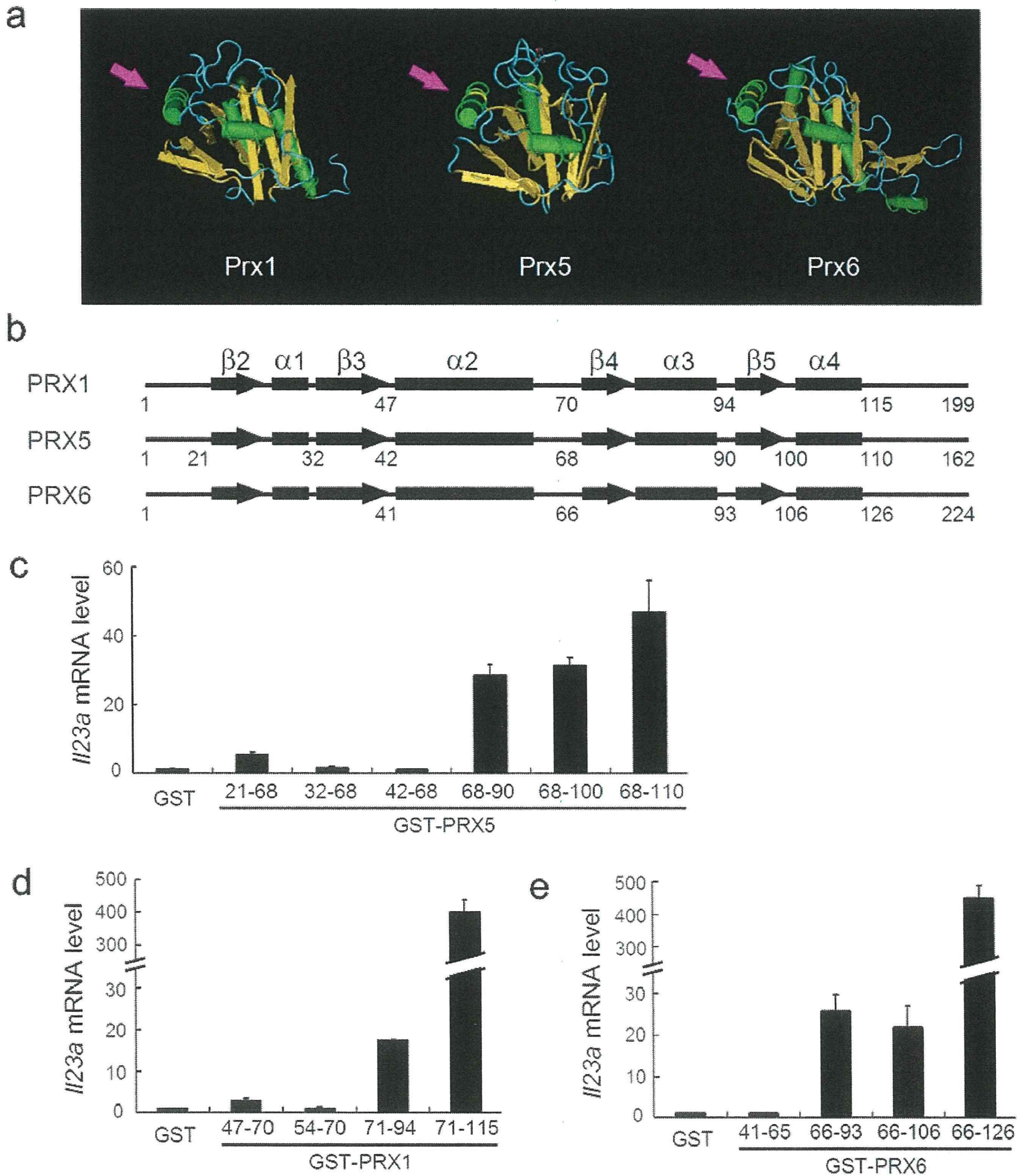
Supplementary Figure 14. The result of anti-rabbit IgG immunohistostaining of ischemic brain tissue on day 1. Positively stained region was observed in the ischemic area (*) and vascular lumen (blue arrow). Anti-Prx1,2,5,6 antibodies (derived from rabbit) were administered just after the induction of brain ischemia.



