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ORIGINAL PAPER

# Hmgb1 Promotes Wound Healing of 3T3 Mouse Fibroblasts via Rage-Dependent ERK1/2 Activation

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**Abstract** HMGb1 is a nuclear protein playing a role in DNA architecture and transcription. This protein has also been shown to function as a cytokine and to stimulate keratinocyte scratch wound healing. Due to the importance of finding new wound healing molecules, we have studied the effects of HMGb1 on fibroblasts, another major skin cell type, using the NIH 3T3 line. HMGb1 expression in these cells was assessed by Western blot, while its nuclear localization was pointed out by confocal immunofluorescence. HMGb1-induced cell proliferation with a maximum at a concentration of 10 nM, and such a dose also stimulated cell migration and scratch wound healing. Western blot analysis showed that HMGb1 activates ERK1/2, while the use of an anti-RAGE receptor-blocking antibody and of the selective MEK1/2 inhibitor PD98059 blocked ERK1/2 activation and wound healing responses to HMGb1. Taken together data show that HMGb1 promotes 3T3 fibroblast wound healing by inducing cell proliferation and migration, and that this occurs through the activation of the RAGE/MEK/ERK pathway. In conclusion, HMGb1 seems a good candidate for the development of medical treatments to be used on chronic or severe wounds.

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**Keywords** Confocal co-localization · Cytotoxicity assays · PD98059 · Scratch wound assay · Transwell chemotaxis assay

## Introduction

High-mobility group (HMG) nuclear proteins were discovered in 1973 in an effort to define the specific regulators of gene expression [1]. This group of non-histone, chromatin-associated proteins are constitutively expressed in the nucleus of eukaryotic cells, and have been found to be involved in DNA organization and transcription. HMGb1 is a member of the HMG family whose nuclear localization and affinity for DNA is regulated through phosphorylation and acetylation. This protein has a dynamic relationship with chromatin and plays an important role in DNA architecture and transcriptional regulation [2].

However, HMGb1 has been also implicated as an endogenous signalling molecule. Wang et al. [3] described its cytokine activity by identifying it as a late mediator of endotoxin-related lethality in mice. HMGb1 is released passively during cellular necrosis by almost all nucleated cells, thereby signalling to neighbouring cells the ongoing damage [4]. In addition, HMGb1 is actively secreted by immune cells such as monocytes, macrophages, and dendritic cells [3, 5]. Specific cellular activities elicited by HMGb1 have been also pointed out. The protein has been shown to function as a chemoattractant for rat vascular smooth muscle cells, by inducing cytoskeletal reorganization and cell migration [6]. It has been also found to promote the migration and proliferation of regenerative cells towards inflammation and injury sites [7]. Moreover, in vitro and in vivo experiments have shown that the induction of migratory responses of several cell type functions in a dose-dependent manner through HMGb1 interaction with

the receptor of advanced glycation end products (RAGE) [8, 9].

Cytokine-induced cell proliferation and migration are fundamental elements of tissue repair. Wound healing is a complex tissue remodelling process entailing several interactions among the injured cells, the extracellular matrix, and blood cells and factors involved in inflammatory responses and vascular repair [10, 11]. Growth factors are thought to be main intercellular signalling that orchestrates the complex sequence of wound healing-related cell activities [12]. However, other cytokines, such as proinflammatory and platelet-derived factors, are also essential, while other players have yet to be determined [13].

Due to the observed pro-inflammatory role of HMGb1, and its ability to induce cell proliferation and motility, it is reasonable to assume that this protein could also play a role in wound healing-related tissue remodelling. HMGb1 has been proven to possess this kind of property in an in vitro study on HaCaT keratinocytes [14] suggesting that the protein could be involved in skin wound healing. In order to confirm such a possibility, we explored the effects of HMGb1 on fibroblasts, which are on a par with keratinocytes as a major cell type involved in skin reparatory processes. We therefore evaluated the in vitro cell responses to extracellular HMGb1 of NIH 3T3 embryonic mouse skin fibroblasts [15], a common fibroblast model that has already been used in this kind of studies [16]. We also tried to highlight the mechanism of action of the protein by focusing on the RAGE–ERK1/2 pathway. ERK1/2 activation is a main signal of cell proliferation and is known to be activated by extracellular HMGb1 [14, 17]. It has been shown that HMGb1 activates ERK1/2 through its binding to RAGE [9, 18], and this latter has been pointed out as the major receptor for the proinflammatory activity of HMGb1 in rodents [19].

