High-Mobility Group Box 1 Protein in Human and Murine Skin: Involvement in Wound Healing

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High-mobility group box 1 (HMGB1) protein is a multifunctional cytokine involved in inflammatory responses and tissue repair. In this study, it was examined whether HMGB1 plays a role in skin wound repair both in normoglycemic and diabetic mice. HMGB1 was detected in the nucleus of skin cells, and accumulated in the cytoplasm of epidermal cells in the wounded skin. Diabetic human and mouse skin showed more reduced HMGB1 levels than their normoglycemic counterparts. Topical application of HMGB1 to the wounds of diabetic mice enhanced arteriole density, granulation tissue deposition, and accelerated wound healing. In contrast, HMGB1 had no effect in normoglycemic mouse skin wounds, where endogenous HMGB1 levels may be adequate for optimal wound closure. Accordingly, inhibition of endogenous HMGB1 impaired wound healing in normal mice but had no effect in diabetic mice. Finally, HMGB1 had a chemotactic effect on skin fibroblasts and keratinoyctes *in vitro*. In conclusion, lower HMGB1 levels in diabetic skin may play an important role in impaired wound healing and this defect may be overcome by the topical application of HMGB1.

Journal of Investigative Dermatology (2008) **128,** 1545–1553; doi:10.1038/sj.jid.5701212; published online 31 January 2008

INTRODUCTION

Skin wound repair is a complex process requiring the coordinated interaction of numerous cell types. Growth factors and cytokines released in the wound play a pivotal role in orchestrating the regenerative process, which occurs through three phases: inflammation, proliferation, and remodeling (Martin, 1997). These events are finely balanced and any alteration in their occurrence results in impaired wound healing. In diabetes, there is evidence that the phases of wound healing are deregulated. Decreased chemotaxis of inflammatory cells into the wound leads to diminished availability of growth factors important for effective wound repair; excessive protease activity and increased microbial load, together with the frequent co-existence of macro- and microangiopathy, delay wound healing (Werner *et al.*, 1994; Shaw and Boulton, 1997; Doxey *et al.*, 1998).

High-mobility group box 1 (HMGB1), a 214 amino-acid protein with 99% amino-acid identity between rodent and humans (Parkkinen *et al.*, 1993; Maher and Nathans, 1996), is a non-histone nuclear protein that binds into the minor groove of DNA and, via this mechanism, is involved in the regulation of gene transcription and in the modulation of nucleosome accessibility (Bianchi *et al.*, 1989; Giese *et al.*, 1992; Agresti and Bianchi, 2003). In addition to its role in the nucleus, HMGB1 can be released into the extracellular space. In the late phase of inflammation and in response to various stimuli, including lipopolysaccharide, tumor necrosis factor-α (TNF-α), or interleukin-1β (IL-1β), HMGB1 is secreted by monocytes and macrophages (Wang *et al.*, 1999; Andersson *et al.*, 2000; Scaffidi *et al.*, 2002). The extracellular protein acts as a cytokine by binding to the receptor for advanced glycation end products (RAGE) and initiates a positive feedback autocrine loop that maintains the inflammatory cascade. HMGB1 also elicits proinflammatory responses in endothelial cells by increasing the expression of vascular adhesion molecules as well as secretion of cytokines (TNF-α) and chemokines (IL-8 and MCP-1 (monocyte chemotactic protein-1)) (Fiuza *et al.*, 2003). High levels of HMGB1 have been detected in serum (Wang *et al.*, 1999) and in the synovial fluid (Kokkola *et al.*, 2002; Taniguchi *et al.*, 2003) of patients with sepsis and rheumatoid arthritis, respectively. Similarly, HMGB1 is increased in atherosclerotic lesions

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Abbreviations: EGF, epidermal growth factor; BoxA, HMGB1-DNA binding A box; HMGB1, high-mobility group box 1; IL, interleukin; PDGF BB, platelet-derived growth factor BB; RAGE, receptor for advanced glycation end products; TNF-α, tumor necrosis factor-α

Received 17 July 2007; revised 10 October 2007; accepted 31 October 2007; published online 31 January 2008

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where it contributes to the inflammatory response (Kalinina *et al.*, 2004; Porto *et al.*, 2006). Extracellular functions of HMGB1 are not limited to inflammation. HMGB1 induces neurite outgrowth in the developing nervous system as well as neuronal differentiation of embryonic stem cells (Huttunen *et al.*, 2002) and smooth muscle cell chemotaxis (Degryse *et al.*, 2001). Moreover, HMGB1 is a strong chemoattractant and mitogen for vessel-associated stem cells (mesoangioblasts) (Palumbo *et al.*, 2004) and for endothelial precursor cells (Chavakis *et al.*, 2007). Recently, we demonstrated that intracardiac HMGB1 injection in a mouse model of myocardial infarction induced newly formed myocytes from resident cardiac stem cells and functional recovery of the infarcted hearts (Limana *et al.*, 2005; Germani *et al.*, 2007).

In this study, we examined the role of endogenous and exogenously administered HMGB1 in skin tissue repair. Lower levels of HMGB1 were detected in the skin of diabetic patients and mice, where repair is impaired. Exogenous HMGB1 directly applied to the wound accelerated healing by enhancing vessel density and granulation tissue deposition in diabetic mice but not in normal mice. HMGB1 inhibitors delayed skin wound closure in normal mice, whereas they had no effect in diabetic mice. Both skin fibroblasts and keratinocytes migrated *in vitro* in response to HMGB1. These results suggest that HMGB1 is required for skin regeneration and that its low level in diabetic patients and in mice may contribute to delayed wound healing.