Supplementary Figure 15. (**a**) Infarct volume on day 4 (bar: 1 mm) and (**b**) neurological scores on days 1 and 3 of IL-23p19 KO mice treated with control IgG or anti-Prx antibody mixture just after the induction of brain ischemia (500 μg/mouse). There were no significant differences between control IgG- and anti-Prx antibody mixture-administered IL-23p19 KO mice (n = 7, each).

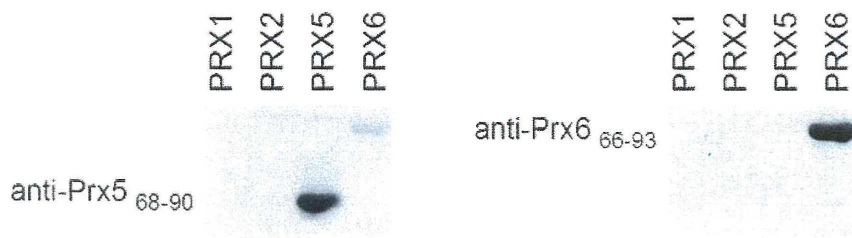


Supplementary Figure 16. The absolute number and the ratio of IFN-γ^{+} T cell on day 3. The administration of anti-Prx antibody mixture significantly increased the ratio of IFN-γ-producing T cells, because the number of IFN-γ-producing T cells was kept in spite of decreased number of total infiltrating T cells, similar to TLR2 and/or TLR4 deficient mice (**Supplementary Fig.11**). $^{*}p < 0.05$ vs. control IgG-administered mice (two-sided Student's *t*-test; the error bars represents s.e.).



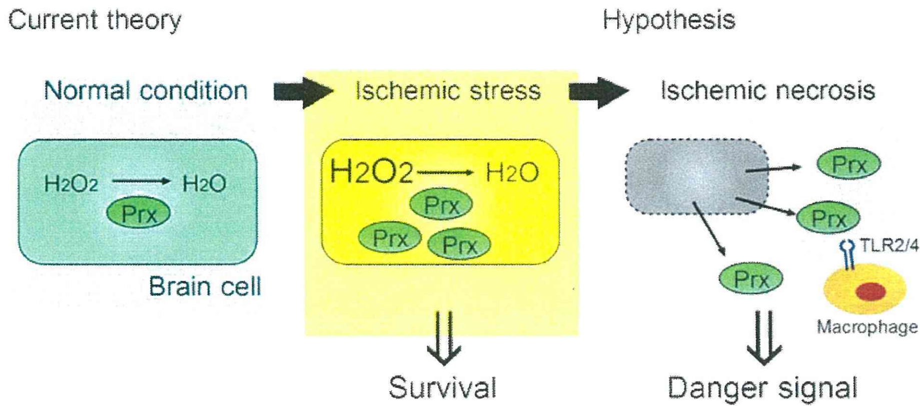
Supplementary Figure 17. (**a**) The crystal structure of Prx1 (Hbp23: rat homologue of Prx1; MMDB ID:11298), Prx5 (human Prx5; MMDB ID:26393), and Prx6 (hORF6: human homologue of Prx6; MMDB ID: 7978). Pink arrows indicate α3-helix of peroxiredoxins. They are relatively

sequestered on the protein surface and they are on almost opposite side of site for peroxidase activity. (**b**) The protein secondary structure of Prx family proteins. Numerals under the bar indicate amino acid residue numbers of each Prx protein. (**c,d,e**) IL-23p19-inducing activities of GST fusion PRX5 (**c**), PRX1 (**d**), and PRX6 (**e**) peptides. The number of amino acid residues contained in each PRX peptide was shown in X-axis. All of the IL-23p19 mRNA expression levels were detected by means of quantitative RT-PCR in BMDC 1 hour after the stimulation.

