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C-C Chemokine Receptor 5 (CCR5) Expression in the Infarct Brain of the Photothrombosis Mouse Model

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Brain ischemic stroke is one of the leading causes of death in developed countries, including Japan. Controlling the neuroinflammation in the penumbra region with mild ischemia is crucial for treating ischemic stroke. C-C chemokine receptor 5 (CCR5), noted for its functions in the progression of neuroinflammation, is considered a promising drug target. We recently found that three CCR5-regulated matrix metalloproteinases (MMPs) are detected in various cell types, including neurons, microglia, and blood vessel endothelial cells, in the ischemic brain of the photothrombosis model mouse. However, it is still unclear whether CCR5 is expressed in these cell types. This study examined the presence of CCR5 in the photothrombotic brain. Preceding the analysis, we evaluated and improved the photothrombosis induction protocol to obtain equable results with lower toxicity. Rose bengal, used to induce thrombosis to cause an infarction, is radicalized by laser and exhibits pancreatic toxicity. Therefore, we changed the administration route from the abdomen to the jugular vein and reduced the required dose of rose bengal. With this improved protocol, we found that the level of CCR5 protein was increased in neurons, microglia, and blood vessels in the ischemic core, in the infarct brain. The increase in CCR5 levels was sensitive to NSAIDs, especially to cyclooxygenase-2-selective etodolac. CD4, a collaborative membrane receptor for CCR5, was also detected in the migrating microglia. These results suggest that CCR5 is dynamically regulated and play diverse roles during ischemic stroke.

Key words brain, ischemic stroke, CCR5, photothrombosis, NSAID

INTRODUCTION

Ischemic stroke is one of the common forms of stroke and is a leading cause of death worldwide, and accounts for more than 80% of all brain strokes.[1)] During an ischemic stroke, the blood flow in certain areas in the brain is reduced. An area becomes the ischemic core when blood flow reduced to below 10%-25% of the normal level; here, oxygen and glucose are immediately depleted, resulting in rapid neuronal necrosis. The area surrounding the ischemic core, known as the penumbra, has mild ischemia. The neurons in the penumbra are also affected by low oxygen and glucose levels, but they remain viable for a while. The survival of the neurons in the penumbra is considered a potential therapeutic target for ischemic stroke.

After the onset of ischemic stroke, many cytokines are produced in the penumbra, which includes tumor necrosis factor-α (TNF-α), interleukins (ILs), and C-C chemokine ligands (CCLs).[2,3)] C-C chemokine receptor 5 (CCR5), a receptor for CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES (Regulated upon activation, normal T cell expressed, and presumably secreted), is upregulated upon neurological injuries, such as ischemic stroke.[4,5)] CCR5 was also recently reported to be a potential therapeutic target for ischemic stroke and traumatic brain injury.[6)] We have also examined the expression of *Ccr5* mRNAs in the ischemic brain by using the photothrombosis model to induce ischemic stroke in mice and found that *Ccr5* was upregulated 24-72 h after photothrombosis induction.[7)] CCR5 protein is expressed in various cell types, including CD4-positive T cells, neurons, astrocytes, microglia, monocytes, and neutrophils.[8-10)] In the infarct brain with ischemic stroke, CCR5 was reported to be upregulated in neurons and protect them from neuronal death.[6)] On the other hand, the expression of CCR5 is increased upon the differentiation of monocytes.[11)] In our previous study, the levels of mRNAs for CCR5-regulated matrix metalloproteinases (MMPs), *Mmp-3*, *Mmp-12*, and *Mmp-13* were upregulated in various cell types, including neurons, microglia, and blood vessel endothelial cells. However, the cell types that possess CCR5 during photothrombotic ischemic stroke remain unclear.

In this study, we examined the existence of CCR5 in the photothrombotic cerebral cortex. The results showed that CCR5 protein, which was detected in the cerebral neurons of a healthy brain, was upregulated in neurons in the penumbra. CCR5 was also detected in blood vessels in the ischemic core and microglia migrating along the white matter upon photothrombosis. In addition, we found that the CCR5 upregulation was sensitive to treatment with non-steroidal anti-inflammatory drugs (NSAIDs), such as etodolac and aspirin. Etodolac was found to be more potent than aspirin, suggesting that cycloox-

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ygenase (COX)-2-produced prostanoids might be involved in the upregulation of CCR5 after ischemic stroke.

MATERIALS AND METHODS

Animals Ten to fourteen-week-old ICR male mice were purchased from Japan SLC, Inc. and individually housed in the animal facility in Kobe Pharmaceutical University. All experimental procedures were conducted following the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan, and protocols were approved by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Photothrombotic Ischemic Stroke Model The photothrombotic ischemic stroke model was created based on a previous method.[12] First, the mice were anesthetized with Avertin to achieve an appropriate level of anesthesia. Then, the mice's scalps were shaved and a 5-mm incision was made from the base of the left ear to the forehead through the midline to expose the skull. Then, 10 mg/mL rose bengal (Waldeck GmbH & Co KG., Münster, Germany) in saline solution was injected intraperitoneally at 15 μL/g body weight or intravenously through the jugular vein at 5 μL/g body weight. After 5 min, the skull's surface was dried by wiping with 70% ethanol, the visual cortical area of the brain was illuminated with a 50 mW laser at 532 nm (Naku Technology Co. Ltd., Zhejiang, China) for 5 min to induce blood coagulation. Next, the mice's scalps were sutured, and the mice were allowed to recover on a 37°C heat pad with protection from light. After regaining consciousness, mice were returned to their home cages with shading.