RESULTS

HMGB1 in normal and diabetic skin wounds

The distribution of HMGB1 during skin wound healing was investigated in skin biopsies from normoglycemic and diabetic patients. Immunohistochemical analysis revealed that HMGB1 distribution both in non-diabetic and diabetic human skin was restricted to the nucleus of both epidermal and dermal cells (Figure 1a). To address whether HMGB1 distribution was modified in chronic, non-healing wounds, skin biopsies were taken from the edge of the ulcers of diabetic and normoglycemic patients. In non-healing ulcers, HMGB1 was localized in the cytoplasm of epidermal cells (Figure 1a) without difference in its localization between diabetic and normoglycemic skin lesions. HMGB1 cytoplasmic relocalization represents a signal for its secretion in inflammatory cells stimulated either with lipopolysaccharide

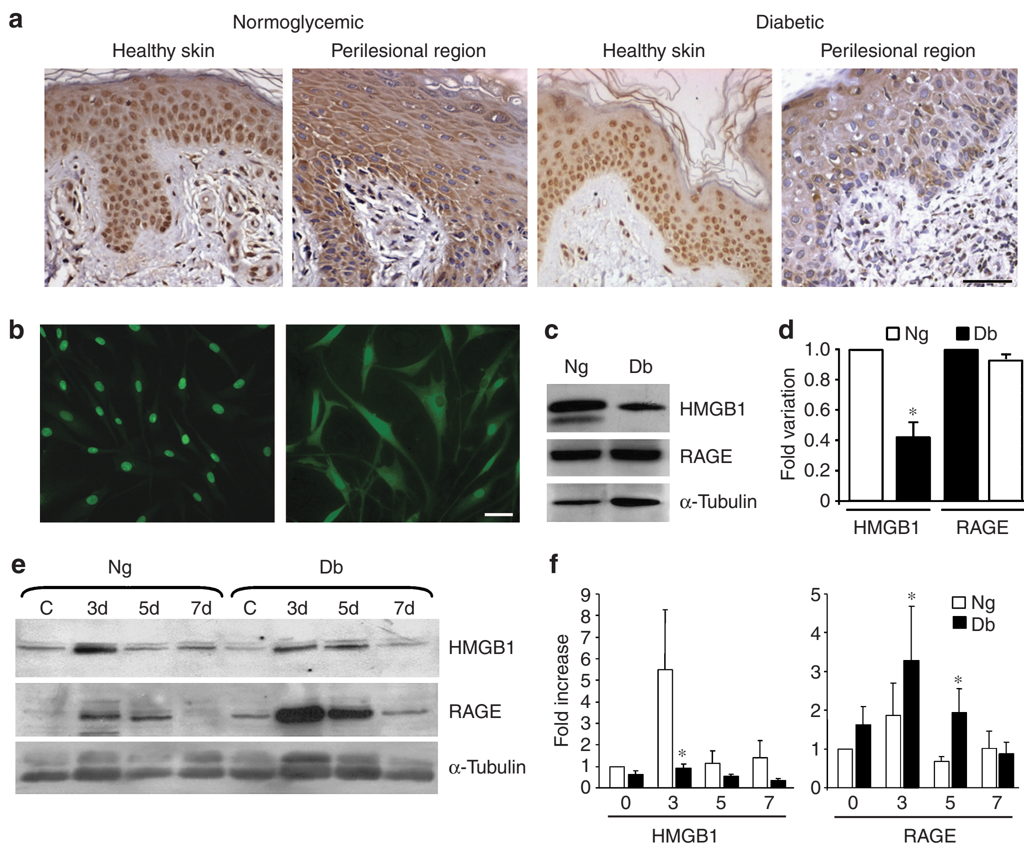

Figure 1. HMGB1 expression in normal and diabetic skin. (**a**) Representative immunohistochemical staining for HMGB1 in human normoglycemic and diabetic skin in the absence of skin lesions (healthy skin) and in the peri-lesional region ($n=4$). Bar = 50 μm. (**b**) Immunofluorescence analysis showing HMGB1 localization in unstimulated (left panel) and TNF-α-stimulated (right panel) human fibroblasts. Bar = 50 μm. (**c–e**) Western blot analysis to detect HMGB1 and RAGE levels in (**c, d**) normoglycemic (Ng) and diabetic (Db) human healthy skin extracts, and (**e, f**) in normoglycemic (Ng) and diabetic (Db) mouse skin prior to (C) and at 3, 5 and 7 days after wounding. To normalize loading, filters were also probed with anti-α-tubulin mAb. (**d, f**) Average results, normalized to α-tubulin, of HMGB1 and RAGE densitometric analyses for the experimental protocols shown in (**c**) and (**e**), respectively ($n=4$, $^*P<0.05$ vs their corresponding Ng values). Values are expressed as the mean ± SE.

or inflammatory mediators, that is, TNF-α (Wang *et al.*, 1999; Bonaldi *et al.*, 2003). To evaluate whether inflammatory stimuli at the wound site may have a similar effect on dermal cells, HMGB1 distribution was analyzed in isolated human fibroblasts and keratinocytes exposed to TNF-α. In both cell types, HMGB1 was detected in the cell nucleus in the absence of stimulation (Figure 1a and data not shown); TNF-α induced HMGB1 cytoplasmic translocation in skin fibroblasts (Figure 1b), whereas its distribution was not affected in keratinocytes (data not shown). Furthermore, HMGB1 was not detected in the conditioned medium of TNF-α-treated human fibroblasts (data not shown).

