Table I

Treatment	Total Cholesterol (mmol/l)	HDL-Cholesterol (mmol/l)	LDL-cholesterol (mmol/l)	Triglycerides (mmol/l)
Control Ab	7.55 ± 0.33	1.27 ± 0.05	5.19 ± 0.22	2.46 ± 0.22
HMGB1 Ab	7.70 ± 0.38	1.30 ± 0.08	5.45 ± 0.29	2.14 ± 0.13

Table II

MONOCYTE/DENDRITIC CELLS*	Control Ab	Anti-HMGB1 Ab
Blood		
BrdU+CD11b^{hi}Ly-6C^{hi}	0.032 ± 0.017 x 10^6	0.044 ± 0.009 x 10^6
CD11b^{hi}Ly-6C^{hi}	0.103 ± 0.045 x 10^6	0.099 ±0.024 x 10^6
CD11b^{hi}	0.137 ± 0.056 x 10^6	0.130 ± 0.024 x 10^6
BrdU+CD11c^{hi}CD11b^{hi}	0.0348 ± 0.016 x 10^6	0.0394 ± 0.008 x 10^6
CD11c^{hi}CD11b^{hi}	0.082 ± 0.035 x 10^6	0.088 ± 0.025 x 10^6
CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}	0.0097 ± 0.0048 x 10^6	0.0111 ± 0.0027 x 10^6
Inguinal Lymph Nodes		
CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}	0.007 ± 0.003 x 10^6	0.008 ± 0.003 x 10^6
Mediastinal Lymph Nodes		
CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}	0.0014 ± 0.004 x 10^6	0.0027 ±0.0021 x 10^6

* All cells are CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}

Figure I

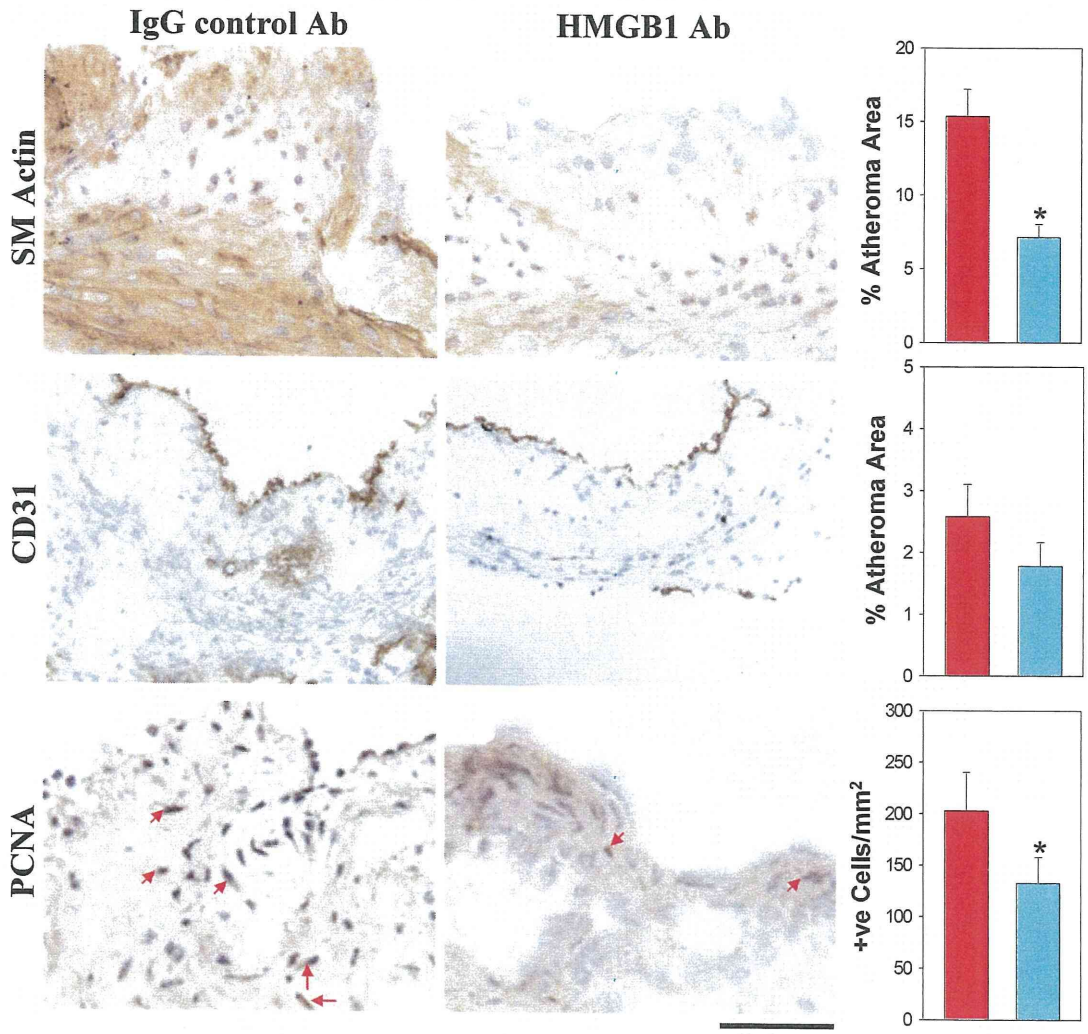


Figure II

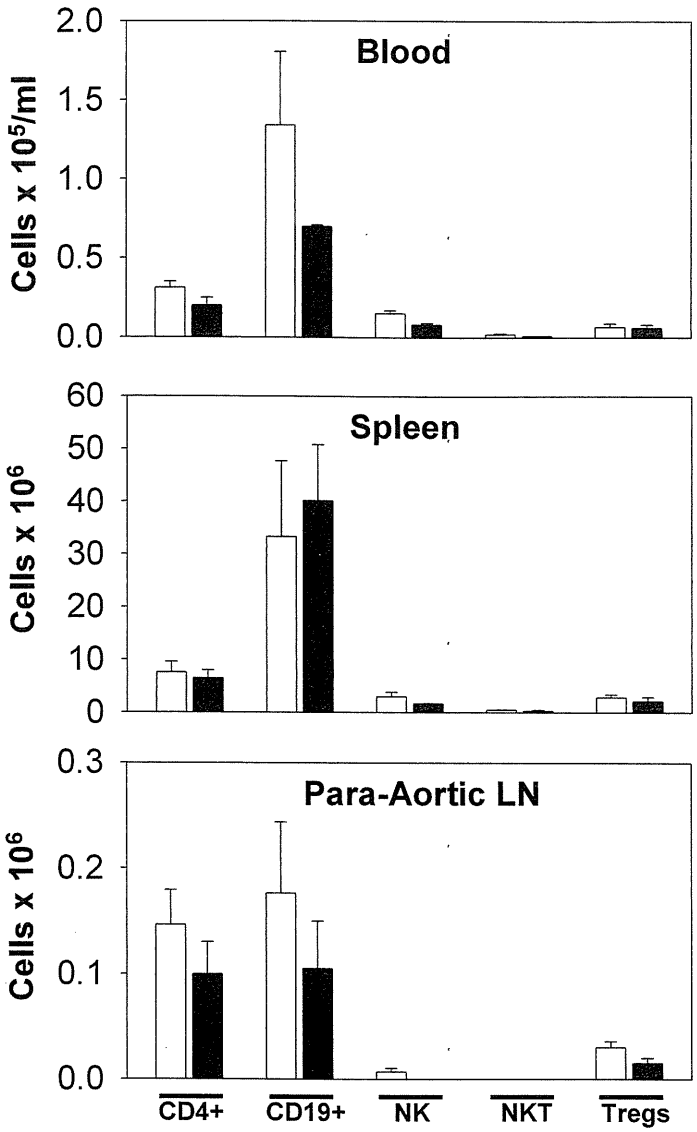
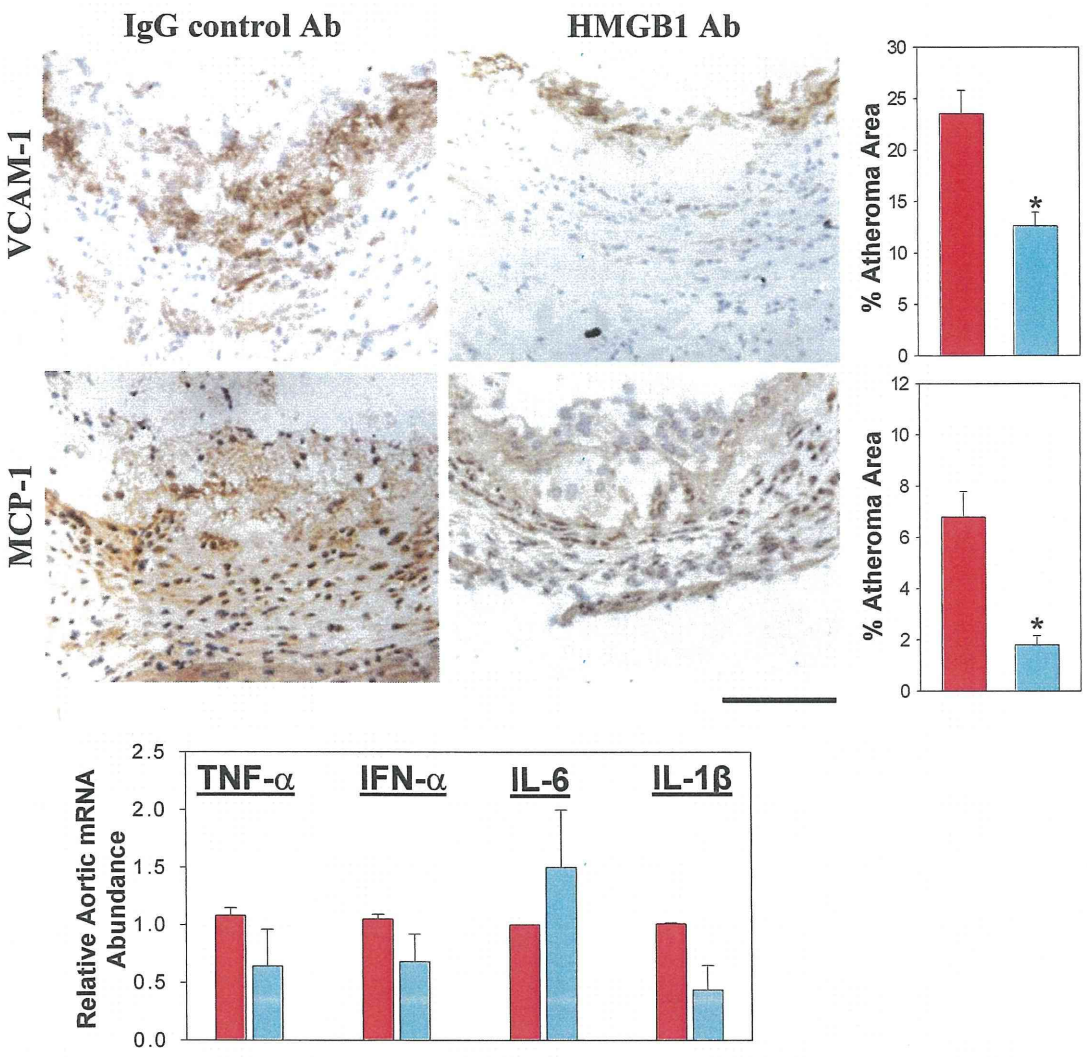