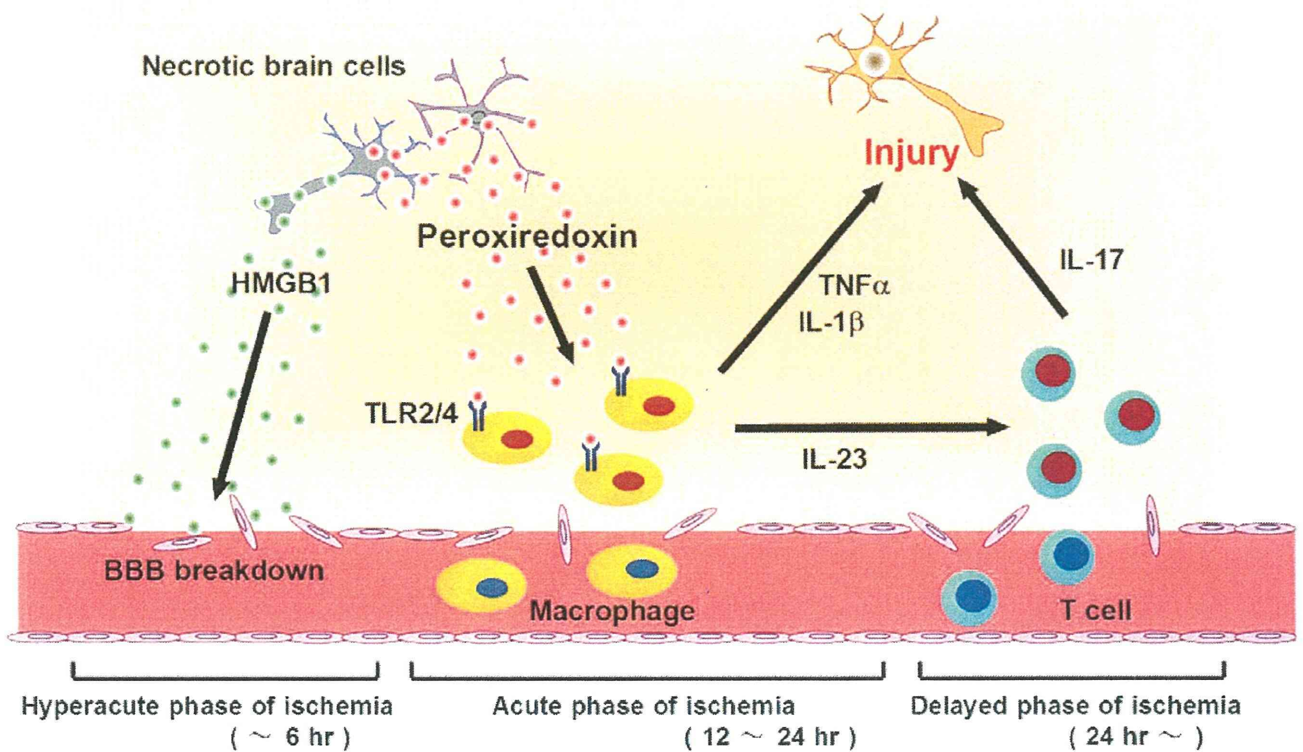


Supplementary Figure 18. Western blot analysis using rabbit polyclonal anti-Prx5 $_{68\text{-}90}$ and anti-Prx6 $_{66\text{-}93}$ antibody.

a



b



Supplementary Figure 19. Schematic model of the roles of DAMP molecules and inflammatory cytokines in the ischemic brain injury. (**a**) Peroxiredoxin (Prx) has two opposing functions: one inside and one outside the brain cells. Ischemic stress increases Prx expression inside brain cells, which may contribute to the survival of brain cells by catabolizing reactive oxygen species (ROS). But when ischemic phenomena finally results in the necrosis, Prx is released from necrotic brain cells into the extracellular compartment where it becomes a strong TLR2/4 stimulator for the infiltrating macrophages. (**b**) At the hyperacute phase of brain ischemia (within 6 hours after stroke onset), HMGB1 is released from ischemic brain cells and promotes

blood-brain barrier breakdown. Thereafter, peripheral blood cells begin to infiltrate into the ischemic brain, however, the extracellular release of HMGB1 is mostly diminished in the ischemic core at the acute phase of ischemia (12 to 24 hours after stroke onset). In this phase, extracellular Prx released from necrotic brain cells activates the infiltrating macrophages via TLR2/4 and induces the inflammatory cytokine expression which promotes ischemic injury. At the delayed phase of brain ischemia (over 24 hours after stroke onset), IL-23 induces IL-17 production from T cells ($\gamma\delta$T cells) and causes the further ischemic damages. Thus, HMGB1 is a hyperacute DAMP (within 6 hours after stroke onset), while Prx is the secondary one in the acute phase (12 to 24 hours after stroke onset) for postischemic inflammation.