HMGB1 protein levels were then evaluated in human and mouse normoglycemic and diabetic skin. In the absence of lesions, HMGB1 protein levels were lower in diabetic than in normoglycemic human skin (Figure 1c and d), and a trend in the reduction of HMGB1 levels was also detected in diabetic mouse skin (Figure 1e and f). Both in normoglycemic and in diabetic mice, skin injury induced a transient increase of HMGB1; however, at day 3 after wounding, HMGB1 levels were higher in normoglycemic mice compared to their diabetic counterpart (Figure 1e and f). The HMGB1 receptor RAGE strongly increased in diabetic skin compared with normoglycemic skin at day 3 after wounding, and returned to control levels by day 7 (Figure 1e and f).

HMGB1 administration promotes wound healing in diabetic mice

As HMGB1 levels were reduced in diabetic skin, in these experiments it was examined whether HMGB1 topical application to murine diabetic skin wounds promoted wound closure. Wound healing in diabetic mice was delayed as expected (Figure 2a) (Goodson and Hung, 1977; Romano Di Peppe *et al.*, 2002). A single dose of 200, 400, and 800 ng HMGB1 was applied to the wound. In response to 200 and 400 ng HMGB1, wound closure was accelerated compared to wounds treated either with saline or 800 ng of protein (Figure 2b). Wound areas shrank, at day 3, by 37.6 ± 5.3% in 400 ng HMGB1-treated mice and by 13 ± 4% in saline-treated mice (Figure 2b); wound closure was 62.7 ± 5 vs 44.5 ± 1.9% at day 5 and 70 ± 4.8 vs 63.3 ± 2% at day 6 (Figure 2b). The effect of HMGB1 was compared to that

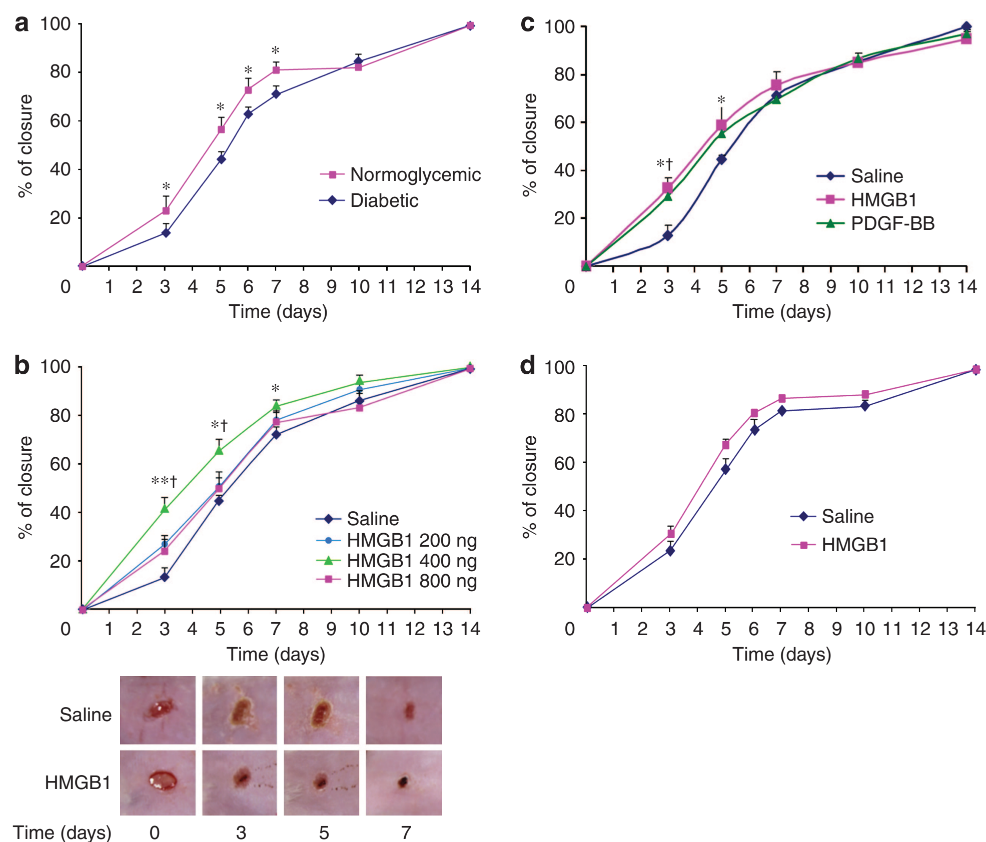

Figure 2. Effect of topical administration of HMGB1 in wound healing. Kinetics of skin wound healing in (**a**) normoglycemic and diabetic mice in the absence of any treatment (12 mice/group; *$P<0.003$ vs control) and (**b**) in diabetic mice that received topical application of HMGB1 ($n=10$ mice per HMGB1 200 ng group, $n=6$ mice/HMGB1 400 and 800 ng groups) or saline ($n=28$, mice; **$P<0.001$ and *$P<0.01$ vs saline; †$P<0.05$ vs HMGB1 200 ng). Lower panel: representative pictures of diabetic wounds treated either with saline solution or a single dose of HMGB1 (400 ng). (**c**) Analysis of wound healing in diabetic mice treated either with HMGB1 ($n=4$ mice per group, 400 ng; *$P<0.05$ vs saline group) or PDGF-BB ($n=4$ mice per group, PDGF-BB 400 ng; †$P<0.05$ vs saline group). (**d**) Kinetics of skin wound healing in normoglycemic and mice treated either with HMGB1 (200 ng) or saline solution ($n=6$ mice per group). Results are presented as percentage of wound closure, that is, wound area at different times after wounding per wound area at time 0, and values expressed as the mean ± SE.

obtained with platelet-derived growth factor BB (PDGF-BB), a growth factor well known to induce wound healing (Greenhalgh *et al.*, 1990), and also approved for clinical use in the treatment of skin ulcers. Both PDGF-BB and HMGB1 applied to wounds in a single dose of 400 ng showed a comparable effect in wounded area reduction (Figure 2c). In normoglycemic mice, HMGB1 application to the wound site did not significantly modify wound healing (Figure 2d).