Figure III



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Experimental paper

Reduction of the infarct size by simultaneous administration of L-histidine and diphenhydramine in ischaemic rat brains☆

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ABSTRACT

Aims: While diphenhydramine is a histamine H_1 receptor antagonist, the agent has been shown to inhibit histamine-*N*-methyltransferase, a histamine inactivating enzyme in the brain. Since an increase in the brain concentration of histamine ameliorates reperfusion injury after cerebral ischaemia, effects of postischaemic administration of diphenhydramine were evaluated in rats treated with L-histidine, a precursor of histamine.
Methods: The right middle cerebral artery was occluded for 2 h, and the infarct size was determined 24 h after reperfusion of cerebral blood flow. Brain oedema was evaluated by comparing the area of the right hemisphere to that of the left hemisphere.
Results: Focal cerebral ischaemia provoked marked damage in saline-treated control rats, and infarct volumes in the striatum and cerebral cortex were 56 (49–63) mm^3 and 110 (72–148) mm^3, respectively (means and 95% confidence intervals, $n = 6$). Administration of L-histidine (1000 mg/kg, intraperitoneal) immediately after reperfusion did not affect the infarct size. Simultaneous administration of diphenhydramine (20 mg/kg, intraperitoneal) with L-histidine reduced the infarct size to 25% and 21% of that in the control group, respectively. The combination therapy completely reduced ischaemia-induced brain oedema.
Conclusion: Because histamine H_1 action does not influence ischaemic brain damage, elevation of the central histamine concentration by blockade of histamine-*N*-methyltransferase may be a likely mechanism responsible for the alleviation.

1. Introduction

We have shown that an increase in the histamine concentration in the brain alleviates reperfusion injury after brain ischaemia by reducing inflammatory responses through histamine H_2 receptors.[1–4] Although a single dose of L-histidine (1000 mg/kg), a precursor of brain histamine, failed to improve the outcome, simultaneous administration of metoprine, a competitive inhibitor of histamine-*N*-methyltransferase, an inactivating enzyme of brain histamine, reduced the size of brain infarction.[3]

There are several compounds that inhibit histamine-*N*-methyltransferase activity, such as amodiaquine, tacrine, chlorpromazine and vecuronium. However, adverse effects of these agents are problematic as well as those of metoprine. Diphenhydramine, a histamine H_1 receptor antagonist, is usually applied to various allergic diseases and has an inhibitory effect on histamine-*N*-methyltransferase.[5,6] Because the agent readily passes the blood–brain barrier, and no serious adverse effect has been reported, we investigated the effect of concomitant administration of diphenhydramine and L-histidine on ischaemic brain damage.

2. Methods

This study was approved by the Committee on Animal Experimentation at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by Okayama Univer-

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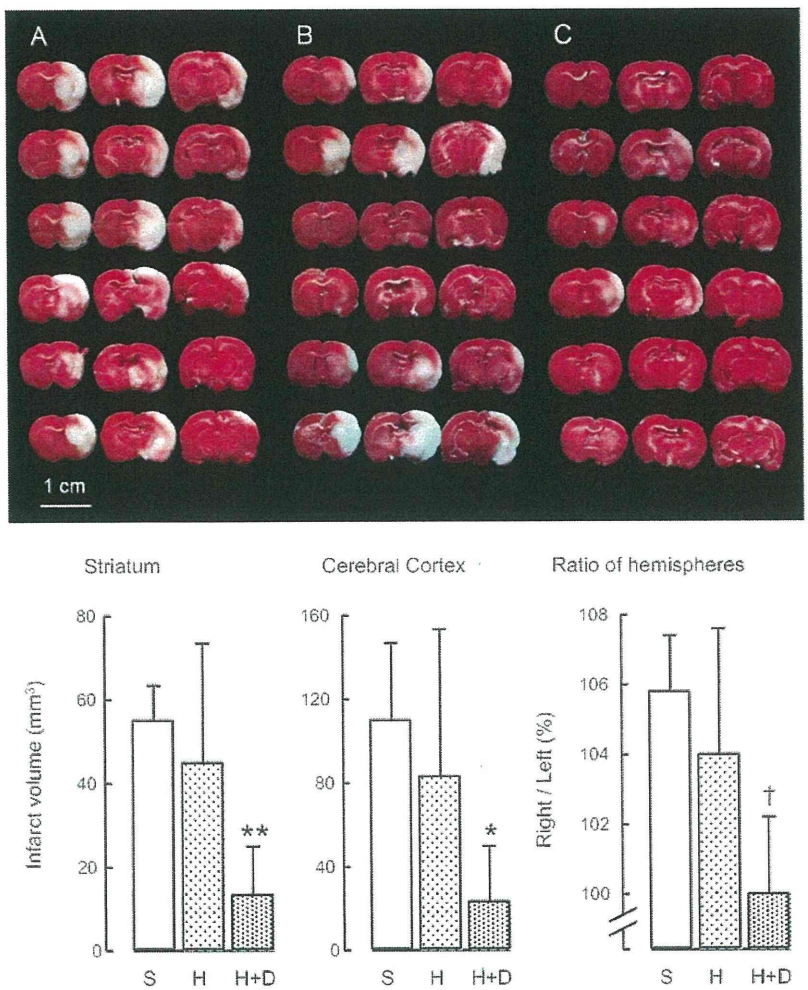

Fig. 1. Effects of postischaemic administration of L-histidine and diphenhydramine. The right middle cerebral artery was occluded for 2 h, and L-histidine (1000 mg/kg) and diphenhydramine (20 mg/kg) were intraperitoneally administered immediately after reperfusion of cerebral blood flow. The effect was evaluated by assessing the size of brain infarction 24 h after reperfusion with 2,3,5-triphenyltetrazolium chloride stain, which turns the viable tissue a deep red colour. All brain slices in the saline-injected control (A), L-histidine (B) and L-histidine plus diphenhydramine groups (C) are shown. The size of brain infarction was measured in the striatum and cerebral cortex. Using these brain slices, the percentage of the area of the right (ischaemic) hemisphere to that of the left (non-ischaemic) hemisphere was calculated as a measure of brain oedema. Values represent means and 95% confidence intervals ($n=6$ each). $^{*}p=0.02$, $^{**}p=0.004$, $^{\dagger}p=0.006$ as compared with the control group. S, saline; H, L-histidine; D, diphenhydramine.

sity Graduate School of Medicine, Dentistry and Pharmaceutical Sciences. Male Wistar rats (Charles River, Yokohama, Japan) weighing 280–300 g were kept in groups in a room controlled at 23 ± 2 °C and maintained under an alternating 12-h light and 12-h dark cycle.