Fraction	No. 2	No. 3
Phosphoglycerate kinase 1 (Pgk1)	11	10
Cofilin-1 (Cfl1)	10	4
Transgelin-3 (Tagln3)	9	5
Peptidyl-prolyl cis-trans isomerase (Ppia)	8	8
Triosephosphate isomerase (Tpi1)	7	10
Peroxiredoxin-5 (Prx5)	7	7
Fatty acid-binding protein, epidermal (Fabp5)	7	5
Carbonyl reductase [NADPH] 1 (Cbr1)	7	5
Alpha-synuclein (Snca)	6	5
Dual specificity protein phosphatase 3 (Dusp3)	6	3
Peroxiredoxin-6 (Prx6)	5	9
Gamma-synuclein (Sncg)	5	2
Alcohol dehydrogenase (Akr1)	5	6

Supplementary Table 1. The protein list identified by LC/MS analysis in No.2 and 3 sucrose gradient fraction.

	WT	TLR2 KO	TLR4 KO	TLR2/4 DKO
MABP (mmHg)	84 ±3	81 ±1	81 ±2	82 ±1
pH	7.33 ±0.01	7.35 ±0.02	7.33 ±0.02	7.33 ±0.01
PaO_2 (mmHg)	123 ±4	118 ±8	125 ±5	133 ±3
$PaCO_2$ (mmHg)	39.3 ±2.7	39.3 ±3.6	38.4 ±1.3	37.7 ±1.0
Hematocrit (%)	40.3 ±2.9	38.3 ±2.2	41.3 ±2.7	40.0 ±0.6

Supplementary Table 2. Physiological data of each type of mouse used in this study (average ± standard error, n = 3). MABP: Mean arterial blood pressure.

CBF reduction (%)	N	before CCA occlusion	after CCA occlusion	after MCA occlusion
WT	6	100	80.9 ± 3.7	14.3 ± 1.2
TLR2 KO	6	100	75.0 ± 4.0	16.5 ± 1.4
TLR4 KO	6	100	75.4 ± 3.1	14.9 ± 1.6
TLR2/4 DKO	6	100	74.2 ± 3.7	17.7 ± 2.2

Supplementary Table 3. The percent of cerebral blood flow (CBF) reduction after common carotid artery (CCA) occlusion and middle cerebral artery (MCA) occlusion (average ± standard error).

CBF reduction (%)	N	before CCA occlusion	after CCA occlusion	after MCA occlusion
WT ←WT BM	6	100	71.4 ± 5.0	13.5 ± 2.0
WT ←TLR2KO BM	6	100	76.2 ± 2.1	12.7 ± 2.6
WT ←TLR4KO BM	6	100	76.0 ± 3.2	14.7 ± 1.1
WT ←TLR2/4DKO BM	6	100	76.2 ± 3.0	13.3 ± 1.7
TLR2KO ←WT BM	6	100	80.4 ± 2.3	16.0 ± 1.0
TLR4KO ←WT BM	6	100	71.9 ± 3.0	15.1 ± 1.8
TLR2/4DKO ←WT BM	6	100	75.2 ± 3.2	13.7 ± 2.2

Supplementary Table 4. The percent of CBF reduction after CCA and MCA occlusion (average ± standard error).

a

CBF reduction (%)	N	before CCA occlusion	after CCA occlusion	after MCA occlusion
Control IgG	6	100	79.6 ± 4.0	16.2 ± 2.4
Anti-Prx1,2,5,6	6	100	75.2 ± 5.7	12.6 ± 0.8
Anti-HMGB1	6	100	78.2 ± 2.8	16.6 ± 1.9
Anti-HMGB1 + Prx	6	100	72.7 ± 2.1	12.9 ± 0.9

b

Survival rate (%)	N	Day 1	Day 4
Control IgG	17	100	94.1
Anti-Prx1,2,5,6	9	100	100
Anti-HMGB1	9	100	100
Anti-HMGB1 + Prx	9	100	100

Supplementary Table 5. (**a**) The percent of CBF reduction after CCA and MCA occlusion and (**b**) the survival rate of each type of mouse used in **Figure 4a,b** (average ± standard error).

Supplementary Methods

Bone marrow-chimeric mice. The recipient mice were given lethal doses of total body radiation with two 5 Gy exposures given four hours apart. The irradiated recipients were rescued by injecting the donor bone marrow cells (1×10^{7}) into the tail vein. Seven weeks after irradiation and bone marrow transplantation, bone marrow-chimeric mice were applied to the focal brain ischemia experiments.

