acute lung inflammation (Abraham et al., 2000) and atherosclerosis (Porto et al., 2006).

It is reported that HMGB1 binds to the RAGE, TLR-2 and TLR-4, a receptor for lipopolysaccharide (LPS) (Hori et al., 1995; Park et al., 2004; van Beijnum et al., 2008). The interaction of HMGB1 and its receptors plays a number of roles in mediating part of the pro-inflammatory response, including pro-inflammatory cytokine production. HMGB1-enhanced activation of macrophages mediates the production of TNF-α, interleukin (IL)-1α, IL-1β, IL-6 and macrophage inflammatory proteins MIP-1α and MIP-1β (Kalinina et al., 2004). However, little is known about the cellular interplay mechanism of HMGB1-induced cytokine production and T-cell activation.

In the present study, we demonstrated, for the first time, the involvement of HMGB1-induced adhesion molecule expression on monocytes and on pro-inflammatory cytokine production and lymphocyte proliferation in human PBMCs. The involvement of RAGE, TLR-2 and TLR-4 in the effects of HMGB1 also was determined, using RAGE, TLR-2 and TLR-4 knockout mice, and we found the predominant involvement of RAGE.

2. Materials and methods

2.1. Reagents and drugs

Recombinant human (rh) HMGB1 was produced as described previously (Wake et al., 2009). In brief, complementary DNA (cDNA) encoding full-length HMGB1 was amplified by the PCR from human microvascular endothelial cell cDNA. The PCR product was subcloned into a pGEX-6p-1 vector (GE Healthcare, Little Chalfont, England) to generate a glutathione S-transferase (GST) fusion protein. Sf9 insect cells (Invitrogen Life Technologies, NY) were transformed with the recombinant plasmid and incubated overnight at 37 °C in Overnight Express Instant TB Medium (Merck, San Diego, CA) to express recombinant GST-HMGB1. A Sf9 cell extract containing GST-HMGB1 fusion proteins was incubated with glutathione-Sepharose 4B for 1 h at room temperature. After washing, the gel bed was incubated with PreScission protease for 3 h at 4 °C. After a brief centrifugation, the supernatant containing HMGB1 with the GST tag removed was collected and purified by gel filtration chromatography using TSK-gel 3000SWXL (Tosoh, Tokyo, Japan). Purified rhHMGB1 protein was identified by western blotting (Wake et al., 2009) with anti-HMGB1 monoclonal Ab (mAb). The LPS content of the purified rhHMGB1 was < 2.0 pg/μg protein.

Anti-human RAGE rabbit IgG was obtained by immunization of rabbits with a recombinant human soluble form of RAGE (sRAGE), which was prepared as described previously (Liu et al., 2009). The purified Ab was digested with immobilized pepsin beads using a kit from Pierce Chemical Co (Rockford, IL) to produce Fab fragments.

2.2. Isolation of PBMCs and monocytes

Normal human PBMCs were obtained from ten healthy volunteers after acquiring Institutional Review Board approval (Okayama Univ. IRB No.106). Each 20–50 ml peripheral blood sample was withdrawn from a forearm vein, after which PBMCs were prepared and monocytes were separated from the PBMCs by counterflow centrifugal elutriation as previously described (Takahashi et al., 2002, 2003).

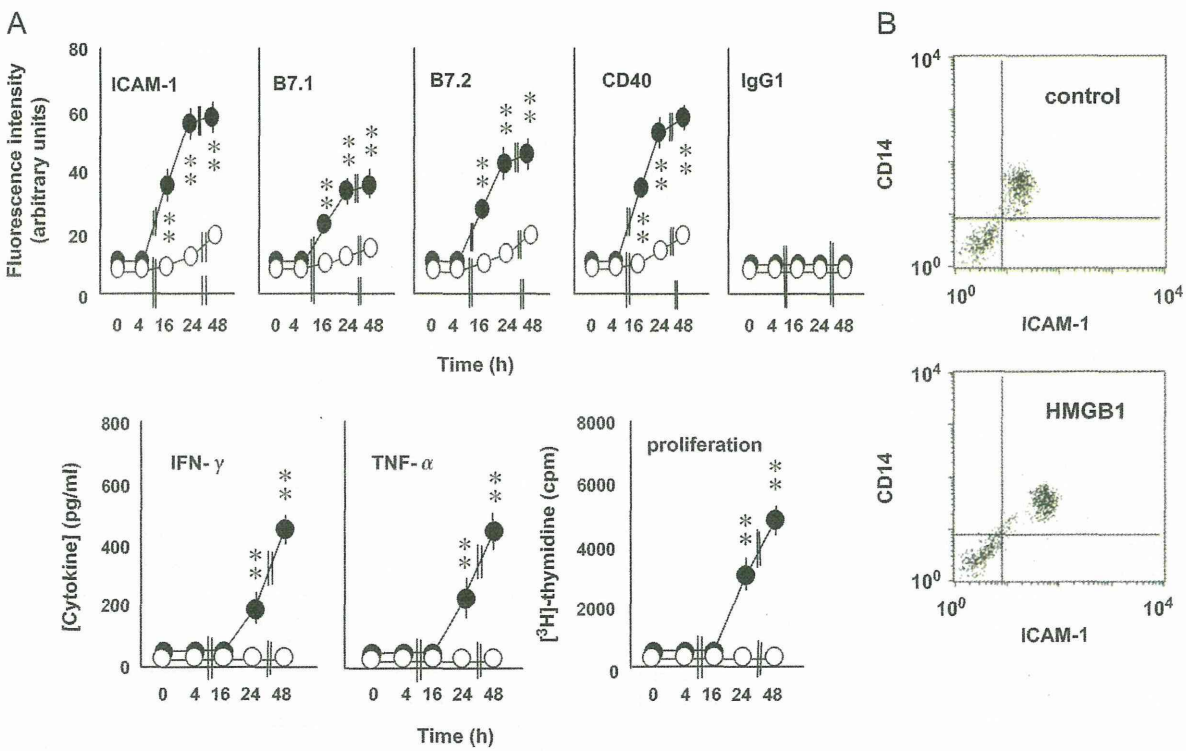

Fig. 1. Effect of HMGB1 on ICAM-1, B7.1, B7.2 and CD40 expression on monocytes, and IFN-γ and TNF-α production and lymphocyte proliferation in PBMCs. (A) PBMCs at 4×10^6 cells/ml were incubated with or without 10 μg/ml HMGB1 for the indicated times, and adhesion molecule expression on monocytes, and cytokine production and lymphocyte proliferation in PBMCs in the absence (open circles; ○) or presence (filled circles; ●) of HMGB1 were determined by flow cytometry, ELISA and [^{3}H]-thymidine uptake, respectively. FITC-conjugated IgG1 was used as an isotype-matched control Ab. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. $^{**}P < 0.01$ compared with the value at 0 h. Error bars within symbols are hidden. (B) PBMCs were incubated with or without 10 μg/ml HMGB1 for 24 h and ICAM-1 expression on monocytes was determined by flow cytometry. The typical data was shown.

2.3. RAGE, TLR-2 and TLR-4 knockout (-/-) mice

Animal experiments were approved by the Animal Care Committee at Okayama University. RAGE knockout (-/-) mice were produced as described previously (Yamamoto et al., 2011). In brief, RAGE-/- mice backcrossed to C57BL/6J mice (Charles River Japan) for eight generations were used. TLR-2 deficient (TLR-2-/-) and TLR-4-/- knockout mice were obtained from Oriental BioService Inc. (Tokyo, Japan). These mice were used for preparation of spleen cells. Samples of 4×10^6 spleen cells/ml were incubated with 10 μg/ml HMGB1 for 24 h at 37 °C in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO), and 5×10^5/ml cultured cells were then prepared for flow cytometric analysis (Takahashi et al., 2002, 2003).

2.4. Flow cytometric analysis of adhesion molecule expression

For flow cytometric analysis, fluorescein isothiocyanate (FITC)-conjugated mouse IgG1 mAb against human ICAM-1/CD54 and R-Phycoerythrin (PE)-conjugated anti-human CD14 mAb were purchased from DAKO (Glostrup, Denmark). FITC-conjugated mouse IgG1 mAb against human B7.2 and CD40 were purchased from Pharmingen (SanDiego, CA), and FITC-conjugated IgG1 class-matched control was purchased from Sigma Chemical. FITC-conjugated mouse anti-mouse ICAM-1 mAb was purchased from DAKO. Changes in the expression of human leukocyte antigens ICAM-1, B7.1, B7.2 and CD40 on monocytes were examined by multi-color flow cytometry using a mixture of anti-CD14 Ab with anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab. Samples of 4×10^6/ml PBMCs were incubated with 0.1–100 μg/ml HMGB1 or 0.01–10 μg/ml anti-RAGE Ab Fab for 24 or 48 h at 37 °C in RPMI 1640 (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 μg/ml kanamycin, 100 μg/ml streptomycin and penicillin, and 5×10^5/ml cultured cells were then prepared for flow cytometric analysis as previously described (Takahashi et al., 2002, 2003) and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA). The data were processed using the CELL QUEST program.