Eighteen rats were divided into three groups ($n=6$ in each); the saline-injected control group, the L-histidine-injected group and the L-histidine plus diphenhydramine-injected group. The animal was anesthetised with 2% halothane in balanced 50% oxygen and 50% nitrous oxide, and kept under spontaneous ventilation. With the rat in the supine position, the right common carotid artery was exposed, and a thermocouple needle probe was inserted into the temporal muscle to maintain the brain temperature. After an intraperitoneal injection of heparin (100 units), the root of the right middle cerebral artery was occluded by insertion of a silicone-coated 4.0 nylon thread from the bifurcation of the internal and external carotid arteries. The tip of the thread was placed 18 mm distal from the bifurcation. After the surgical incision was sutured, the animal was allowed to recover from anesthesia. During surgery, the temperature of the temporal muscle was maintained at 37.0 ± 0.1 °C with a heating lamp. All rats showed paralysis of the contralateral limbs after recovery from anesthesia. The animal was anesthetised again 5 min before reperfusion of the blood flow, and the skin was reopened. Cerebral blood flow was resumed 2 h after middle cerebral artery occlusion by pulling the thread by 5 mm. Then, the animal was intraperitoneally injected with saline, L-histidine (1000 mg/kg) or L-histidine (1000 mg/kg) plus diphenhydramine (20 mg/kg). The surgical incision was sutured, and the animal was allowed to recover from anesthesia.

The animal that underwent 24 h of reperfusion was anesthetised with an intraperitoneal injection of sodium pentobarbital, and the brain was perfused with saline. Brain slices, 2-mm thick, between the coronal planes at the optic chiasma and caudal edge of the mammillary body were incubated for 30 min with 2% 2,3,5-triphenyltetrazolium chloride in 0.1 mol/L phosphate buffer (pH 7.4) at 37 °C. 2,3,5-Triphenyltetrazolium chloride is reduced by dehydrogenase enzymes, which exist in viable cells and result in a formazan precipitate, thereby turning the tissue a deep red colour. In contrast, nonviable cells in the infarcted area show a pale gray colour with the procedure. The infarct size in the striatum and cerebral cortex was then determined, using computer-aided planimetry by an investigator who was unaware of the particular treatment group. Using these brain slices, the ratio of the area of the right

hemisphere to that of the left hemisphere was obtained in each slice. Then, the average percentage of three slices was calculated in each animal.

The data were evaluated by Scheffé's tests, and p values less than 0.05 were considered statistically significant.

3. Results

Focal cerebral ischaemia for 2 h provoked marked damage in the striatum and surrounding cerebral cortex in saline-treated control animals (Fig. 1). The infarct volumes in the striatum and cerebral cortex were 56 (49–63) mm^3 and 110 (72–148) mm^3, respectively (means and 95% confidence intervals, $n = 6$). Postischaemic administration of L-histidine (1000 mg/kg) affected the infarct size in neither the striatum ($p = 0.64$) nor the cerebral cortex ($p = 0.65$). Simultaneous administration of diphenhydramine (20 mg/kg) with L-histidine reduced the infarct volume in both the striatum ($p = 0.004$) and cerebral cortex ($p = 0.02$), and the values were 25% and 21% of those in control animals, respectively.

The ratio of the area of the right hemisphere to that of the left hemisphere was 105.8% (104.0–107.6%) in the control group. Although a single dose of L-histidine did not affect the ratio ($p = 0.54$), simultaneous administration significantly reduced it ($p = 0.006$).

4. Discussion

In the present study, L-histidine administration did not ameliorate the size of brain infarction. The finding is consistent with our previous reports that L-histidine provides no benefits at any doses by a single dose after reperfusion.[3,7] Simultaneous administration of diphenhydramine reduced the size of brain infarction, and the magnitude of the decrease is similar to that obtained from combination therapy with L-histidine and metoprine.[3] The treatment with L-histidine and diphenhydramine markedly reduced ischaemia-induced brain oedema as well as the infarct size.

In our previous study, brain infarction produced by occlusion of the middle cerebral artery for 2 h was alleviated by postischaemic administration of L-histidine (1000 mg/kg × 2), immediately and 6 h after reperfusion.[2] The beneficial effect was completely abolished by topical administration of ranitidine, a histamine H_2 receptor antagonist, indicating that an increase in the histamine level contributes to the improvement through histamine H_2 receptors. Considering that diphenhydramine is a potent H_1 antagonist and has negligible affinity for histamine H_2 receptors, it is unlikely that the improvement of the outcome by diphenhydramine attributes to its blocking action on histamine H_1 receptors.

Methylation by the specific enzyme, histamine-*N*-methyltransferase, is the predominant pathway of histamine inactivation in the brain, and no high-affinity uptake for histamine has been reported in brain slices, homogenates and cultured neurones.[8–10] In a study on purified histamine-*N*-methyltransferase from the mouse brain, diphenhydramine showed a biphasic effect on histamine-*N*-methyltransferase activity.[5] At histamine concentrations below 10 μmol/L, diphenhydramine inhibited the enzyme activity. On the other hand, the agent markedly augmented it at histamine concentrations in excess of 10 μmol/L. Although the extracellular concentration of histamine increases in ischaemic brains, the concentration does not attain 10 μmol/L.[7,11] Therefore, diphenhydramine may show an inhibitory action on histamine-*N*-methyltransferase in cerebral ischaemia. Facilitation of the brain concentration of histamine by simultaneous administration may provide benefits by suppressing inflammatory responses during reperfusion through histamine H_2 receptors.

5. Conclusion

Since both L-histidine and diphenhydramine are readily transported to the brain across the blood–brain barrier, simultaneous administration of these compounds may be a new strategy for stroke.

Conflict of interest

N. Adachi, K. Liu, A. Motoki and M. Nishibori are concerned with patent applications/registrations related to reperfusion therapies with L-histidine. K. Ninomiya, E. Matsuoka and Y. Irisawa have no conflicts of interest.

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Original Article

Gene Expression and Localization of High-mobility Group Box Chromosomal Protein-1 (HMGB-1) in Human Osteoarthritic Cartilage

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We investigated the expression and localization of high-mobility group box chromosomal protein-1 (HMGB-1) in human osteoarthritic (OA) cartilage in relation to the histopathological grade of cartilage destruction, and examined the role of HMGB-1 in the regulation of proinflammatory cytokine expression in chondrocytes. An immunohistochemical study demonstrated that total HMGB-1-positive cell ratios increase as the Osteoarthritis Research Society International (OARSI) histological grade increased. The population of cytoplasmic HMGB-1-positive chondrocytes was especially increased in the deep layers of higher-grade cartilage. The ratios and localization of receptors for advanced glycation end products (RAGE) expression by chondrocytes in Grade 2, 3, and 4 were significantly higher than those in Grade 1. *In vitro* stimulation with IL-1β, but not TNFα, significantly upregulated the expression of HMGB-1 mRNA by human OA chondrocytes. Both IL-1β and TNFα promoted the translocation of HMGB-1 from nuclei to cytoplasm. IL-1β and TNFα secretions were stimulated at higher levels of HMGB-1. The results of our study suggest the involvement of HMGB-1 in the pathogenesis of cartilage destruction in OA.

Key words: HMGB-1, RAGE, chondrocyte, osteoarthritis, cartilage

Osteoarthritis (OA) is a proliferative joint disease characterized by articular cartilage degeneration, osteophyte formation, subchondral bone sclerosis, and secondary induced synovitis [1]. Although numerous studies have revealed the contribution of genetic factors, growth factors and cytokines, mechanical stress, proteinase, or altered responses of chondrocytes in the progression of OA, the pathogenesis of OA has not been fully understood.

High-mobility group box chromosomal protein (HMGB-1), previously called HMG-1 or amphoterin, named for its rapid mobility on electrophoresis gels, is a ubiquitous, approximately 27-kDa nonhistone protein with a highly conserved amino acid sequence identity between rodents and humans [2-4]. Nuclear HMGB-1 has been widely studied as a deoxyribonucleic acid (DNA)-binding protein. It participates in the maintenance of nucleosomal structure and stability and facilitates the binding of transcription factors to their cognate DNA sequences [5]. HMGB-1 also has func-

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tions in DNA transcription, recombination [6, 7], repair, cell replication, cell migration, and tumor growth [8, 9].

In contrast to its intranuclear role, HMGB-1 plays a critical role outside the cell as a proinflammatory cytokine mediating delayed endotoxin lethality as well as acute lung injury in mice [10, 11]. Moreover, high levels of HMGB-1 have been detected in the blood of patients with sepsis and in the synovial fluid of rheumatoid arthritis (RA) patients [12]. Proinflammatory mediators, such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), dose-dependently induce the release of HMGB-1 from monocytes and macrophages [10]. Furthermore, once released, HMGB-1 activates an additional downstream cascade by stimulating monocytes to produce proinflammatory cytokines and chemokines [13].