Inhibition of endogenous HMGB1 impairs wound healing in normoglycemic mice

The lower HMGB1 level in diabetic mice and the improvement in wound healing observed in response to HMGB1 in diabetic mice, but not in normoglycemic mice, suggested that normoglycemic mice may already have an optimal amount of HMGB1 for wound closure. Thus, we tested the effect of HMGB1 blockade in normoglycemic mice using the HMGB1-DNA binding A box (BoxA), a truncated form of the protein that acts as a competitive antagonist by inhibiting HMGB1 binding to its receptor RAGE (Kokkola *et al.*, 2003; Yang *et al.*, 2004). BoxA was administered topically in the full excisional wound every 2 days from day 0 to day 14 after wounding. Between days 3 and 7 after wounding, BoxA slowed down wound closure in normal mice (Figure 3a). At day 3, wound areas were reduced by 29 ± 4.6 and 14.6% ± 2.6% in saline- and BoxA-treated mice, respectively. Statistically significant differences in wound closure persisted at day 5 (54.6 ± 5.4 vs 37.6 ± 4.1%) and at day 6 (69.3 ± 4.2 vs 52.2 ± 4.6%) (Figure 3a). In contrast, no delay in wound closure in response to BoxA was observed in diabetic mice (Figure 3b). Similar results were obtained both in diabetic and normoglycemic mice with glycyrrhizin, a different HMGB1 inhibitor (Sitia *et al.*, 2006) (Figure S1).

Taken together, these data suggest that endogenous HMGB1 is adequate for wound healing in normoglycemic mice, but is not sufficient in diabetic mice.

HMGB1 promotes granulation tissue formation and increases vessel density in diabetic skin wounds

Histologic evaluation of diabetic wounds performed 5 days after wounding revealed that HMGB1 administration (400 ng) significantly increased wound re-epithelialization, whereas the administration of BoxA had no effect (Figure 4a). Granulation tissue area measurement at day 5 after wound and HMGB1 treatment revealed an increase in granulation tissue vs saline-treated wounds, and the amount of granulation tissue was comparable to that present in normoglycemic mouse wounds treated with saline (Figure 4b and c). In contrast, the granulation tissue area in BoxA-treated wounds was similar to that observed in saline-treated wounds (Figure 4b). Notably, wounds that received saline solution appeared poorly cellularized with low amount of deposited collagen (Figure 4c). Morphometric analysis indicated that the density of arterioles 4–41 μm in diameter, identified by staining with antibodies against anti-smooth muscle actin and von Willebrand factor, was significantly increased in diabetic HMGB1-treated wounds compared both with saline- or BoxA-treated wounds (16.8 ± 1.5 vs saline 8.8 ± 2 mm mm^{-3}) (Figure 4d–f). The enhanced vascularization observed in HMGB1-treated wounds was not associated with increased stem cell recruitment; c-kit^{+} and CD34^{+} cell number was similar in HMGB1- and saline-treated diabetic wounds (data not shown).

HMGB1 has a chemotactic effect on fibroblasts and keratinocytes

As rapid induction of keratinocyte and fibroblast migration and proliferation into wounds are necessary for tissue repair, we examined HMGB1's ability to modulate these cells' function *in vitro*. Under our experimental conditions, HMGB1 promoted fibroblast and keratinocyte migration in a concentration-dependent manner. Fibroblast and keratinocyte peak