2.5. Flow cytometric analysis and immunofluorescence staining of RAGE, TLR-2 and TLR-4 expression

Mouse anti-human RAGE mAb (MAB11451) was purchased from R&D Systems (Minneapolis, MN), and FITC-conjugated mouse anti-human TLR-2 (TL2.3) and anti-human TLR-4 (HTA125) Abs were purchased from Abcam (Cambridge, MA). Changes in the expression of RAGE, TLR-2 and TLR-4 on monocytes were examined by multi-color flow cytometry using a mixture of anti-CD14 Ab with anti-RAGE, anti-TLR-2 or anti-TLR-4 Ab. Samples of 4×10^6/ml PBMCs were incubated with 0.1–100 μg/ml HMGB1 or 0.01–10 μg/ml adhesion molecule Abs for 24 h at 37 °C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 μg/ml kanamycin, 100 μg/ml

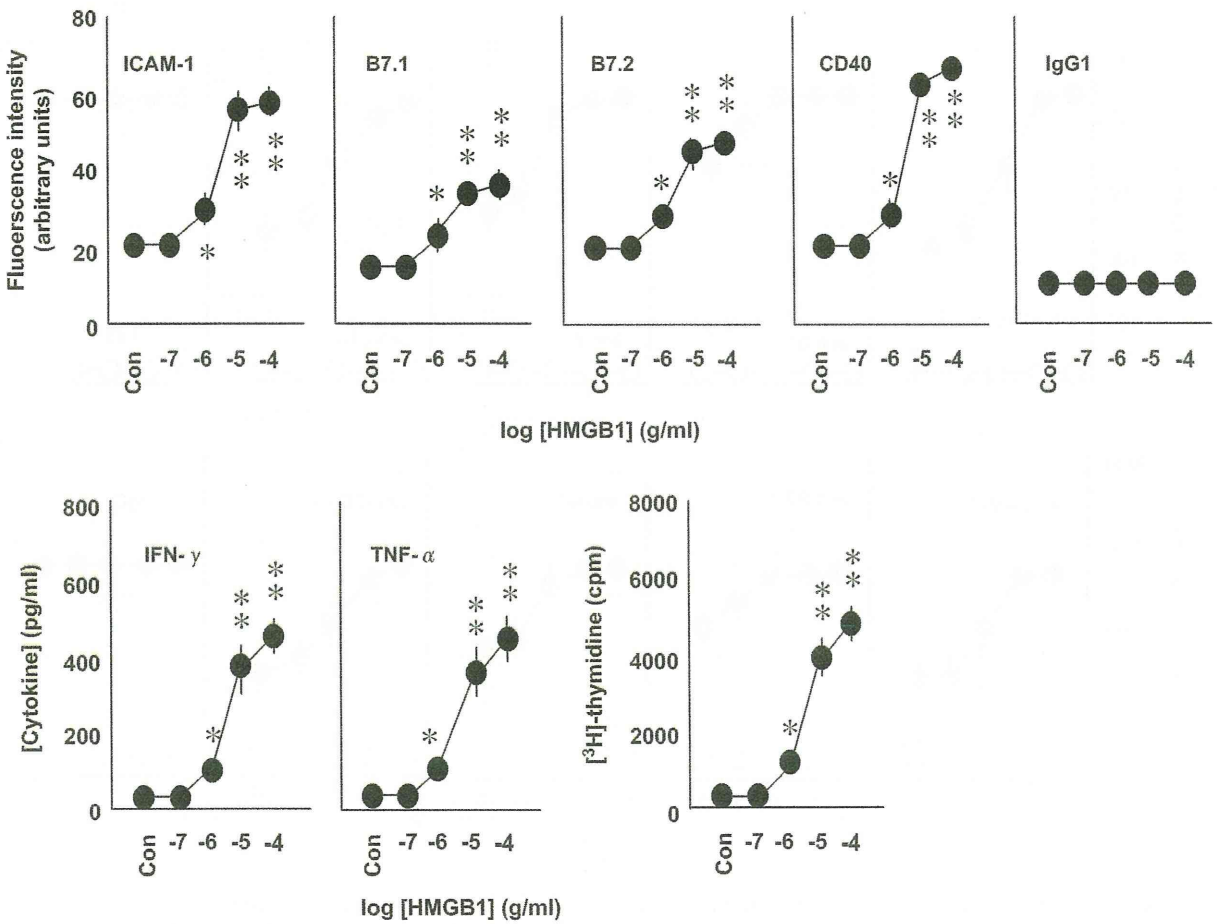

Fig. 2. Dose-response of the HMGB1 effect on ICAM-1, B7.1, B7.2 and CD40 expression, IFN-γ and TNF-α production and lymphocyte proliferation in PBMCs. PBMCs at 4×10^6 cells/ml were incubated with 0.1–100 μg/ml HMGB1 for 24 h. The effect of HMGB1 on adhesion molecule expression on monocytes, and cytokine production and lymphocyte proliferation in PBMCs was determined. Control (Con) means 0 μg/ml HMGB1. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. $^*P < 0.05$, $^{**}P < 0.01$ compared with the value for medium alone. Error bars within symbols are hidden.

streptomycin and penicillin, and 5×10^5/ml cultured cells were then prepared for flow cytometric analysis. The cells were reacted with anti-RAGE Ab followed by FITC-conjugated goat anti-mouse IgG before staining with PE-conjugated anti-human CD14 Ab (DAKO), or the cells were reacted with FITC-conjugated anti-TLR-2 and anti-TLR-4 Abs. After Ab treatment, the cells were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA) and the data were processed using the CELL QUEST program.

The expression of RAGE on human monocytes was examined by multi-color immunocytochemical staining. Samples of 4×10^6/ml PBMCs were incubated with 10 μg/ml HMGB1 for 24 h at 37 °C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 μg/ml kanamycin, 100 μg/ml streptomycin and penicillin, and 5×10^5/ml cultured cells were then prepared for immunocytochemical staining as previously described (Takahashi et al., 2009). The cells were reacted with anti-human CD14 mouse IgG (DAKO) followed by Alexin555-conjugated anti-mouse IgG rabbit IgG (Chemicon International; Temecula, CA) or with anti-human RAGE rabbit Ab followed by Alexin488-conjugated anti-rabbit IgG goat IgG (Chemicon International). Stained cells were mounted and viewed by fluorescence confocal microscopy (Keyence, Osaka, Japan).

2.6. Enzyme-linked immunosorbent assay

Samples of 4×10^6/ml PBMCs were used for analyzing IFN-γ and TNF-α production. After incubation at 24 h at 37 °C in a 5%