The receptor for advanced glycation end products (RAGE) is a transmembrane protein that belongs to the immunoglobulin superfamily and is expressed in vascular smooth muscle cells, neurons, and certain phagocytes such as monocytes and macrophages [14]. Recently, the expression of RAGE has been reported in articular chondrocytes, synoviocytes, and RA synovial macrophages [15–17]. Membrane-associated HMGB-1 mediates cellular proliferation and growth by signaling through the receptor for RAGE [18].

Previous reports have suggested the HMGB-1 upregulation in OA cartilage and induction of inflammatory cytokines such as IL-6 or IL-8 [19]. In the current study, we investigated the expression and localization of nuclear and cytoplasmic HMGB-1 in normal human cartilage and OA cartilage. We further investigated the effect of HMGB-1 stimulation on TNFα and IL-1β production, as well as the effect of TNFα and IL-1β on HMGB-1 production by human OA chondrocytes. Our data strongly suggest the involvement of HMGB-1 in proinflammatory cytokines in cartilage destruction.

Materials and Methods

Production of HMGB-1-specific monoclonal antibody. Rats were immunized with HMGB-1/HMGB-2 (Sigma, St. Louis, MO, USA) emulsified with Freund's complete adjuvant. A booster injection with incomplete adjuvant was administered to the rats 3 weeks later. After confirming the elevation of anti-HMGB-1 antibody, we produced hybridomas as previously described [20]. The epitope recognized by each monoclonal antibody was determined by dot blotting, using synthetic overlapping peptides derived from a human HMGB-1 sequence 15 amino acids long. The clone (Nos. 10–22, subclass IgG2a) used for the experiments recognized the C-terminal sequence of the HMGB-1 molecule, DEDEEEE, as specific for HMGB-1 but not for HMGB-2.

Recombinant human HMGB-1 (rHMGB-1). rHMGB-1 was produced in Sf9 cells to obtain LPS-free HMGB-1. In brief, full-length human HMGB-1 DNA was amplified by PCR using Cap Site cDNA dT from human microvascular endothelial cells (Nippon Gene, Tokyo, Japan) and primers (forward 5'-GCA GAA TTC ATG GGC AAA GGA GAT CCT A-3', reverse 5'-CAT CTC GAG TCA TTA TTC ATC ATC ATC ATC-3'). The fragment was digested with EcoRI and XhoI and cloned into a pFastBacHTA (Invitrogen, Carlsbad, CA, USA) expression vector. The transfection of the Sf9 cells with the pFastBacHTA-HMGB-1 bacmid was performed according to the manufacturer's instructions (Bac-to-Bac Baculovirus Expression System, Invitrogen). The infected SF9 cell extract containing His-tagged HMGB-1 protein was applied to Ni-NTA agarose (Qiagen, Hilden, Germany) and incubated for 3 hours at room temperature. After extensive washing, rHMGB-1 was eluted with imidazole buffer. The rHMGB-1 was collected and dialyzed overnight at 4℃ against PBS. Purified rHMGB-1 protein was identified by SDS-PAGE and Western blotting with anti-HMGB-1 mAb. The final HMGB-1 preparation contained LPS of less than 2.0 pg/μg protein.

Human cartilage samples. Thirty-one human OA cartilage samples were obtained at the time of total knee arthroplasty (TKA) surgery from 27 OA patients (aged 48 to 82 years; average, 70.7 years), who lacked a medical history of other inflammatory diseases. Normal cartilage was obtained from 4 patients with osteosarcoma (aged 9 to 29 years; average 15.5 years) who lacked any inflammatory diseases at the time of amputation surgery. Written informed consent was obtained from the patients or their parents.

RNA isolation from OA cartilage. OA cartilage samples from OA patients (n = 4) and normal cartilage from osteosarcoma patients (n = 4) were

divided in half; one half was immediately frozen in liquid nitrogen and stored at −180℃ until required for RNA isolation. At that point the frozen articular cartilage (10–40 mg wet weight) was milled with Micro Smash (Tomy Seiko Co, Tokyo, Japan) at 2 cycles of 1 min at 4,500 rpm and was suspended in 1 mL Isogen (Nippon Gene Co, Tokyo, Japan). Total RNA was isolated according to the manufacturer's protocol.

cDNA synthesis and Quantitative Real-Time PCR. First-strand cDNA was synthesized from 1 μg total RNA using the ReverTra Ace-α according to the manufacturer's (Toyobo Co., Ltd., Osaka, Japan) instructions. cDNA (1.5 μL) was amplified by real-time PCR using Brilliant® II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA). Quantitative PCR was performed on a 25-μL samples. Mixtures were preincubated at 95℃ for 10 min, followed by 40 cycles of PCR at 95℃ for 30 sec and 60℃ for 30 sec, and normalized to GAPDH. In every case, the cycle threshold (C_T) taken for quantitation was in the linear portion of the amplification range. PCR primers were HMGB-1 sense, 5'-TAT GAA AAG AAG GCT GCG AAG-3'; and HMGB-1 antisense, 5'-CTG CGC TAG AAC CAA CTT ATT C-3', GAPDH sense, 5'-CAT CAA GAA GGT GGT GAA GCA G-3'; and GAPDH; antisense, 5'-CTG CAA AGG TGG AGG AGT GG-3'. All PCRs were performed in triplicate using the Mx3000P quantitative PCR system (Stratagene). RNA levels are reported as fold changes compared with the control using the comparative quantitation analysis software available with the Mx3000P. Changes in expression (fold) for HMGB-1 were calculated as $2^{-\Delta(\Delta CT)}$, where $\Delta C_T = C_T(\text{target}) - C_T(\text{housekeeping})$, and $\Delta(\Delta C_T) = \Delta C_T\ (\text{treated}) - \Delta C_T(\text{control})$. Reaction product purity was confirmed by examination of the melting curves for a single peak.

Immunohistochemistry for HMGB-1 and RAGE. Twenty-seven OA and 4 normal cartilage specimens were used in an immunohistochemical study for HMGB-1 and RAGE. The samples were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), decalcified in 0.3 M EDTA (pH 7.5) for 2–3 weeks, and embedded in paraffin. All sections were used for the histological and immunohistochemical studies.

The paraffin sections were soaked in xylene to be deparaffinized, then dehydrated in a graded alcohol series (50–100%). Antigen retrieval was performed by autoclaving for 5 min at 97℃. After it was cooled, endogenous peroxidase activity was blocked by treatment with 0.3% H_2O_2 in PBS at room temperature for 30 min. It was then incubated with either rat monoclonal anti-HMGB-1 antibody (10 μg/mL) or goat anti-RAGE polyclonal antibody (10 μg/mL) (Chemicon, Temecula, CA, USA) with PBS/bovine serum albumin (BSA) overnight at 4℃. After washing, the slides were incubated with biotinylated anti rat IgG antibody diluted 1 : 200 with PBS. After rinsing in PBS, the specimens were incubated with avidin-biotinylated enzyme complex (ABC) reagent (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), then washed with distilled water. For RAGE, a chemical reagent marketed as Histofine Simple Stain MAX PO (G) (Nichirei Biosciences, Tokyo, Japan) was applied as a secondary antibody at room temperature for 30 min. The reaction was visualized by diaminobenzidine, which turned brown, after which the samples were counterstained with hematoxylin.

Quantitative analysis for HMGB-1 and RAGE-positive cells. Immunoreactivity was evaluated by light microscopy. The chondrocytes with definite, diffusely stained cytoplasm or nuclei were regarded as positively stained. The populations of HMGB-1-positive, or RAGE-positive cells in the superficial, middle, and deep layers of OA cartilage, were quantified by counting the number of the cells within the 3 layers. Cell counts were performed in at least 3 fields, at × 100 magnification, and then averaged. Judgment was based on the consensus of at least 2 of the authors (C.T. and A.Y.) without reference to the patients' clinical information. The number of positive chondrocytes was divided by the total number of chondrocytes within all 3 layers to calculate the positive chondrocyte ratio. The marginal areas between each layer were avoided for cell counting to reduce inter-observer errors. All sections were also stained with Safranin O and observed by light microscopy. Histological classification of the severity of OA lesions was graded 1–6, using the OARSI cartilage OA histopathology grading system reported by Pritzker *et al.* [21].

Chondrocyte isolation from OA cartilage. For the *in vitro* study, OA cartilage samples were obtained from another 6 sets of patients at TKA. The

ages of patients ranged from 62 to 88 years (mean ± SEM 72.1 ± 8.2 years). Chondrocytes were isolated by digestion of cartilage specimens in 0.1% chymotrypsin (Wako Pure Chemical Industries, Osaka, Japan) and 0.2% collagenase (Sigma, St. Louis, MO, USA) following the method of Bruckner *et al.* [22]. Chondrocytes were seeded in 6 - well plates (5×10^4/mL), cultured in 3mL Dulbecco's modified Eagle medium (Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum, 100U/mL penicillin (Wako Pure Chemical Industries, Osaka, Japan), and 100μg/mL streptomycin (Wako Pure Chemical Industries, Osaka, Japan), and incubated in a 5% CO_2 humidified incubator at 37℃ for 14 days before starting the experiments.

Quantification of IL-1β and TNFα production by HMGB-1-stimulated chondrocytes. After exposure of the cells by recombinant human HMGB-1 (10, 100, and 1,000ng/mL) (Biological Industries, Kibbutz, Israel) for 6, 12, and 24h, the concentrations of IL-1β and TNFα in the supernatant were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocol.

Quantitative Real-Time PCR for HMGB-1 mRNA in cytokine-stimulated chondrocytes. Recombinant human IL-1β and TNFα were purchased from R&D Systems. The cytokines were stored at −180℃ and diluted in a culture medium immediately before being used. Total RNA was isolated from primary OA chondrocytes after treatment with IL-1β (10ng/mL) and TNFα (1ng/mL) for 0, 12, and 24h. HMGB-1 mRNA expression was analyzed by real-time PCR as previously described.

Immunocytochemistry for HMGB-1 in cytokine-stimulated chondrocytes. Primary cultured OA chondrocytes were treated with IL-1β (10ng/mL) and TNFα (1ng/mL) for 48h, and the distribution of the HMGB-1 protein was investigated by immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 10min, blocked with 1% BSA for 10min at room temperature, then incubated with an anti-HMGB-1 antibody (1μg/mL) for 30min at room temperature. A BSA solution without the primary antibody was used as a negative control. Alexa Fluor 488-conjugated antibody (Molecular Probes, Eugene, OR, USA) and Alexa-Fluor 594-conjugated phalloidin (Molecular Probes), both incubated for 30min at room temperature, and Hoechst 33342 (ICN Biomedicals, Aurora, OH, USA), incubated for 5min at room temperature, were used for detection. Fluorescence images were captured using an inverted Leica DMRIII microscope (Leica, Bannockburn, IL, USA) equipped with epifluorescence filters and a charge-coupled device camera using Leica CW4000 software.

Statistical analysis. Statistical comparisons were made by Mann-Whitney *U* test using Stat-view software. All values are expressed as means ± SEM. *P* values less than 0.05 were considered significant.

Results

Expression of HMGB-1 in OA cartilage. HMGB-1 was detected in normal and OA cartilage at both the mRNA and protein level. HMGB-1 mRNA expression was significantly higher in OA cartilage than in normal cartilage (Fig. 1).

Expression and localization of HMGB-1 in OA cartilage. The protein expression of HMGB-1 was seen in all 27 OA cartilage samples (Fig. 2). HMGB-1-positive cell ratios relevant to the OA histopathology were 12.6% in Grade 1, 20.1% in Grade 2, 21.8% in Grade 3, and 34.2% in Grade 4 (Fig. 3A). In Grades 2, 3, and 4, HMGB-1-positive cell ratios were significantly higher than that in Grade 1. There was no significant difference between ratios in Grade 2 and 3. In Grade-4 OA, the HMGB-1-positive cell

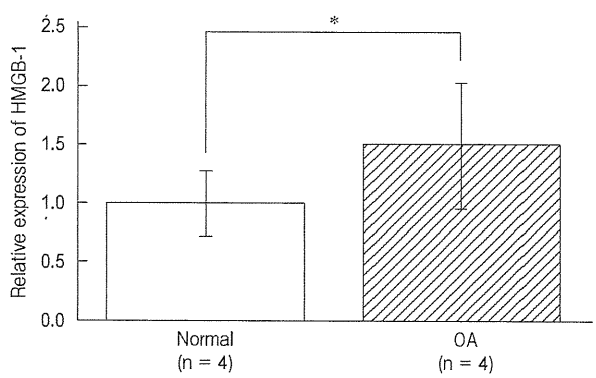

Fig. 1 Expression of HMGB-1 in OA cartilage. HMGB-1 expression in normal cartilage (n = 4) and OA cartilage (n = 4). HMGB-1 mRNA was significantly expressed. $^*p < 0.05$ vs. normal ($p = 0.043$).

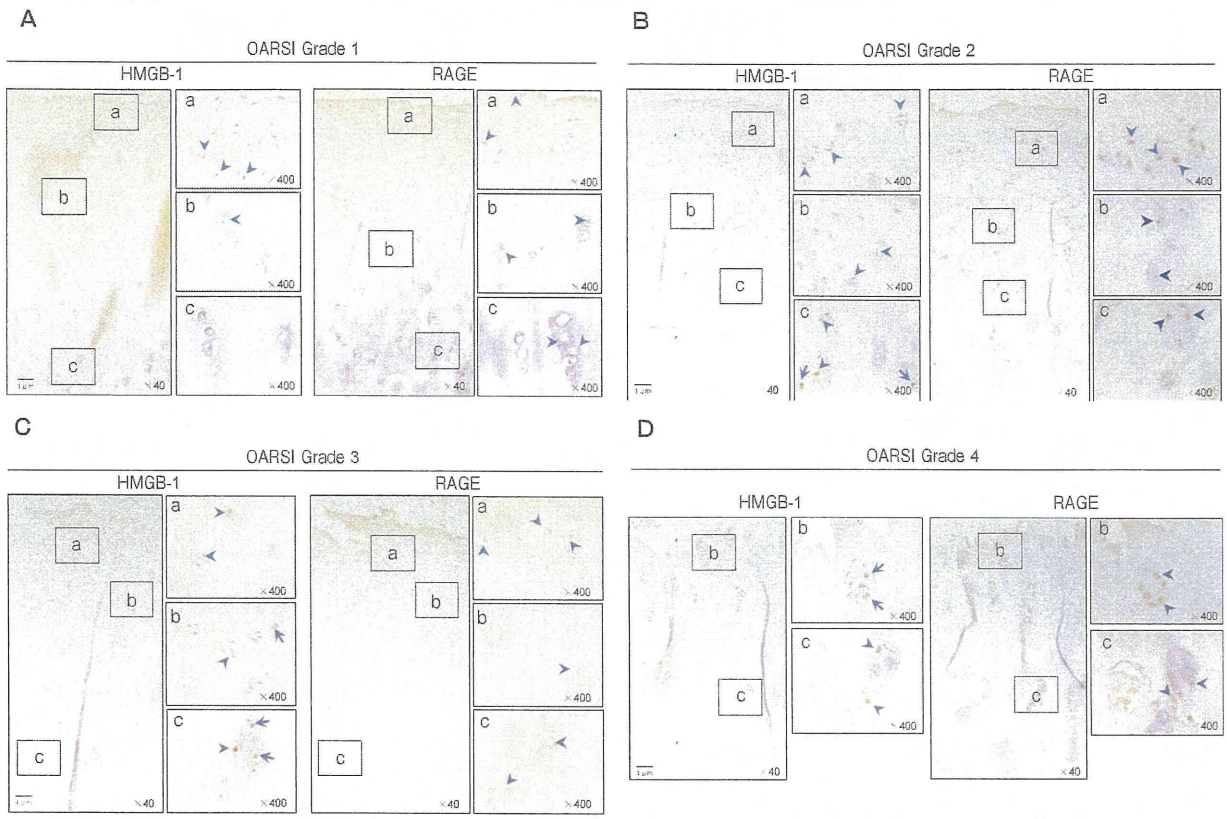

Fig. 2 Immunohistochemistry for HMGB-1 and RAGE. Expression and localization of HMGB-1 and RAGE in OA cartilage in relation to the level of cartilage destruction. Twenty-seven OA and 4 normal cartilage specimens were used for the immunohistochemical study of HMGB-1 and RAGE. HMGB-1 was expressed in the nucleus. The cytoplasmic HMGB-1-positive cell rate tended to increase as the OARSI grade increased. RAGE was expressed in the cytoplasm, and the RAGE-positive cell ratio tended to be higher according to the OARSI grade, as with HMGB-1. Arrow heads indicate nuclear HMGB-1- and RAGE-positive cells, and arrows indicate cytoplasmic HMGB-1-positive cells. Inset a, b and c show the superficial, middle and deep layer, respectively.

ratio was significantly higher than those in Grade 2 or 3 (Fig. 3A). The cytoplasmic HMGB-1-positive cell rates were 1.4% in Grade 1, 2.3% in Grade 2, 11.1% in Grade 3, and 13.4% in Grade 4. In Grades 3 and 4, the cytoplasmic HMGB-1-positive cell ratios were significantly higher than those in Grade 1 and 2, respectively (Fig. 3A). There was no significant difference in ratios between Grade 1 and 2, or between Grade 3 and 4.