Our data showed that 3T3 fibroblasts express HMGb1, while they do not release appreciable amounts of the protein under normal conditions. However, exogenously supplied HMGb1 was able to induce cell proliferation, cell migration, and scratch wound closure, and these activities were mediated by a RAGE-dependent activation of the ERK1/2 signalling cascade.

## Materials and Methods

### Cell Culture and Reagents

All reagents were from Sigma-Aldrich, unless otherwise indicated. NIH 3T3 cells were obtained from American Type Culture Collection (Cat. no. ATCC CRL-1658, http://www.lgcstandards-atcc.org) maintained at 37°C, 5% $CO_2$, in DMEM supplemented with 10% foetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% antibiotic mixture.

### Purification of HMGb1

Eukaryotic recombinant HMGb1 protein was produced using the SF9 cells/*Baculovirus* system, and was purified as previously described [20, 21].

### Immunofluorescence Analysis

Cells were cultured on glass coverslips on the bottom of 12-well plates, washed three times with phosphate buffered saline (PBS) solution, fixed with 3.7% paraformaldehyde in PBS, and then permeabilized with 0.5% Triton X-100 in PBS. Non-specific interactions were blocked by 30-min incubation in 5% BSA in PBS. Coverslips were then incubated for 1 h with 1:200 of anti-HMGb1 mouse antibody (a kind gift of prof. B. Sparatore, DiMES, University of Genoa) [22] in PBS containing 0.5% Triton X-100, as primary antibody, and then with a FITC-conjugated secondary antibody (Cell Signaling Technology, Celbio S.p.A, Mi, Italy). Nuclear staining was achieved by 10-min incubation in 1-μg/ml propidium iodide. Double-stained cells were observed under a Zeiss LSM 510 confocal system interfaced with a Zeiss Axiovert 100-M microscope (Carl Zeiss Inc., Thornwood, NY, USA). Confocal micrographs were captured by imaging each fluorophore individually to prevent cross excitation. The confocal settings for the FITC channel consisted of 488-nm Argon laser excitation and BP 505–550 emission; while for the propidium channel they were 543-nm HeNe laser excitation and LP 560 emission. The Argon laser power was reduced to 15% to lower probe bleaching. Confocal imaging was performed with a resolution of 1024 × 1024 pixels at 256 intensity values, using a Zeiss oil-immersion Plan-Neofluar Ph3 100×/1.3 NA objective.

### Neutral Red Uptake (NRU) and Crystal Violet (CV) Assays

The NRU assay is a cell viability test based on the incorporation of neutral red dye into the lysosomes of viable cells after incubation with the test agent [23]. Cells were seeded on 96-well plates (20,000 cells/well), grown in DMEM with 10% FBS, and starved overnight in the absence of serum prior to treatments [24]. Cells were then exposed to HMGb1 at various concentrations for 24 h. After removing the medium, a 0.05% solution of neutral red was added to each well, followed by incubation for 3 h at 37°C. Cells were then washed with PBS 1x, followed by the addition of a solution of 1% glacial acetic acid in 50%

ethanol, to fix the cells and extract the neutral red dye incorporated into the lysosomes. Thereafter, plates were shaken and the absorbance was measured at 540 nm in a plate reader (Sirio S, SEAC, Florence, Italy).

For the CV assay, cells were plated and exposed to HMGb1 as above, washed with PBS 1x, stained for 10 min with 0.5% crystal violet in 145 mmol/l; NaCl, 0.5% formal saline, 50% ethanol, and washed thrice with water. Crystal violet was eluted from cells with 33% acetic acid and the absorption of the supernatant was measured at 540 nm in the plate reader.

### Scratch Wound and Cell Migration Assays

Cells were seeded in 12-well plates and allowed to grow until confluence. Thereafter, scratch wounds of about 700–750-μm width were created in cell monolayers (two wounds for each well) using a sterile 0.1–10-μl pipette tip. After washing away suspended cells, cultures were refed with medium in the presence or in the absence of HMGb1. After 6- or 24-h incubations, cells were fixed in 3.7% formaldehyde in PBS for 30 min, and then stained with 0.1% toluidine blue at room temperature for 30 min. Wound width was estimated at 0, 6, and 24 h after wounding with image analysis, using an inverted Televal microscope (Carl Zeiss Inc.) equipped with a digital camera, and the NIH ImageJ software. In a typical experiment, each group consisted of three different plates, i.e. a total of six wounds. Four measurements of wound width were made for each wound at randomly chosen points. All measurements were made by a single observer unaware of the treatments.