For the experiments with NSAIDs, aspirin in saline (5 mg/kg body weight; Tokyo Chemical Industry Co. Ltd, Tokyo, Japan), etodolac in saline (5 mg/kg body weight; Tokyo Chemical Industry Co. Ltd.), or normal saline was administered by an intraperitoneal injection immediately after the model induction and every 24 h until analysis.

Histological Analyses The mice were then sacrificed 24, 48 or 72 h after the ischemic stroke model induction. Their brain, pancreas, and intestine were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C for 6 h. After cryoprotection in 30% sucrose, they were embedded in OCT compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) as previously described.[7] Then, 20 or 30-μm tissue sections were prepared in a cryostat (SLEE medical GmbH, Mainz, Germany).

Hematoxylin and eosin (H&E) staining was performed with a standard protocol. The tissue sections were washed in tap water and immersed in hematoxylin solution at room temperature (R.T.) for 10 min. They were then rinsed in tap water for 5 min, stained with eosin solution at R.T. for 2 min, washed in tap water, dehydrated, and mounted with Entellan® New (EMD Millipore Corp., Billerica, MA, USA).

For Giemsa staining, the tissue sections were washed in tap water and immersed in the Giemsa staining solution (Nacalai tesque, Inc., Kyoto, Japan) for 60 min at R.T. They were differentiated in diluted acetic acid and dehydrated in isopropanol. After the permeation procedure in xylene, they were mounted with Entellan® New.

Immunohistochemistry was performed as described in our previous manuscript with minor modifications.[13] The tissue sections were fixed in 4% paraformaldehyde in PBS at R.T. for 5 min, briefly washed with PBS, and incubated in citrate buffer pH 6.0 at 70°C for 40 min for antigen retrieval. They were then cooled to R.T., washed in PBS three times, and incubated in 0.3% hydrogen peroxide in methanol at R.T. for 15 min to inactivate the endogenous peroxidases. After three washes in PBS, the sections were blocked with 1.5% fetal bovine serum in PBS and then incubated with rabbit antibodies against CCR5 (Novus Biologicals, LLC., Centennial, CO, USA) or CD4 (Abcam plc., Cambridge, UK) at 4°C overnight. The sections were then washed with 0.1% Tween-20 in high-salt, 10 mM phosphate buffer pH 7.4 with 0.5 M NaCl and incubated with horseradish peroxidase-conjugated donkey antibody against rabbit immunoglobulin G (Thermo Fisher Scientific, MA, USA) diluted in low-salt, 10 mM phosphate buffer containing 0.05% Tween-20 at R.T. for 2 h. Next, a peroxidase-positive signal was developed with the ImmPACT DAB substrate (Vector Laboratories, Burlingame, CA). The sections were dehydrated and mounted with Entellan® New.

All images were captured under an Axio Scope.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and processed in the GNU Image Manipulation Program (GIMP), an open resource software for manipulating images.

RESULTS

An Improved Method to Induce Photothrombosis During our previous analysis, the mice with induced photothrombosis sometimes suffered from sedimentation, decreased body temperature, and even death within 48 h after the operation. We hoped to solve this problem before starting the next analysis. Laser application without the injection of rose bengal did not induce brain ischemia or other tissue abnormality in the pancreas and intestine (Fig. 1A, D, and G). The old procedure caused infarct change of the brain, but the infarct region's size was smaller than expected from the diameter of the laser, 5.5 mm (Fig. 1B).[14] Observation of the mice after photothrombosis with the previous protocol showed an intense staining of various abdominal tissues, with the most prominent staining of pancreas and intestine (data not shown). The analysis of the pancreas revealed that the acinar cells were swollen and degenerated becoming whitish (Fig. 1E). The structure of pancreatic blood vessels was also destroyed; in comparison, the Islet of Langerhans was relatively normal. The intestine also underwent a considerable morphological change. The diameter of the intestine was much bigger compared with control (data not shown). The H&E staining of the intestinal sections showed the destruction of villi (Fig. 1H). Thus, the systemic abnormality after the photothrombosis may be due to the defects in the digestive organs.

Rose bengal has been reported to have toxicity because it tended to be radicalized.[15] Therefore, we tried to reduce the dose of rose bengal by changing its administration route. We injected one-third of the original dose of rose bengal through jugular vein; then, we applied a laser on the brain as described before. This method resulted in a much larger infarct region in the brain, which was almost comparable to the laser's diameter (Fig. 1C). Also, the pancreas was almost normal in this condition (Fig. 1F). Although the intestine was slightly affected, the entire structure of villi was intact (Fig. 1I). These results indicate that the reduced dose of rose bengal administered through the jugular vein was safe and sufficient to induce ischemic stroke in the brain.