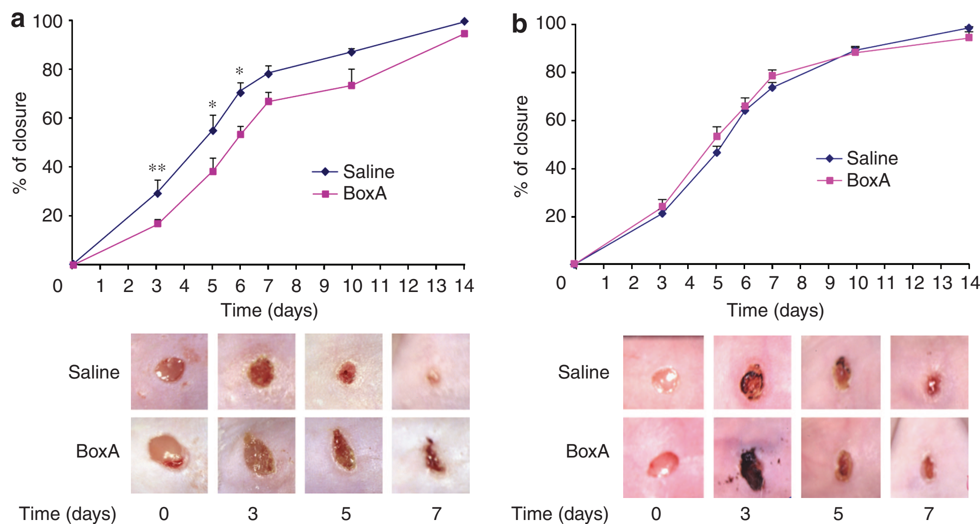

Figure 3. Effect of HMGB1 inhibition on wound healing. Analysis of wound closure in normoglycemic (**a**) and diabetic (**b**) mice treated either with a saline solution or BoxA (1 μg). BoxA was administered to wounds every 2 days. Lower panels: Representative pictures of wound healing in saline solution- and BoxA-treated normoglycemic (**a**) and diabetic mice (**b**) ($n=15$ mice per saline groups and $n=10$ mice per BoxA-treated groups. $^{**}P<0.05$ and $^{*}P<0.01$ vs BoxA). Values are expressed as the mean ± SE.

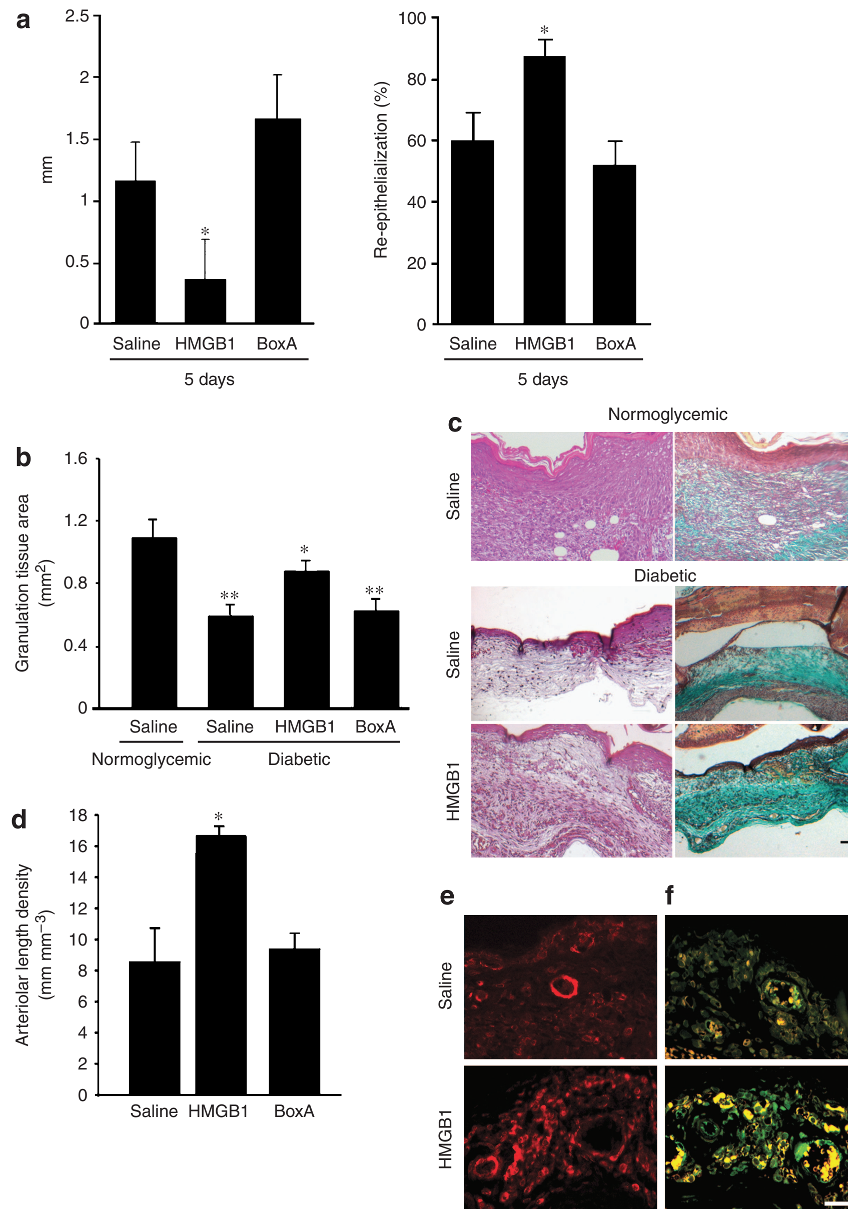

Figure 4. Effect of HMGB1 on re-epithelialization, granulation tissue, and vessel density. (**a**) HMGB1 administration to diabetic wounds reduced the epithelial gap (left panel) and enhanced re-epithelialization (right panel) at day 5 after injury ($n=6$ mice per group, $*P<0.05$ vs saline and BoxA-treated wounds). (**b**) HMGB1, but not BoxA, enhanced granulation tissue area. Central sections of wound area were examined at day 5 after wounding ($n=6$ mice per group; $*P<0.05$ vs db saline-treated wounds; $**P<0.01$ vs normoglycemic saline-treated wounds). Values are expressed as the mean ± SE. (**c**) Representative images of the granulation tissue area stained with hematoxylin–eosin (H&E) (left panels) and of the collagen deposition stained with Masson's Trichrome (right panels) in normoglycemic wounds which received saline (upper panel) and in diabetic wounds treated either with saline (middle panel) or HMGB1 (lower panel). Bar = 50 μm. (**d**) Arteriole length density in 5 days of diabetic wounds measured by smooth muscle actin staining ($n=6$ mice per group; $*P<0.01$). Values are expressed as the mean ± SE. (**e, f**) Representative images of vessels stained with smooth muscle actin (red fluorescence) (**e**) and vWF (**f**) (green fluorescence). Bar = 25 μm.