Cell chemoattraction by HMGb1 was assessed by a cell migration assay using transwell plates (8-μm pore size, Costar, Cambridge, MA). A total of $1 \times 10^5$ cells were seeded in the upper compartment of filters, while culture medium containing 10-nM HMGb1 was placed in the lower chamber. After 6 h filters were removed, stained with 0.5% crystal violet (145-mmol/l NaCl, 0.5% formal saline, 50% ethanol) for 10 min, and washed thrice with water. The upper side of filters was scraped using a cotton swab to remove cells that had attached but not migrated. Following PBS washing of filters, the dye was eluted from cells with 33% acetic acid, and measured at 540 nm.

### Western Blot Experiments

Cells were lysed with buffer containing 10-mM NaCl, 10-mM $MgCl_2$, 10-mM Tris-HCl, pH 7.3, 1% Triton X-100, 1% Na-deoxycholate, 1-mM DTT, 5-mM NaF, 2-mM $Na_3VO_4$, and protease inhibitor cocktail. The crude cell extract was clarified at 4°C at 15,000×*g* for 10 min and the amount of protein in the supernatant was quantified by the bicinchoninic acid (BCA) protein assay.

Amounts of 30 μg of protein, were loaded on gel, subjected to SDS-PAGE (12% gel), and then transferred onto nitrocellulose membranes using a Bio-Rad Mini Trans Blot Electrophoretic Transfer Unit. Membranes were blocked for non-specific protein with 5% non-fat dry milk in PBS and then probed overnight at 4°C with anti-p-ERK1/2 (1:1000, Db Biotech, Kosice, Slovakia) followed by stripping and reprobing with anti-ERK1/2 (1:1000, Db Biotech). In other cases, membranes were probed with anti-RAGE (1:1000, Chemicon, Millipore, Billerica, MA, USA). After probing with each primary antibody, membranes were washed thrice (10 min per wash) with PBS additioned with 0.05% Tween-20 to remove unbound antibodies, and then further incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000, Bethyl Laboratory, Montgomery, TX, USA). Membranes were developed by an ECL kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol, digitized with the Quantity One Image Software (ChemiDoc XRS, Bio-Rad Laboratories, Hercules, CA), and normalized against proper loading controls. Band intensities were quantified by densitometric analysis using the Adobe Photoshop 7.0.1 software (Adobe Systems Inc., San Jose, CA).

### Evaluation of Extracellular HMGb1

Extracellular HMGb1 was determined essentially as previously described [17, 22]. Briefly, cells were treated with 10-μg/ml heparin (5 min), and the conditioned media were collected, clarified by centrifugation (13,000×*g* for 10 min) and additioned with Triton X-100 (0.1% final concentration). Cells were lysed with 1% Triton X-100 in DMEM + BSA, followed by 1:10 dilution in the same medium. Aliquots of the media and cells underwent protein precipitation in 10% trichloroacetic acid (TCA), followed by SDS-PAGE and Western blot analysis as described above, using 1:4000 of the above anti-HMGb1 antibody and 1:1000 of HRP-conjugated anti-mouse antibody (Santa Cruz Biotechnology, CA, USA). Recombinant HMGb1 was added to DMEM + BSA and processed in parallel.

### Statistics

Data were analysed by ANOVA and the Tukey's post hoc test using the Instat software package (GraphPad Software, Inc, San Diego, CA).

## Results

### Analysis of HMGb1 Expression and Release

The expression of the HMGb1 protein has been detected in many cell types [25], but little is known about its role and regulation in the NIH 3T3 cell line, or more in general in fibroblasts. In order to verify the expression and localization of HMGb1 in 3T3 fibroblasts, we carried out confocal immunofluorescence and Western blot analyses. In confocal images, protein labelling with a primary monoclonal anti-HMGb1 and a secondary fluoresceinated antibody showed a good colocalization with propidium iodide nuclear staining, indicating that the protein is prevalently localized in the nucleus (Fig. 1a). HMGb1 detection in cell lysates was achieved through Western blot analysis using anti-HMGb1 antibody, as revealed by lane-to-lane comparison with the purified protein (Fig. 1b).

In order to examine the potential secretory release of HMGb1 by cells, we sought for the occurrence of the protein in the cell medium. After overnight cell incubation in serum-free medium, the immunoblotting detection of HMGb1 in the medium was negligible, whereas the induction of cell necrosis by $H_2O_2$ resulted in the release of the protein (Fig. 1c).

