Activation of A431 human carcinoma cell motility by extracellular high-mobility group box 1 protein and epidermal growth factor stimuli

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HMGB1 (high-mobility group box 1) protein, a pleiotropic cytokine released by several cell types under physiological and pathological conditions, has been identified as a signal molecule active on A431 cells. Although extracellular HMGB1 itself does not trigger any detectable signalling effect on these cells, it induces an increased susceptibility to EGF (epidermal growth factor) stimulation. Specifically, at concentrations of EGF which promote undetectable or limited cell responses, the addition of sub-nanomolar concentrations of HMGB1 potentiates the effect of EGF by specifically activating a downstream pathway that leads to enhanced

cell motility through an increase in Ca^{2+} influx, activation of extracellular-signal-regulated kinase 1/2 and remodelling of the actin cytoskeleton. These results, which identify extracellular HMGB1 as an activator of human tumour cell migration operating in concert with EGF, have important implications in the search for novel strategies to control tumour progression and metastatic invasion.

Key words: cell calcium influx, cytoskeletal reorganization, extracellular-signal-regulated kinase 1/2 activation, high-mobility group box 1 cytokine (HMGB1 cytokine), wound-repair assay.

INTRODUCTION

HMGB1 (high-mobility group box 1) is a conserved eukaryotic protein that does not harbour a secretory signal peptide, but can undergo active cell export by constitutive or inducible mechanisms [1]. Extracellular HMGB1 displays signalling activities on several cell types, such as endothelial and smooth muscle cells, enterocytes, macrophages, monocytes and tumour cells of nervous and haematopoietic origin [2–7]. Specifically, HMGB1 causes autocrine or paracrine pro-inflammatory responses, cell differentiation or cytoskeletal reorganization, depending on the cell phenotype [8–10]. Protein leakage from necrotic endothelial cells can also produce a local increase in the level of extracellular HMGB1, which results in enhanced smooth muscle cell migration, thus suggesting a potential role for this cytokine in vasculopathies [11]. Furthermore, a chemotactic activity of HMGB1 has been also shown to play important roles in inflammation and tumour cell migration [12,13]. Hence HMGB1 has recently been proposed as a member of a new class of protein molecules that show chemokine-like functions (CLFs) [14].

The molecular mechanisms that mediate cell responses to extracellular HMGB1 have not yet been completely characterized, partly on account of the still uncertain role of a proposed cell receptor for this protein. Engagement of the RAGE (receptor for advanced glycation end-products) by HMGB1 has been indicated to be involved in the activation of metastatic potency of primary tumours in mice [13]. Conversely, only a minor role for RAGE has been demonstrated in HMGB1-induced activation of macrophages and microvascular endothelial cells [15,16]. Moreover, in a murine erythroleukaemia cell line, extracellular HMGB1 stimulated erythroid differentiation independently of RAGE [17], leaving open the question of the existence of different cell-binding sites for the HMGB1 cytokine.

In the A431 human epidermoid carcinoma cell, proliferation and migration responses to extracellular stimuli require activation of distinct pathways [18,19]. These cells are therefore considered to be a good model for analysing the biochemical mechanisms of cytokine signalling. In the present study, we demonstrate that, although A431 cells express HMGB1, they do not export appreciable amounts of the cytokine, even when subjected to different secretion-activating agonists. However, exogenously supplied HMGB1 binds to the cell surface and induces EGF (epidermal growth factor) signalling at a concentration of the growth factor that is scarcely effective. HMGB1/EGF combined stimuli result in an increased cell calcium influx and in a more sustained phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1/2). The activation of these intracellular events by HMGB1 culminates in enhanced cell migration in the presence of concentrations of EGF that are sufficient to induce only limited cell motility. Taken together, these observations suggest that HMGB1 may promote human tumour cell invasiveness by potentiating specific EGF signalling cascades.