chemotactic response was achieved with 100 and 10 ng ml^{-1} HMGB1, respectively (Figure 5a and b). PDGF-BB (15 ng ml^{-1}) and epidermal growth factor (EGF, 10 ng ml^{-1}) were used as positive controls for fibroblast and keratinocyte migration assays, respectively (Figure 5a and b). In additional experiments, we examined HMGB1 chemotactic effects on fibroblasts and keratinocytes isolated from diabetic patients (Figure 5c and d). We were limited by the difficulty of obtaining a large number of fibroblasts and keratinocytes from diabetic biopsies, and we therefore tested migration only in response to 100 and 10 ng ml^{-1} HMGB1, respectively. Under all conditions tested, migration of diabetic fibroblasts and keratinocytes was markedly impaired compared to their normoglycemic counterparts (Figure 5a and b) (Lerman *et al.*, 2003). However, as in normoglycemic cells, 100 ng ml^{-1} HMGB1 induced a ~3-fold increase in the number of

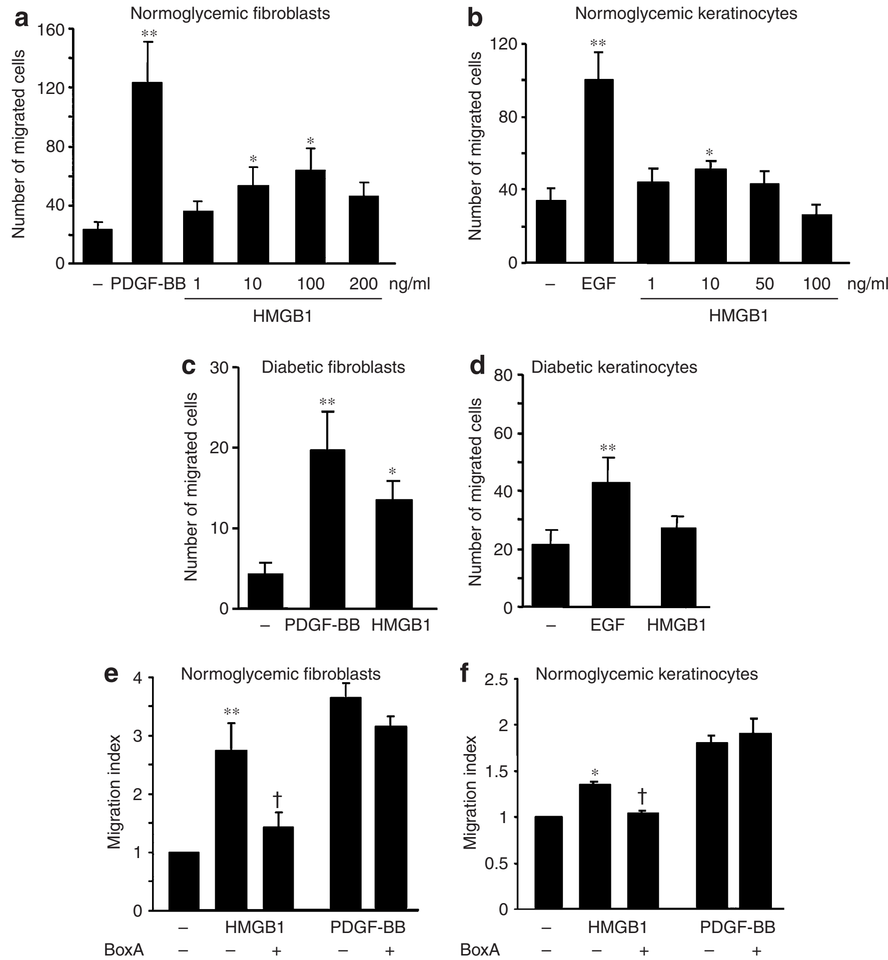

Figure 5. Effect of HMGB1 on human fibroblast and keratinocyte migration. (**a, b**) Normoglycemic human fibroblasts and keratinocytes were subjected to chemotaxis assays with the indicated concentrations of HMGB1; PDGF-BB (15 ng ml^{-1}) and EGF (10 ng ml^{-1}) were used as positive controls for fibroblast and keratinocyte migration, respectively. DMEM containing 0.1% BSA was used as negative control (–). Optimal HMGB1 concentrations were 100 and 10 ng ml^{-1} for fibroblasts and keratinocytes, respectively. (**c**, **d**) Chemotactic activity of 100 and 10 ng ml^{-1} HMGB1 on diabetic fibroblasts and keratinocytes, respectively. Data were obtained from four independent experiments performed in triplicate (* and **$P<0.01$ and $P<0.001$, respectively, vs negative control). (**e**) Fibroblasts were induced to migrate in the presence of 100 ng ml^{-1} of HMGB1 alone or in the presence of BoxA (500 ng ml^{-1}). (**f**) Keratinocytes migrated in response to 10 ng ml^{-1} HMGB1 and BoxA (50 ng ml^{-1}) blocked this effect. The data are expressed either as number of migrated cells or as the fold increase in the number of migrated cells relative to the number of migrated cells in the absence of factor (migration index) and are the means ± SE of at least four independent experiments performed in triplicate with cells derived from three different patients (* and **$P<0.01$ and $P<0.001$, respectively, vs negative control; †$P<0.05$ vs HMGB1). Values are expressed as the mean ± SE.

migrating fibroblasts (Figure 5c). Keratinocytes from diabetic patients migrated in response to EGF, whereas there was no significant chemotactic effect of 10 ng ml^{-1} HMGB1 on these cells (Figure 5d). HMGB1-induced migration of both normoglycemic fibroblasts and keratinocytes was inhibited by BoxA (Figure 5e and f). It is noteworthy that BoxA had no inhibitory effect on PDGF-BB- and EGF-mediated fibroblast and keratinocyte migration, respectively (Figure 5e and f). Finally, HMGB1 (10, 100, and 200 ng ml^{-1}) did not significantly affect fibroblast and keratinocyte proliferation, as it failed to increase cell number vs control after 24 and 48 hours in culture (data not shown).