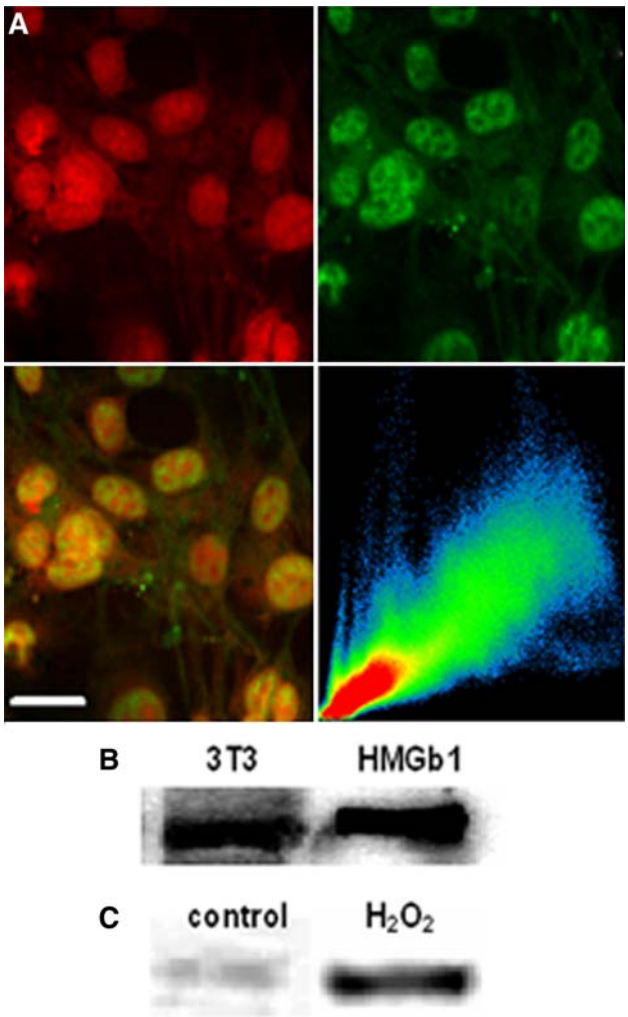



**Fig. 1** Immunofluorescence and immunoblotting detection of HMGb1. **a** Confocal immunofluorescence microscopy of 3T3 fibroblasts labelled with propidium iodide (*upper left*) and with anti-HMGb1 (*upper right*), dual channel image (*lower left*), and colocalization scatter diagram generated with the Zeiss LSM 510 software (*lower right*). *Bar* = 20 μm. **b** Western blot of HMGb1 in 3T3 fibroblasts (*left lane*) (50-μg protein) and of the purified protein (*right lane*). **c**. Western blot of HMGb1 in 3T3 conditioned medium after overnight incubation of cells under control conditions or in the presence of 10-mM $H_2O_2$. The blots are representative of three different experiments

### Effect of Extracellular HMGb1 on Cell Proliferation, Migration, and Scratch Wound Closure

In order to assess HMGb1 effects on 3T3 fibroblasts, we exposed cells to HMGb1 in the absence of serum since various authors have reported that HMGb1 can interact with serum proteins [14, 22, 26]. The responsiveness of 3T3 to HMGb1 was first evaluated by investigating the ability of HMGb1 to modulate cell viability. Cell exposures to increasing concentrations (0–100 nM) of HMGb1 for 24 h, followed by the NRU or the CV assays, showed that the protein induces no toxicity at the used doses, but in contrast it increases cell viability and proliferation, with a maximum at a concentration of 10 nM (Fig. 2). Such a result was similar to what has been reported for keratinocytes [14], and therefore we chose to carry out cell migration and scratch wound experiments at the 10-nM dose.

Cell migration plays an essential role in tissue wound closure, and we therefore investigated whether HMGb1 influences cell migration using a transwell chemotaxis assay. Cell incubation with 10-nM HMGb1 for 6 h increased cell migration rates respect to controls by a chemoattraction mechanism (Table 1). Accordingly, exposure of scratch wounded 3T3 cells to 10-nM HMGb1 induced a significant increase of wound closure rates respect to controls (Fig. 3). The acceleration of wound closure was observed already at 6 h, and it was still clearly detectable at 24 h (Table 1).