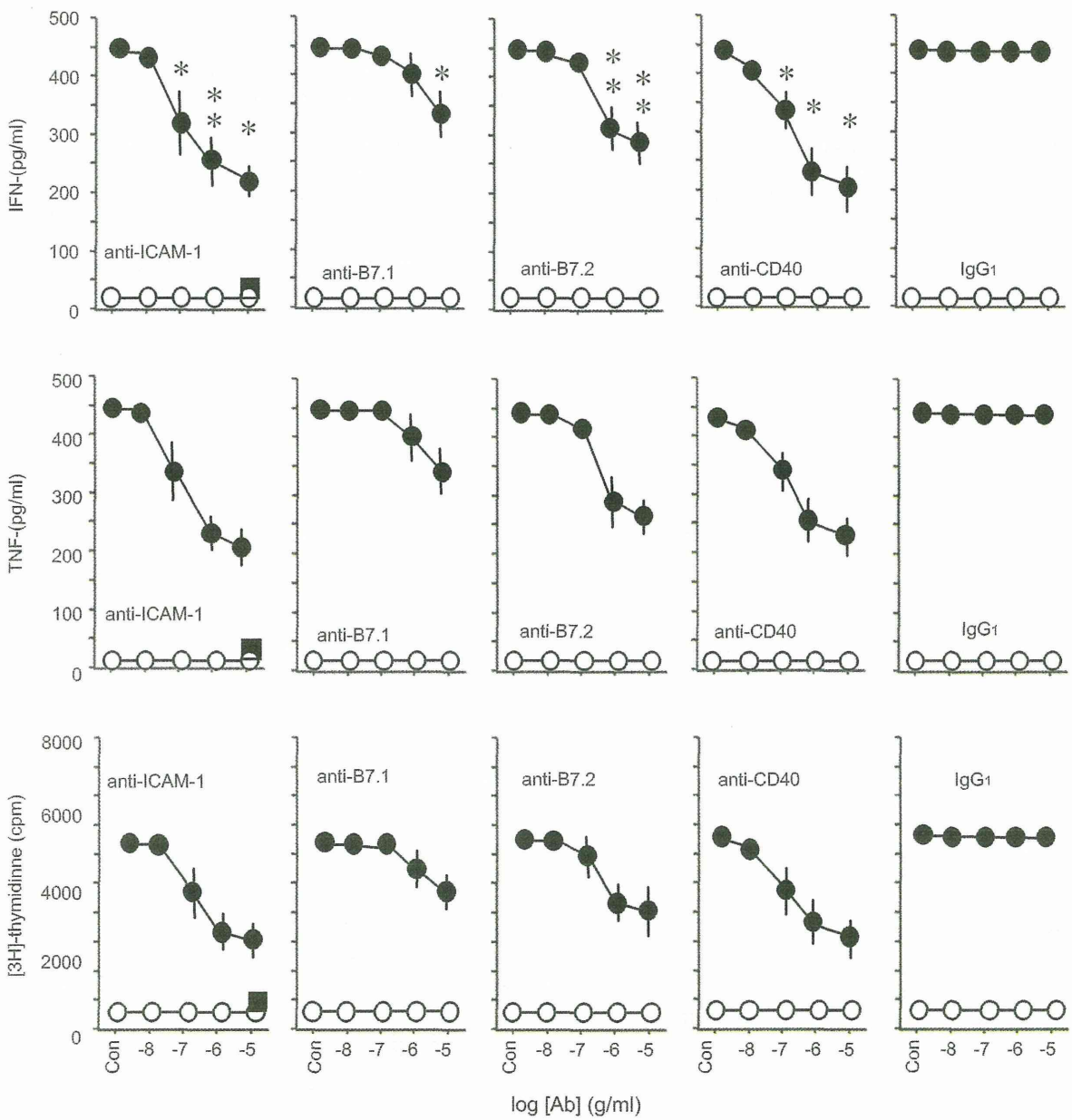

Fig. 3. Involvement of adhesion molecules in the effect of HMGB1 on cytokine production and lymphocyte proliferation in PBMCs. PBMCs at 4×10^6 cells/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab at 0.01–10 μg/ml in the absence (open circles; ○) or presence (filled circles; ●) of 10 μg/ml HMGB1 for 24 h, and IFN-γ and TNF-α production and lymphocyte proliferation were determined by ELISA and [^{3}H]-thymidine uptake, respectively. The effect of incubation in a mixture of 10 μg/ml anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs on HMGB1 activity was determined (filled squares; ■ in the left panels of Fig.3). Control (Con) means 0 μg/ml Abs. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. $^*P < 0.05$, $^{**}P < 0.01$ compared with the value for HMGB1 alone. Error bars within symbols are hidden.

CO2/air mixture, cell-free supernatants were assayed for IFN-γ and TNF-α proteins by enzyme-linked immunosorbent assay (ELISA) using the multiple Abs sandwich principle (R&D Systems, Minneapolis, MN). The ELISA detection limit for both IFN-γ and TNF-α was 10 pg/ml.

2.7. Western blot analysis

The effect of HMGB1 on expression of RAGE, TLR-2 and TLR-4 was determined by western blot analysis using anti-human RAGE rabbit IgG, anti-human TLR-2 rabbit IgG (ab24192) (Abcam) and anti-human TLR-4 rabbit IgG (ab13867) (Abcam). Samples of 4×10^6 isolated human monocytes/ml were incubated with 10 μg/ml HMGB1 for 24 h at 37 °C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 μg/ml kanamycin, 100 μg/ml streptomycin and penicillin. Lysates of the human monocytes were then analyzed by SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes and blotted. Blotting of β-actin was used as a loading control.

2.8. Proliferation assay

Samples of 4×10^6/ml PBMCs were treated with 0.1–100 μg/ml HMGB1, 0.01–10 μg/ml anti-RAGE Ab Fab or 0.01–10 μg/ml adhesion molecule Abs and incubated for 24 h at 37 °C in RPMI 1640 supplemented with 10% heat-inactivated FCS, 20 μg/ml kanamycin, 100 μg/ml streptomycin and penicillin, during which they were pulsed with [^{3}H]-thymidine (3.3 Ci/well) for the final 16 h. The cells were then dispensed into 96-well microplates, 200 μl/well, resulting in 1 μ Ci [^{3}H]-thymidine per well, and harvested with a Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science Inc., Boston, MA, USA). Thymidine incorporation was measured with a Matrix 9600 β-counter (Perkin Elmer Life Science Inc., Yokohama, Japan).

2.9. Statistical analysis

Statistical significance was evaluated using ANOVA followed by Dunnet's test. A probability value < 0.05 was considered to indicate statistical significance. Each data point was expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors.

3. Results

3.1. Effect of HMGB1 on ICAM-1, B7.1, B7.2 and CD40 expression, IFN-γ and TNF-α production and lymphocyte proliferation

The kinetics of adhesion molecule expression on monocytes incubated in HMGB1 at 10 μg/ml was determined by flow cytometry. ICAM-1, B7.1, B7.2 and CD40 expression began to increase at 16 h after HMGB1 addition, increased significantly up to 24 h, and then leveled off (Fig. 1A). Fig. 1(B) showed the typical pattern of the expression of ICAM-1 on monocytes (CD14) in human PBMCs stimulated with 10 μg/ml HMGB1 for 24 h. HMGB1 also induced IFN-γ and TNF-α production and lymphocyte proliferation in PBMCs in a time-dependent manner, starting at 24 h and continuing through the 48 h incubation period (Fig. 1A). In the

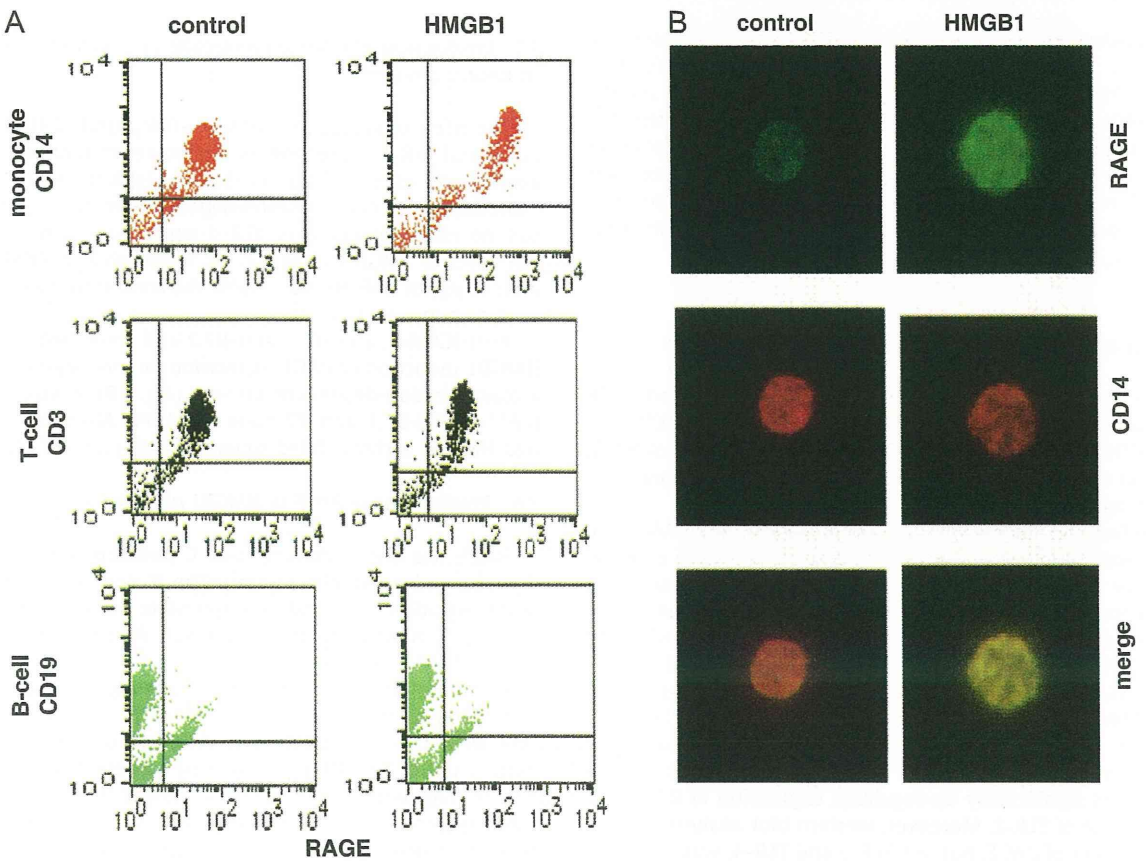

Fig. 4. Expression of RAGE on monocytes, T-cells and B-cells in PBMCs. (A) RAGE expression on monocytes (CD14), T-cells (CD3) and B-cells (CD19) in PBMCs treated with 10 μg/ml HMGB1 for 24 h was analyzed by multi-color flow cytometry. (B) RAGE expression on monocytes (CD14) in PBMCs treated with 10 μg/ml HMGB1 was analyzed by multi-color fluorescence microscopy. Immunocytochemical staining was performed using anti-RAGE Ab. Original magnification X40 for all panels.

absence of HMGB1, there was a small increase in ICAM-1, B7.1, B7.2 and CD40 expression starting at 16 h and continuing to 48 h, but no increase in IFN-γ or TNF-α production or lymphocyte proliferation during the 48 h incubation period (Fig. 1A).

3.2. Dose-response of the HMGB1 effect on ICAM-1, B7.1, B7.2 and CD40 expression, IFN-γ and TNF-α production and lymphocyte proliferation

The effect of 0.1–100 μg/ml HMGB1 on ICAM-1, B7.1, B7.2 and CD40 expression on monocytes and on IFN-γ and TNF-α production and lymphocyte proliferation in PBMCs was determined after a 24 h incubation (Fig. 2). HMGB1 induced adhesion molecule expression, IFN-γ and TNF-α production and lymphocyte proliferation in a concentration-dependent manner. Adhesion molecule expression, cytokine production and lymphocyte proliferation increased at 1 μg HMGB1/ml and continued to increase up to 10 and 100 μg HMGB1/ml. The amount of IFN-γ and TNF-α production induced by 10 μg/ml HMGB1 was 395 ± 42 and 397 ± 46 pg/ml, respectively. Assuming that the effects of HMGB1 were maximal at 10 μg/ml, the EC_{50} of HMGB1 for induction of ICAM-1 expression and TNF-α production were 2 and 4 μg/ml, respectively. These EC_{50} values were almost the same as those for HMGB1 activity in the presence of polymyxin B (data not shown), as previously reported (Andersson et al., 2000).

3.3. Involvement of adhesion molecules in HMGB1 induction of cytokine production and lymphocyte proliferation

The involvement of ICAM-1, B7.1, B7.2 and CD40 in HMGB1-induced IFN-γ and TNF-α production and lymphocyte proliferation in human PBMCs was examined using the respective and specific Abs. Anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs inhibited HMGB1-induced IFN-γ and TNF-α production and lymphocyte proliferation in PBMCs in a concentration-dependent manner (Fig. 3). A mixture of anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs completely inhibited HMGB1 activity (filled square in left panels of Fig. 3).

3.4. Effect of HMGB1 on RAGE, TLR-2 and TLR-4 expression

Flow cytometry showed that 10 μg/ml HMGB1 induced RAGE expression on monocytes (CD14), but not on T-cells (CD3) or B-cells (CD19) (Fig. 4A). HMGB1-induced up-regulation of RAGE expression on the cell surface was also shown by immunocytochemical staining (Fig. 4B).

To confirm the binding specificity of anti-RAGE Ab to RAGE, we used a combination of anti-RAGE Ab and soluble form of RAGE (sRAGE) for flow cytometry and immunocytochemical staining in the presence and absence of HMGB1. The expression of RAGE was not detected in the presence of sRAGE (data not shown), indicating that anti-RAGE Ab recognized the extracellular domain of RAGE.

RAGE, TLR-2 and TLR-4 expression on monocytes isolated from human PBMCs was determined by flow cytometry (Fig. 5A). Expression of RAGE, but not TLR-2 and TLR-4, was increased significantly by 24 h incubation with 10 μg/ml HMGB1, while 10 ng/ml LPS significantly up-regulated expression of RAGE and TLR-4, but not of TLR-2. Moreover, western blot analysis showed that expression of RAGE, but not TLR-2 and TLR-4, was increased significantly by 24 h incubation with 10 μg/ml HMGB1 (Fig. 5B).

In addition, no effect of HMGB1 on RAGE, TLR-2 or TLR-4 expression was shown by quantitative PCR (data not shown).

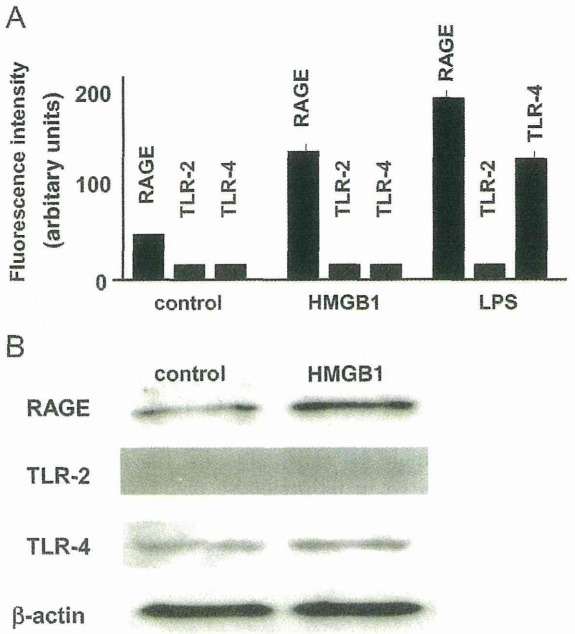

Fig. 5. Effect of HMGB1 on RAGE expression on isolated monocytes. (A) The expression of RAGE, TLR-2 and TLR-4 on monocytes isolated from PBMCs stimulated with 10 μg/ml HMGB1 or 10 ng/ml LPS for 24 h was analyzed by flow cytometry. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. Error bars within symbols are hidden. (B) The expression of RAGE, TLR-2 and TLR-4 on monocytes isolated from PBMCs stimulated with 10 μg/ml HMGB1 for 24 h was analyzed by western blotting.

3.5. Involvement of adhesion molecules in the effect of HMGB1 on RAGE expression

The effect of incubation with 0.1–100 μg/ml HMGB1 on RAGE, TLR-2 and TLR-4 expression on monocytes in human PBMCs was determined after a 24 h incubation. HMGB1 increased RAGE expression in a concentration-dependent manner (Fig. 6A), but had no effect on TLR-2 or TLR-4 expression (data not shown). The level of RAGE expression on monocytes in PBMCs treated with 10 μg/ml HMGB1 was double the level in isolated monocytes, as shown in Fig. 5(A).

Anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs inhibited HMGB1 induction of RAGE expression on monocytes in PBMCs in a concentration-dependent manner (Fig. 6B). A mixture of anti-ICAM-1, anti-B7.1, anti-B7.2 and anti-CD40 Abs completely inhibited HMGB1 activity (filled squares in left panel of Fig. 6B).

3.6. Involvement of RAGE in HMGB1 activity

RAGE has one V domain, two C domains, a transmembrane domain, and a cytoplasmic tail. The V domain consists of two *N*-glycosylation sites and is responsible for extracellular ligand binding. It is reported that anti-RAGE Ab Fab fragments inhibit RAGE-HMGB1 interaction (Hori et al., 1995). To investigate the involvement of RAGE on HMGB1 activity, adhesion molecule expression on monocytes, and cytokine production and lymphocyte proliferation in PBMCs were determined 24 h after incubation with 10 μg/ml HMGB1 and increasing concentrations of anti-RAGE Ab Fab fragments. Flow cytometry showed that the anti-RAGE Ab Fab fragments inhibited HMGB1-induced expression of ICAM-1, B7.1, B7.2 and CD40 on human monocytes in a concentration-dependent manner (Fig. 7). Moreover, the anti-RAGE Ab Fab fragments inhibited HMGB1-induced IFN-γ and TNF-α production and lymphocyte proliferation in human PBMCs (Fig. 7). However,

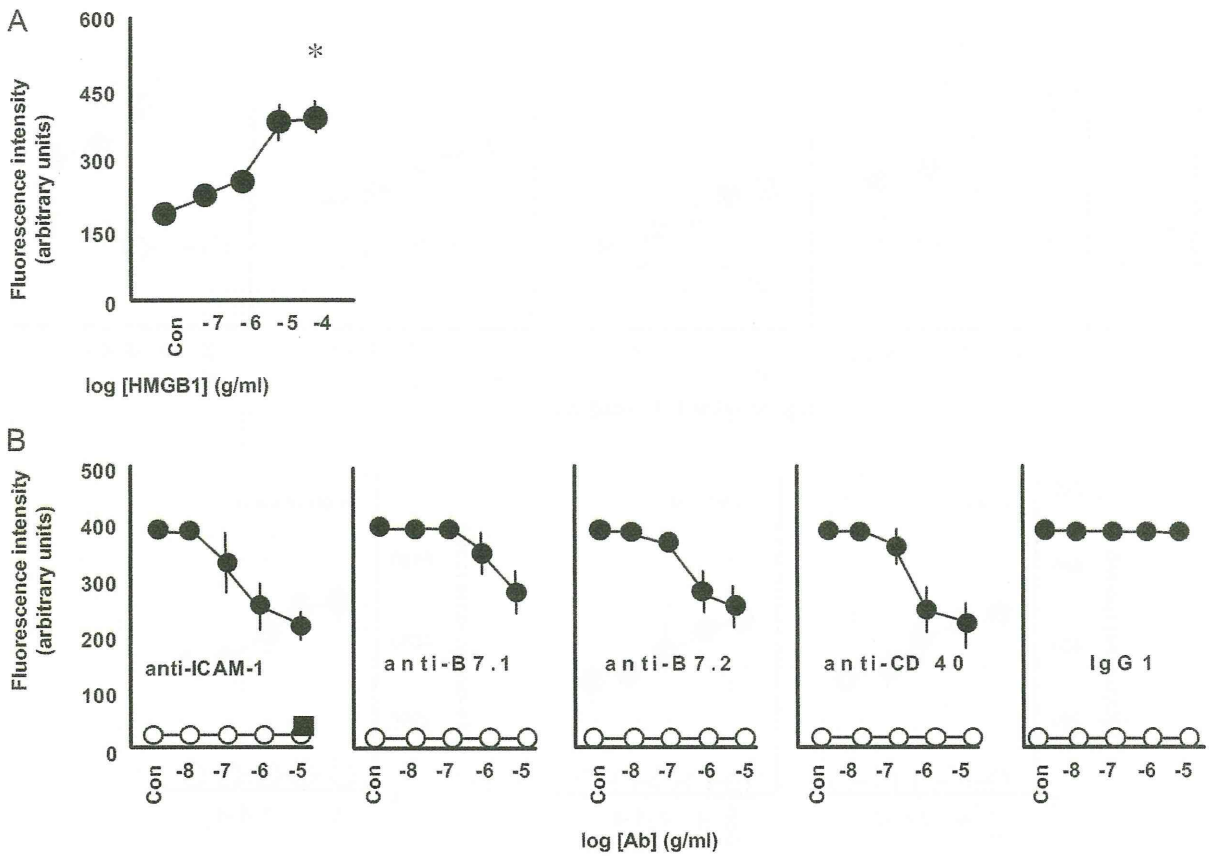

Fig. 6. Involvement of adhesion molecules in RAGE expression. RAGE expression was determined by flow cytometry after 24 h incubation. (A) 4×10^6 PBMCs/ml were incubated with 0.1–100 μg/ml HMGB1. Control (Con) means 0 μg/ml HMGB1. $^{**}P < 0.01$ compared with the value for medium alone. (B) 4×10^6 PBMCs/ml were incubated with anti-ICAM-1, anti-B7.1, anti-B7.2 or anti-CD40 Ab at 0.01–10 μg/ml in the absence (open circles; ○) or presence (filled circles; ●) of 10 μg/ml HMGB1. The effect of a mixture of the Abs at 10 μg/ml was shown in the left panel (filled square; ■). Control (Con) means 0 μg/ml Abs. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the value for HMGB1 alone. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. Error bars within symbols are hidden.

anti-TLR-2 and TLR-4 Abs had no effect on HMGB1 activity (data not shown).

In addition, 10 μg/ml HMGB1 had no effect on the expression of ICAM-1 on macrophages (CD68) in spleen cells of RAGE knockout mice, whereas HMGB1 induced expression of ICAM-1 in spleen cells of wild-type mice and of TLR-2 and TLR-4 knockout mice (Fig. 8).

4. Discussion

It has been demonstrated that macrophages/monocytes and T-cells play roles in the immune responses of patients with inflammatory diseases. For example, pro-inflammatory cytokines released from activated macrophages/monocytes and T-cells induce initial events and liver damage during viral and autoimmune hepatitis (Koziel, 1999; McFarlane, 1999). Intrahepatic recruitment of macrophages/monocytes and T-cells contributes to HMGB1-induced inflammation in an HBV hepatitis model mice (Sitia et al., 2007). In addition, it has been reported that HMGB1 induces inflammatory responses, including maturation and migration of monocytes/macrophages (Rauvala and Rouhiainen, 2010), leading to activation of naïve T-cells in the promotion and induction of Th1 responses and to clonal expansion of antigen-specific T-cells (Messmer et al., 2004; Dumitriu et al., 2005). HMGB1 is necessary for CD4 T-cell proliferation after activation by DCs (Bustin, 1999). 1 μg/ml HMGB1 has been shown to induce production of TNF-α, but not of IL-10 or IL-12 in normal human PBMCs (Andersson et al., 2000). However, little is known about the cellular mechanism of monocyte/macrophage-induced T-cell activation. The present study is the first report that HMGB1 induced ICAM-1, B7 and CD40 expression on monocytes, and IFN-γ and TNF-α production and T-cell proliferation in human PBMCs (Figs. 1 and 2). Involvement of adhesion molecules on monocytes and T-cells was required for HMGB1-induced IFN-γ and TNF-α production and T-cell proliferation (Fig. 3). Therefore, cell-cell interactions might play roles in HMGB1-induced activation of monocytes and T-cells, perhaps with involvement of adhesion molecules to regulate cellular responses in the area of local inflammation in an HMGB1 concentration-dependent manner.

Putative receptors for HMGB1 on monocytes/macrophages and defined pathways activated by HMGB1 after binding to cell-surface receptors have been investigated. It is reported that RAGE expression is at low levels in normal tissues, but is up-regulated at sites where its ligands accumulate (Yan et al., 2003). In the present study, HMGB1 was shown to induce expression of RAGE on monocytes, but not of TLR-2 and TLR-4 (Figs. 4 and 5). Moreover, involvement of adhesion molecules on monocytes and T-cells was required for HMGB1-induced RAGE expression (Fig. 6B). RAGE has been reported to be the major receptor for the pro-inflammatory activity of HMGB1 in ex vivo macrophages of rats and mice (Kokkola et al., 2005). On the other hand, HMGB1 can interact directly with TLR-4, inducing cytokine production in peritoneal macrophages of mice (Yang et al., 2010). In the present study, we found that RAGE, but not TLR-2 or TLR-4, played a role

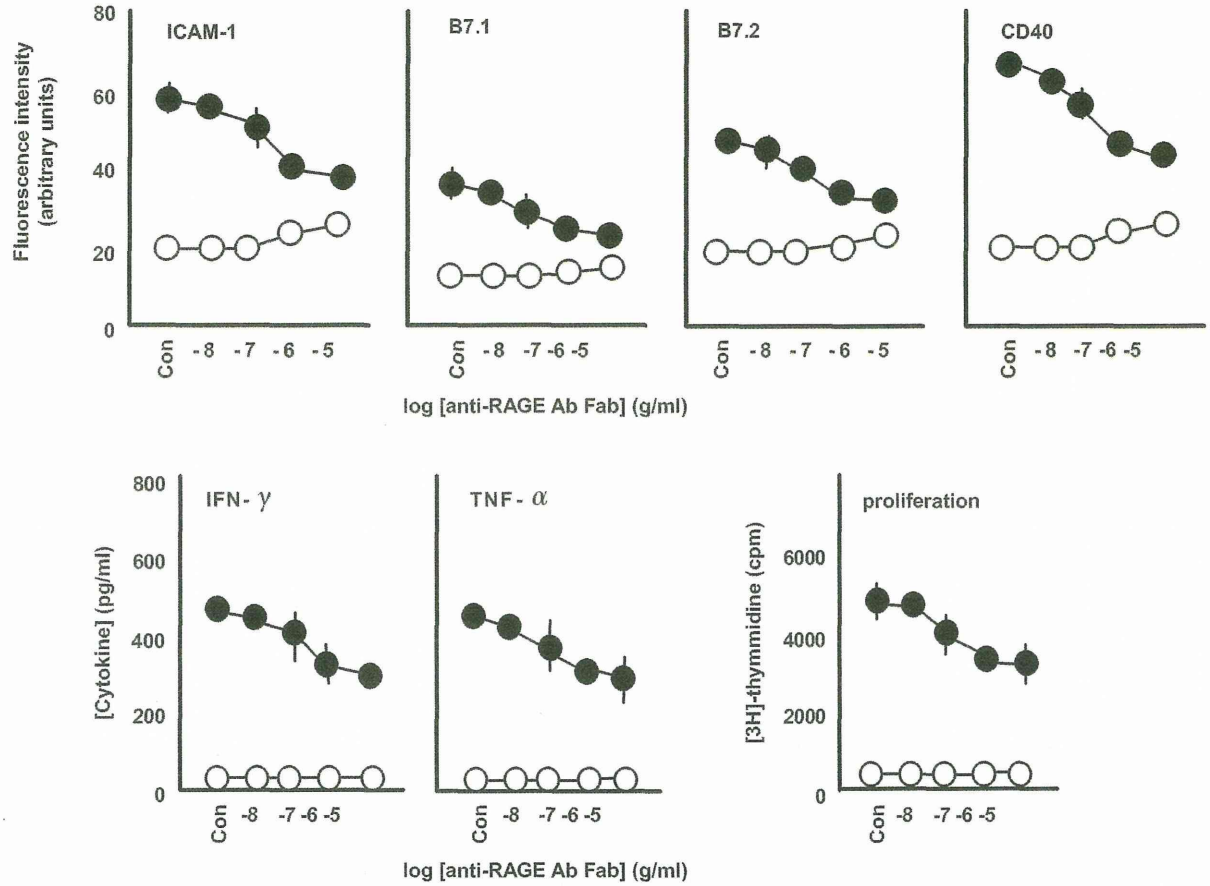

Fig. 7. Involvement of RAGE in HMGB1 activity. PBMCs at 4×10^6 cells/ml were incubated with 10 μg/ml HMGB1 and 0.01–10 μg/ml anti-RAGE Ab Fab, and ICAM-1, B7.1, B7.2 and CD40 expression on monocytes, and IFN-γ and TNF-α production and lymphocyte proliferation in human PBMCs were determined 24 h later by flow cytometry, ELISA and [^{3}H]-thymidine uptake, respectively. Open circles (○) represent the results in the absence of HMGB1, and filled circles (●) represent those in the presence of HMGB1. Control (Con) means 0 μg/ml anti-RAGE Ab Fab. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. $^{**}P < 0.01$ compared with the value for HMGB1 alone. Error bars within symbols are hidden.

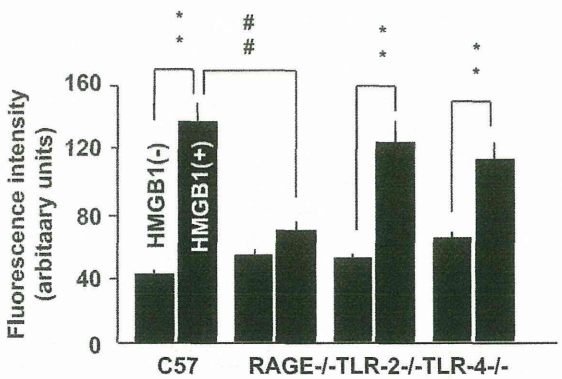

Fig. 8. Effect of HMGB1 on ICAM-1 expression on macrophages of spleen cells of RAGE-/-, TLR-2-/- and TLR-4-/- knockout mice. Spleen cells of control mice (C57BL/6 J), RAGE-/-, TLR-2-/- and TLR-4-/- knockout mice at 4×10^6 cells/ml were incubated with or without 10 μg/ml HMGB1. The expression of ICAM-1 on macrophages (CD68) was analyzed by flow cytometry. Each data point is expressed as the mean ± S.E.M. of triplicate determinations of samples from five donors. $^{**}P < 0.01$ compared with the value for medium alone. $\#\#P < 0.01$ compared with the value for HMGB1 in macrophages of control mice. Error bars within symbols are hidden.

in HMGB1 activity (Figs. 7 and 8). In addition, the previous study by Yamamoto et al. (2011) has shown that stimulation of RAGE by LPS induced TNF-α production in mice macrophages. Together with these results, both RAGE and TLR-4 might be involved in the pathogenesis of a wide range of inflammatory disorders via recruitment of ligands. Further studies are needed on the types of HMGB1 receptors that mediate HMGB1 activity.

Administration of agents that specifically inhibit HMGB1 activity in animals with ischemia and inflammatory diseases has been reported to interrupt progression of tissue injury and suppress inflammatory responses (Andersson and Tracey, 2011). We have proposed that the inhibition of HMGB1 activity is a target for treatment of inflammatory diseases, including brain ischemia (Liu et al., 2007) and atherosclerosis (Kanellakis et al., 2011). Therefore, we suggest that a functional property of such a drug should be inhibition of HMGB1-induced adhesion molecule expression on monocytes.

5. Concludions

The results presented here provide new insight into the mechanism of HMGB1 effects on the innate immune response. HMGB1 activation of monocytes and T-cells involves adhesion molecules on both types of cells.

Acknowledgements

This work was supported in part by grants from the Japan Society for the Promotion of Science [Grants 18590509, 20590539](H.K.T.),

[Grants 17659159, 19659061, 21390071, 21659141](M.N.), [Grant 21590594](K.L.); from the Scientific Research from Ministry of Health, Labour and Welfare of Japan [09156274](M.N.); from the Takeda Science Foundation (H.K.T.).

The authors thank Ms. Miyuki Shiotani and Mr. Yukinari Isomoto for technical assistance.

References

Abraham, E., Arcaroli, J., Carmody, A., Wang, H., Tracey, K.J., 2000. HMG-1 as a mediator of acute lung inflammation. J. Immunol. 165, 2950–2954.

Albayrak, A., Uyanik, M.H., Cerrah, S., Altas, S., Dursun, H., Demir, M., Uslu, H., 2010. Is HMGB1 a new indirect marker for revealing fibrosis in chronic hepatitis and a new therapeutic target in treatment? Viral Immunol. 23, 633–638.

Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H., Tracey, K.J., 2000. High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. J. Exp. Med. 192, 565–570.

Andersson, U., Tracey, K.J., 2011. HMGB1 is a therapeutic target for sterile inflammation and infection. Annu. Rev. Immunol. 29, 139–162.

Bustin, M., 1999. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol. Cell. Biol. 19, 5237–5246.

Camacho, S.A., Heath, W.R., Carbone, F.R., Sarvetnick, N., LeBon, A., Karlsson, L., Peterson, P.A., Webb, S.R., 2001. A key role for ICAM-1 in generating effector cells mediating inflammatory responses. Nat. Immunol. 2, 523–529.

Degryse, B., Bonaldi, T., Scaffidi, P., Muller, S., Resnati, M., Sanvito, F., Arrigoni, G., Bianchi, M.E., 2001. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J. Cell Biol. 152, 1197–1206.

Dumitriu, I.E., Baruah, P., Valentinis, B., Voll, R.E., Herrmann, M., Nawroth, P.P., Arnold, B., Bianchi, M.E., Manfredi, A.A., Rovere-Querini, P., 2005. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. J. Immunol. 174, 7506–7515.

Durie, F.H., Foy, T.M., Masters, S.R., Laman, J.D., Noelle, R.J., 1994. The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol. Today 15, 406–411.

Fiuza, C., Bustin, M., Talwar, S., Tropea, M., Gerstenberger, E., Shelhamer, J.H., Suffredini, A.F., 2003. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood 101, 2652–2660.

Gardella, S., Andrei, C., Ferrera, D., Lotti, L.V., Torrisi, M.R., Bianchi, M.E., Rubartelli, A., 2002. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. EMBO Rep. 3, 995–1001.

Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J.X., Nagashima, M., Lundh, E.R., Vijay, S., Nitecki, D., Morser, J., Stern, D., Schmidt., A.M., 1995. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J. Biol. Chem. 270, 25752–25762.

Kalinina, N., Agrotis, A., Antropova, Y., DiVitto, G., Kanellakis, P., Kostolias, G., Ilyinskaya, O., Tararak, E., Bobik, A., 2004. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. Arterioscler. Thromb. Vasc. Biol. 24, 2320–2325.

Kanellakis, P., Agrotis, A., Kyaw, T.S., Koulis, C., Ahrens, I., Mori, S., Takahashi, H.K., Liu, K., Peter, K., Nishibori, M., Bobik, A., 2011. High-mobility group box protein 1 neutralization reduces development of diet-induced atherosclerosis in apolipoprotein e-deficient mice. Arterioscler. Thromb. Vasc. Biol. 31, 313–319.

Kokkola, R., Sundberg, E., Ulfgren, A.K., Palmblad, K., Li, J., Wang, H., Ulloa, L., Yang, H., Yan, X.J., Furie, R., Chiorazzi, N., Tracey, K.J., Andersson, U., Harris, H.E., 2002. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. Arthritis Rheum. 46, 2598–2603.

Kokkola, R., Andersson, A., Mullins, G., Ostberg, T., Treutiger, C.J., Arnold, B., Nawroth, P., Andersson, U., Harris, R.A., Harris, H.E., 2005. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. Scand. J. Immunol. 61, 1–9.

Koziel, M.J., 1999. Cytokines in viral hepatitis. Semin. Liver Dis. 19, 157–169.

Liu, K., Mori, S., Takahashi, H.K., Tomono, Y., Wake, H., Kanke, T., Sato, Y., Hiraga, N., Adachi, N., Yoshino, T., Nishibori, M., 2007. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. FASEB J. 21, 3904–3916.

Liu, R., Mori, S., Wake, H., Zhang, J., Liu, K., Izushi, Y., Takahashi, H.K., Peng, B., Nishibori, M., 2009. Establishment of in vitro binding assay of high mobility group box-1 and S100A12 to receptor for advanced glycation end products: heparin's effect on binding. Acta Med. Okayama 63, 203–211.

Luster, A.D., Alon, R., von Andrian, U.H., 2005. Immune cell migration in inflammation: present and future therapeutic targets. Nat. Immunol. 6, 1182–1190.

McDonnell, M., Liang, Y., Noronha, A., Coukos, J., Kasper, D.L., Farraye, F.A., Ganley-Leal, L.M., 2011. Systemic toll-like receptor ligands modify B-cell responses in human inflammatory bowel disease. Inflamm. Bowel Dis. 17, 298–307.

McFarlane, I.G., 1999. Pathogenesis of autoimmune hepatitis. Biomed. Pharmacother. 53, 255–263.

Messmer, D., Yang, H., Telusma, G., Knoll, F., Li, J., Messmer, B., Tracey, K.J., Chiorazzi, N., 2004. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. J. Immunol. 173, 307–313.

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A., Abraham, E., 2004. Involvement of toll like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J. Biol. Chem. 279, 7370–7377.

Porto, A., Palumbo, R., Pieroni, M., Aprigliano, G., Chiesa, R., Sanvito, F., Maseri, A., Bianchi, M.E., 2006. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. FASEB J. 20, 2565–2566.

Ranger, A.M., Das, M.P., Kuchroo, V.K., Glimcher, L.H., 1996. B7-2 (CD86) is essential for the development of IL-4-producing T cells. Int. Immunol. 8, 1549–1560.

Rauvala, H., Rouhiainen, A., 2010. Physiological and pathophysiological outcomes of the interactions of HMGB1 with cell surface receptors. Biochim. Biophys. Acta 1799, 164–170.

Scaffidi, P., Misteli, T., Bianchi, M.E., 2002. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418, 191–195.

Sitia, G., Iannacone, M., Müller, S., Bianchi, M.E., Guidotti, L.G., 2007. Treatment with HMGB1 inhibitors diminishes CTL-induced liver disease in HBV transgenic mice. J. Leukoc. Biol. 81, 100–107.

Steinman, R.M., Kaplan, G., Witmer, M.D., Cohn, Z.A., 1979. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. J. Exp. Med. 149, 1–16.

Takahashi, H.K., Iwagaki, H., Yoshino, T., Mori, S., Morichika, T., Itoh, H., Yokoyama, M., Kubo, S., Kondo, E., Akagi, T., Tanaka, N., Nishibori, M., 2002. Prostaglandin E(2) inhibits IL-18-induced ICAM-1 and B7.2 expression through EP2/EP4 receptors in human peripheral blood mononuclear cells. J. Immunol. 168, 4446–4454.

Takahashi, H.K., Iwagaki, H., Tamura, R., Xue, D., Sano, M., Mori, S., Yoshino, T., Tanaka, N., Nishibori, M., 2003. Unique regulation profile of prostaglandin E1 on adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. J. Pharmacol. Exp. Ther. 307, 1188–1195.

Takahashi, H.K., Mori, S., Wake, H., Liu, K., Yoshino, T., Ohashi, K., Tanaka, N., Shikata, K., Makino, H., Nishibori, M., 2009. Advanced glycation end products subspecies-selectively induce adhesion molecule expression and cytokine production in human peripheral blood mononuclear cells. J. Pharmacol. Exp. Ther. 330, 89–98.

van Beijnum, J.R., Buurman, W.A., Griffioen, A.W., 2008. Convergence and amplification of toll-like receptor (TLR) and receptor for advanced glycation end products (RAGE) signaling pathways via high mobility group B1 (HMGB1). Angiogenesis 11, 91–99.

Wake, H., Mori, S., Liu, K., Takahashi, H.K., Nishibori, M., 2009. High mobility group box 1 complexed with heparin induced angiogenesis in matrigel plug assay. Acta Med. Okayama. 63, 249–262.

Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J.M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K.R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P.E., Abumrad, N.N., Sama, A., Tracey, K.J., 1999. HMG-1 as a late mediator of endotoxin lethality in mice. Science 285, 248–251.

Yamamoto, Y., Harashima, A., Saito, H., Tsuneyama, K., Munesue, S., Motoyoshi, S., Han, D., Watanabe, T., Asano, M., Takasawa, S., Okamoto, H., Shimura, S., Karasawa, T., Yonekura, H., Yamamoto, H., 2011. Septic shock is associated with receptor for advanced glycation end products (RAGE) ligation of LPS. J. Immunol. 186, 3248–3257.

Yan, S.F., Ramasamy, R., Naka, Y., Schmidt, A.M., 2003. Glycation, inflammation, and RAGE: a scaffold for the macrovascular complications of diabetes and beyond. Circ. Res. 93, 1159–1169.

Yang, H., Wang, H., Czura, C.J., Tracey, K.J., 2005. The cytokine activity of HMGB1. J. Leukoc. Biol. 78, 1–8.

Yang, H., Hreggvidsdottir, H.S., Palmblad, K., Wang, H., Ochani, M., Li, J., Lu, B., Chavan, S., Rosas-Ballina, M., Al-Abed, Y., Akira, S., Bierhaus, A., Erlandsson-Harris, H., Andersson, U., Tracey, K.J., 2010. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. Proc. Nat. Acad. Sci. U.S.A. 107, 11942–11947.

日薬理誌（*Folia Pharmacol. Jpn.*）143，5～9（2014）

抗HMGB1抗体治療の可能性
外傷性脳障害と神経因性疼痛に対する抗HMGB1抗体治療

大熊　佑[1,2]，伊達　勲[2]，西堀　正洋[1]

要約：脳外傷後に必発する脳浮腫は，しばしば脳ヘルニアを生じ致命的となる．脳浮腫はまた，脳低酸素症を増悪し，その結果脳障害を助長する．脳外傷急性期における患者の救命と神経後遺症の軽減化のために，急性期の脳浮腫を制御することは極めて重要である．しかし現在，エビデンスを伴う薬物治療法はない．最近，脳虚血時の炎症惹起物質として核内DNA結合タンパク質であるhigh mobility group box-1（HMGB1）が注目されている．HMGB1を標的とする抗体治療は虚血性脳障害に有効であったが，最近外傷性脳障害にも著効することが動物実験で明らかにされた．さらに神経因性疼痛の動物モデルでも抗体治療の有効性が示された．抗HMGB1抗体の作用機序について概説する．

1．はじめに

脳外傷は，交通事故や転落事故を最大の原因とし，脳外科臨床あるいは救急医療において最も頻繁に経験される病態の一つである．高齢化の進行するわが国において，交通事故の犠牲となる高齢者の割合も高い．また若年層においては，不慮の事故は死因のトップの位置にある．脳外傷の急性期に脳浮腫が必発することはよく知られた事実である．しかし，患者の全身管理法が進歩した今日でも，脳外傷によって生じる脳浮腫と随伴する神経障害に対しエビデンスのある有効な治療法は，存在しない．脳外傷急性期における広範囲の脳浮腫は，脳ヘルニアを生じ直接的な死亡の原因になる．また，急性期を乗り越えた場合でも，脳外傷は高次脳神経機能の低下，運動・知覚障害，人格変化，外傷後てんかん等，種々の神経後遺症を残してしまうことが多い．したがって，脳外傷急性期の脳障害の軽減化と神経後遺症回避の観点から，急性期脳浮腫治療の重要性が理解される．

我々の研究グループは，これまで脳虚血後の脳内炎症の研究を行ない，虚血後早期の脳内イベントとして神経細胞核に局在するhigh mobility group box-1（HMGB1）の細胞質から細胞外へと至る移動について明らかにしてきた(1, 2)．HMGB1の細胞外への遊離についてはそれまで壊死細胞からの受動的遊離とマクロファージ系細胞からの活性化依存的分泌の二様式が報告されてきた(3, 4)が，神経細胞からも組織障害依存的に放出される可能性が示されたわけである．これらの研究が嚆矢となり，脳内炎症におけるHMGB1の動態と機能について関心が高まってきた．特に脳虚血後急性期における起炎性サイトカインとしてのHMGB1の働きは，病態形成において極めて重要であることが明らかになってきた．本総説では，脳虚血時と同様のHMGB1動態を生じる脳外傷を中心に中枢神経障害時におけるHMGB1のトランスロケーションとその細胞外での機能，それに対する抗HMGB1抗体治療の有効性について概説する．

2．脳虚血障害とHMGB1

Liuら(1)は，脳梗塞急性期の脳内炎症の進展が最終的に形成される脳梗塞巣の増大に繋がるとの仮説のもとに，抗HMGB1単クローン抗体によるターゲットバリデーションを試みた．複数種類作製された単クローン抗体のうち，HMGB1タンパク質にのみ存在するC末端配列（DEEEDDDDE）を認識し，HMGB1活性を中和する抗体が試験用抗体として選ばれた．ラットのMCAO 2時間閉塞－再灌流後の脳梗塞形成に対する本抗体の投与効果は，再灌流直後とさらにその6時

キーワード：high mobility group box-1（HMGB1），脳外傷，神経因性疼痛，血液－脳関門（BBB），receptor for advanced glycation endproduct（RAGE）
岡山大学大学院 医歯薬学総合研究科[1] 薬理学，[2] 脳神経外科学（〒700-8558 岡山市北区鹿田町 2-5-1）
E-mail: mbori@md.okayama-u.ac.jp　原稿受領日：2013年8月30日，依頼原稿
Title: Anti-HMGB1 antibody therapy for traumatic brain injury and neuropathic pain
Author: Yu Okuma, Isao Date, Masahiro Nishibori

間後の末梢尾静脈内投与で検討された(1)．つまり，臨床での使用と同様に卒中発作後の投与プロトコールで効果が評価された．その結果，対照動物では虚血側半球のかなりの範囲に広がる梗塞巣が形成されるのに対し，抗HMGB1抗体投与群では24時間後には90%，48時間後でも75%の抑制が観察された．再灌流後24時間まで測定したRota-rod上での歩行時間を指標にした運動麻痺機能の評価でも，著明な運動機能改善効果が証明された．以上の結果から，抗HMGB1単クローン抗体は，卒中発作後の投与によっても脳梗塞サイズの縮小と運動麻痺症状の軽減化を図れる治療法であることが示唆された．

虚血脳におけるHMGB1の局在が詳細に検討され，虚血コア領域では，虚血中ならびに再灌流早期から神経細胞内でHMGB1のトランスロケーションが生じることが明らかにされた(2)．このような素早いHMGB1の移動現象は，神経細胞内でのシグナル伝達を介して制御されていることを想像させるが，現在のところその詳細は明らかになっていない．時系列的に考えると，古典的サイトカインの発現誘導に先行する現象であるかもしれない．再還流後24時間までは，アストログリアやミクログリアではこのようなトランスロケーションは殆ど認められないので，神経細胞に特有の反応であると考えられる．その後，複数のグループ(5-7)から脳虚血あるいは低酸素負荷によるHMGB1の細胞内トランスロケーションならびに消失に関し詳細な報告が相次いだ．

Liuら(1)とZhangら(2)の抗HMGB1抗体の作用機序検討の結果を総合すると，以下のようにまとめることができる．抗HMGB1抗体の投与によって，①エバンスブルー漏出の測定で評価される脳血管の透過性亢進が著明に抑制される，②脳血管の透過性亢進測定時点で脳を固定し透過型電子顕微鏡で血液－脳関門を観察すると，構成要素であるアストログリア細胞のエンドフィートの腫脹やエンドフィートの内皮細胞基底膜からの遊離が抑制される，③起炎性サイトカインTNF-αのmRNA発現が抑制される．脳虚血後急性期のイベントとして，血液－脳関門の機能的ならびに構造的破綻は極めて重要である．この最初期応答としての脳血管透過性亢進を抗HMGB1抗体による治療は抑制することができるかもしれない．同様に，抗体投与は虚血領域のミクログリアの活性化，MMP-9タンパク質発現など脳内炎症の進行を促す種々の要因を抑制した(2)．

Hayakawaら(8)は，ミノサイクリンの脳梗塞抑制作用を，HMGB1の発現抑制と関連づけた．また，Kikuchiら(9)は，わが国における脳梗塞治療薬のエダラボンが，HMGB1のトランスロケーションを抑制することを見出した．このように，既存薬の中にもその作用の一部にHMGB1の発現や遊離抑制が関与するものが存在するようであり，今後さらに検討を続ける必要がある．HMGB1の受容体の一つはreceptor for advanced glycation endproduct（RAGE）であると考えられている(10)．RAGEのノックアウトマウスを用いた脳梗塞実験結果によると，RAGEノックアウトでは野生型マウスに比較し生じる梗塞巣が小さく，マクロファージ系細胞の活性化が減弱していた(11)．またこの報告の中で，抗HMGB1ウサギポリクローナル抗体の腹腔内投与が野性型マウスの脳梗塞を縮小すること，さらに抗体は脳虚血部位に到達していることが免疫組織化学的に示された．このように，脳梗塞急性期におけるHMGB1-RAGE系の役割が，徐々に明らかにされてきている．

Shichitaら(12)は，樹状細胞の活性化を指標に脳組織由来の新規DAMPの精製による探索を行い，peroxiredoxin familyを同定した．彼らは，脳虚血後6時間までの時間帯におけるHMGB1の役割と，12時間以降24時間までの時間帯におけるperoxiredoxinの役割を合わせて見出し，虚血後の時間依存的なメディエーターの役割を示唆した(12)．peroxiredoxinは，マクロファージ系細胞の活性化によるIL-23の産生とその下流でのIL-17産生が炎症惹起に重要であるとされた．HMGB1とperoxiredoxinそれぞれに対する抗体治療法が提案されている．

3. 脳外傷とHMGB1

Okumaら(13)は，ラットのfluid percussionモデルを用いて，脳外傷におけるHMGB1の動態と外傷性脳炎症の病態解析ならびに抗HMGB1抗体の効果を詳細に解析した．その結果，脳外傷の受傷部位である大脳皮質と海馬錐体細胞における神経細胞内HMGB1のトランスロケーションの時間ならびに移行パターンが，脳虚血障害の場合と酷似していることを明らかにした（図1）．彼らは，受傷脳部位からはHMGB1が消失していくこと，神経から放出されたHMGB1の一部は循環血中へ出て血漿HMGB1の上昇につながること，抗HMGB1抗体の投与によってこのトランスロケーションそのものが強く抑制され，血漿中のHMGB1上昇が消失することを示した．さらに，抗体投与が受傷直後であった場合には，脳外傷に伴う脳血管透過性亢進と脳浮腫は85%抑制されることを示した．T2強調MRIでの撮像で（図2）受傷後の脳浮腫を経時的に観