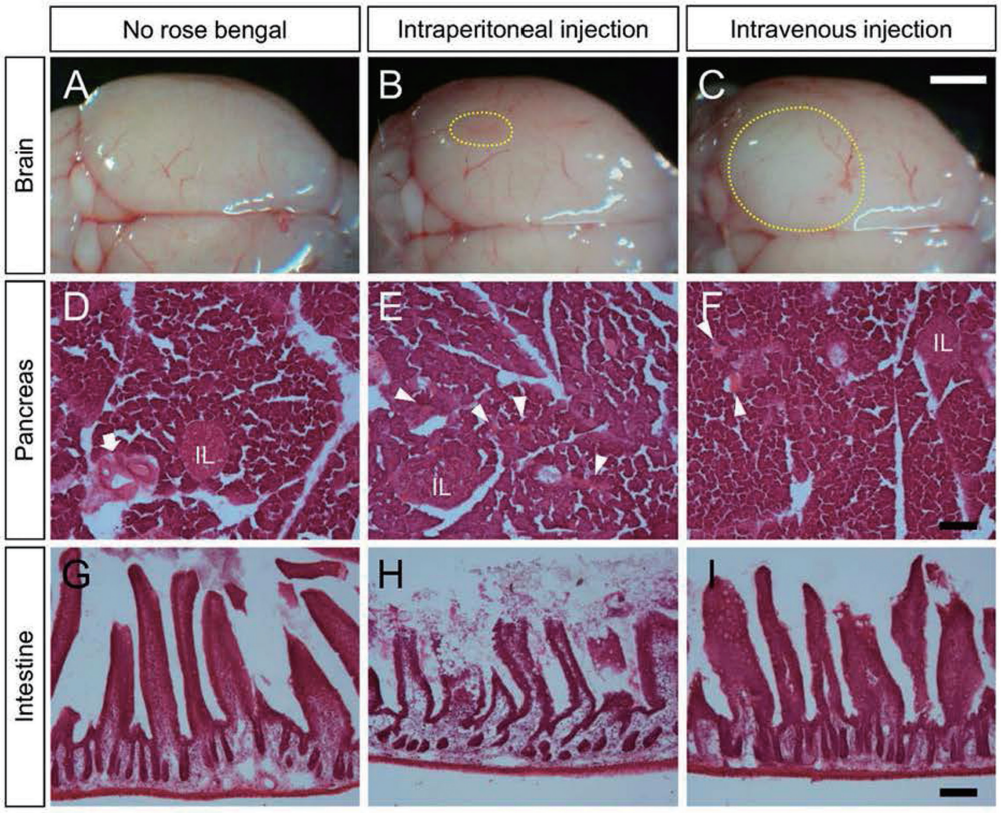

Fig. 1. Brain Ischemic Stroke and Damage of Digestive Tissues Activated by Rose Bengal

A-C: The whole mouse brain without (A), with intraperitoneal injection (B), or with intravenous injection (C) of rose bengal. The yellow dotted lines indicate the infarct region. Scale bar: 2 mm. D-F: Pancreatic sections stained by H&E. Photothrombosis was not induced (D), or induced with intraperitoneal injection (E) or with intravenous injection (F) of rose bengal. The arrow and arrowheads in panels D-F indicate pancreatic duct and resting rose bengal, respectively. IL; Islet of Langerhans. Scale bar: 100 μm. G-I: Intestinal sections stained by H&E. Photothrombosis was not induced (G), or induced with intraperitoneal injection (H) or with intravenous injection (I) of rose bengal. Scale bar: 100 μm.