### Analysis of the Signalling Pathway Elicited by HMGb1

After having assessed the ability of HMGb1 to stimulate 3T3 proliferation, migration, and wound healing, we tried to highlight the pathway that mediate these activities. Given that RAGE is a main target of HMGb1, we first verified the expression of this receptor in our cells by Western blotting (Fig. 4a). Thereafter, we tried to highlight

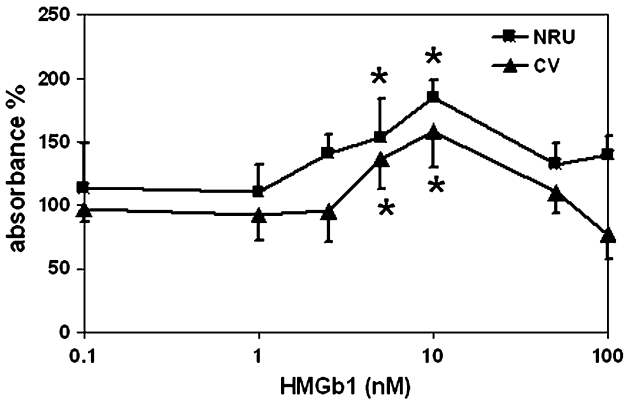



**Fig. 2** Dose–response curves derived from exposure of 3T3 fibroblast to increasing concentrations of HMGb1 (0.1, 1, 2.5, 5, 10, 50, 100 nM), and determined by the NRU and CV assays (see "Methods"). Data are mean absorbances ± SD ($n = 8$), expressed as percent of control (0 nM, not plotted) on a semilogarithmic scale. * Significantly different from control ($P < 0.01$, Dunnett's test)

**Table 1** Effects of HMGb1 on scratch wound closure and cell migration of 3T3 fibroblasts

| | Wound closure | | | Cell migration |
|---|---|---|---|---|
| | 0 h | 6 h | 24 h | |
| Control | 729 ± 5 | 414 ± 23 | 384 ± 27 | 100 ± 11 |
| HMGb1 (10 nM) | 729 ± 7 | 339 ± 14* | 317 ± 18* | 130 ± 12* |

Wound closure data are means ± SD ($n = 24$), expressed as microns. Cell migration data are means ± SD ($n = 5$) expressed as percent absorbance at 540 nm (see "Methods")

* $P < 0.01$ vs. control, according to $t$ test

RAGE activation through the detection of the phosphorylated form of its downstream effector ERK1/2. Western blot analysis of 3T3 monolayers subjected to multiple scratch wounds showed that wounding did not induce per se ERK1/2 phosphorylation, whereas in the presence of 10-nM HMGb1 a significant activation of ERK1/2 was observed (Fig. 4b). This activation was completely abolished by pre-incubation with an anti-RAGE antibody able to block receptor–ligand interaction (R&D Systems, Minneapolis, MN) (Fig. 4b). In addition, we also blocked HMGb1-dependent ERK1/2 activation by acting midway along the RAGE-dependent pathway, using the selective MEK1/2 inhibitor PD98059 (10 μM) (Fig. 4c). The PD98059 vehicle alone (0.1% DMSO) was uneffective (not shown).

### Involvement of the RAGE-Dependent Pathway in HMGb1 Activities on 3T3 Cells

We sought for a final confirmation of the HMGb1 mechanism of action by trying to block HMGb1 activities using the above inhibitors of the RAGE pathway. A cause–effect relationship between RAGE activation and the induction of 3T3 proliferation was demonstrated using the NRU and CV tests. These two analyses consistently showed that the increase in cell viability and proliferation induced by 10-nM HMGb1 can be abolished using 10 μg/ml of the anti-RAGE receptor-blocking antibody (Fig. 5a) or 10-μM PD98059 (Fig. 5b). In contrast, inhibitor alone did not affect normal cell viability and proliferation rate (Fig. 5).

According to these latter results, experiments of scratch wound carried out in the presence of anti-RAGE antibody or PD98059 at the same doses as above, showed that both the inhibitors were able to completely abolish the acceleration effect of HMGb1 on wound closure rates, while the inhibitors alone did not affect the wound closure rate of controls (Fig. 6a, b). Similarly to what observed in scratch wound tests, PD98059 blocked the stimulation of cell migration induced by HMGb1, but it did not alter control migration rates (Fig. 6c).