DISCUSSION

HMGB1 is a multifunctional cytokine known to play a key role in inflammation. Recently, it has been shown that HMGB1 administration to skeletal muscle induces stem cell recruitment in the absence of damage (Palumbo *et al.*, 2004) and regeneration either after acute hindlimb ischemia (De Mori *et al.*, 2007) or myocardial infarction (Limana *et al.*, 2005) through a mechanism that involves activation of resident stem cells. These experimental evidences prompted us to investigate HMGB1's role in cutaneous wound healing. Further, as skin wound repair is impaired in diabetes, we examined HMGB1 expression and function both in normoglycemic and diabetic human and mice skin. HMGB1 is expressed in skin cells: it is located in the nucleus of normoglycemic and diabetic epidermal cells and, in the presence of skin ulcers, it accumulates in the cytoplasm of cells located in the peri-lesional regions. The role and mechanisms of the cytoplasmic redistribution of HMGB1 in these cells is currently not understood. During wound repair,

several pro-inflamamtory cytokines are upregulated (Grellner, 2002); among these cytokines, TNF-α has been shown to promote HMGB1 secretion from monocytes. It is noteworthy that in TNF-α-stimulated human fibroblasts, HMGB1 was accumulated in the cytoplasm, but was not detected in the culture medium. Nevertheless, it is possible that HMGB1 secretion from fibroblasts may occur *in vivo* where the activation of multiple pathways as well as enhanced membrane permeability, which occurs during necrosis, could direct the protein to the extracellular space.

In this study, it was found that HMGB1 levels were reduced in the skin of both diabetic humans and mice. In mouse skin wounds, HMGB1 and RAGE expression increased; in normoglycemic animals, HMGB1 and RAGE expression peaked 3 days after wounding and were back to baseline by day 5. A similar pattern of HMGB1 and RAGE expression was detected during wound repair in diabetic mice, although these mice displayed lower HMGB1 and higher RAGE levels compared to their normoglycemic counterparts. Therefore, we suggest that reduced HMGB1 in diabetic skin may account, at least in part, for the altered wound healing process in diabetes. In agreement with this hypothesis, blocking HMGB1 either with competitor BoxA or glycyrrhizin slowed wound healing in normoglycemic mice, whereas HMGB1 administration enhanced wound healing in diabetic mice. Furthermore, HMGB1 administration did not significantly modify wound closure in normal mice, where endogenous HMGB1 levels may already be optimal.

There are several mechanisms by which HMGB1 may promote skin wound repair. HMGB1 induces human fibroblast and keratinocyte migration; such migration is reduced in cells from diabetic patients. Although HMGB1 has been shown to have a proliferative effect on murine 3T3 fibroblasts (Zimmermann *et al.*, 2004), proliferation of skin fibroblast and keratinocyte was not affected by HMGB1 treatment. HMGB1 also induces angiogenesis *in vitro* and *in vivo* (Schlueter *et al.*, 2005; Mitola *et al.*, 2006). Indeed, HMGB1 increased the density of arterioles during the healing of diabetic wounds, and this effect likely contributed to increased granulation tissue formation. This process most likely did not involve stem cell recruitment to the wound site, as neither endothelial precursor cells expressing CD34 antigen nor bone marrow-derived cells expressing c-kit increased after HMGB1 administration to diabetic wounds. Finally, HMGB1 may act on macrophages, whose role in wound repair is well established (DiPietro, 1995; Doxey *et al.*, 1998; Wetzler *et al.*, 2000; Zykova *et al.*, 2000).

Our results also suggest that HMGB1 topical administration to diabetic wounds may have a therapeutic potential. However, at high concentration, HMGB1 has detrimental effects; it exacerbates inflammation and it is known to contribute to several inflammatory and infectious disorders (Ulloa and Messmer, 2006). Indeed, 500 μg of HMGB1 delivered intraperitoneally causes shock in mice (Wang *et al.*, 1999), whereas intra-tracheal administration of 10–100 μg HMGB1 produces inflammatory injury including neutrophil accumulation and lung edema (Ulloa and Messmer, 2006). The amount of HMGB1 that showed a beneficial effect in wound healing, 200–800 ng, is well below the HMGB1 level known to have a detrimental effect and is in the concentration range that has been shown to induce skeletal muscle and cardiac regeneration following myocardial infarction (Limana *et al.*, 2005; De Mori *et al.*, 2007). In conclusion, the results of this study show that endogenous HMGB1 is critical for skin tissue repair and that the reduced levels of HMGB1 in diabetic skin may impair wound healing; this specific defect can be corrected by exogenous HMGB1 topical administration.