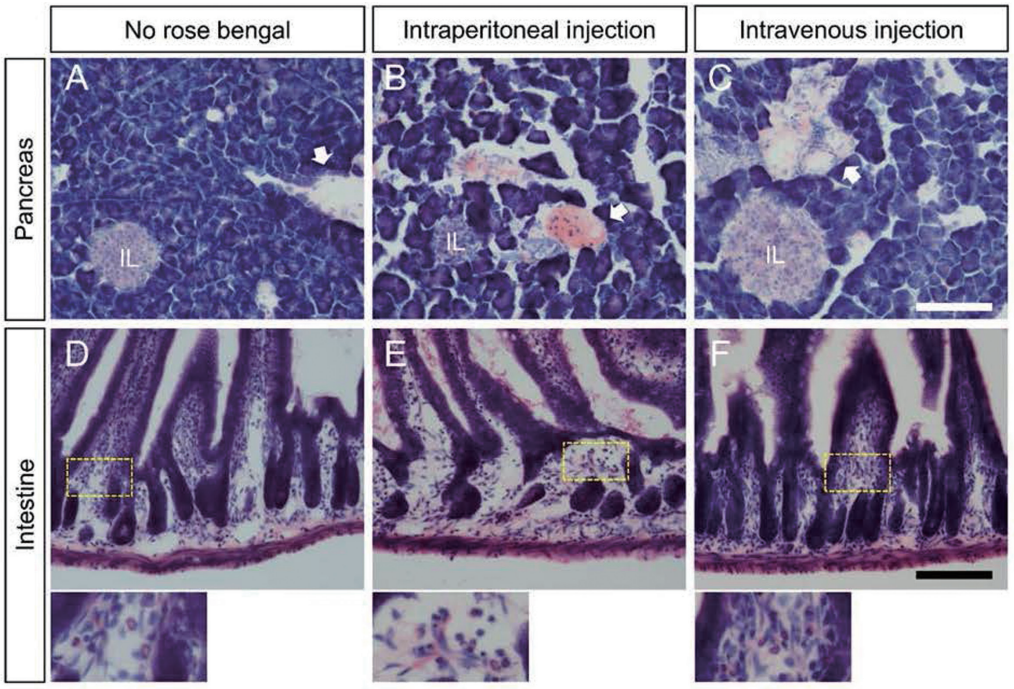

Fig. 2. The Histological Properties of Pancreas and Intestine After Rose Bengal Treatment

A-C: Tissue sections of the pancreas from the mice without (A), or with intraperitoneal injection (B), or with intravenous injection (C) of rose bengal were stained by Giemsa solution. The white arrows indicate pancreatic duct. IL; Islet of Langerhans. Scale bar: 100 μm. D-F: Tissue sections of the intestine from the mice without (D), with intraperitoneal injection (E), or with intravenous injection (F) of rose bengal were stained by Giemsa solution. The yellow square regions were enlarged in the lower panels. Scale bar: 100 μm.

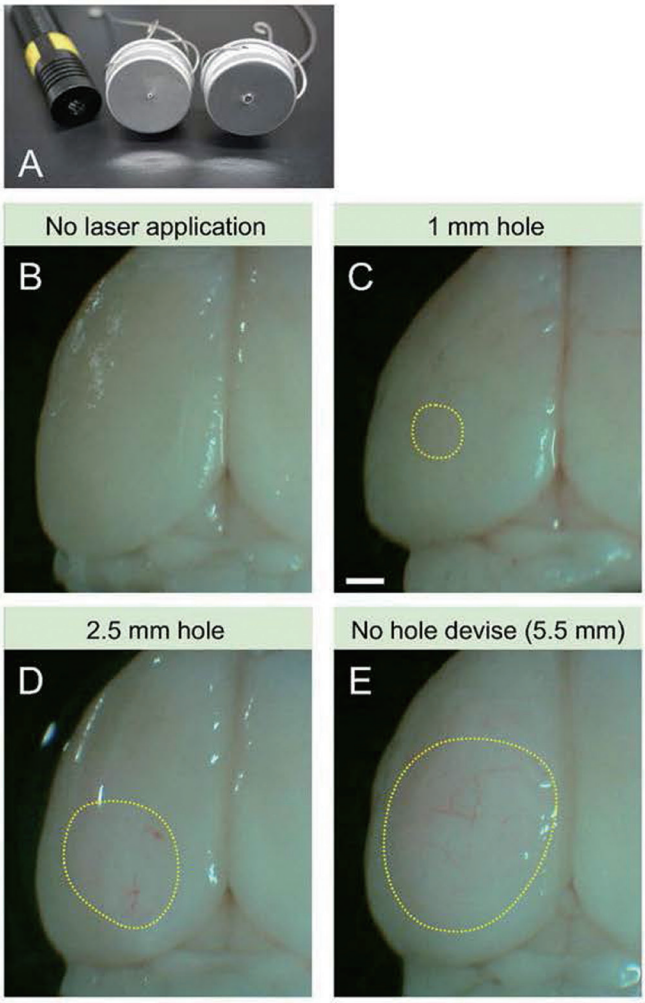

Fig. 3. Controlling the Size of the Infarct Region by Photothrombosis

A: The laser and devices used for inducing photothrombosis. B-E: The whole mouse brain with no laser application (B), a 1-mm hole (C), a 2.5-mm hole (D), or no hole device (5.5 mm) (E) 48 h after photothrombosis. Scale bar: 1 mm.

We stained the tissue sections by the Giemsa method to determine the histological characteristics in the pancreas and intestine after induced photothrombosis. In the pancreas, with the intraperitoneal injection of rose bengal, the pancreatic fluid in the ducts was reddish with eosin-staining (Fig. 2B). In contrast, the inside of pancreatic ducts showed almost no color regardless of the intravenous injection of rose bengal (Fig. 2A and C). These results suggest that the pancreatic fluid under the intraperitoneal injection of rose bengal contains cytosolic fraction released from the damaged acinar cells. In the intestine, we did not observe any significant change in the Giemsa staining pattern, except the reduction of cell number in the intestinal wall, in the mice with the intraperitoneal rose bengal injection. The number of eosin-stained immune cells was almost comparable among the different samples, indicating that the increased inflammation was not likely to be a cause of intestinal defects.