## Discussion

The first part of this study was devoted to check the expression and distribution of HMGb1 in our 3T3 experimental model. As expected, we found that the protein is fairly expressed in these cells and that it is mostly concentrated in the nucleus. HMGb1 does not seem to operate as an autocrine factor in fibroblasts, since it is not exported outside the cell. However, we showed that it behaves as a

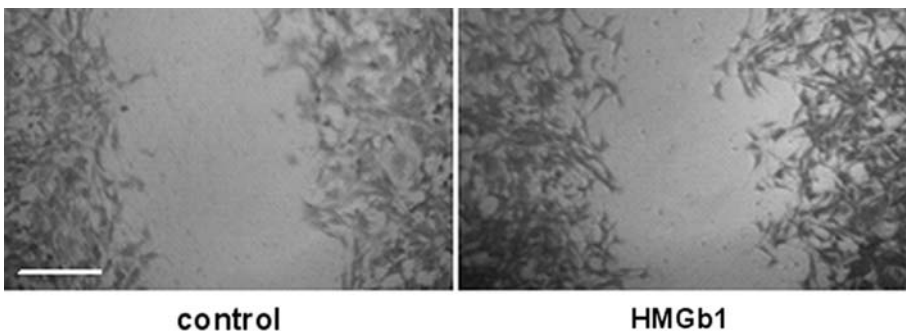
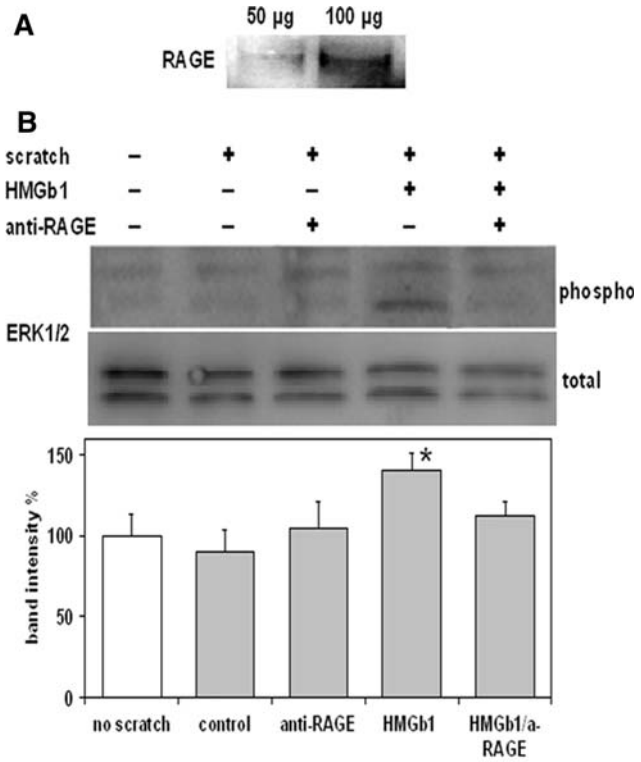



**Fig. 3** Phase-contrast micrographs of blue toluidine-stained, scratched 3T3 monolayers observed at 6 h after wounding in the presence or in the absence of 10-nM HMGb1. *Scale bar*, 200 μm




◀ **Fig. 4** HMGb1-dependent activation of the RAGE-ERK1/2 pathway in 3T3 fibroblasts. **a** Western blot detection of the RAGE protein in total cell lysates separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and labelled with anti-RAGE antibody. **b** Effect of extracellular HMGb1 (10 nM) on the activation of ERK1/2 in 3T3 fibroblast monolayers and inhibitory effect of anti-RAGE receptor-blocking antibody. Confluent 3T3 cells grown in 12-well plates were subjected or not to multiple scratch wounding, exposed or not to 10-nM HMGb1 for 30 min, in the presence or in the absence of 10-µg/ml anti-RAGE antibody, lysed, separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, labelled with anti-phospho-ERK1/2, and then stripped and reprobed with anti-ERK1/2 as internal control. Commercial anti-RAGE receptor-blocking antibody (anti-mouse RAGE, R&D Systems, Minneapolis, MN) was prepared for experiments as follows: 0.5 ml of anti-RAGE was added to prewashed PD 10 desalting column, equilibrated in PBS buffer pH 7.4, and allowed to enter the gel bed. The column was then washed with 10 ml of PBS buffer and the eluted peak containing the protein was recovered, yielding a final stock solution of 200 µg/ml, as evaluated by a Bradford assay. The graph shows the ratio between the optical density of the bands of the phosphorylated form and the bands of the corresponding total protein. Data are mean ± SD ($n = 3$) expressed as percent of control. * Significantly different from other groups ($P < 0.01$, Tukey's test). **c** Inhibitory effect of the MEK1/2 inhibitor PD98059 on the ERK1/2 activation induced by HMGb1. Cells were exposed to 10-nM HMGb1 for 6 h in the presence or in the absence of 10-µM PD98059 and then analysed as above. Data on graph as above