MATERIALS AND METHODS

Reagents

Expression and purification of both HMGB1 and its truncated form, BoxA, were performed by HMGBiotech (Milan, Italy). Endotoxins were removed by passage through Detoxy-Gel columns (Pierce Biotechnology Inc., Rockford, IL). Recombinant HMGB1 was diluted in PBS and stored at −80 °C. Glycyrrhizin was from Minophagen Pharmaceutical (Tokyo, Japan). PDGF-BB and EGF were purchased by R&D Systems (Minneapolis, MN).

Human studies—skin biopsies, cell isolation, and culture

After Local Ethical Committee approval and signed informed consent, skin biopsies were obtained from normoglycemic controls and type II diabetic patients aged 45–70 years. The study was conducted according to the Declaration of Helsinki Principles. A punch biopsy, 4 mm in diameter, was performed under local anesthesia in the peri-lesional area of the skin ulcer or in the skin edge of a surgical incision during routine surgery. For fibroblast isolation, biopsies were seeded on 6-mm-diameter tissue culture dishes containing DMEM with 10% fetal bovine serum (Euroclone Inc., Milan, Italy), 20 mM glutamine, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin (Gibco BRL, Paisley, UK). Fibroblasts started growing out of the explants after 7–10 days and became confluent within 3–5 weeks. Normal and diabetic epidermal keratinocytes were cultured on a feeder layer of lethally irradiated 3T3-J2 cells as described (Pellegrini *et al.*, 1999). Cells were used at passages 2–4.

Immunofluorescence and HMGB1 detection in the culture medium

Human fibroblasts were kept in DMEM overnight and then TNF-α (50 ng ml^{-1}) was added at the culture medium for 20 hours. Cells were fixed in PBS with 4% paraformaldehyde and permeabilized in PBS with 0.1% Triton X-100. Coverslips were rinsed and blocked 10 minutes in PBS with 0.2% BSA before incubation with antibodies. Fixed cells were incubated with primary antibody anti-HMGB1 polyclonal antibody (1 μg ml^{-1}) followed by incubations with goat anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) (1:40). The coverglasses were mounted and analyzed with a Zeiss microscope equipped for epifluorescence. HMGB1 detection in the culture medium was performed either by western blot or ELISA assay (Oxford Biosystems, Oxford, UK).

Migration assays

Chemotaxis was performed in 48-microwell chemotaxis chambers (Neuroprobe, Cabin John, MD) using 8 μm pore size polycarbonate filters (Costar Scientific Corporation, Cambridge, MA) coated with

murine collagen type IV (Becton-Dickinson, Bedford, MA) (Supplementary Materials and Methods).

Animal skin wound model

CD1 male mice were obtained from Charles River (Calco, Lecco, Italy). Mice were rendered diabetic by intraperitoneal injection of a single dose of streptozotocin (200 mg per kg weight, Sigma-Aldrich, St Louis, MO). After 2 weeks, glycemia was measured, and animals with a blood glucose level of 200–400 mg per 100 ml were selected for further studies. Three weeks later, mice were anesthetized with an intraperitoneal injection of 2.5% Avertin (Sigma) (100% Avertin: 10 g 2,2,2-tribromoethyl alcohol in 10 ml tert-amyl alcohol). Their dorsum was clipped free of hair and a full-thickness wound of 3.5 mm diameter was created using a biopsy punch. HMGB1 was applied at the indicated concentration in 20 μl of saline solution, directly in the wound area at time 0, in a single dose. Another group of diabetic and non-diabetic mice was treated by placing 20 μl of saline solution on the wound. The HMGB1 inhibitor BoxA and glycyrrhizin were administered in the wound area at the concentration of 1 and 250 μg per mouse in 20 μl of PBS, respectively. To compare the rate of wound closure between HMGB1-treated and saline-treated mice, animals were photographed at day 0, 3, 5, 6, 7, 10, and 14 after treatment.

At different time points after treatment, pictures were digitally processed and wound areas were calculated using the KS300 system (Zeiss, Jena GmbH, Germany). For each sample, the rate of the healing process was measured as a ratio of the area at each time point divided by the area at time 0 (that is, immediately after the wound). All experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

Histology and wound analysis

Human and mouse biopsies were fixed in formalin and embedded in paraffin (Bio-plast special; melting point 52–54 °C). For immunohistochemical staining, 3-μm-thickness sections incubated with specific antibodies (Supplementary Materials and Methods).

Hematoxylin–eosin staining was performed to quantify granulation tissue as described (Romano Di Peppe *et al.*, 2002). The epithelial gap, which represents the distance between the leading edge of migrating keratinocytes, was measured by hematoxylin–eosin staining and reported in millimeter. The percent of re-epithelialization was calculated with the following formula: $\%\,\mathrm{Re-epithelialization} = \left[1 - \frac{\mathrm{Wound\,size}}{\mathrm{Original\,wound\,size}}\right] \times 100$ (Cheng *et al.*, 2007).

Data collections and statistics

Immunohistochemistry analyses were examined blindly. Comparisons among different groups for continuous variables were carried out by analysis of variance. Statistical significance between two measurements was evaluated by a two-tailed unpaired Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr G. Tinelli for providing human skin biopsies, and Dr A. Rossini and Dr T. Odorisio for helpful suggestions and criticism. We also thank D. Avitabile for helping in statistical analysis and M. Inzillo of the Medical Imaging Service of Istituto Dermopatico dell' Immacolata for figure preparation. This research was supported by grants from the Italian Ministry of Health.

SUPPLEMENTARY MATERIAL

Figure S1. Effect of HMGB1 inhibition with glycyrrhizin on wound healing.

Supplementary Materials and Methods.

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