We next examined whether the size of the infarct region can be controlled in the improved protocol of photothrombosis. We prepared simple devices with a hole size of 1 and 2.5 mm, respectively, to reduce the diameter of the laser application (Fig. 3A). The photothrombotic operation with these devices produced infarction according to each device's hole size (Fig. 3B-E). Thus, the improved protocol of photothrombosis with reduced rose bengal injection with the devices with various hole sizes would help to analyze various pathological situations of ischemic stroke.

The Detection of CCR5 Protein in the Photothrombotic Infarct Brain Our previous results indicate the upregulation of *Ccr5* mRNA in the infarct cerebral cortex.[7] Here, we performed immunohistochemistry to detect the localization of CCR5 protein in the ischemic brain. In the healthy normal cerebral cortex, CCR5 was weakly detected in layer II/III and V neurons and undetectable or almost undetectable in layer IV and VI (Fig. 4A). On the other hand, a small number of neurons in the oriens layer of the hippocampus had a high level of CCR5 (Fig. 4A). The pattern of CCR5 protein levels was not changed in the contralateral region after the induction of photothrombosis (Fig. 4B and Ba).

In the ischemic core, CCR5 protein was detected in the blood vessels with an elongated shape. Although the small rounded cells in the ischemic core, which might be migrated neutrophils, were also stained, only the secondary antibody gave the similar signals, preventing us to evaluate the presence of CCR5 in those cells (Fig. 4Ca and D). In the penumbra, the CCR5 signals were exclusively observed in neurons. However, the pattern of CCR5 signals in neurons in this region was different from that in the healthy cerebral neurons. The CCR5 signals in the neurons in the healthy brain region distant from the ischemic core were observed only in the cell periphery (open arrowheads in Fig. 4Cb). In contrast, entire cells were stained for CCR5 protein in the neurons in the penumbra region (white arrowheads in Fig. 4Cb). In addition, the neurites in the penumbra region were positive for CCR5, whereas those in control regions were almost negative. These results indicate that the level of CCR5 protein is upregulated in the penumbra neurons. Migrating microglia in the white matter were also positive for CCR5 (Fig. 4Cc). Thus, the level of CCR5 protein is increased in various types of cells in the ischemic brain.

CCR5 plays a role in the progression of inflammation and previous studies have indicated that prostanoids could modulate the expression of CCR5.[16-18] Therefore, we examined the effect of NSAID treatment on the level of CCR5 in the ischemic brain. The increased CCR5 levels in the penumbra neurons were observed in the aspirin- or etodolac-treated brain (Fig. 5A-D), although the signal intensity in the penumbra neurons was weaker in the aspirin- and etodolac-treated brains, compared with the saline-treated ones. The signals in the blood vessels were not found in the aspirin-or etodolac-treated samples (Fig. 5E-G). In the lateral cortex, strong CCR5 signals were observed in the migrating microglia in the white matter in the saline-treated samples. On the other hand, there were very few CCR5-positive migrating microglia in the aspirin-treated samples and almost no CCR5-positive microglia was observed in the etodolac-treated samples (Fig. 5H-K).

CCR5 has been known to interact with CD4 and its cell surface expression is controlled.[19,20] We performed immunohistochemistry using the anti-CD4 antibody to investigate whether the localization of CCR5 protein in the infarct brain was correlated with CD4. No CD4 signal was observed in the contralateral side (Fig. 6A). In the ipsilateral side, CD4 signals were weakly detected in the migrating microglia in the white matter (Fig. 6B and Ba), in addition to the T cells along the surface