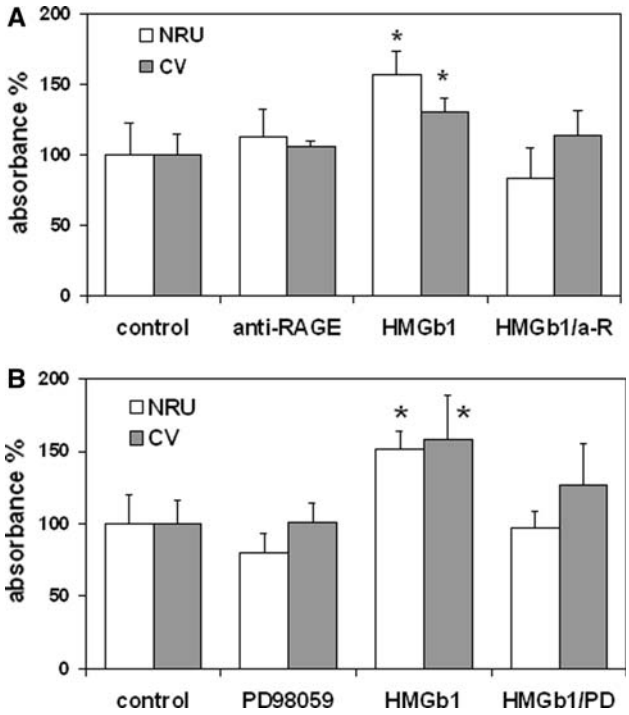



**Fig. 5** Effect of 10-nM HMGb1 on cell viability in the presence or in the absence of 10-µg/ml anti-RAGE receptor-blocking antibody (**a**) or 10-µM PD98059 (**b**), as determined by the NRU and CV assays. Data are mean absorbances ± SD ($n = 8$) expressed as percent of control. For each series, * significantly different from other groups ($P < 0.01$, Tukey's test)

cytokine when it is supplied extracellularly. This was demonstrated by the stimulation of cell proliferation in quiescent 3T3 cells induced by exogenously supplied HMGb1 in nanomolar amounts. Such a result is a confirmation of previous data concerning different cell types, including the same 3T3 fibroblasts [27], the WI-38 human lung fibroblast cell line [28], and HaCaT keratinocytes [14]. Moreover, our finding that HMGb1 exerts a chemoattractant activity on 3T3 cells is in line with the chemotactic response to HMGb1 reported for fibroblasts [29],

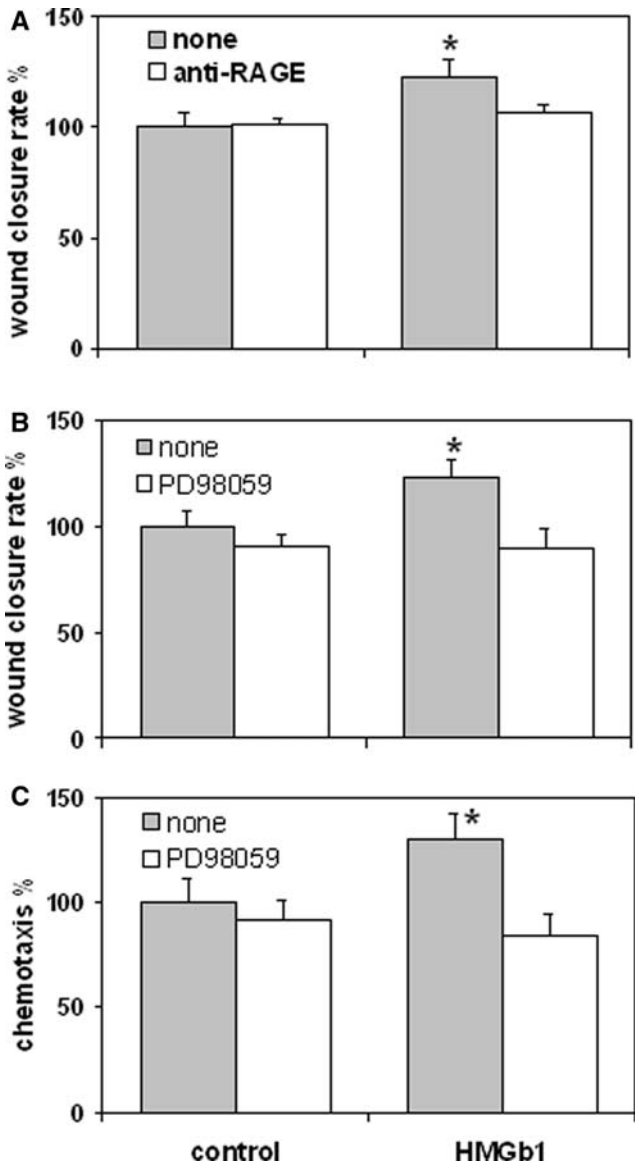



**Fig. 6** Effect of 10-nM HMGb1 on wound closure rates in the presence or absence of 10-μg/ml anti-RAGE receptor-blocking antibody (**a**) or 10-μM PD98059 (**b**). Wound closure rates after scratch wounding of 3T3 monolayers were calculated as the difference between wound width at 0 and 6 h (see "Methods" for further details). Data are means ± SD ($n = 24$) expressed as percent of control. For each series, * significantly different from other groups ($P < 0.01$, Tukey's test). **c** Inhibitory effect of 10-μM PD98059 on the chemotactic attraction exerted by 10-nM HMGb1 on 3T3 cells, evaluated after 6 h incubation using a transwell chemotaxis assay (see "Methods"). Data are mean absorbances ± SD ($n = 5$) expressed as percent of control. Statistics as above

keratinocytes [14], smooth muscle cells [30], and dendritic cells [31]. Cell proliferation and motility are two key elements of tissue repair processes, and therefore, their induction could explain the ability of HMGb1 to promote the scratch wound healing of 3T3 cells, similarly to what reported for keratinocytes [14].

Besides showing the activity of HMGb1 on 3T3 wound healing, we have also demonstrated that the mechanism of action depends on the activation of RAGE–ERK1/2 pathway. Different kinds of evidence argue for the involvement of this pathway. First, HMGb1 was able to induce ERK1/2 phosphorylation. Second, it was possible to block such an effect by acting on either the RAGE receptor with an anti-RAGE antibody, or on the ERK1/2 activator MEK1/2, using its selective inhibitor PD98058. Third, the above inhibitors prevented the effects of HMGb1 on cell proliferation and wound healing. Although we cannot exclude that in 3T3 fibroblasts, HMGb1 could signal through other kinds of receptors, such as toll-like receptor Tlr4 and Tlr2 [32], our data demonstrate that RAGE is deeply involved in the protein effects on cell proliferation, migration, and wound healing. These results are quite consistent with previous findings showing that HMGb1 induces migratory responses mediated by the RAGE receptor in rat smooth muscle cells [6] and human dendritic cells [31].

Our findings on the scratch wound healing of 3T3 fibroblasts provide evidence of a novel activity of HMGb1 related to its cytokine role. However, the physiological meaning of this property in cutaneous wound healing remains to be ascertained. In animal models, the topical application of HMGb1 to diabetic mice has been found to accelerate cutaneous wound healing, while the inhibition of HMGb1 signalling in normal mice has been reported to slow wound healing [29]. These results, together with the present and previous data from our laboratory, support the view of a physiological role of HMGb1 in tissue repair. The supposed cytokine role of HMGb1 entails the in vivo occurrence of tissue sources allowing for the accumulation of the protein at wounded sites. We have shown that in 3T3 fibroblasts HMGb1 is a nuclear protein that is not released under normal conditions, while the same has been found in keratinocytes [14]. However, it is conceivable that a passive release by necrotic cells may occur at wounded sites, as suggested by the detection of the protein in the cell medium after experimentally induced cell necrosis. It is also possible that HMGb1 release may occur in vivo from skin cells as the result of their activation by other cytokines, possibly including HMGb1 itself. After its release in the wounded area, HMGb1 could operate the recruitment of different cell types, and influence keratinocyte and fibroblast proliferation and migration. Hence, according to our data, HMGb1 could represent an important element of the healing cascade, and this could be relevant to long-term effects on wound healing and tissue repair.

Cytokines are having currently a limited role in clinical practice for wound management, but there is a growing interest in developing cytokine-based medicaments, especially for the healing of problematic wounds. Even though the only available commercial product proven to be

efficacious in randomized, double-blind studies is platelet-derived growth factor (PDGF), other cytokines are under study in vitro, including transforming growth factor beta (TGF-b), epidermal growth factor (EGF), and IGF-1 [33]. Our findings suggest that HMGb1 could represent a novel, biologically active and easily available factor, which could be clinically manipulated to promote correct tissue healing and regeneration.

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