EXPERIMENTAL

Cell culture and wound-repair assay

A431 cells were obtained from A.T.C.C. (Laboratory for the Government Chemist Promochem) and cultured in Dulbecco's modified Eagle's medium containing 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 4 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FCS (foetal calf serum) (Gibco). Cells were starved for 48 h in the same medium in the absence of FCS. Cell cultures were free of mycoplasma contamination, as established by a routine assay with VenorGeM (Minerva Biolabs). To measure the extent of wound closure, 10^5 A431 cells were

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; ERK 1/2, extracellular-signal-regulated kinase 1/2; FCS, foetal calf serum; GST, glutathione S-transferase; HMGB1, high-mobility group box 1; HS, heparan sulphate; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; RAGE, receptor for advanced glycation end-products; TCA, trichloroacetic acid; TNF α , tumour necrosis factor α ; TRITC, tetramethylrhodamine β -isothiocyanate.

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seeded in 24-well plates, cultured for 24 h and then starved for 48 h. Confluent monolayers were wounded by means of a pipette tip and stimuli were added as specified elsewhere. After 18 h of culture, cells were washed with 20 mM sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl (PBS) and fixed in 4% (w/v) paraformaldehyde for 30 min at 4°C. Following three washes with PBS containing 30 mM glycine, cells were stained with 0.1% (w/v) Toluidine Blue for 15 min at 4°C.

Production of an anti-HMGB1 monoclonal antibody

The antibody was produced by means of the method described previously [9], using a recombinant fusion protein, consisting of GST (glutathione S-transferase) and a 6 kDa polypeptide corresponding to the N-terminus of HMGB1 [20], as an immunogen. A monoclonal IgG1a antibody (Immunotype Mouse Monoclonal Antibody Isotyping kit; Sigma–Aldrich) toward HMGB1 was obtained from the hybridoma cell clone termed BSD1 and was purified from mouse ascitic fluids by gel filtration as described in [9].

Analysis of HMGB1 release from A431 cells and purification of recombinant HMGB1

Semi-confluent A431 cells (2×10^6) were starved for 48 h and subjected to different stimuli for 16 h, followed by 5 min of treatment with 10 µg/ml heparin (Sigma–Aldrich). Conditioned media (10 ml) were then collected, detached cells were removed by centrifugation at 200 g for 10 min, and aliquots of the clear supernatants were used to assay LDH (lactate dehydrogenase) activity [21,22]. To measure the total level of LDH activity and HMGB1, cells were lysed by sonication in 1 ml of ice-cold PBS. Extracellular amounts of HMGB1 were evaluated by the addition of 20 µg/ml BSA to the clear conditioned media and protein precipitation with 10% (v/v) TCA (trichloroacetic acid) [23]. Proteins were then subjected to SDS/PAGE and immunoblotting, using 0.25 µg/ml anti-HMGB1 monoclonal antibody (clone BSD1). Horseradish-peroxidase-conjugated anti-mouse antibody (Santa Cruz Biotechnology) diluted 1:2000 was used as a secondary antibody, and membranes were developed with an ECL[®] (enhanced chemiluminescence) detection system (Amersham Biosciences). Recombinant prokaryotic GST–HMGB1 fusion protein and eukaryotic HMGB1s were obtained and purified as reported previously [9,24].

Confocal microscopy

Cells were cultured and starved on glass coverslips. Following specific treatments, cells were fixed by 15 min of incubation in 10% (v/v) TCA [25] or paraformaldehyde [26] and, where indicated, were permeabilized with 0.1% (v/v) Triton X-100. Non-specific interactions were blocked by 30 min of incubation in PBS containing 5% (v/v) FCS. Fixed cells were then exposed for 16 h at 4°C to the primary antibody, diluted 1:200, and then incubated for 1 h at 4°C with a secondary antibody conjugated to Alexa Fluor[®] 488 (Molecular Probes), diluted 1:500. TRITC (tetramethylrhodamine β -isothiocyanate)-conjugated phalloidin (0.5 µg/ml) was used to visualize F-actin, and propidium iodide (1 µg/ml) (Sigma–Aldrich) was used to stain chromatin. Images were collected by means of a Bio-Rad MRC 1024 confocal microscope attached to a Nikon Diaphot 200 by using a 60× Plan Apo oil-immersion lens with numerical aperture 1.4.

