inhibitor and siRNA of p38MAPK), but not U-0126, significantly abrogated HMGB1 release. This suggested the involvement of p38MAPK in CRP-induced HMGB1 release by RAW264.7 cells. Our findings are consistent with a previous report demonstrating that HMGB1 release is mediated through the p38MAPK signaling pathway [40]. However, it has also been reported that HMGB1 release can occur through the activity of MAPKs other than p38MAPK [21,28]. This discrepancy of results might be due to differences in the cell types and stimulants examined.

The level of CRP, a key inflammatory cytokine, is a strong predictor of cardiovascular events [1]. CRP has a prognostic value for predicting the activity and vulnerability of atheromatous plaque rupture [31,41–43]. Highly elevated CRP in atherosclerosis patients not only serves as a biomarker for cardiovascular disease risk but also functions as an active mediator of atherosclerosis by promoting arterial endothelial activation and macrophage recruitment [44]. Recent studies have also shown that CRP is expressed in macrophages and VSMCs in atheromatous plaques [2–5] and plays a role in the progression and vulnerability of atherosclerotic lesions. Several investigators have suggested that therapeutic inhibition of CRP is a new approach for the treatment of cardiovascular diseases [13,14]. Atherosclerosis is now considered a chronic inflammatory disease of the arterial system. Although endothelial injury and dysfunction remain central to the initiation and pathogenesis of the disease, accumulating evidence suggests that inflammation evoked by injury plays a pathogenic role in all stages of the disease, from initiation to plaque rupture and associated thrombotic complications. On the other hand, HMGB1, which is released from macrophage lineage cells in response to the inflammatory cytokines TNF-α, IL-1β, IFN-γ, and transforming growth factor-β of acute-phase inflammation, plays a pivotal role in chronic inflammatory diseases and also acts as a late-phase lethal mediator in endotoxin shock [15,19,20,27,40,45,46]. HMGB1 acts on its receptor, RAGE, and activates NF-κB signaling, resulting in the expression of proinflammatory cytokines, including IL-1 and TNF-α. HMGB1 has also been shown to stimulate human umbilical vascular endothelial cells, thereby up-regulating adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, inducing granulocyte colony-stimulating factor expression and IL-8 release [45]. CRP induces the expression of ICAM-1, VCAM-1, and E-selectin, in addition to the chemokine MCP-1 [10,47]. These findings suggest that CRP promotes endothelial cell activation and dysfunction, indicating that CRP may enhance and amplify atherosclerosis by promoting the inflammatory component of atherosclerosis by both activated macrophages. We have recently demonstrated that CRP is colocalized with the proinflammatory cytokine HMGB1 in macrophages and VSMCs in atherosclerotic lesions [14]. Kalinina et al. [21] have recently reported that HMGB1 expression levels are up-regulated in atherosclerotic lesions and that macrophages are the major cell type responsible for the production of HMGB1. They also suggested that HMGB1 plays a role in the pathogenesis of plaque formation and progression [21]. Thus, others, as well as our previous reports and the findings of the present study, provide evidence for potential links among CRP, HMGB1, and atherosclerosis. In the current study, we used a murine macrophage cell line, RAW264.7, the cells of which are often used as fair substitutes for macrophages in analyzing the production of inflammatory mediators/cytokines, including HMGB1, in response to various inflammatory stimuli [48,49]. It has been reported that murine macrophage RAW 264.7 cells, human or mouse alveolar macrophages, and monocytes, when differentiated into macrophages, exhibit almost similar patterns of proinflammatory mediator production [50–52]. Similar levels of HMGB1 release by LPS or IFN-γ-activated macrophage RAW264.7 cells and human peripheral blood monocytes have also been reported [45,53]. However, we cannot rule out the possibility that CRP may exhibit some influence on human monocytes/macrophages to release HMGB1. Further study will be needed to clarify this important issue. Indeed, we hope to continue our investigations into the release of HMGB1 by human and animal monocytes/macrophages (by in vitro and in vivo studies) in response to the purified CRP.

Taken together, to the best of our knowledge, this is the first study to have demonstrated that CRP triggers an active release of HMGB1 by macrophage RAW264.7 cells through the Fcγ receptor and p38MAPK signal transduction pathways. Our findings suggested that CRP plays a potentially important role in the induction, amplification, and prolongation of inflammatory processes, including atherosclerosis, by inducing the release of the key inflammatory mediator HMGB1 and thus presents a potential target for the treatment of cardiovascular diseases.

5. Summary

The interaction between proinflammatory cytokines, CRP, and HMGB1 is unclear. Here, we show that CRP induced HMGB1 release by macrophage (RAW264.7) cells through the Fcγ receptor and the activation of p38MAPK, suggesting that CRP plays an important role in the propagation and prolongation of inflammation.

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Mechanism of HMGB1 release inhibition from RAW264.7 cells by oleanolic acid in *Prunus mume Sieb. et Zucc.*

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Abstract. High mobility group box-1 protein (HMGB1), primarily from the nucleus, is released into the extracellular milieu either passively from necrotic cells or actively through secretion by monocytes/macrophages. Extracellular HMGB1 acts as a potent inflammatory agent by promoting the release of cytokines such as tumor necrosis factor (TNF)-α, has procoagulant activity, and is involved in death due to sepsis. Accordingly, HMGB1 is an appropriate therapeutic target. In this study, we found that an extract of *Prunus mume Sieb. et Zucc.* (Ume) fruit (Ume extract), an abundant source of triterpenoids, strongly inhibited HMGB1 release from lipopolysaccharide (LPS)-stimulated macrophage-like RAW264.7 cells. The inhibitory effect on HMGB1 release was enhanced by authentic oleanolic acid (OA), a naturally occurring triterpenoid. Similarly, the HMGB1 release inhibitor in Ume extract was found to be OA. Regarding the mechanisms of the inhibition of HMGB1 release, the OA or Ume extract was found to activate the transcription factor Nrf2, which binds to the antioxidative responsive element, and subsequently the heme oxygenase (HO)-1 protein was induced, indicating that the inhibition of HMGB1 release from LPS-stimulated RAW264.7 cells was mediated via the Nrf2/HO-1 system; an essentially antioxidant effect. These results suggested that natural sources of triterpenoids warrant further evaluation as 'rescue' therapeutics for sepsis and other potentially fatal systemic inflammatory disorders.

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Key words: high mobility group box 1, Ume extract, oleanolic acid, Nrf2, heme oxygenase 1

Introduction

The high mobility group box 1 protein (HMGB1), a nuclear protein, has two distinct functions in cellular systems. In the nucleus, HMGB1 acts as an intracellular regulator of the transcription process with a crucial role in the maintenance of DNA functions (1). In the extracellular space, HMGB1 is released by all eukaryotic cells upon necrosis or by macrophages in response to inflammatory stimuli (2-4) such as endotoxins, tumor necrosis factor (TNF)-α, and C-reactive protein. Extracellular HMGB1 can act as a potent inducer of pro-inflammatory cytokines including TNF-α, interleukin (IL)-6, and IL-1β from macrophages, thus playing a major role in various inflammatory diseases such as sepsis, rheumatoid arthritis, disseminated intravascular coagulation, periodontitis, xenotransplantation and atherosclerosis (2-10). Therefore, agents capable of inhibiting HMGB1 can be considered to possess therapeutic potential.

Hitherto, studies have shown two approaches toward the inhibition of HMGB1; one comprises a blockade of its activity and the other involves the inhibition of HMGB1 release (2). In mouse models of endotoxemia or septic shock, treatment with a neutralizing anti-HMGB1 antibody increased survival even when treatment was started 24 h after the onset of endotoxemia or infection (2). Similarly, a blockade of HMGB1 activity by administering thrombomodulin, an anti-

coagulant protein, to rodents along with thrombin ameliorated the clinical and histological features of the disease (6,7,11), and the inhibition of HMGB1 release was found to be protective against experimental sepsis (2). Several other substances, including the N-terminal kinase activation (JNK) inhibitors and heat shock protein (HSP) 72, are known to inhibit HMGB1 release from lipopolysaccharide (LPS)-stimulated macrophages (13-22). Additionally, natural substances, such as green tea and deep ocean water (DOW), have been shown to inhibit HMGB1 release in RAW264.7 cells (23,24). Therefore, in view of the apparent pathological roles of HMGB1, natural substances capable of blocking HMGB1 release from activated cells might prove to be valuable therapeutic agents.

Prunus mume Sieb. et Zucc. is a variety of Japanese apricot and is known as Ume in Japan (25). The health benefits of Ume are now being widely recognized and strengthened by recent studies that have shown Ume extracts to have strong anticancer and antiproliferative effects in *in vivo* and *in vitro* settings (25-28). These studies have also indicated that Ume might possess strong anti-inflammatory effects (25-28). A recent study by Adachi *et al* (25) isolated 7 triterpenoids from Ume extract. However, the exact mechanism(s) of actions of Ume against pathological processes are yet to be described.

Recently, endogenous heme oxygenase (HO)-1 expression has been shown to protect against the lethal effects of sepsis (29). HO-1 is a cytoprotective enzyme that plays a critical role in defending the body against oxidant-induced injury during inflammation (29,30). In purely inflammatory models of disease, such as endotoxin exposure, HO-1-deficient mice are susceptible to oxidant-induced tissue injury followed by death (30). HO-1 expression has been found to be essential for the activation of the transcription factor Nrf2, which is also a regulator of survival during experimental sepsis (31), suggesting that the Nrf2/HO-1 pathway might be active in the suppression of HMGB1 release. However, to the best of our knowledge, there have been no reports demonstrating a link between the inhibition of HMGB1 release and the Nrf2/HO-1 pathway. In the present study, we showed that the incubation of LPS-stimulated RAW264.7 cells (murine macrophage-like cells) with oleanolic acid (OA), which was extracted from Ume extract, strongly inhibits HMGB1 release and that the mechanism of this inhibition is mediated by the Nrf2/HO-1 pathway.

Materials and methods

Cell culture. Murine macrophage-like RAW264.7 cells were obtained from the American Type Cell Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine.

Fractionation of Ume extract. Isolation of Ume extract was performed as described previously (25-28). Briefly, Ume extract was concentrated and dried with a rotary evaporator. The dried product was dissolved in diethyl ether and fractionated by using a silica gel column elution procedure (methanol:chloroform, 9:1). By this method, we obtained four fractions (F1, F2, F3 and F4).

Stimulation of RAW264.7 cells. RAW264.7 cells ($2x10^6$ cells/6-cm dish) were starved for 2 h in serum-free Opti-MEM-I medium (Invitrogen, Carlsbad, CA) with or without Ume extract (Japan Apricot, Takasaki, Gunma, Japan) and authentic OA (Sigma). Then, 500 ng/ml of LPS (O111, Alexis, Switzerland) was added to the medium. Authentic OA was used as a positive control against Ume extract.

Preparation of HMGB1 samples for Western blot analysis. Preparations of HMGB1 samples were performed as previously described (4). Following treatment with LPS, the culture supernatant (2 ml) was incubated with 50 μl of heparin-Sepharose 6B (heparin beads) for 4 h and washed 3 times with 10 mM phosphate buffer (pH 7.0). Next, a 50-μl aliquot of buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol and 0.002% bromophenol blue] was added to the washed heparin beads and boiled for 5 min.

Western blotting. HMGB1 samples (40 μl) were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the separated proteins were transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences KK, Piscataway, NJ). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS; pH 7.4) containing 0.02% Tween-20 (TBST) for 1 h at room temperature (RT) and then incubated with anti-HMGB1 antibody (Shino-Test), Nrf2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or HO-1 antibody (Biomol International, L.P., Plymouth Meeting, PA) in TBST containing 1% non-fat dry milk for 3 h at RT. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal antibody (Invitrogen) diluted 1:3000 in TBST containing 2.5% non-fat dry milk for 1 h at RT. The membrane was washed again, and the immunoreactive bands were visualized by using the ECL detection system (GE Healthcare Bio-Sciences KK).

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as previously described (4). Briefly, $5x10^5$ RAW264.7 cells per well were cultured in 4-well BioCoat Collagen I culture slides (Becton Dickinson Labware). After stimulation, slides were washed with phosphate-buffered saline (PBS) and fixed with 250 μl of OptiLyse C (Becton Dickinson) containing 0.1% Triton X-100 (Sigma). The slides were blocked with 1% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 (PBST) for 1 h, incubated with 1 μg/ml of rabbit anti-HMGB1 antibody for 1 h at RT, and washed with PBST. The slides were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Invitrogen) for 1 h, and were then washed with PBST. After washing, the slides were examined under an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. The intensity of the protein bands on the Western blots was quantified by using the National Institutes of Health (NIH, Bethesda, MD) Image 1.63 software. The statistical significance of differences in band intensities was determined by applying the Student's t-test, and $P<0.05$ was considered significant.

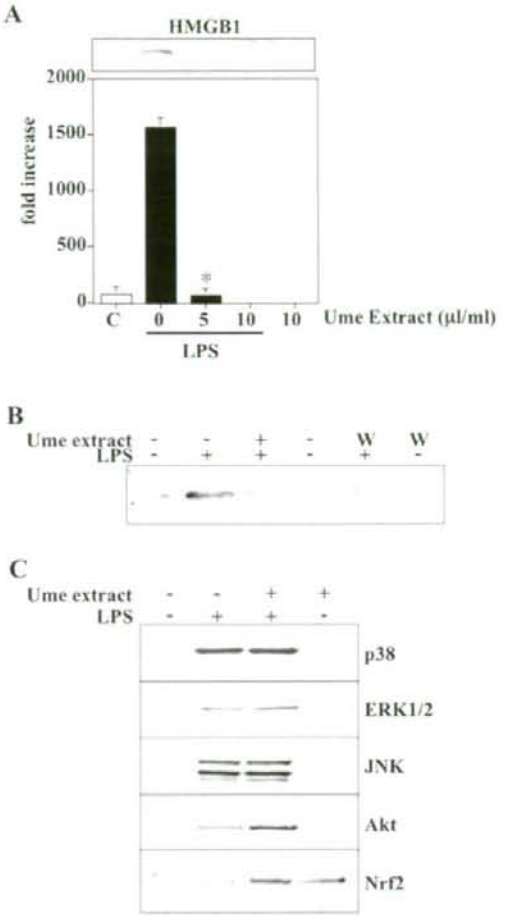

Figure 1. Inhibition of HMGB1 release by Ume extract. (A) Dose-dependent effect of Ume extract in LPS-treated RAW264.7 cells. RAW264.7 cells were incubated with Ume extract (0, 5, or 10 μl/ml) for 16 h. HMGB1 was measured by Western blotting. *P<0.05. (B) Transient exposure of RAW264.7 cells to Ume extract inhibited LPS-induced HMGB1 release. RAW264.7 cells were incubated with Ume extract for 2 h, and the cells were washed extensively (lane 5) and exposed for 16 h to LPS (500 ng/ml). HMGB1 was detected by Western blotting. (C) Nrf2 activation by Ume extract in RAW264.7 cells. RAW264.7 cells were pretreated with Ume extract for 2 h and incubated with LPS for 1 h. Inhibition of ERK1/2, p38MAPK and JNK activation, or activation of Nrf2 by Ume extract was detected by Western blotting.

Figure 2. Identification of the Ume fraction with the HMGB1 release inhibitory effect in LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with Ume extract and 4 fractions (F1, F2, F3, and F4) as described in Materials and methods for 2 h, and a 500-ng/ml aliquot of LPS was added to the cells. After incubation for 16 h, the released HMGB1 was detected by Western blotting.

Results

Inhibition of LPS-induced HMGB1 release by Ume extract. In this study, we focused on determining whether an Ume extract would inhibit HMGB1 release in LPS-stimulated macrophage-like RAW264.7 cells. RAW264.7 cells were incubated with or without Ume extract (0-10 μl/ml) for 2 h. LPS (500 ng/ml) was then added to the cells and incubated for a further 16 h. The medium was analyzed with Western blotting by using an anti-HMGB1 antibody. As shown in Fig. 1A, Ume extract inhibited HMGB1 release from the stimulated RAW264.7 cells in a dose-dependent manner, clearly indicating that Ume extract contains an inhibitor against HMGB1 release. No cell death by Ume extract was observed under conditions examined in this study (unpublished data).

Transient exposure to Ume extract inhibited LPS-induced HMGB1 release. We sought to determine whether or not transient exposure of cells to Ume extract leads to a sustained inhibition of LPS-induced HMGB1 release. RAW264.7 cells were pretreated with Ume extract (10 μl/ml) for 2 h (Fig. 1B), and the cells were washed extensively before being exposed for 16 h to LPS. HMGB1 was then measured in the medium. As was the case with sustained exposure to Ume extract (Fig. 1B, lane 3), even transient incubation with Ume extract strongly inhibited LPS-induced HMGB1 release (Fig. 1B, lane 5).

Induction of Nrf-2 activation by Ume extract. It is known that HMGB1 release is mediated by the mitogen-activated protein kinase (MAPK) pathways, particularly p38MAPK and JNK (4,16). We examined whether the Ume extract suppressed MAPK activation by LPS by Western blot analysis. As shown in Fig. 1C, Ume extract failed to suppress the activation of MAPKs, including ERK1/2, JNK and p38MAPK in the LPS stimulation system. Recently, endotoxin shock was reported to be enhanced in Nrf2 knockout mice; Nrf2 is known to be an antioxidant protein inducer (31). We hypothesized that Ume extract might induce Nrf2 activation. Our investigation revealed that Ume extract indeed activated Nrf2, strongly suggesting that Ume extract might be a rich source of anti-inflammatory protein inducers by *de novo* synthesis. In our conditions, although the molecular weight of Nrf2 is 72 kDa, Nrf2 in the nucleus is ~100 kDa. As reported previously, Nrf2 may migrate slowly (41).

The HMGB1 release inhibitory fraction of Ume extract contained OA. We wished to identify the inhibitory fraction of the Ume extract against HMGB1 release among the 4 fractions described in Materials and methods. RAW264.7 cells were incubated with 10 μg/ml of F1, F2, F3 or F4, and then 500 ng/ml of LPS was added. As shown in Fig. 2, both unfractionated Ume extract and F4 showed inhibitory activity against HMGB1 release; F1, F2 and F3 showed no inhibitory action. Additionally, we found that only F4 contained the triterpenoid of OA (unpublished data). Therefore, this investigation strongly suggested that OA might be the inhibitor of HMGB1 release in LPS-treated RAW264.7 cells.

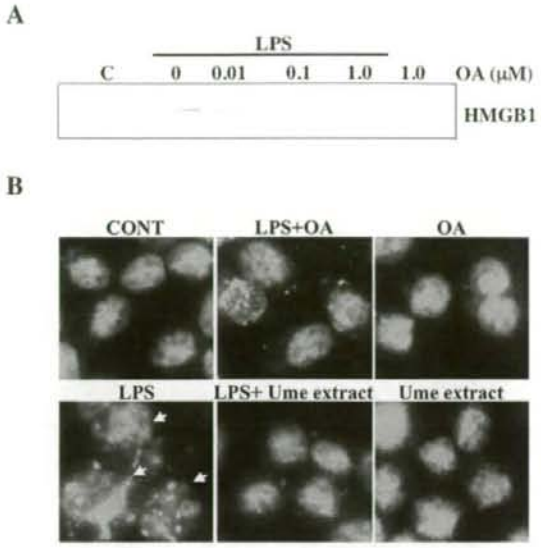

Figure 3. Activation of the Nrf2/HO-1 pathway in OA-treated RAW264.7 cells. (A) Dose-dependent effect of OA on HMGB1 release from LPS-treated RAW264.7 cells. After RAW264.7 cells were incubated with OA (0, 0.01, 0.1 or 1.0 μM), the cells were incubated with LPS for 16 h. The released HMGB1 was detected by Western blotting. (B) Translocation of HMGB1 from the nuclear to extracellular space in response to Ume extract and OA. RAW264.7 cells were incubated with or without Ume extract (5 μl/ml) and OA (1.0 μM) for 16 h. The fixed cells were incubated with rabbit anti-HMGB1 polyclonal antibody, followed by incubation with FITC-labeled anti-rabbit IgG as a secondary antibody. Original magnification, x400. Arrows indicate translocation of HMGB1 from the nuclear to the extracellular space.

OA in Ume extract inhibited LPS-induced HMGB1 release. Since triterpenoids are abundant in Ume extract (25) and have anti-inflammatory properties (32), we hypothesized that OA, one of the triterpenoids from the Ume extract, might inhibit HMGB1 release. Thus, we examined whether authentic OA inhibits HMGB1 release in LPS-stimulated RAW264.7 cells. As shown in Fig. 3A, OA (0-1.0 μM) inhibited HMGB1 release by LPS-stimulated RAW264.7 cells in a dose-dependent manner similar to F4 of Ume extract.

Inhibition of HMGB1 translocation in LPS-treated RAW264.7 cells by Ume extract. Additionally, we examined whether the cell nucleus was the source of HMGB1 in stimulated RAW264.7 cells. As shown in Fig. 3B, the released HMGB1 was from the nuclei of the LPS-stimulated RAW264.7 cells (Fig. 3B, LPS), and its release was inhibited by both Ume extract (LPS+Ume extract) and by OA (LPS+OA).

Activation of the Nrf2/HO-1 pathway by OA in Ume extract. To further confirm the above results, we hypothesized that OA induces the HO-1 protein in RAW264.7 cells. This hypothesis was verified by tests that showed OA, in fact, induced the expression of the HO-1 protein in RAW264.7 cells. As shown in Fig. 4, OA (0-1.0 μM) was added to RAW264.7 cells and incubated for 5 h; subsequently, both the cytoplasm and the nuclear proteins of the cells were extracted as described in Materials and methods. As shown in Fig. 4, OA

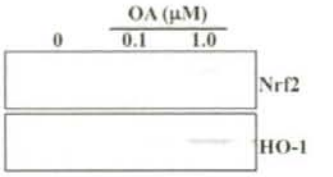

Figure 4. Nrf2 and HO-1 induction by OA in RAW264.7 cells. RAW264.7 cells were incubated with OA (0, 0.1 or 1.0 μM) for 3 h, and Nrf2 protein from the nuclear fraction of the cells was analyzed by Western blotting (upper panel). Additionally, RAW264.7 cells were incubated with OA (0, 0.1 or 1.0 μM) for 5 h, and HO-1 protein (lower lane) of the cytoplasmic protein fraction of the cells was detected by Western blotting.

induced HO-1 in the cytoplasm of RAW264.7 cells in a dose-dependent manner. Similarly, the activation of Nrf2 in the nuclear proteins of RAW264.7 cells by OA was also detected in a dose-dependent manner.

Discussion

A recent study (25) found Ume extract to be a very rich natural source of triterpenoids, which have strong health-enhancing and disease-suppressing effects (25,32). In the present study, we used LPS-stimulated RAW264.7 cells as a model for HMGB1 release and found that Ume extract has a potent inhibitory effect on HMGB1 release. The effects of the Ume extract were dose-dependent and were mimicked by OA, a naturally occurring triterpenoid that we extracted from the Ume extract. The actions of Ume extract-derived OA were confirmed by using authentic OA. Furthermore, we demonstrated that the inhibitory action of the Ume extract occurred via the activation of Nrf2 and induction of HO-1 protein (the Nrf2/ HO-1 pathway) in RAW264.7 cells, thus essentially being an antioxidant effect.

Hitherto, studies have shown that JNK inhibition and an increase in the expression of HSP72 suppress HMGB1 release by LPS-stimulated RAW264.7 cells (16,17). Contrary to these reports, both Ume extract and authentic OA failed to affect JNK activation, induction of HSP72 protein, or anticoagulant activity (data not presented). However, JNK inhibition, HSP72, and substances with strong antioxidant actions, such as green tea and DOW, inhibit HMGB1 release and also protect against death in animal models of sepsis involving oxidative stress (33-39). These observations support our notion that Ume extract contains substances with strong antioxidant effects (described below).

Ume extract is known to be highly effective in the prevention and treatment of cancer in many animal models and for apoptosis induction in tumour cells. Despite these reports, Ume substances have not yet been used to treat disease in clinical settings (25-28); possibly, such use is dependent on the outcome of future clinical trials in humans. In this study, the Ume extract fraction (F4), abundant in triterpenoid/OA, inhibited HMGB1 release, suggesting that an antioxidant effect of triterpenoid is involved in the inhibition of HMGB1 release. Since many triterpenoids are widely used in Asian medicine and are known to occur in natural products, akin to OA/ triterpenoids in Ume extract which has multiple health

benefits including antioxidant, anti-inflammatory and anticancer effects (32), clinical trials to fully understand the therapeutic potentials of Ume extracts are warranted. Furthermore, in this study, we demonstrated the antioxidant activity to be associated with the activation of the transcription factor Nrf2. In response to oxidative stress, Nrf2 is released from KEAP1 and then activates an antioxidant response element on the promoter of phase 2 response genes, which include HO-1, glutathione synthesis and quinone reductase. One consequence of activating the phase 2 response is a reduction in the reactive oxygen species. Thus, Nrf2 is required for such an attenuation of oxidative stress.

In endotoxemia, the upregulation of HO-1 is thought to be beneficial in combating the detrimental consequences of exacerbated inflammation. Administration of HO-1 inhibitors at high doses (decreasing HO enzyme activity below basal levels) made rats more susceptible to LPS-induced death (40). Similarly, mouse cells lacking HO-1 were susceptible to LPS-induced oxidative injury. Hence, the induction of endogenous HO-1 counteracts increased inflammation and oxidative injury associated with endotoxemia via antioxidant action.

In conclusion, the present study provides evidence that *Prunus mume Sieb. et Zucc* extract and oleanolic acid inhibit HMGB1 release from stimulated RAW264.7 cells via the Nrf2/HO-1 pathway and thereby play major roles in the regulation of cell survival in endotoxemia and other inflammatory conditions. Clinical trials are required to fully understand the therapeutic potential of Ume extracts.

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Intraocular expression and release of high-mobility group box 1 protein in retinal detachment

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High-mobility group box 1 (HMGB1) protein is a multifunctional protein, which is mainly present in the nucleus and is released extracellularly by dying cells and/or activated immune cells. Although extracellular HMGB1 is thought to be a typical danger signal of tissue damage and is implicated in diverse diseases, its relevance to ocular diseases is mostly unknown. To determine whether HMGB1 contributes to the pathogenesis of retinal detachment (RD), which involves photoreceptor degeneration, we investigated the expression and release of HMGB1 both in a retinal cell death induced by excessive oxidative stress *in vitro* and in a rat model of RD-induced photoreceptor degeneration *in vivo*. In addition, we assessed the vitreous concentrations of HMGB1 and monocyte chemoattractant protein 1 (MCP-1) in human eyes with RD. We also explored the chemotactic activity of recombinant HMGB1 in a human retinal pigment epithelial (RPE) cell line. The results show that the nuclear HMGB1 in the retinal cell is augmented by death stress and upregulation appears to be required for cell survival, whereas extracellular release of HMGB1 is evident not only in retinal cell death *in vitro* but also in the rat model of RD *in vivo*. Furthermore, the vitreous level of HMGB1 is significantly increased and is correlated with that of MCP-1 in human eyes with RD. Recombinant HMGB1 induced RPE cell migration through an extracellular signal-regulated kinase-dependent mechanism *in vitro*. Our findings suggest that HMGB1 is a crucial nuclear protein and is released as a danger signal of retinal tissue damage. Extracellular HMGB1 might be an important mediator in RD, potentially acting as a chemotactic factor for RPE cell migration that would lead to an ocular pathological wound-healing response.

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KEYWORDS: danger signal; high-mobility group box 1 protein; photoreceptor degeneration; retinal detachment; tissue damage; wound healing

Cell death is the predominant event of degenerative tissue damage and can be a trigger that activates the immune system and repair program. Recently, there has been much interest in the pivotal role of endogenous danger signals released during cell death.[1] High-mobility group box 1 (HMGB1) protein is a prototypic innate danger signal, and appears to be crucial in this context because extracellular HMGB1[2] can modulate inflammation, proliferation, and remodeling, which are involved in the wound-healing process.[3]

HMGB1 was originally described as an abundant and ubiquitous nuclear DNA-binding protein that had multiple functions dependent on its cellular location.[2,4] In the nucleus, HMGB1 binds to DNA and is critical for proper transcriptional regulation. It is also called amphoterin and accelerates cellular motility on the cell surface.[5] HMGB1 is reported to be passively released into the extracellular milieu by necrotic cells, but not by apoptotic cells,[6] or is exported actively by monocytes/macrophages[7] and neural cells[8] upon receiving appropriate stimuli. In damaged tissue, extracellular HMGB1 acts as a necrotic signal, which alerts the surrounding cells and the immune system.[2] Although extracellular HMGB1 can contribute to normal tissue development and repair, it is also implicated in the pathogenesis of several diseases (including lethal endotoxemia,[7] disseminated intravascular coagulation,[9] ischemic brain,[10] tumor,[11] atherosclerosis,[12] rheumatoid arthritis,[13] and periodontitis[14]).

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Retinal detachment (RD), the physical separation of photoreceptors from the underlying retinal pigment epithelium (RPE), is one of the main causes of visual loss. Photoreceptor degeneration due to RD is thought to be executed by apoptosis[15,16] and necrosis,[17] which usually occur after tissue damage. Although retinal cell death and the following reactive responses must occur in almost all forms of retinal disease including RD,[18] data regarding the relationship among cell death, danger, and responses in the eye, have been very limited, especially in terms of danger signals. We previously reported that HMGB1 was significantly elevated in inflamed eyes with endophthalmitis, and suggested a possible link between HMGB1 and ocular inflammatory diseases.[19] On the other hand, considering the properties of HMGB1, we hypothesized that HMGB1 might have some roles in photoreceptor degeneration and subsequent damage-associated reactions in RD.

To investigate whether HMGB1 is involved in the pathogenesis of RD, we first examined the expression and release of HMGB1 both in a retinal cell death *in vitro* and in a rat model of RD-induced photoreceptor degeneration *in vivo*. To focus on human RD, we assessed the intravitreous concentrations of HMGB1 in human eyes affected by RD. Monocyte chemoattractant protein 1 (MCP-1), which was recently documented to be a potential proapoptotic mediator in RD,[20] was also measured in the same vitreous samples. We further analyzed the effects of recombinant HMGB1 (rHMGB1) on chemotactic activity in a RPE cell line *in vitro*. Our findings suggest that extracellular HMGB1 is evident in eyes with RD as a danger signal, potentially acting as a chemotactic factor for RPE cell migration that would lead to ocular pathological wound healing.

MATERIALS AND METHODS

Reagents

Full-length, LPS-free rat rHMGB1 protein, which is 99% identical to human HMGB1 and is fully functional on cells of mammalian origin,[21] was purchased from HMGBiotech (Milan, Italy). Human recombinant MCP-1 (rMCP-1) was purchased from Peprotec (Rocky Hill, NJ). Rabbit polyclonal antibody against HMGB1 was provided by Shino-Test Corporation (Kanagawa, Japan). Antibodies against phospho- and total extracellular signal-regulated kinase (ERK)-1/2 were obtained from Cell Signaling Technology (Beverly, MA). U0126 was obtained from Calbiochem (La Jolla, CA).

Human Vitreous Samples

This study was approved by our institutional ethical committee (Kagoshima University Hospital), and was performed in accordance with the Declaration of Helsinki. All surgeries were performed at Kagoshima University Hospital. All patients gave informed consent before treatment. The clinical histories of all patients were obtained from their medical records. Undiluted vitreous fluid samples (0.5–0.7 ml) were obtained by pars plana vitrectomy. Vitreous humor was collected in sterile tubes, placed immediately on ice, centrifuged to remove cells and debris, and stored at −80°C until analysis.

Animals

All animal experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and the approval of our institutional animal care committee (Kagoshima University). Adult male Brown Norway rats (250–300 g; KBT Oriental, Saga, Japan) were housed in covered cages and kept at constant temperature and relative humidity with a regular 12-h light–dark schedule. Food and water were available *ad libitum*.

Surgical Induction of RD

Rat experimental RD was induced as described previously.[22] Briefly, the rats were anesthetized with an intramuscular injection of ketamine and xylazine, and their pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. The retinas were detached using a subretinal injection of 1% sodium hyaluronate (Opegan; Santen, Osaka, Japan) with an anterior chamber puncture to reduce intraocular pressure. Sodium hyaluronate (0.05 ml) was slowly injected through the sclera into the subretinal space to enlarge the RDs. These procedures were performed only in the right eye, with the left eye serving as a control. Eyes with lens injury, vitreous hemorrhage, infection, and spontaneous reattachment were excluded from the following analysis. The rats were killed at 3, 7, and 14 days after treatment, with six animals per each time point.

Cell Culture

The rat immortalized retinal precursor cell line R28, a kind gift from Dr GM Siegel (The State University of New York, Buffalo), was cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), 10 mM non-essential amino acids, and 10 μg/ml gentamicin as described previously.[23] The human immortalized RPE cell line ARPE-19, obtained from American Type Culture Collection (Manassas, VA), was grown in DMEM/F12 supplemented with 10% FBS, 2% penicillin–streptomycin, and 1% fungizone (all products were obtained from Invitrogen-Gibco, Rockville, MD). Cells were incubated at 37°C in a 5% CO_2 incubator and subcultured with 0.05% trypsin-EDTA. Subconfluent cultures were trypsinized and seeded for the following experiments. ARPE-19 cells were obtained at passage 21 and used at passages 24–30. Increased passage did not alter the following experimental results up to this passage number.

Cell Viability Assay

Cell viability was analyzed by mitochondrial respiratory activity measured using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide) assay (Wako Chemicals,

Osaka, Japan), as described previously.[24] Briefly, 2×10^5 R28 cells were cultured in 24-well plates (500 μl medium per well) with or without hydrogen peroxide (1 mM; Merck, Darmstadt, Germany) for 24 h. Then the cells were incubated with MTT (0.5 mg/ml; final concentration) for 3 h. Formazan product was solubilized by the addition of dimethyl sulfoxide for 16 h. Dehydrogenase activity was expressed as absorbance at a test wavelength of 570 nm and at a reference wavelength of 630 nm. Assays were performed in triplicate and repeated three times in independent experiments.

Immunofluorescence for HMGB1 and TUNEL

Indirect immunofluorescence was carried out as described previously,[19,25] with some modifications. The eyes were harvested and fixed in 4% paraformaldehyde at 4°C overnight. The anterior segment and the lens were removed, and the remaining eye cup was cryoprotected with 10–30% sucrose in phosphate-buffered saline. The eye cups were then frozen in an optimal cutting temperature compound (Sakura Finetech, Tokyo, Japan). Sections were cut at 8 μm with a cryostat (Leica Microsystems, Wetzler, Germany). After being incubated with blocking buffer containing 10% goat serum, 1% bovine serum albumin (BSA), and 0.05% Tween-20 for 1 h, the slides were incubated with rabbit polyclonal anti-HMGB1 antibody (1 μg/ml). After overnight incubation, sections were washed and probed with Alexa-Fluor 594-conjugated goat anti-rabbit IgG F(ab')2 fragment (Molecular Probes, Carlsbad, CA) for 1 h. In some experiments, TUNEL co-staining was also performed according to the manufacturer's protocol (ApopTag Fluorescein *In situ* Apoptosis Detection kit; Chemicon, Temecula, CA) as previously described.[22] Slides were counterstained with DAPI, mounted with Shandon PermaFlour (Thermo Scientific, Waltham, MA), and viewed with a Zeiss fluorescence microscope. Images were captured using the same exposure time for each comparative section. To examine the specificity of immunostaining, the primary antibody was replaced with normal rabbit IgG (1 μg/ml). Control slides were invariably negative under the same setting (data not shown). For all experiments, at least three sections from each eye were evaluated. To demonstrate the expression patterns of HMGB1 in retinal cells under oxidative stress *in vitro*, R28 cells (2×10^5 cells/500 μl medium per well) were seeded on four-well glass coverslips and challenged with or without hydrogen peroxide (1 mM) for 1 h. Slides were fixed in 4% paraformaldehyde for 1 h, permeabilized with Triton X-100, and then examined by the same methods as described above.

ELISAs

HMGB1 and MCP-1 were quantified in each human vitreous sample using commercial ELISAs; HMGB1 ELISA kit (Shino-Test Corporation) and Human CCL2/MCP-1 Immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturers' protocols. The detection limits of these kits were 0.2 ng/ml for HMGB1 and 5.0 pg/ml for MCP-1. Concentrations below the limits were taken as zero in subsequent analyses. Each sample was run in duplicate and compared with a standard curve. All samples were assessed in a masked manner. The mean concentration was determined per sample. For *in vitro* study, HMGB1 levels in culture supernatants were measured by the same ELISA.

Migration Assay

Cell migration was assayed using a modified Boyden chamber assay as previously described.[26] In brief, 5×10^4 ARPE-19 cells resuspended in 200 μl control medium (1% FBS-DMEM/F12) were seeded onto the upper compartment of the BD Falcon® culture inserts (BD Bioscience, San Jose, CA) with an 8-μm diameter pore size membrane in a 24-well companion plate. The lower chamber was filled with control medium (negative control) and those containing 50, 100, or 200 ng/ml rHMGB1. Because MCP-1 was reported to display a potent chemotactic activity on RPE cells,[27] a control medium containing 10 ng/ml rMCP-1 was used as a positive control. After 8-h incubation, cells remaining on the upper surface of the filters were removed mechanically, and those that had migrated to the lower surface were fixed with methanol, stained with Diff-Quick (Dade-Behring, Deerfield, IL), and counted in five randomly selected high-power fields (× 100) per insert. Migration index (% of control) was calculated by dividing the number of migrating cells in the presence of chemoattractants by the cells that migrated in response to the negative control. To inhibit ERK-1/2 activity, the cells were pretreated with 1, 5, or 10 μM U0126, or vehicle (0.1% dimethyl sulfoxide) for 30 min, prior to the addition of rHMGB1. U0126 is an inhibitor of active and inactive MEK-1/2, the MAPK kinase that activates ERK-1/2. These concentrations of U0126 and dimethyl sulfoxide had no effect on ARPE-19 cell viability determined by MTT assay in our study (data not shown) and in a previous report.[28] Assays were performed in triplicate and repeated three times in independent experiments.

Immunoblotting

ARPE-19 cells (5×10^5) were subcultured on 6-cm tissue culture dishes. Then, the cells were serum starved overnight in DMEM/F12 and stimulated with 100 ng/ml rHMGB1 for the indicated times. Activation of ERK-1/2 was analyzed as described previously.[24] In brief, after treatment, whole cells were lysed with SDS sample buffer and an equal volume of protein extracts was loaded onto 12% SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane. The membrane was blocked by incubation with 5% non-fat dry milk plus 1% BSA in TBST (0.02% Tween-20 in Tris-buffered saline, pH 7.4) for 1 h at room temperature. The membrane was then incubated with the antibody against phospho-ERK-1/2 (diluted 1/1000) at 4°C overnight. The blots were subsequently probed with secondary anti-rabbit antibodies conjugated to horseradish peroxidase (diluted 1/3000 in TBST), and images were developed using the en-

hanced chemiluminescence system (GE Healthcare). The membrane was stripped and reprobed with an antibody against total ERK-1/2 (diluted 1/1000).

Statistical Analysis

The vitreous HMGB1 and MCP-1 concentrations in each group were compared using the Mann–Whitney *U*-test. The correlation between HMGB1 and MCP-1 in RD samples was analyzed using a simple linear regression analysis and Spearman's rank correlation coefficient. All *in vitro* data are presented as mean ± s.d. and the significance of differences between groups was determined by Student's *t*-test. *P*-value less than 0.05 was considered significant.

RESULTS

HMGB1 is Present in Cultured Retinal Cell and Released Extracellularly by Oxidative Stress-Induced Cell Death

We first evaluated the expression patterns and cellular distribution of HMGB1 in an R28 retinal cell line with or without oxidative stress, a known cause of neurodegeneration.[29] Excessive reactive oxygen species can lead to the destruction of cellular components and ultimately induce cell death through apoptosis or necrosis. To induce oxidative stress, we used a toxic dose (1 mM) of hydrogen peroxide, which was reported to stimulate monocytes/macrophages to release HMGB1 actively and passively.[30] As shown in Figure 1a, HMGB1 immunoreactivity was stably present in the nucleus of unstimulated R28 cells, and relatively weak staining was observed in the cytoplasm. By contrast, 1 h after exposure to 1 mM hydrogen peroxide, some cells seemed to present rather high levels of HMGB1 in their nucleus as well as their cytoplasm compared with those under an unstimulated condition. However, in the other cells, the nuclear HMGB1 was diminished or appeared to be released into the cytoplasm. These results indicate that the nuclear HMGB1 could be varied by death stress and be released into the cytoplasm according to the degree of stress. Hydrogen peroxide (1 mM) treatment for 24 h, in which about 90% of the cells lost their viability (Figure 1b), induced a massive release of HMGB1 from the cells to the cell supernatants (Figure 1c). Taken together, these findings suggested that HMGB1 could relocate from the nucleus to the cytoplasm for eventual release in dying retinal cells, and that the extracellular release of HMGB1 in the eye might be increased dependent on the extent of retinal cell death.

HMGB1 is Abundantly Expressed in Rat Retina and Released after RD

As the above findings indicated that HMGB1 was of relevance to retinal cell death, we investigated whether HMGB1 was maintained in the rat retina and how HMGB1 would vary after RD. As it was reported that HMGB1 in rat photoreceptors had a light-sensitive circadian rhythmic expression,[25] we performed all animal studies on a regular time schedule, and all eyes were set to be almost equally exposed to light. As shown in Figure 2, HMGB1 immunoreactivity was well represented in sections of the normal control rat retina and, as expected, colocalized with DAPI-positive nuclei (Figure 2a, d and g). HMGB1 staining in the normal rat retina was prominent in the nuclei of ganglion cell layer, inner nuclear layer, outer nuclear layer, and RPE, and was also apparent in the photoreceptor inner segments. In particular, HMGB1 was localized in photoreceptor at the nuclear periphery, and HMGB1 levels were higher in the inner nuclear layer than the outer nuclear layer as opposed to DAPI staining, which preferred to bind to heterochromatic DNA. This was consistent with the previous report[25] that HMGB1 was preferentially colocalized with euchromatin, which was often under active transcription and was stained less by DAPI. Interestingly, HMGB1 appeared to be robustly upregulated in both the photoreceptors and the other retinal cells at day 3 after RD inductions, and DAPI staining was inversely downregulated at the same time (Figure 2b, e and h). As previous reports demonstrated that dramatic alterations of retinal gene expression occurred after RD,[31] this high level of HMGB1 expression might be related to the active gene transcription. HMGB1 in the nucleus might be stress responsive and necessary for proper transcription after RD tissue damage. Afterwards, the nuclear HMGB1 expression in the photoreceptors seemed to subside at day 7, while still clearly remained in the inner segments (Figure 2c, f and i), gradually decreasing along with the thinning of the outer nuclear layer due to photoreceptor degeneration by day 14 (data not shown).

Although HMGB1 expression was increased in the photoreceptors of the detached retina at day 3, it was not homogeneous, but was rather heterogeneous. To clarify the relationship between the upregulation of HMGB1 and photoreceptor cell death, especially with DNA damage, the RD retina at day 3 was co-stained with TUNEL, which could detect apoptotic and potentially necrotic cell death by labeling the damaged DNA (Figure 3a–c). Previous studies indicated that HMGB1 could not be released from apoptotic cells[6] and the apoptotic photoreceptors were prominent in this RD model at day 3 after RD.[22] We also confirmed remarkable numbers of apoptotic photoreceptors in the detached retina at day 3 (Figure 3b), and found that the early faint TUNEL-positive nuclei had relatively low levels of HMGB1 and fragmented nuclei, which were brightly stained by TUNEL, had almost no apparent HMGB1 immunoreactivity (Figure 3c), suggesting that apoptotic dying cells might lose the expression of HMGB1 to maintain the proper gene transcription. It might be indispensable for the surviving photoreceptors to maintain and/or boost the nuclear HMGB1 in RD.

In the subretinal space of RD at day 7, HMGB1-positive and TUNEL-negative debris could be observed (Figure 3d, arrows), which might be released by necrotic photoreceptors and/or degradated inner segments, and spread diffusely into the vitreous cavity if a retinal break was present. It was also

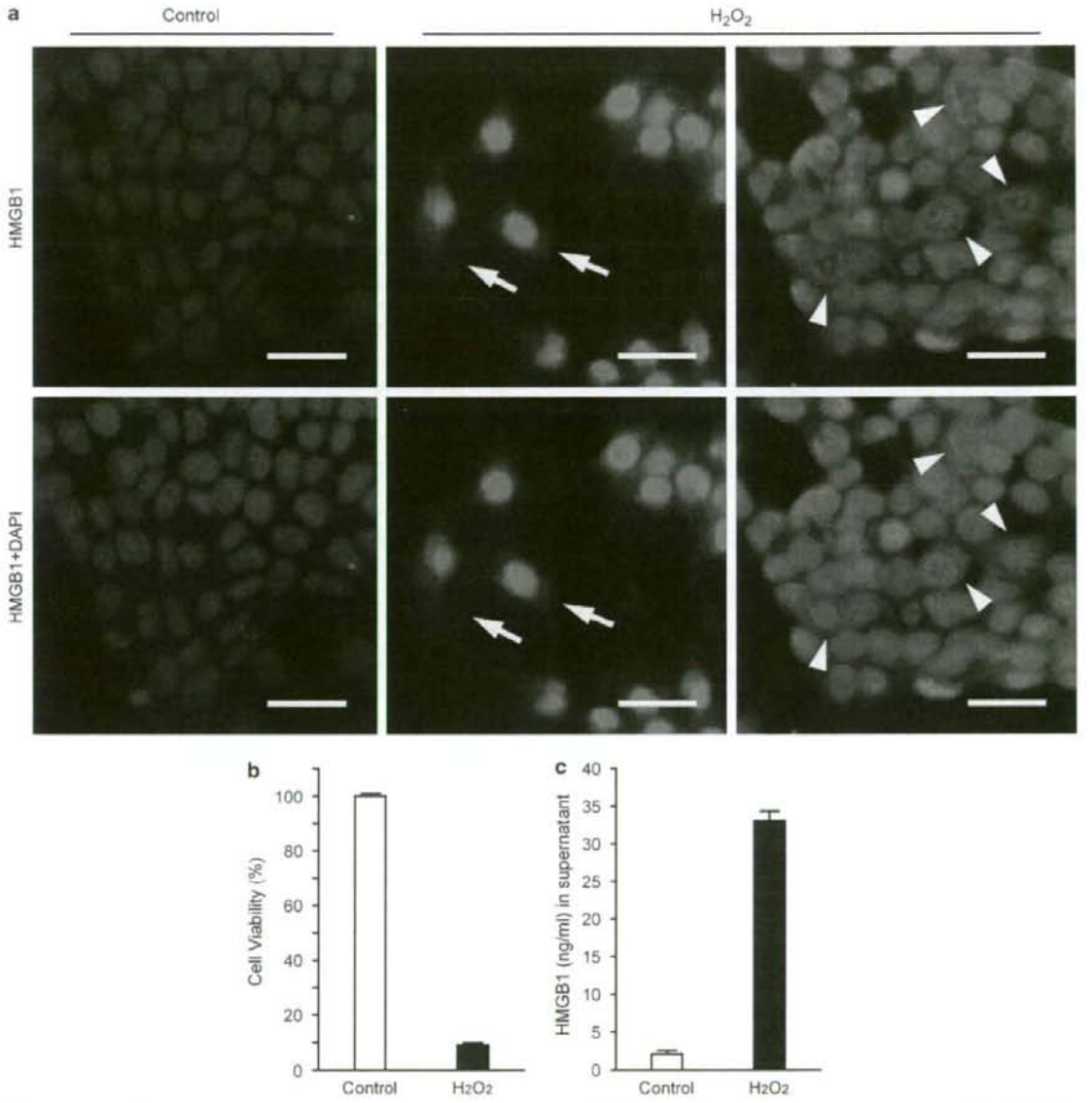
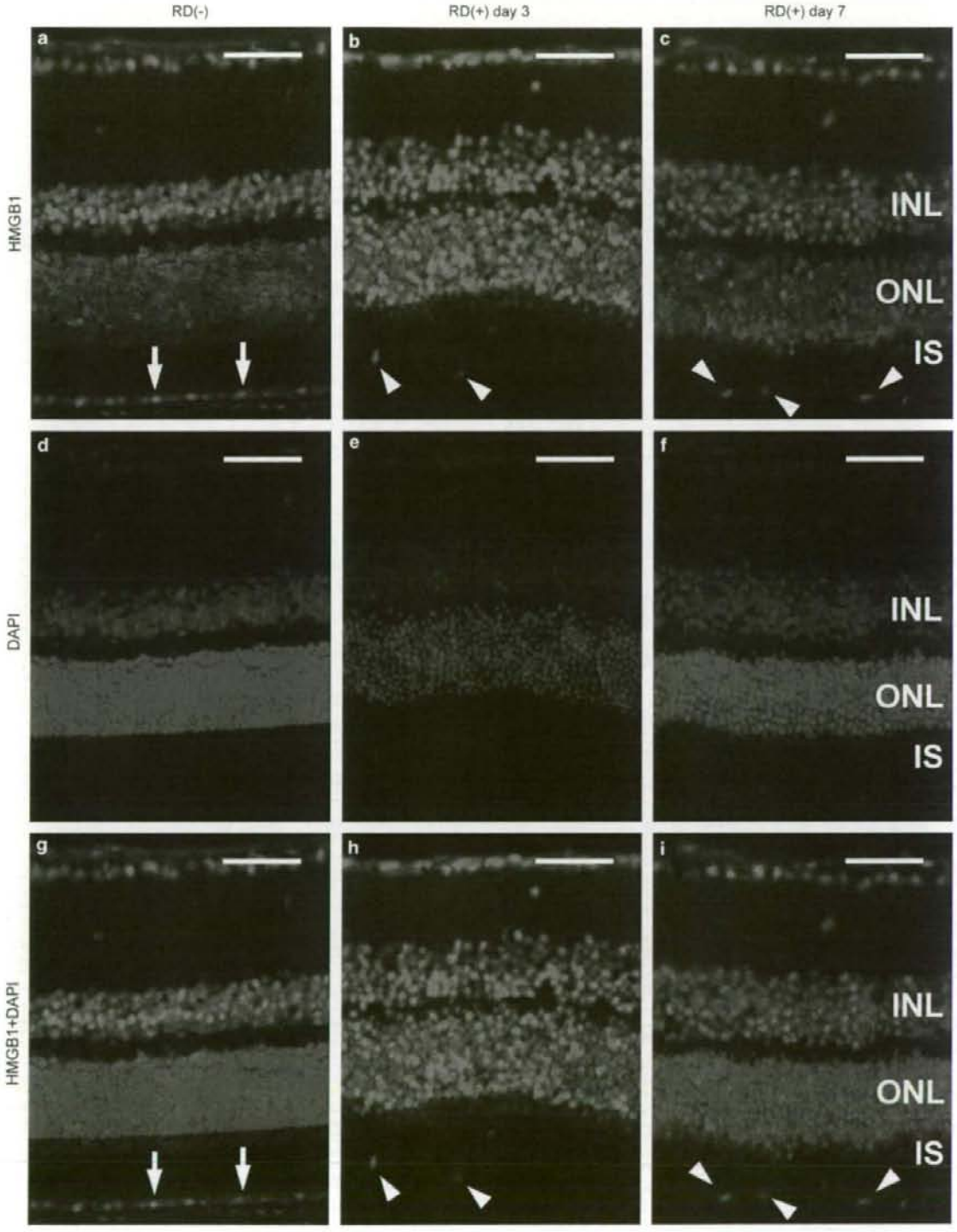


Figure 1 Release of HMGB1 from R28 retinal neuronal cells exposed to excessive oxidative stress. (**a**) Immunofluorescence was performed with anti-HMGB1 antibody (red) and DAPI (blue). HMGB1 is predominantly present in the nuclei of unstimulated R28 cells (left column). Some cells present robust upregulation of HMGB1 in the nuclei, as well as relocation into the cytoplasm (middle column; arrows) on 1 h exposure to a toxic dose of hydrogen peroxide (1 mM). In the other cells, the nuclear HMGB1 is found to be diminished or released into the cytoplasm (right column; arrowheads). Scale bars: 20 μm. (**b**) After 24 h exposure to 1 mM hydrogen peroxide, the cell viability analyzed by MTT assay is decreased to about 10% compared with the control. (**c**) Massive HMGB1 release into the culture supernatant was determined by ELISA after the same treatment as (**b**). The data represent the mean ± s.d. ($n = 3$). Similar results were obtained from three independent experiments.

Figure 2 Immunofluorescence analysis of HMGB1 in a rat model of RD. Representative photomicrographs of retinal sections labeled with anti-HMGB1 antibody (red; **a**–**c**) and DAPI (blue; **d**–**f**). Merged images (**g**–**i**) are also presented. The retinal sections were derived from the control eye (**a**, **d**, **g**), those at 3 days (day 3; **b**, **e**, **h**), or 7 days after RD (day 7; **c**, **f**, **i**). Arrows point to retinal pigment epithelium (**a**, **g**), and arrowheads indicate subretinal macrophages (**b**, **c**, **h**, **i**). Note that expression of HMGB1 is augmented especially in ONL at day 3 after RD, whereas the upregulation in ONL appears to be subside by day 7 ($n = 6$ for each time point). Scale bars: 50 μm. INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer.


RD(-)
RD(+) day 3
RD(+) day 7
HMGB1
DAPI
HMGB1+DAPI
a
b
c
d
e
f
g
h
i
INL
ONL
IS
INL
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reported that macrophages migrated into the subretinal space of this RD model.[32] The migrating macrophages also had abundant HMGB1 expression (Figure 3d, arrowheads), and might have released HMGB1 actively in this space. In line with these data, a large amount of extracellular HMGB1 must be present at least in the subretinal space after RD.

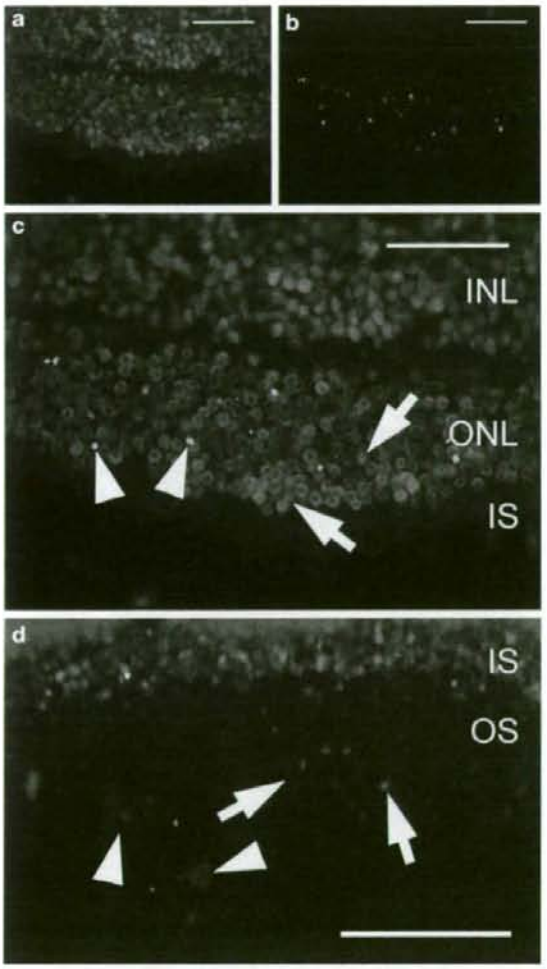

Figure 3 Expression of HMGB1 in DNA-damaged photoreceptors (**a–c**) and release of HMGB1 in the subretinal space (**d**). Representative photomicrographs of anti-HMGB1 antibody (red; **a**), TUNEL (green; **b**), and merged image (**c**) from rat retinal sections at 3 days after RD ($n=6$). The early faint TUNEL-positive nuclei (**c**; arrows) have relatively low levels of HMGB1 and the fragmented nuclei (**c**; arrowheads) have almost no apparent HMGB1 immunoreactivity. (**d**) Representative photomicrograph of a merged image of anti-HMGB1 (red), DAPI (blue), and TUNEL (green) obtained from rat retinal sections at 7 days after RD ($n=6$). HMGB1-positive and TUNEL-negative debris (**d**; arrows) and migrating macrophages with abundant HMGB1 expression (**d**; arrowheads) can be observed in the subretinal space. Scale bars: 50 μm. INL, inner nuclear layer; IS, inner segment; ONL, outer nuclear layer; OS, outer segment.

Vitreous HMGB1 and MCP-1 Levels in Patients with RD

The result obtained from the rat model of RD is the first evidence to our knowledge that HMGB1 is involved in RD-induced photoreceptor degeneration. Next, we tested whether extracellular HMGB1 could also be detected in human vitreous samples of RD. Samples were harvested from 35 eyes with RD, including rhegmatogenous RD, RD with macular hole, and atopic RD and 19 eyes with control diseases, including idiopathic epiretinal membrane and idiopathic macular hole (Table 1). The vitreous HMGB1 and MCP-1 levels were significantly higher in the eyes with RD than in those with control diseases (Figure 4). The median HMGB1 level was 1.4 ng/ml (range, 0–28.3) in the eyes with RD and 0.6 ng/ml (range, 0–1.3) in those with control diseases ($P<0.001$; Figure 4a). The median MCP-1 level was 1383.2 pg/ml (range, 39.8–5436.1) in the RD eyes and 404.4 pg/ml (range, 17.9–1168.9) in the control eyes ($P<0.0001$; Figure 4b). The vitreous concentration of HMGB1 was correlated significantly with that of MCP-1 in the 35 eyes with RD by a simple linear regression ($r=0.593$, $P<0.001$; Figure 4c) and by Spearman's rank correlation coefficient ($r=0.613$, $P<0.001$). On the other hand, there was no significant relationship between the vitreous concentrations of HMGB1 and MCP-1 in the 19 eyes of control patients (data not shown). Although there was no significant difference, the HMGB1 levels in the eyes with proliferative vitreoretinopathy (PVR), a condition of retinal fibrosis that follows severe long-standing RD, tended to be lower than those without PVR (Figure 4d). These findings showed that HMGB1 could be released not only in the subretinal space but also in the vitreous cavity after RD-induced photoreceptor degeneration, and that the HMGB1 release was coincident with vitreous MCP-1 expression.

Table 1 Characteristics of the patients

Characteristics	Retinal detachment ($n=35$)	Control diseases ($n=19$)
Age (years)	57.3 ± 16.3	68.2 ± 8.7
Female sex, no. (%)	19 (54)	10 (53)
Patients with PVR, no. (%)	6 (17)	—
Subgroups, no. (%)		
Rhegmatogenous retinal detachment	28 (80)	—
Retinal detachment with macular hole	5 (14)	—
Atopic retinal detachment	2 (6)	—
Idiopathic epiretinal membrane	—	7 (37)
Idiopathic macular hole	—	12 (63)

PVR, proliferative vitreoretinopathy.
Values are expressed as mean ± s.d. Dashes denote not applicable.

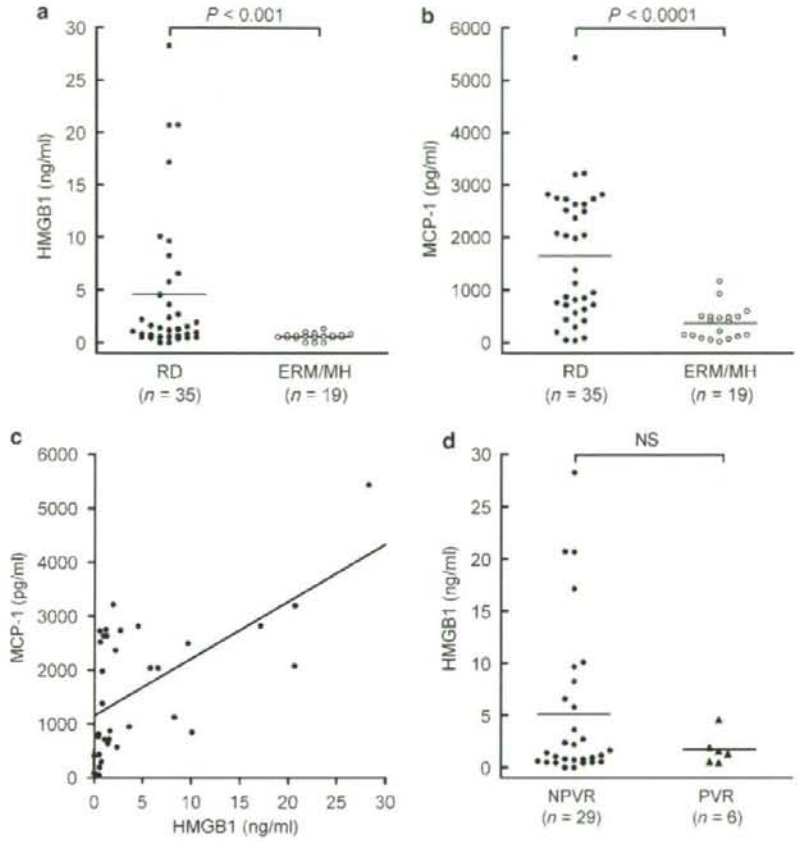

Figure 4 Vitreous levels of HMGB1 and MCP-1. The vitreous HMGB1 (**a**) and MCP-1 (**b**) levels are significantly higher in eyes with RD than in those with control diseases (idiopathic epiretinal membrane or idiopathic macular hole). Each bar indicates the average value. (**c**) Scatter plot for the correlation between vitreous levels of HMGB1 and MCP-1 in eyes with RD (simple linear regression, $r = 0.593$, $P < 0.001$; Spearman's rank correlation coefficient, $r = 0.613$, $P < 0.001$). (**d**) The HMGB1 levels in the eyes with PVR tend to be lower than those without PVR. ERM/MH, epiretinal membrane/macular hole; NPVR, no PVR; PVR, proliferative vitreoretinopathy.

RPE Cells Respond Chemotactically to Extracellular HMGB1 through an ERK-Dependent Mechanism

Previous reports have shown that extracellular HMGB1 is a chemoattractant for a variety of cell types.[21,33,34] We investigated whether HMGB1 is also a chemoattractant for RPE cells. Extracellular HMGB1 has been reported to engage multiple receptors, including the receptor for advanced glycation end products (RAGE) and Toll-like receptors 2 and 4.[2,4] In particular, RAGE has been thought to be a crucial receptor for HMGB1-induced cell migration through ERK activation.[33] The expression of RAGE at the RNA and protein level was identified in human RPE[35] and ARPE-19 cells[36,37] in previous studies. It was also shown that the expression of RAGE and HMGB1 was colocalized in the proliferative membrane from an eye with proliferative retinal disease.[38] We, therefore, performed a migration assay using modified Boyden chambers with various concentrations of rHMGB1. The representative photographs in Figure 5 show that rHMGB1 was capable of inducing a significant level of migration (Figure 5b) above that obtained with the control medium (Figure 5a). HMGB1 stimulated the migration of RPE cells in a concentration-dependent manner with a 2.7-fold maximal response at 100 ng/ml (Figure 5c). This maximal response to rHMGB1 was slightly stronger than that induced by rMCP-1 (10 ng/ml). Next, we investigated whether HMGB1 induced phosphorylation of ERK-1/2 in ARPE-19 cells; we stimulated cells with 100 ng/ml rHMGB1 for various time periods and used western blotting with an anti-phospho-ERK-1/2 antibody on whole-cell lysates (Figure 6a). Little phosphorylation of ERK-1/2 could be observed in unstimulated ARPE-19 (at 0 min), but a prominent increase was detected after 5 min of stimulation with rHMGB1. Figure 6a shows that phosphorylation of ERK-1/2 was augmented from 5 to 60 min after rHMGB1 stimulation in comparison

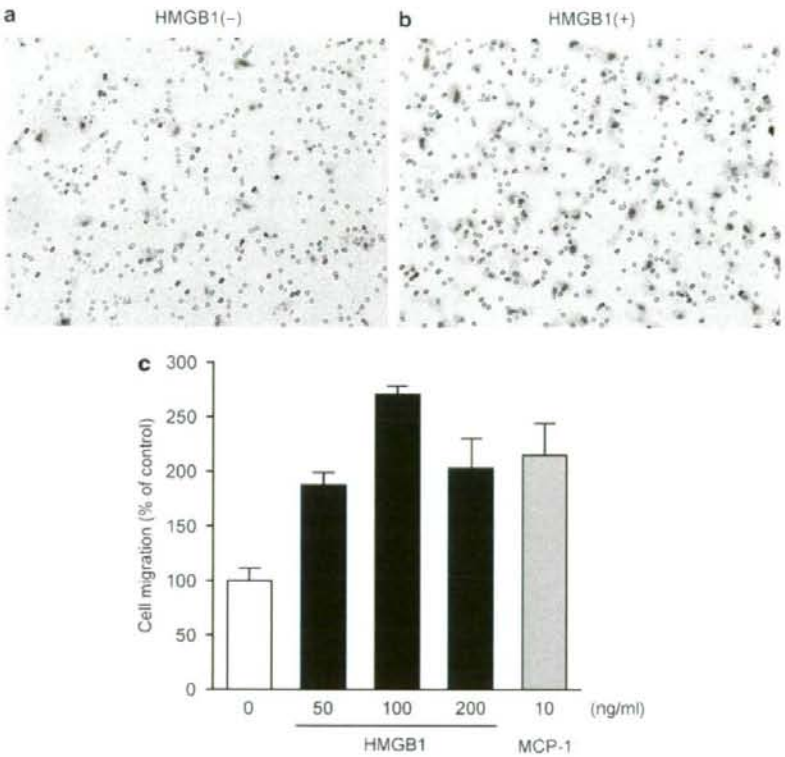

Figure 5 RPE cells migrate in response to HMGB1. Representative photographs of ARPE-19 cells stained with Diff-Quick after migration toward control medium (**a**) or 100 ng/ml HMGB1 (**b**). Original magnification: × 100. (**c**) HMGB1 stimulated ARPE-19 cell migration in a concentration-dependent manner with a 2.7-fold maximal response at 100 ng/ml. The data represent the mean ± s.d. ($n = 3$). All treatments increase the migratory response relative to the control ($P < 0.01$ in Student's *t*-test). Similar results were obtained from three independent experiments.

with unstimulated ARPE-19 (time 0). To demonstrate that the ERK signaling induced by HMGB1 was in fact linked to the migration of RPE cells, we next inhibited ERK-1/2 and assessed cell migration to HMGB1. Pretreatment of ARPE-19 with U0126 abrogated the migration toward rHMGB1 (Figure 6b). Thus, the ERK pathway appears to play an essential role in HMGB1-induced RPE cell migration.

DISCUSSION

Our findings suggest a possible role of HMGB1 in RD, as an essential nuclear protein and a principal danger signal for photoreceptor degeneration. Using an *in vitro* assay of retinal cell death induced by excessive oxidative stress, we found that HMGB1 was augmented in the nucleus by the stress and released into the extracellular space during cell death. On the basis of immunohistochemical analyses of a rat model of RD-induced photoreceptor degeneration, augmentation of HMGB1 in the nucleus is also observed *in vivo* and appears to be crucial for the proper transcription of photoreceptors after RD. Moreover, double labeling with TUNEL reveals defects of upregulation of the nuclear HMGB1 in the DNA-damaged photoreceptors, which are presumably programmed dying photoreceptors. Therefore, we propose that the nuclear HMGB1 in the retinal cells might be critical for retinal cell survival under death stresses both in the *in vivo* RD and *in vitro* retinal cell death. These results for ocular HMGB1 are compatible with previous reports that HMGB1 is a vital nuclear protein and has a protective role in the nucleus.[2,4]

In a previous animal study, Erickson *et al*[17] reported that a loss of photoreceptors in a cat model of RD occurred due to necrosis. During studies on RD, photoreceptor degeneration after RD had been thought to be mainly caused by apoptosis.[15,16] Hisatomi *et al*[32] demonstrated the presence of apoptotic debris in the subretinal space of rat RD. In the present study, considering our immunohistochemistry results from the same rat model of RD, so-called necrotic debris, which is HMGB1 positive and TUNEL negative, was found to be present. On the basis of the previous finding of the preferential release of HMGB1 from necrotic cells,[6] this suggests that necrosis might still be a fundamental type of photoreceptor cell death after RD.

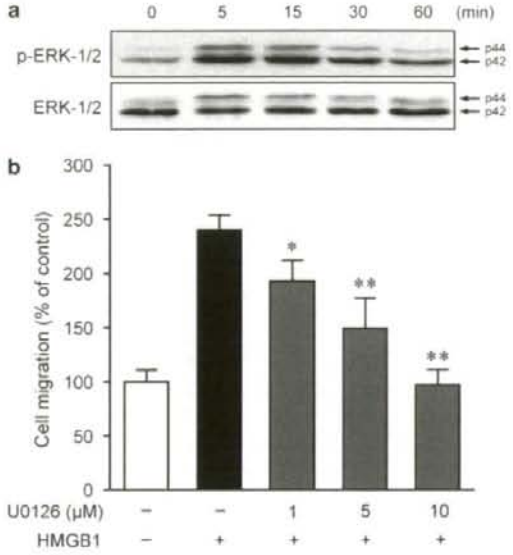

Figure 6 The phosphorylation of ERK is induced by HMGB1 and linked to HMGB1-induced migration of RPE cells. (**a**) ARPE-19 cells were stimulated with HMGB1 (100 ng/ml) for 5, 15, 30, or 60 min, and total cell lysates were analyzed by western blot. ERK-1/2 activation was detected with anti-phospho-ERK-1/2 antibody (p-ERK-1/2). Stripped membrane was reprobed with the antibody against total ERK-1/2 (ERK-1/2). Results are representative of three independent experiments. HMGB1 augments the ERK-1/2 phosphorylation from 5 to 60 min after stimulation. (**b**) Pretreatment of ARPE-19 with U0126 inhibits the cell migration toward HMGB1 (100 ng/ml) in a dose-dependent manner. The data represent the mean ± s.d. ($n = 3$). Similar results were obtained from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with vehicle-treated control.

Furthermore, exploring human vitreous samples by ELISA, we found that both HMGB1 and MCP-1 are increased significantly in eyes with RD. Although MCP-1 is a well-known mediator for RD,[39] to our knowledge, this is the first report indicating that extracellular HMGB1 might also be of relevance to human RD. HMGB1 concentration tended to be high in the eye without PVR, but not so with PVR. One possible explanation for this tendency is that HMGB1 might be sequestered and/or masked in PVR, the advanced stage of RD. HMGB1 binds tightly to heparin and proteoglycans with heparan sulfate,[5] and it is also reported that such proteoglycans are abundantly present as the ocular extracellular matrix, even in RD.[40] Hence, these molecules might affect the HMGB1 concentration in the vitreous humor. Nevertheless, this possibility does not negate the presence of HMGB1. Considering the results obtained with the rat RD model, extracellular HMGB1 could be present at much higher levels, at least in the subretinal fluid of RD, and it might serve as a persistent signal adhering to the local damaged retina and/or surrounding matrix as previously described.[5]

It is also of importance that HMGB1 is significantly correlated with MCP-1 in RD vitreous. The secretion of MCP-1 might parallel the extent of photoreceptor degeneration of RD. Nakazawa *et al*[20] recently suggested that MCP-1 is a potential proapoptotic mediator during RD through the activation of microglias and/or macrophages. In their study, Müller-glial cells were observed to upregulate MCP-1, leading to activation and increased infiltration of microglias/macrophages in the detached retina. These cells induced further photoreceptor apoptosis through local oxidative stress. Corresponding to this report, RAGE was also reported to be prominently expressed in the Müller-glial cells.[41] Therefore, HMGB1 might influence MCP-1 expression through Müller-glial cells. Conversely, HMGB1 is known to be released by activated monocytes/macrophages.[7] MCP-1 is a potent stimulator and chemoattractant for monocytes/macrophages,[42] and these cells were observed in the subretinal space of RD with abundant HMGB1 expression. This would also be another possible explanation for the parallel increases of HMGB1 and MCP-1. Nevertheless, the positive correlation of these molecules indicates that cell death-related mediators might be highly orchestrated in ocular degenerative tissue damage. Several studies suggest that extracellular HMGB1 can aggravate tissue damage in neuronal tissues.[10,43] In these studies, extracellular HMGB1 plays a key role in the development of neuronal injury through the induction of inflammation, microglial activation, and neuronal excitotoxicity. According to these recent reports, the presence of extracellular HMGB1 concomitantly with MCP-1 is a possible deteriorating factor for RD, in spite of its essential role in the nucleus.

PVR is one of the most threatening complications of RD. It is thought to be a reactive process to retinal injury, in other words, it is one of the wound-healing responses in the eye. RPE cells are known to be detectable in the fibrotic proliferative membranes of PVR, and play an important role in the pathogenesis of PVR.[44] Thus, the effects of a molecule on PVR formation could be traced to RPE migration, at least in part. Here, we demonstrate that extracellular HMGB1 promotes RPE cell migration by chemotaxis *in vitro*. This result is consistent with previous reports of HMGB1-induced cell migration in various cell types, such as smooth muscle cells,[21,33] fibroblasts,[45] and chondrocytes.[34] We also found that HMGB1 activated phosphorylation of ERK-1/2 in RPE cells and the migration induced by HMGB1 was dependent on ERK phosphorylation. The phosphorylation of ERK is associated with cell proliferation and cell migration through effects on cell–matrix contacts.[46] It was also reported to be found in Müller-glial cells after RD.[47] Taken together, our results suggest that extracellular HMGB1 from dying ocular cells might affect retinal cells through ERK phosphorylation and potentially serve to promote the formation of PVR, which is wound healing, but has a pathological meaning in the eye. Several new strategies for prevention of ocular fibrosis, especially targeting specific signaling pathways, have been proven to be beneficial in animal models.[48–50] We propose that the identification and further characterization of danger signals, including HMGB1, would provide a novel

perspective for better understanding the molecular pathogenesis of PVR before applying these promising therapeutic manipulations to human subjects.

It has been suggested that post-transcriptional modifications of HMGB1, such as acetylation, methylation, and phosphorylation, might influence its activity.[51] Some recent reports also demonstrate that the proinflammatory activity of HMGB1 is due to combined action with other molecules.[52] The present data are mostly limited to the presence of HMGB1 rather than its biological activity, and we do not address what modifications or molecules are involved in intraocular HMGB1. However, we identify for the first time that HMGB1 is evident in a typical retinal injury of human RD, in which nuclear HMGB1 is a crucial nuclear protein and extracellular HMGB1 is a danger signal that might be required for the ocular wound-healing response. Our findings might have relevance for the underlying mechanisms of degenerative neuronal diseases. Further detailed studies will be needed to obtain more accurate knowledge and therapeutic value of HMGB1 in human diseases.

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