Preparation of infiltrating immune cells. The mice were perfused with PBS transcardially. The forebrain was removed and well-suspended with RPMI-1640. The suspension was digested with type IV collagenase (1 mg ml^{-1}, Sigma-Aldrich) and DNase I (50 µg ml^{-1}, Roche) at 37°C for 45 minutes in a shaker. Infiltrating immune cells were isolated by 37%-70% Percoll (GE Healthcare) density gradient centrifugation and were removed from the interphase for further analysis.

FACS analysis. The method for surface and intracellular cytokine staining was described previously[2]. FACS analysis was performed on a FACSCantoII instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Generation of BMDC. Bone marrow was collected from tibias and femurs of C57BL/6 mice, TLR2- and/or TLR4-KO mice. Bone marrow cells were passed through a nylon mesh and cultured for eight days in RPMI-1640 medium with 10% fetal calf serum (FCS) and 1% supernatant from a granulocyte macrophage colony stimulating factor (GM-CSF) expressing cell line (J558L). The cytokine production was determined by quantitative real-time PCR or ELISA (eBioscience) at the indicated time points.

Quantitative real-time PCR. BMDCs or infiltrating inflammatory cells prepared by Percoll gradient centrifugation were lysed in RNAiso (Takara). Real-time PCR was performed on cDNA samples using KAPA SYBR Fast qPCR kit (Kapa Biosystems). The relative quantitation value is expressed as $2^{-\Delta Ct}$, where ΔCt is the difference between the mean Ct value of duplicate measurements of the sample and the endogenous hypoxanthine phosphoribosyltransferase 1 (HPRT1) control.

Immunohistochemistry of ischemic brain tissue. At the indicated time points after reperfusion, the brain section at the caudoputamen level was evaluated for immunohistochemistry.

For terminal deoxynucleotidyl transferase–mediated dUTP-biotin in situ nick end labeling (TUNEL) staining, the sections were treated with proteinase K (20 $\mu g\ ml^{-1}$) and 0.3% hydrogen peroxide. After blocking with normal goat serum, TUNEL staining was performed by using a kit (In Situ Cell Death Detection Kit, POD and Fluorescein, Roche) as manufacturer's protocol. Coloring reactions were performed with hydrogen peroxide containing 3, 3'-diaminobenzidine tetrahydrochloride (Histofine DAB Substrate Kit, Nichirei) and the sections were counterstained with hematoxylin. Positively-stained cells were counted on the cortex in the peri-infarct area, which was considered to extend 3 mm lateral from the midline as previously described method[3]. The number of positive cells within 3 different areas, each 0.1 mm square, was expressed as an averaged value.

For immunostaining of peroxiredoxins, the sections were treated with proteinase K (20 $\mu g\ ml^{-1}$) and blocked with normal goat serum. Sections were washed with PBS and then incubated overnight at 4°C with rabbit anti-mouse peroxiredoxin (Prx1/2, Prx5, and Prx6) antibody or rat anti-mouse F4/80 antibody (clone A3-1, Serotec). After three times washes with PBS, the sections were incubated with Alexa Fluor 488-labeled anti-rabbit IgG, Alexa Fluor 546-labeled anti-rat IgG (Invitrogen), and Hoechst 33342 (Invitrogen) for 30 min at room temperature.

The sections were observed and captured under a fluorescence microscope (BZ-8000, Keyence) or a confocal laser microscope (LSM510 META, Carl Zeiss).

For immunostaining of rabbit IgG, the sections were treated with proteinase K (20 $\mu g\ ml^{-1}$) and blocked with Blocking One (Nacalai Tesque). Sections were washed with PBS and then incubated overnight at 4°C with goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). After three times washes with PBS, the sections were incubated with the biotinylated anti-goat IgG antibody at room temperature as manufacturer's protocol (VECTASTAIN ABC Kit, Vector Laboratories). They were incubated with horseradish peroxidase-conjugated avidin-biotin complex (ABC) and visualized with DAB, as previously described method[4].

NF-κB luciferase assay. Human HEK293-MD2-CD14-TLR4 and HEK293 were transiently transfected using polyethyleneimine with a total amount of 1 μg DNA per well comprising of NF-κB luciferase plasmid, β-galactosidase plasmid, and empty vector as filler DNA. 24 hours after transfection, cells were treated with LPS (100 ng ml^{-1}) or recombinant GST or Prx5 protein (1 μM) for 1 hour. Cell lysates were assayed for luciferase activity using the luciferase assay system and chemiluminescent reagents (Promega). A plasmid containing the β-galactosidase gene was used to normalize for transfection efficiency.

References

1 Jin MH, *et al.* Characterization of neural cell types expressing peroxiredoxins in mouse brain. *Neurosci Lett.* **381**, 252-257 (2005)

2 Shichita T, *et al.* Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat Med.* **15**, 946-950 (2009)

3 Takada J *et al.* Postischemic gene transfer of midkine, a neurotrophic factor, protects against focal brain ischemia. *Gene Ther.* **12**, 487-493 (2005)

4 Li Z, Terada N, Ohno N, Ohno S. Immunohistochemical analyses on albumin and immunoglobulin in acute hypertensive mouse kidneys by "in vivo cryotechnique". *Histol Histopathol.* **20**, 807-816 (2005)

Accepted Article

Anti-High Mobility Group Box-1 Antibody Therapy for Traumatic Brain Injury

Running head: Anti-HMGB1 mAb therapy for TBI

Yu Okuma, M.D.[1,2], Keyue Liu, M.D., Ph.D.[1], Hidenori Wake, Ph. D.[1], Jiyong Zhang, Ph.D.[1], Tomoko Maruo, M.D.[2], Isao Date, M.D., Ph.D[2], Tadashi Yoshino, M.D., Ph.D[3], Aiji Ohtsuka, M.D., Ph.D[4], Naoki Otani, M.D., Ph.D[5], Satoshi Tomura, M.D., Ph.D[5], Katsuji Shima, M.D., Ph.D[5], Yasuhiko Yamamoto, M.D., Ph.D[6], Hiroshi Yamamoto, M.D., Ph.D[6], Hideo K Takahashi, M.D., Ph.D[1], Shuji Mori,Ph.D.[7], and Masahiro Nishibori, M.D., Ph.D[1].

Departments of Pharmacology[1], Neurosurgery[2], Pathology[3] and Human Morphology[4], Okayama University Grduate School of Medicine, Dentistry & Pharmaceutical Sciences, Okayama, Japan. Department of Neurosurgery[5], National Defense Medical College, Tokorozawa, Japan. Department of Biochemistry and Molecular Vascular Biology[6], Kanazawa University Graduate School of Medical Science, Kanazawa, Japan. Department of Pharmacology[7], School of Pharmacy, Shujitsu University, Okayama, Japan.

Corresponding author: Masahiro Nishibori, M.D., Ph.D. Department of Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. Tel/FAX: +81-86-235-7140. E-mail: mbori@md.okayama-u.ac.jp

Present address: Department of Pharmacology, Kinki University School of Medicine. Osakasayama, Japan.

The authors have declared that no conflict of interest exists.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ana.23602

Accepted Article

Abstract

Objective: High mobility group box-1 (HMGB1) plays an important roles in triggering inflammatory responses in many types of diseases. In this study, we examined the involvement of HMGB1 in traumatic brain injury (TBI) and evaluated the ability of intravenously administered neutralizing anti-HMGB1 monoclonal antibody (mAb) to attenuate brain injury.

Methods: Traumatic brain injury was induced in rats or mice by fluid percussion. Anti-HMGB1 mAb or control mAb was administered intravenously after TBI.

Results: Anti-HMGB1 mAb remarkably inhibited fluid percussion-induced brain edema in rats, as detected by T2-weighted MRI, associated with inhibition of HMGB1 translocation, protection of blood-brain barrier (BBB) integrity, suppression of inflammatory molecule expression and improvement of motor function. In contrast, intravenous injection of recombinant HMGB1 dose-dependently produced the opposite effects. Experiments using RAGE-/-, TLR-4-/- and TLR-2-/- mice suggested the involvement of RAGE as the predominant receptor for HMGB1.

Interpretation: Anti-HMGB1 mAb may provide a novel and effective therapy for TBI by protecting against BBB disruption and reducing the inflammatory responses induced by HMGB1.

Accepted Article

Introduction

Traumatic brain injury (TBI) due to accidents is one of the main causes of death and disability in young individuals worldwide. The varied extents of brain edema, associated with blood-brain barrier (BBB) disruption[1] and the inflammatory response,[2] develops in acute phase of TBI as a consequence of secondary responses to the initial mechanical damage to brain tissue.[3] The marked increase in intracranial pressure due to brain edema reduces the perfusion pressure in capillary blood vessels and which leads to hypoxia and further damage to brain tissue through ischemic injury. The vicious cycle causing extensive and progressive brain edema may ultimately induce brain herniation, leading to a poor prognosis for brain-injured patients. Many kinds of cytokines and other inflammatory mediators can be produced at local lesion sites in patients with TBI via neuro-glial or neuro-vascular communication as well as through the interaction between activated vascular endothelial cells and blood cells.[4, 5] Much attention has been paid to the factors involved in the breakdown of the BBB in TBI: i.e., digestion of the lamina propria and protein complexes in tight junctions; astrocyte swelling and endothelial disorder occurring because the disruption of the BBB triggers an inflammatory cascade that exacerbates brain edema. At the present time, efficient therapies for brain injury and brain edema targeting the specific molecules are lacking.[6]

High mobility group box-1 (HMGB1), originally identified as a non-histone chromatin DNA-binding protein, is now recognized as a representative of damage-associated molecular patterns (DAMPs).[7, 8] Once released into the extracellular space from necrotic or activated cells, HMGB1 triggers the inflammatory

Accepted Article

response[9-11] through the activation of multiple receptors such as the receptor for advanced glycation endproduct (RAGE)[12, 13] and toll-like receptor-4/2 (TLR-4/2).[14-16] It has been reported that HMGB1 contributes to the pathophysiology of sepsis,[9] acute respiratory distress syndrome,[10] arthritis,[17] and acute transplant rejection.[18] Recently, we found that the translocation and release of HMGB1 from neuronal nuclei to the extracellular space occurred in ischemic brain regions induced in the middle cerebral artery occlusion/reperfusion model in rats, and that a neutralizing monoclonal antibody (mAb) against HMGB1 significantly ameliorated the resultant brain infarction by protecting the BBB from ischemia-induced breakdown.[19, 20] Using an in vitro reconstituted BBB system, we also observed that the recombinant HMGB1 directly affected the vascular endothelial cells and pericytes inducing the increase in BBB permeability, which was inhibited by the mAb against HMGB1.[20]

In the present study, we found that anti-HMGB1 mAb dramatically inhibited fluid percussion-induced brain injury and edema in rats by maintaining the integrity of the BBB and by reducing the inflammatory responses. HMGB1 appears to play a crucial role in the induction of secondary responses following primary mechanical injury of brain. Anti-HMGB1 mAb therefore represents a potential and novel therapy for TBI.

Accepted Article

Methods

Animals and surgical procedures

All experimental procedures were approved by our University's committee on animal experimentation. Adult male Wister rats 9–11 weeks-old and weighing 250–350 g were used for the experiments. Procedures for fluid percussion injury have been described previously.[21] Under anesthesia with 3.0% isoflurane in a mixture of 50% oxygen and 50% nitrous oxide gas, a 4.8-mm craniectomy was performed on the right parietal cortex (3 mm posterior and 3 mm lateral from the bregma). A plastic cylinder 4.8-mm in diameter was fixed at the craniectomy site.

On the following day, the rats were subjected to fluid percussion injury of moderate severity (2.2–2.6 atm, 16 ms in duration) using a Dragonfly fluid percussion device (model HPD-1700; Dragonfly R&D, Silver Spring, MD), maintaining the rectal temperature at 37.0 ± 0.5°C with a heating pad. Basic physiological parameters were monitored (Table 1). The rats were randomly assigned to 2 groups after the TBI operation, and an anti-HMGB1 mAb (#10-22, IgG2a subclass, 1 mg/kg) or class-matched control mAb (anti-*keyhole limpet* hemocyanin) was administered intravenously at 5 minutes and 6 hours after injury. Sham control rats were subjected to all of the same procedures except for the actual insult.

RAGE knockout (-/-) mice were produced as described previously.[22] RAGE-/- mice backcrossed to C57BL/6J (Charles River Japan) for 8 generations were used. Mice deficient in toll-like receptor 2 (TLR-2-/-) and TLR-4-/- were obtained from Oriental BioService Inc. (Tokyo, Japan).