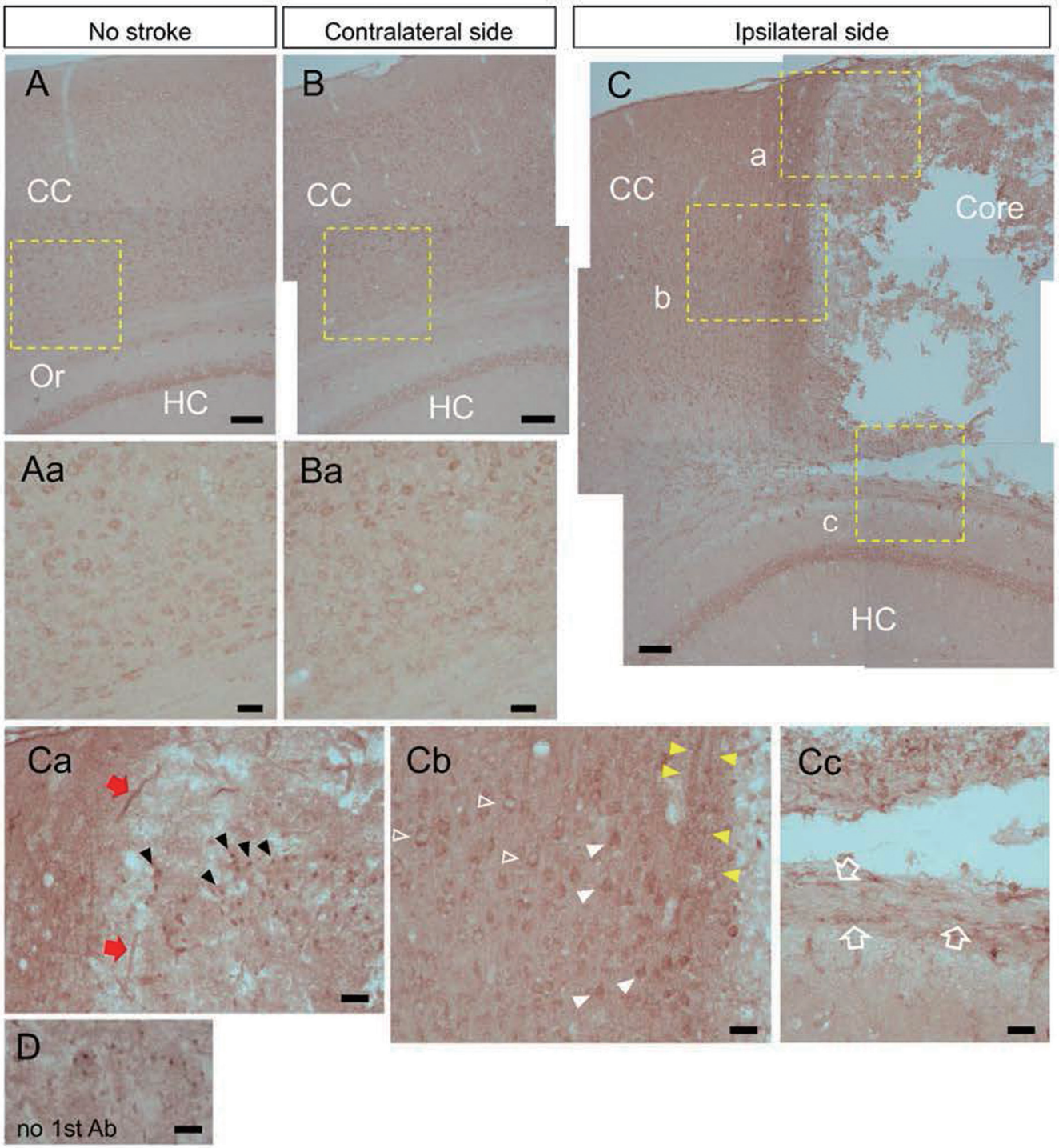

Fig. 4. The Expression of CCR5 in the Infarct Cerebral Cortex

A-C: The coronal brain sections from the mice without any treatment (A) or 72 h after photothrombosis (B, C) were immunostained with the anti-CCR5 antibody. CC, cerebral cortex; HC, hippocampus; Or, oriens layer. The yellow square regions in the control brain (A) and contralateral side (B) was enlarged in panels Aa and Ba, respectively. The yellow square regions in the ipsilateral side (C) were enlarged in panels Ca, Cb, and Cc. The red arrows and black arrowheads in panel Ca indicate blood vessels and non-specific signals in invading immune cells in the ischemic core, respectively. The open and white-filled arrowheads in panel Cb indicate CCR5-positive neurons with circular signal and with filled signal, respectively. The yellow arrowheads in panel Cb indicate CCR5-positive neurites. The open arrows in panel Cc indicate CCR5-positive migrating microglia. D: Ischemic core of the photothrombotic brain (a tissue section adjacent to C) stained only with secondary antibody. Scale bars: 100 μm (A, B, C) or 30 μm (Aa, Ba, Ca, Cb, Cc, D).

of the brain (data not shown). In contrast, CD4 signal was not detected in the neurons in both healthy and penumbra regions (Fig. 6Bb).

DISCUSSION

Rose bengal has been used as a coloring food additive (Red No.105) in Japan since 1948. On the other hand, its antimicrobial effect upon photoactivation has been suggested due to its photo-reactivity.[21,22] Rose bengal produces a singlet oxygen