Immunoprecipitations

Recombinant eukaryotic HMGB1 (5 µg) was diluted in 100 µl of 20 mM Hepes buffer, pH 7.4, containing 0.14 M NaCl, 10 µg

of leupeptin (Sigma–Aldrich) and 10 µg/ml aprotinin (Sigma–Aldrich), and was incubated at 4°C for 1 h with 20 µg of anti-HMGB1 monoclonal antibody (clone BSD1) or anti-calpastatin monoclonal antibody (clone 35.23) [27]. Protein G–Sepharose (15 µl) was then added and, after 1 h at 4°C, the Sepharose beads were discarded and the supernatants were used to evaluate wound-repair activity on A431 cells.

Immunoblotting

Cells were fixed in 10% (v/v) TCA and subjected to SDS/10% PAGE. The separated proteins were transferred on to nitrocellulose membranes (Bio-Rad) [26]. Membranes were blocked in PBS containing 5% (w/v) BSA, 0.1% (v/v) Tween 20 and 1% (v/v) poly(ethylene glycol) 4000, and were incubated with one of the following antibodies for 1 h at 4°C: anti-phospho-EGFR (EGF receptor), 1:2000; anti-phospho-ERK1/2, 1:4000; anti-ERK1/2, 1:4000; anti-phospho-p38, 1:2000; anti-p38, 1:4000; anti-phospho-c-Jun (Ser⁶³), 1:1000; anti-c-Jun, 1:2000 (Cell Signaling Technology) or anti-actin, 1:2000 (Santa Cruz Biotechnology). Immunoreactivity was revealed as described in [25], and signals were quantified by means of a CS9000 dual-wavelength flying-spot scanner (Shimadzu Corporation).

Cell Ca²⁺ influx

A431 cells were grown to semi-confluence in 24-well plates. Following 48 h of starvation, cells were washed and incubated at 37°C in an assay buffer containing 25 mM Hepes, pH 7.4, 0.11 M NaCl, 5 mM KCl, 1 mM MgSO₄, 25 mM glucose and 0.1 mM CaCl₂. Cytokines and 3.3 µCi of ⁴⁵CaCl₂ (Amersham Biosciences) were finally added, and cells were incubated in 0.5 ml (final volume) at 37°C. The metal ion fluxes were blocked with 2 ml of ice-cold blocking buffer (10 mM Hepes buffer, pH 7.4, containing 0.1 M MgCl₂ and 1 mM EGTA). Parallel control experiments were carried out in the presence of 200 µM LaCl₃ as a calcium antagonist and inhibitor of calcium flux [28]. Wells were then washed twice with 2 ml of blocking buffer, and the amount of cell-associated ⁴⁵Ca²⁺ was measured as described previously [28]. Values were normalized for the protein content in each well [29].

Binding of ¹²⁵I-labelled HMGB1 to A431 cells

Eukaryotic recombinant HMGB1 and prokaryotic GST–HMGB1 were labelled with Na¹²⁵I as described previously [5], and their specific radioactivities were 232 000 c.p.m./pmol and 486 000 c.p.m./pmol respectively. Starved A431 cells were incubated with different concentrations of radiolabelled HMGB1 for 15 min at 4°C in 10 mM Hepes buffer, pH 7.4, containing 10 mM glucose, 0.14 M NaCl, 5 mM KCl and 1 mM CaCl₂. Cells were then washed twice with 1 ml of assay buffer and solubilized in 0.2 ml of 0.5 M NaOH for the counting of cell-bound radioactivity. Non-specific binding was determined in parallel experiments in the presence of 100-fold excess of unlabelled protein and was approx. 30% of the total bound radioactivity. Where indicated, the binding assay was carried out using radiolabelled HMGB1 pre-treated with a 100-fold molar excess of heparin. Values were normalized for the protein content present in each well [29]. