a

| | 0 | 5 | 15 | 30 | 60 | (min) |

p-ERK-1/2 — p44, p42

ERK-1/2 — p44, p42

b

| U0126 (µM) | − | − | 1 | 5 | 10 |
| HMGB1 | − | + | + | + | + |

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.

1. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007;81:1–5.
2. Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: friend and foe. Cytokine Growth Factor Rev 2006;17:189–201.
3. Martin P. Wound healing—aiming for perfect skin regeneration. Science 1997;276:75–81.
4. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 2005;5:331–342.
5. Huttunen HJ, Rauvala H. Amphoterin as an extracellular regulator of cell motility: from discovery to disease. J Intern Med 2004;255:351–366.
6. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002;418:191–195.
7. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999;285:248–251.
8. Passalacqua M, Patrone M, Picotti GB, et al. Stimulated astrocytes release high-mobility group 1 protein, an inducer of LAN-5 neuroblastoma cell differentiation. Neuroscience 1998;82:1021–1028.
9. Ito T, Kawahara K, Nakamura T, et al. High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. J Thromb Haemost 2007;5:109–116.
10. Kim JB, Sig Choi J, Yu YM, et al. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 2006;26:6413–6421.
11. Campana L, Bosurgi L, Rovere-Querini P. HMGB1: a two-headed signal regulating tumor progression and immunity. Curr Opin Immunol 2008;20:518–523.
12. Inoue K, Kawahara K, Biswas KK, et al. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. Cardiovasc Pathol 2007;16:136–143.
13. Taniguchi N, Kawahara K, Yone K, et al. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. Arthritis Rheum 2003;48:971–981.
14. Morimoto Y, Kawahara KI, Tancharoen S, et al. Tumor necrosis factor-alpha stimulates gingival epithelial cells to release high mobility-group box 1. J Periodontal Res 2008;43:76–83.
15. Cook B, Lewis GP, Fisher SK, et al. Apoptotic photoreceptor degeneration in experimental retinal detachment. Invest Ophthalmol Vis Sci 1995;36:990–996.
16. Arroyo JG, Yang L, Bula D, et al. Photoreceptor apoptosis in human retinal detachment. Am J Ophthalmol 2005;139:605–610.
17. Erickson PA, Fisher SK, Anderson DH, et al. Retinal detachment in the cat: the outer nuclear and outer plexiform layers. Invest Ophthalmol Vis Sci 1983;24:927–942.
18. Vazquez-Chona F, Song BK, Geisert Jr EE. Temporal changes in gene expression after injury in the rat retina. Invest Ophthalmol Vis Sci 2004;45:2737–2746.
19. Arimura N, Ki IY, Hashiguchi T, et al. High-mobility group box 1 protein in endophthalmitis. Graefes Arch Clin Exp Ophthalmol 2008;246:1053–1058.
20. Nakazawa T, Hisatomi T, Nakazawa C, et al. Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. Proc Natl Acad Sci USA 2007;104:2425–2430.
21. Porto A, Palumbo R, Pieroni M, et al. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. FASEB J 2006;20:2565–2566.
22. Hisatomi T, Sakamoto T, Murata T, et al. Relocalization of apoptosis-inducing factor in photoreceptor apoptosis induced by retinal detachment in vivo. Am J Pathol 2001;158:1271–1278.
23. Neekhra A, Luthra S, Chwa M, et al. Caspase-8, -12, and -3 activation by 7-ketocholesterol in retinal neurosensory cells. Invest Ophthalmol Vis Sci 2007;48:1362–1367.
24. Biswas KK, Sarker KP, Abeyama K, et al. Membrane cholesterol but not putative receptors mediates anandamide-induced hepatocyte apoptosis. Hepatology 2003;38:1167–1177.
25. Hoppe G, Rayborn ME, Sears JE. Diurnal rhythm of the chromatin protein Hmgb1 in rat photoreceptors is under circadian regulation. J Comp Neurol 2007;501:219–230.
26. Hinton DR, He S, Graf K, et al. Mitogen-activated protein kinase activation mediates PDGF-directed migration of RPE cells. Exp Cell Res 1998;239:11–15.
27. Han QH, Hui YN, Du HJ, et al. Migration of retinal pigment epithelial cells in vitro modulated by monocyte chemotactic protein-1: enhancement and inhibition. Graefes Arch Clin Exp Ophthalmol 2001;239:531–538.
28. Glotin AL, Calipel A, Brossas JY, et al. Sustained versus transient ERK1/2 signaling underlies the anti- and proapoptotic effects of oxidative stress in human RPE cells. Invest Ophthalmol Vis Sci 2006;47:4614–4623.
29. Klein JA, Ackerman SL. Oxidative stress, cell cycle, and neurodegeneration. J Clin Invest 2003;111:785–793.
30. Tang D, Shi Y, Kang R, et al. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. J Leukoc Biol 2007;81:741–747.
31. Hollborn M, Francke M, Iandiev I, et al. Early activation of inflammation- and immune response-related genes after experimental detachment of the porcine retina. Invest Ophthalmol Vis Sci 2008;49:1262–1273.
32. Hisatomi T, Sakamoto T, Sonoda KH, et al. Clearance of apoptotic photoreceptors: elimination of apoptotic debris into the subretinal space and macrophage-mediated phagocytosis via phosphatidylserine receptor and integrin alphavbeta3. Am J Pathol 2003;162:1869–1879.
33. Degryse B, Bonaldi T, Scaffidi P, et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol 2001;152:1197–1206.
34. Taniguchi N, Yoshida K, Ito T, et al. Stage-specific secretion of HMGB1 in cartilage regulates endochondral ossification. Mol Cell Biol 2007;27:5650–5663.
35. Yamada Y, Ishibashi K, Ishibashi K, et al. The expression of advanced glycation endproduct receptors in rpe cells associated with basal deposits in human maculas. Exp Eye Res 2006;82:840–848.

36. Howes KA, Liu Y, Dunaief JL, *et al.* Receptor for advanced glycation end products and age-related macular degeneration. Invest Ophthalmol Vis Sci 2004;45:3713–3720.

37. Ma W, Lee SE, Guo J, *et al.* RAGE ligand upregulation of VEGF secretion in ARPE-19 cells. Invest Ophthalmol Vis Sci 2007;48: 1355–1361.

38. Pachydaki SI, Tari SR, Lee SE, *et al.* Upregulation of RAGE and its ligands in proliferative retinal disease. Exp Eye Res 2006;82: 807–815.

39. Elner SG, Elner VM, Jaffe GJ, *et al.* Cytokines in proliferative diabetic retinopathy and proliferative vitreoretinopathy. Curr Eye Res 1995;14:1045–1053.

40. Wang JB, Tian CW, Guo CM, *et al.* Increased levels of soluble syndecan-1 in the subretinal fluid and the vitreous of eyes with rhegmatogenous retinal detachment. Curr Eye Res 2008;33:101–107.

41. Barile GR, Pachydaki SI, Tari SR, *et al.* The RAGE axis in early diabetic retinopathy. Invest Ophthalmol Vis Sci 2005;46: 2916–2924.

42. Matsushima K, Larsen CG, DuBois GC, *et al.* Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. J Exp Med 1989;169:1485–1490.

43. Pedrazzi M, Raiteri L, Bonanno G, *et al.* Stimulation of excitatory amino acid release from adult mouse brain glia subcellular particles by high mobility group box 1 protein. J Neurochem 2006;99:827–838.

44. Pastor JC, de la Rua ER, Martin F. Proliferative vitreoretinopathy: risk factors and pathobiology. Prog Retin Eye Res 2002;21:127–144.

45. Straino S, Di Carlo A, Mangoni A, *et al.* High-mobility group box 1 protein in human and murine skin: involvement in wound healing. J Invest Dermatol 2008;128:1545–1553.

46. Lawrence MC, Jivan A, Shao C, *et al.* The roles of MAPKs in disease. Cell Res 2008;18:436–442.

47. Nakazawa T, Takeda M, Lewis GP, *et al.* Attenuated glial reactions and photoreceptor degeneration after retinal detachment in mice deficient in glial fibrillary acidic protein and vimentin. Invest Ophthalmol Vis Sci 2007;48:2760–2768.

48. Saika S. TGFbeta pathobiology in the eye. Lab Invest 2006;86 :106–115.

49. Saika S, Yamanaka O, Nishikawa-Ishida I, *et al.* Effect of Smad7 gene overexpression on transforming growth factor beta-induced retinal pigment fibrosis in a proliferative vitreoretinopathy mouse model. Arch Ophthalmol 2007;125:647–654.

50. Saika S, Yamanaka O, Sumioka T, *et al.* Fibrotic disorders in the eye: targets of gene therapy. Prog Retin Eye Res 2008;27:177–196.

51. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. Immunol Rev 2007;220:35–46.

52. Sha Y, Zmijewski J, Xu Z, *et al.* HMGB1 develops enhanced proinflammatory activity by binding to cytokines. J Immunol 2008;180:2531–2537.