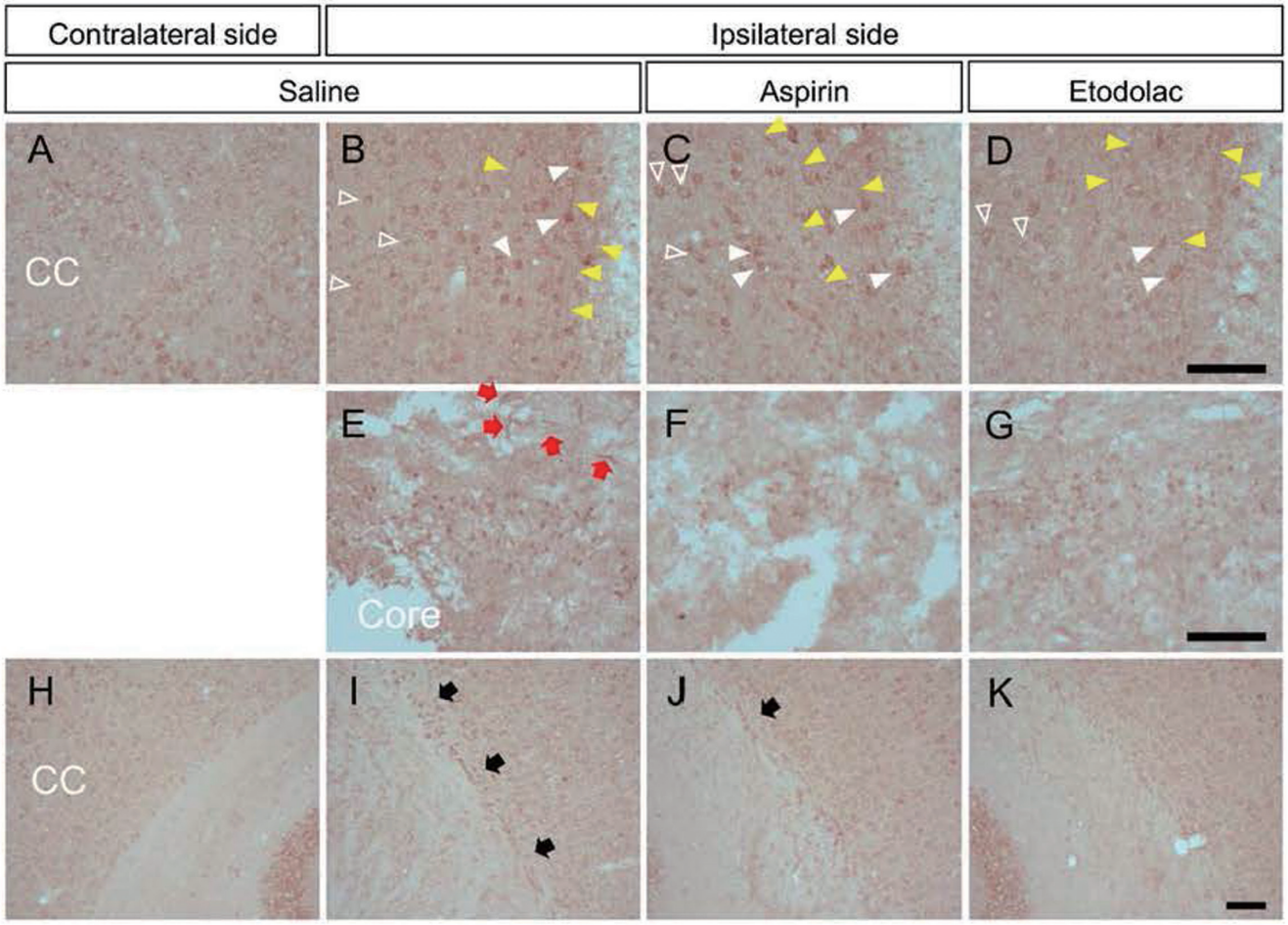

Fig. 5. The Effect of NSAIDs on the Expression of CCR5

The coronal brain sections from the mice 72 h after photothrombosis treated with saline (A, B, E, H, I), aspirin (C, F, J), or etodolac (D, G, K) were immunostained with the anti-CCR5 antibody. A-D: Dorsal cerebral cortex neat the infarct region (B-D) or contralateral side at the similar dorsoventral (D-V) level as a control (A). The white-open, white-closed, and yellow-closed arrowheads indicate neurons with circular CCR5 signal, neurons with filled CCR5 signal, and CCR5-positive neurites, respectively. E-G: The ischemic core from the mice 72 h after photothrombosis treated with saline (E), aspirin (F), or etodolac (G) were immunostained with the anti-CCR5 antibody. The red arrows indicate CCR5-positive blood vessels. H-K: The lateral cerebral cortex away from the infarct region (I-K) and equivalent region of the contralateral side (H). The black allows indicate migrating microglia. CC, cerebral cortex. Scale bars: 100 μm.

with visible light at the wavelength between 450 nm-580 nm.[23] The mouse model of photothrombotic ischemia is a powerful experimental tool that utilizes rose bengal's photo-reactivity.[12,14] However, our previous method sometimes resulted in systemic injuries and even death in mice after the induction of photothrombosis. This study revealed that the cause of the systemic injuries might be due to the harmful effect of radicalized rose bengal in the pancreas (Figs. 1 and 2). Matthews and Cui have reported that photoactivated rose bengal caused toxicity in the pancreatic acini and released digestive enzymes; however, the authors only analyzed isolated pancreatic acini and applied rose bengal *in vitro*.[15] Nevertheless, our results are consistent with their observations and further presented *in vivo* evidence for the pancreatic toxicity of photoactivated rose bengal.

Our previous study has implicated the induced mRNA expression of CCR5-regulated MMPs in various cell types, including neurons, microglia, and blood vessel endothelial cells, in the photothrombosis-induced infarct brain.[7] However, existence of CCR5 in these cell types has not yet to be confirmed. CCR5 protein was detected in the neurons, microglia, and blood vessel endothelial cells after photothrombosis (Fig. 4). This finding supports the hypothesis that CCR5 is a master regulator of tissue remodeling during an ischemic stroke by regulating these MMPs.