Expression of RAGE in OA cartilage. We also investigated RAGE expression in OA cartilage by immunohistochemical analysis. The RAGE-positive cell ratios relevant to OA histopathology were 20.4% in Grade 1, 36.6% in Grade 2, 37.3% in Grade 3, and 35.3% in Grade 4 (Fig. 3B). The ratios of RAGE expression by chondrocytes in Grade 2, 3, and 4 were significantly higher than those in Grade 1 (Fig. 3B). There was no significant difference among ratio in Grades 2, 3, and 4. (Fig. 3B).

Effect of proinflammatory cytokines on HMGB-1 expression and localization in OA chondrocytes. IL-1β (10ng/mL) significantly up-regulated the expression of HMGB-1 mRNA up to 24h by OA chondrocytes (Fig. 4A); however, TNFα (1ng/mL) up-regulation of HMGB-1 mRNA did not reach significance. The change in localization of HMGB-1 after treatment with IL-1β and TNFα was examined by immunocytochemistry. In the unstimulated group, HMGB-1 was localized in the nucleus (Fig. 4B). However, HMGB-1 was translocated from

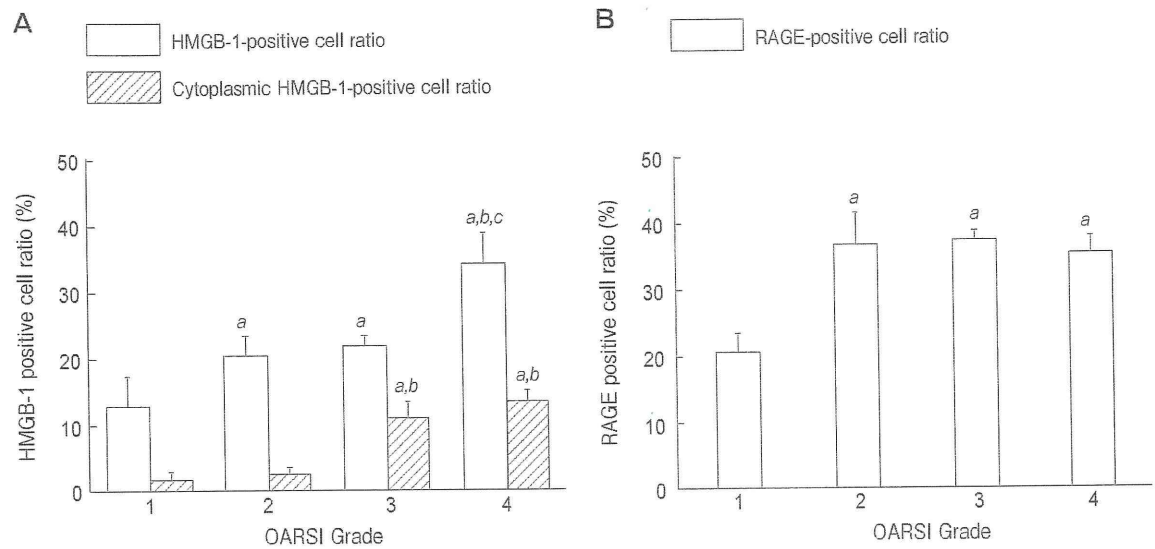

Fig. 3 Population of HMGB-1-and RAGE positive chondrocytes. (A) Cytoplasmic HMGB-1-positive cell ratios tended to be higher in the specimens having the higher OARSI grade. (a, $p < 0.05$ vs. Grade 1; b, $p < 0.05$ vs. Grade 2; c, $p < 0.05$ vs. Grade 3.), (B) The ratios and localization of RAGE expression by chondrocytes in Grades 2, 3 and 4 were significantly higher than those in Grade 1. There was no significant difference between Grades 2/3 and 4, nor was there any significant difference between Grades 3 and 4.

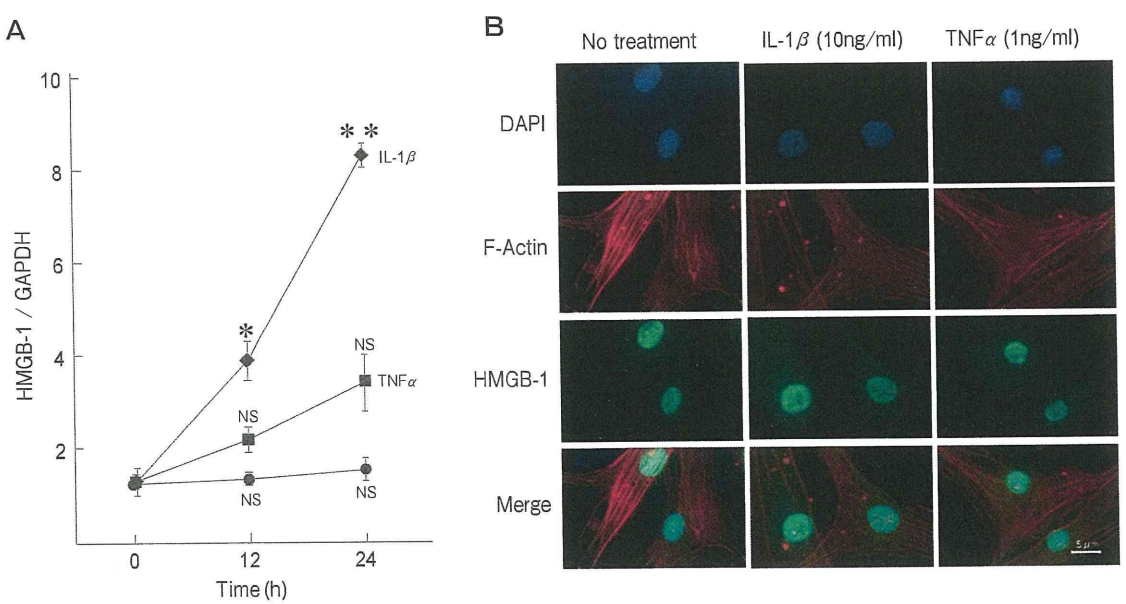

Fig. 4 Effect of IL-1β and TNFα on HMGB-1 and localization in OA chondrocyte. Expression of HMGB-1 mRNA by real-time PCR. Cultured chondrocytes were stimulated with IL-1β (10ng/μL), and total RNA was isolated. Up-regulation of HMGB-1 was still increasing after 12h, but not TNFα (A). ($^{*}p < 0.05$ vs. 0h ($p = 0.033$), $^{**}p < 0.05$ vs. 12h ($p = 0.002$), NS = not significant.) Translocation of HMGB-1 in chondrocytes after IL-1β and TNFα stimulation. The immunofluorescence assay indicated that HMGB-1was translocated from the nucleus to the cytosol following stimulation with both IL-1β and TNFα (B). Chondrocytes were stimulated with IL-1β (10ng/mL) and TNFα (1ng/mL) for 48h and then compared with unstimulated chondrocytes. Chondrocytes stimulated with IL-1β or TNFα exhibited HMGB-1 staining in the cytosol. Note that unstimulated chondrocytes exhibited HMGB-1 staining only in the nucleus.

the nucleus to the cytosol after stimulation by IL-1β (10ng/mL) and TNFα (1ng/mL) at 48h.

Effect of HMGB-1 on IL-1β and TNFα release by chondrocytes. The effects of HMGB-1 on IL-1β and TNFα production by chondrocytes were analyzed by ELISA. HMGB-1 stimulated the production of IL-1β up to 24h (Fig. 5A), and 1,000ng/mL of HMGB-1 significantly up-regulated the production of IL-1β by OA chondrocytes at 12h (Fig. 5B). Peak TNFα levels after stimulation with HMGB-1 (100ng/mL) occurred at 12h and tended to decrease for 24h thereafter. (Fig. 5C). At 12h, the amount of TNFα released into the cultured medium increased in a dose-dependent manner after stimulation by HMGB-1 (Fig. 5D).

Discussion

A previous report demonstrated the significant increase of HMBG-1 mRNA expression in human OA cartilage as compared with normal cartilage [19]. In the present study, we examined the expression and localization of HMGB-1 in OA cartilage. Our results confirmed that HMGB-1 expression in OA cartilage was significantly higher than that in the normal cartilage for both mRNA and protein levels. In the immunohistochemical analysis, it was revealed that the HMGB-1-positive cell ratio and cytoplasmic HMGB-1 positive cell ratio tended to increase along with the OA histological grade, but the nuclear HMGB-1 positive cell ratio was unchanged. These results coincide with a report of Heinola *et al.* in which they studied bovine

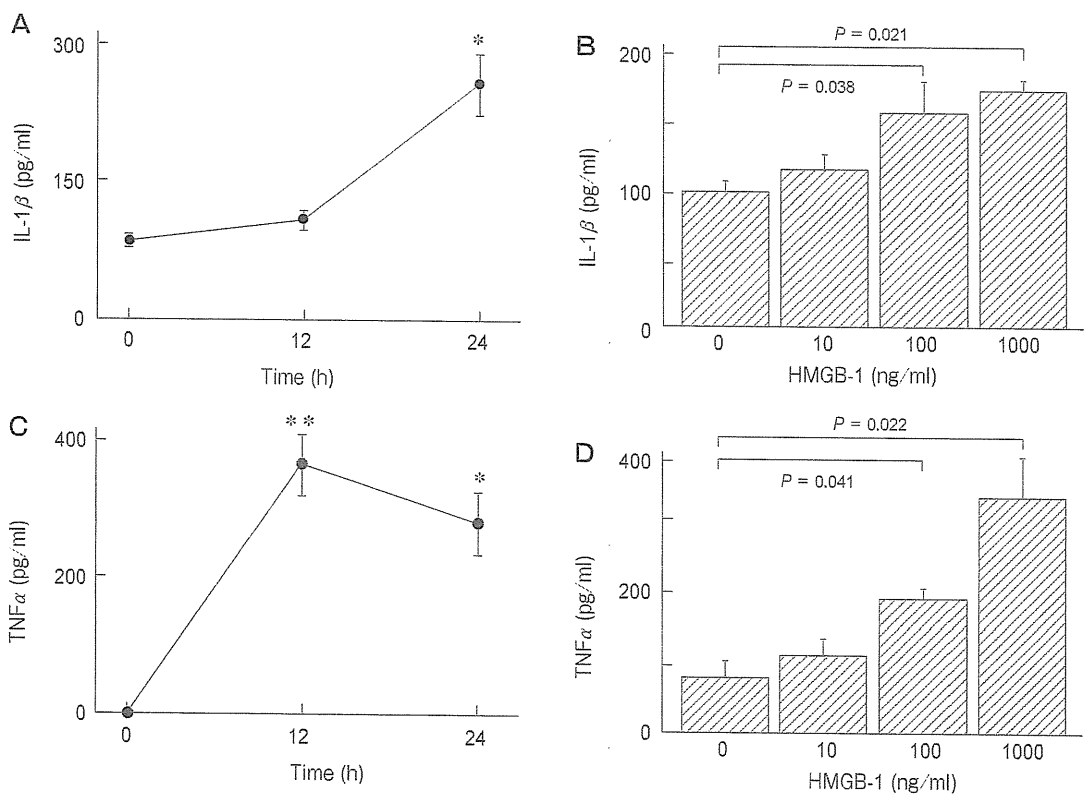

Fig. 5 Effect of HMGB-1 on IL-1β and TNFα expression by human chondrocytes. Chondrocytes were stimulated with HMGB-1, and ELISA was used to measure levels of IL-1β and TNFα. After stimulation with HMGB-1 (100ng/mL), IL-1β levels increased in a time-dependent manner (**A**). IL-1β was released in a dose-dependent manner for 24h after stimulation with HMGB-1, and the amount of IL-1β and TNFα production increased significantly at doses of > 100ng/mL (**B**). The peak TNFα levels after stimulation with HMGB-1 (100ng/mL) occurred at 12h and tended to decrease at 24h (**C**). TNFα was released in a dose-dependent manner after stimulation for 12h (**D**). (A) $^{*}p < 0.05$ vs. 0h ($p = 0.025$). (C) $^{*}p < 0.05$ vs. 0h ($p = 0.039$), $^{**}p < 0.05$ vs. 0h ($p = 0.018$).

cartilage [23].

It is not fully understood what stimulates HMGB-1 production or cytoplasmic translocation from the nucleus in chondrocytes. In normal joint cartilage, HMGB-1 has been reported to be located in the nuclei of chondrocytes and synovial cells [12]. However, in inflammatory synovial cells, HMGB-1 shows both nuclear and cytoplasmic distribution [12]. It was also shown that TNFα stimulation caused translocation of HMGB-1 from the nucleus to the cytosol in CD68-positive cells [14]. In the present study, we demonstrated that IL-1β but not TNFα up-regulated the expression of HMGB-1 at mRNA levels. Treatment with both IL-1β and TNFα caused translocation of HMGB-1 from the nucleus to the cytosol 48h after stimulation.

On the other hand, previous studies have demonstrated that extracellular HMGB-1 induced the production of IL-1β, TNFα, and IL-6 in macrophages [10, 14]. Intra-articular injection of HMGB-1 into murine knee joints resulted in synovitis, but mice rendered deficient in the IL-1 receptor did not develop synovitis after injection. These results suggest that HMGB-1 is not a mere expression of the inflammatory response, but a trigger for joint inflammation by activating macrophages and inducing production of IL-1 via NF-κB activation [13]. In the current study, IL-1β and TNFα production by OA chondrocytes was stimulated by HMGB-1 in a dose-dependent manner. It is speculated that proinflammatory cytokines stimulate the expression and release of HMGB-1 by chondrocytes and that the HMGB-1 released by chondrocytes further promotes the expression of proinflammatory cytokines [14]. This speculation is supported by our current result that the HMGB-1-positive cell ratio increases along with the OA cartilage grade. It is reported that the combination of HMGB-1 and heparin induced marked angiogenesis in a heparin-dependent manner [24]. The HMGB-1 increase in the deep layers of OA cartilage might be related to angiogenesis at the osteochondral junction.

RAGE and toll-like receptors 2 and 4 are known ligands of HMGB-1 [25]. RAGE has been found to be expressed in cartilage [16] and RA synovium [14]; however, RAGE and HMGB-1 do not have a one-to-one correspondence. A study reported by Loeser *et al.* [16] showed that RAGE expression was higher in OA cartilage than in normal cartilage. Our immunohistochemical results also demonstrated that expression of RAGE was significantly higher in cartilage with Grades 2, 3, and 4 OA than that in Grade 1 cartilage. In a separate experiment we used 5 other human OA cartilage specimens to perform double immunohistochemistry for HMGB-1 and RAGE to clarify the interrelationship of the 2 proteins. The result showed that some populations of chondrocytes showed a positive reaction only for HMGB-1 or for RAGE, whereas some were positive for both HMGB-1 and RAGE (data not shown). These findings suggest that HMGB-1 might act on chondrocytes in both an autocrine and paracrine manner. Stimulation of RAGE signaling from HMGB-1 might lead to upregulation of catabolic enzymes such as matrix metalloproteinase 13 production and contribute to cartilage matrix degradation in arthritis [16].

In conclusion, we demonstrated the increased expression of HMGB-1 in OA cartilage relevant to the histological OA grade. *In vitro* experiments using human OA chondrocytes suggested the close interrelationship between HMGB-1 and IL-1β. These results indicate the possible involvement of the proinflammatory cytokine-HMGB-1-RAGE cascade in the development of human osteoarthritis. Whether the blockade of this cascade could contribute to the moderation of cartilage degeneration requires further investigation.

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ORIGINAL ARTICLE

Deleterious Role of Anti-high Mobility Group Box 1 Monoclonal Antibody in Retinal Ischemia-reperfusion Injury

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ABSTRACT

Purpose: To investigate the effect of anti-high mobility group box 1 (HMGB1) monoclonal antibody (mAb) against ischemia-reperfusion injury in the rat retina.

Materials and Methods: Retinal ischemia was induced by increasing and then maintaining intraocular pressure at 130 mmHg for 45 min. An intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Retinal damage was evaluated at 7 days after the ischemia. Immunohistochemistry and image analysis were used to measure changes in the levels of reactive oxygen species (ROS) and the localization of anti-HMGB1 mAb. Dark-adapted full-field electroretinography (ERG) was also performed.

Results: Pretreatment with anti-HMGB1 mAb significantly enhanced the ischemic injury of the retina. HMGB1 expression increased at 6–12 h after ischemia in the retina. After the ischemia, production of ROS was detected in retinal cells. However, pretreatment with anti-HMGB1 mAb increased the production of ROS. On the seventh postoperative day, the amplitudes of both the ERG a- and b-waves were significantly higher in the vehicle group than in the groups pretreated with anti-HMGB1 mAb.

Conclusions: The current *in vivo* model of retinal injury demonstrated that anti-HMGB1 mAb plays a large deleterious role in ischemia-reperfusion injury. In order to develop neuroprotective therapeutic strategies for acute retinal ischemic disorders, further studies on anti-HMGB1 mAb function are needed.

Keywords: Anti-HMGB1 mAb, Retinal ischemia, Reactive oxygen species, Electroretinogram, Retinal thickness

INTRODUCTION

Ischemic injury to the retina is a major cause of visual loss and morbidity. As these morbidities are difficult to treat, research into various potential treatments is currently ongoing.[1–5] Ischemia-reperfusion injury involves many signaling mechanisms that ultimately result in necrotic and apoptotic cell death.[6] Delayed neuronal cell death in the brain and retina secondary to transient ischemic injury occurs, in part, by apoptosis.[7,8] During or after ischemia, reactive oxygen species (ROS) can be produced in large quantities and act as cytotoxic metabolites.[9] ROS can provoke cell death by reacting with cell components that lead to necrosis, or by activating specific targets that trigger apoptosis.

High-mobility group box-1 (HMGB1) protein was originally described 30 years ago as a nonhistone DNA-binding protein with high-electrophoretic mobility.[10] HMGB1 is a nuclear protein involved in nucleosome stabilization and gene transcription.[11] It is known that these functions are essential for survival, as HMGB1-deficient mice have been shown to die of hypoglycemia within 24 h of birth.[12] HMGB1 is

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found in almost all eukaryotic cells, and its presence has been confirmed in the rodent retina.[13] HMGB1 has also been implicated in the mechanism of ischemic brain damage.[14–20] In a stroke model, short hairpin (sh) RNA-mediated HMGB1 down-regulation in the brain reduces the severity of lesions.[15] Intravenous injection of the anti-HMGB1 monoclonal antibody (mAb) causes a dramatic reduction in the infarct size in the stroke model.[17]

The purpose of the present study was to investigate the role of anti-HMGB1 and its specific expression in retinal ischemia-reperfusion injury.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats weighing 200–250 g were obtained from Charles River Japan (Yokohama, Japan). Rats were permitted free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal care and all experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Anti-HMGB mAb or IgG2a was injected by three different methods. Intraperitoneal injection of anti-HMGB1 monoclonal antibody (mAb) (200 μg)[17] or class-matched control mAb (IgG2a) (200 μg) against *Keyhole Limpet* hemocyanin was administered 30 min before ischemia. Anti-HMGB mAb (200 μg) or IgG2a (200 μg) was administered intravenously immediately and 6 h after reperfusion. The pupil was dilated with topical phenylephrine hydrochloride and tropicamide; anti-HMGB mAb (20 μg) or IgG2a (20 μg) was injected into the vitreous space 30 min before ischemia.

Ischemia

Rats were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Abbott, Abbott Park, IL) followed by a topical administration of 0.4% oxybuprocaine hydrochloride. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a reservoir containing normal saline. The intraocular pressure (IOP) was raised to 130 mmHg for 45 min by elevating the saline reservoir. Only the right eye of each rat was subjected to ischemia. Retinal ischemia was indicated by whitening of the iris and fundus. The left eyes served as the sham-treated controls, with these eyes undergoing a similar procedure that did not include elevation of the saline bag, thus maintaining normal ocular tension. Rectal and tympanic temperatures were maintained during the operation at approximately 37°C via the use of a feedback-controlled heating pad (BRC, Nagoya, Japan). After restoration of blood flow, temperature was maintained continuously at 37°C.

HISTOLOGICAL EXAMINATION

For histological examination, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) 7 days after ischemia. Eyes were enucleated and stored in a 4% paraformaldehyde solution for 24 h at room temperature. The retinas were removed and embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Each retina was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin (HE).

Morphometric analysis was performed to quantify ischemic injury. These sections were selected randomly in each eye. Light microscopic examination was performed by a person with no prior knowledge of the treatments. A microscopic image of sections obtained within 0.5–1 mm of the optic disc was scanned. For each computer image, the number of cells in the ganglion cell layer (GCL) was counted. The thicknesses of the inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and outer plexiform layer (OPL) for the entire frame were measured. The number of cells in the GCL was normalized as linear cell density (cells per millimeter). Thicknesses of the IPL, INL, ONL, and OPL were obtained by calculating the mean value of seven measurements in each eye. Similarly, the linear cell density in the GCL was also determined by calculating the mean value of seven measurements. For each animal, the right eye parameter was normalized to that of the intact left eye and shown as a percentage.

ELECTRORETINOGRAMS (ERGS)

ERG responses were measured after overnight dark adaptation (at least 6 h) using a recording device (Mayo Corporation, Aichi, Japan) 7 days after ischemia. Rats were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride eye drops (Santen Pharmaceuticals, Osaka, Japan). All procedures were performed in dim red light, with all rats kept warm during the procedure. The LED corneal electrode was set vertical to the cornea center. A reference electrode was set subcutaneously on the forehead and the ground connection was set on the base of the tail. An LED stimulator LS-W controlled the stimulus duration and intensity during the 11-step intensity series, which ranged from 0.0003–30 cds/m^2. The ERG response was amplified using an AC amplifier ML135 (Bio Amplifier, AD Instruments, NSW, Australia) with a bandwidth of 0.3–500 Hz and amplification of 2,000 times. The ischemic damage to the retina was

evaluated as the percentage of the a- and b-wave amplitudes of the ischemic right eyes as compared to the non-ischemic left eyes.

IMMUNOHISTOCHEMISTRY

Eyes were enucleated at 6, 12 or 24 h after 45 min of ischemia. Eyes were then fixed in 4% paraformaldehyde in the PBS and embedded in paraffin. Retinal sections (5 μm) were rinsed in 100% ethanol twice for 5 min each, followed by a separate 95% ethanol and 90% ethanol rinse for 3 min each. The sections were then washed using PBS, pH 7.4, three times for 10 min each and treated with 0.3% Triton X-100 in PBS, pH 7.4, for 1 h. After further washing three times for 10 min each with PBS, pH 7.4, sections were then blocked in 3% normal horse serum and 1% bovine serum albumin (BSA) in PBS for 1 h in order to reduce nonspecific labeling. Sections were incubated overnight at 4°C in PBS with either 2.0 mg/mL of monoclonal antibody against HMGB1[17] which served as the primary antibody. After washing in PBS for 50 min, sections were then immersed in the second antibody conjugated to horseradish peroxidase for 1 h at room temperature. Images were acquired using 40× objective lenses (DXM 1200; Nikon, Tokyo, Japan). Adobe PhotoShop v. 5.0 was used to adjust the brightness and contrast of the images.

FLUORESCENT LABELING OF ROS

To investigate the production of ROS, we intraperitoneally injected 5 mg/kg dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO) in 5% dimethyl sulfoxide (DMSO) in PBS 15 min before ischemia. A 0.3-mL aliquot of distilled water, 200 μg anti-HMGB1 mAb, or 200 μg IgG2a was administered intraperitoneally 30 min before ischemia. Eyes were enucleated 15 min after ischemia and then embedded in OCT compound (Sakura Finetek, Torrance, CA), after which cryosections (20 μm) were prepared. Sections were examined with a microscope (Radiance 2100/Rainbow, Carl Zeiss, München, Germany) using a laser set (excitation laser 514 nm; emission laser >580 nm).

Statistical Analysis

Fluorescence was quantified by automated image analysis with Image-Pro Plus software (version 4.0, Media Cybernetics, The Imaging Expert, Bethesda, MD). For each section, mean fluorescence was calculated from five separate high-power fields per eye. A threshold was set to define positive labeling.

All data are presented as the mean ± SD. Data were analyzed using an independent Student's *t*-test and ANOVA followed by Tukey-Kramer post-hoc testing corrected for multiple comparisons. Statistical analysis was performed using SPSS for Windows (SPSS Inc, Chicago, IL). A p value of < 0.05 was considered statistically significant.

RESULTS

Histologic Changes in the Retina after Ischemia with Anti-HMGB1

Figure 1A shows a normal retina. Light microscopic photographs were taken 7 days after ischemia and treatment with IgG2a (Figure 1B) or anti-HMGB1 (Figure 1C). In animals pretreated with IgG2a, the GCL cell number was reduced to 73.9 ± 16.2% of the control; the IPL thickness was reduced to 67.7 ± 14.6% of the control; the INL thickness was reduced to 82.8 ± 13.5% of the control; the OPL thickness was reduced to 88.6 ± 30.8% of the control; and the ONL thickness was reduced to 88.1 ± 13.0% of the control ($n = 7$; Figure 1D). In animals pretreated with anti-HMGB1 mAb, the GCL cell numbers were 67.7 ± 16.8% of the control ($p = 0.50$); the IPL thickness was 51.6 ± 12.3% of the control ($p = 0.02$); the INL thickness was 69.0 ± 6.8% of the control ($p = 0.03$); the OPL thickness was 51.9 ± 10.8% of the control ($p = 0.01$); and the ONL thickness was 72.4 ± 13.7% of the control ($p = 0.049$) ($n = 7$; Figure 1D).

Treatment with intravenous injection of IgG2a or anti-HMGB1 mAb twice (immediately and 6 h after reperfusion) reduced the retinal thickness dramatically (n = 4, each group) (Figure 2).

Treatment with local administration of IgG2a or anti-HMGB1 mAb 30 min before ischemia was similar to the results with intraperitoneal injection of anti-HMGB1 mAb ($n = 4$, each group) (Figure 3).

EFFECT OF ANTI-HMGB1 ON NORMAL RETINA

Animals were killed at 7 days after the intraperitoneal injection of anti-HMGB1 mAb or IgG2a. Treatment with anti-HMGB1 did not affect the retinal thickness in normal rat (Figure 4) ($n = 4$, each group).

EFFECT OF ANTI-HMGB1 ON RETINAL FUNCTION

Scotopic ERG was recorded to evaluate anti-HMGB1 mAb effects on retinal function. A representative example of function is seen in Figure 5A. Mean amplitudes of the a- and b-wave are shown in Figure 5B. We observed a statistically significant difference between the three groups ($n = 5$, each group).