Prostanoids play diverse roles in the disease progression of ischemic stroke.[24] COX-2, an inducible isoform responsible for acute prostanoid synthesis, has been implicated in the progression of ischemic stroke and neuroinflammation. In this study, we utilized NSAIDs to elucidate the role of prostanoids in the induction of CCR5 during ischemic stroke. Although most NSAIDs target both COX-1 and COX-2, they have some preference to the isoform specificity: aspirin inhibits COX-1 more effectively than COX-2, whereas etodolac is more selective to COX-2.[25] Here, etodolac was observed to inhibit the increase in the CCR5 levels in the penumbra neurons more effectively than aspirin (Fig. 5), suggesting that COX-2 played a role in the induction of CCR5. *Ccr5* expression has been induced by ILs, including IL-2, IL-6, IL-10, and IL-12.[26-28] Prostanoids may indirectly regulate *Ccr5* expression through these ILs. In contrast to its level in neuronal cell body, CCR5's localization in neurites was not affected by NSAIDs (Fig. 5). Although the plasma membrane localization of CCR5 is affected by cytokine-stimulation,[29] the induced membrane transport

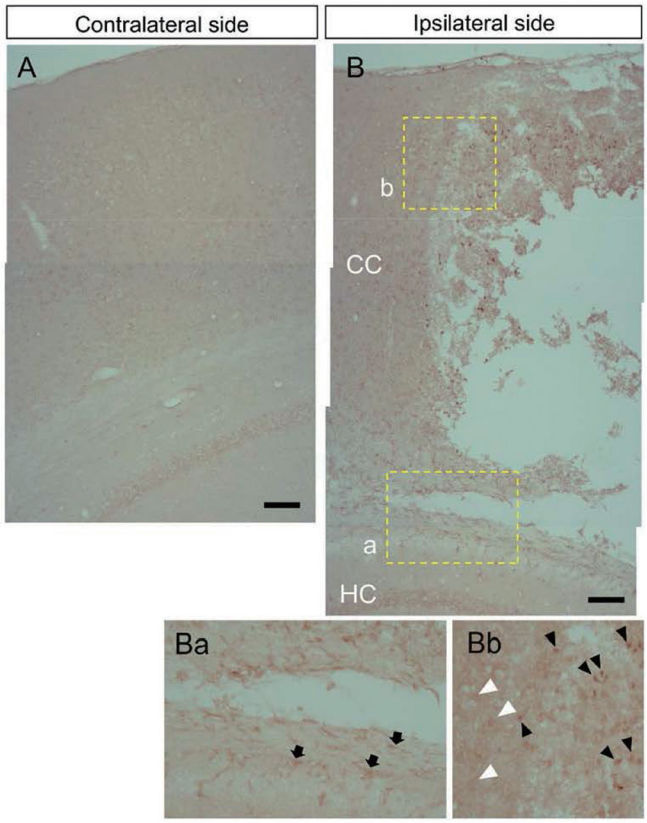

Fig. 6. The Localization of CD4 in the Infarct Region of the Brain 72 h After Photothrombosis

A: The contralateral side of the infarction immunostained with the anti-CD4 antibody. B: The ipsilateral side immunostained with the anti-CD4 antibody. The yellow squares a and b were enlarged in panels Ba and Bb, respectively. The black arrows, white arrowheads, and black arrowheads indicate CD4-positive migrating microglia, cerebral neurons with back ground-level of CD4 signals, and non-specifically stained invading immune cells. CC, cerebral cortex; HC, hippocampus. Scale bars: 100 μm.

of CCR5 may be free from the regulation by prostanoids. The level of CCR5 in migrating microglia in the lateral cortex was also reduced by NSAID treatment, possibly due to the direct or indirect regulation of *Ccr5* expression in microglia by prostanoids. However, NSAIDs may also inhibit the migration of CCR5-positive microglia upon the infarction.

CCR5 interacts with CD4 and functions as its co-receptor.[19] Although CD4 is mainly found in T lymphocytes, the detection of CD4 protein in activated microglia has been implicated in rat virus-injection and EAE models.[30,31] Our results also demonstrated a low level of CD4 in the migrating microglia in the white matter after the induction of photothrombosis (Fig. 6). CD4 facilitates the binding of gp120 human immunodeficiency virus (HIV) protein to CCR5,[32] probably by increasing CCR5's membrane translocation efficiency by its interaction with CD4.[19,33] Thus, CD4 modulates the function of CCR5. It would be interesting to study how CD4 in activated microglia modulates the signaling by CCLs and CCR5. Notably, CD4-positive signal in neurons was not detected in our study (Fig. 6); therefore, CCR5 signaling in neurons and microglia may be differentially modulated. This diversity in CCR5 modulation may explain the different cell type-specific responses to CCLs.

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Conflict of interest The authors declare no conflict of interest.

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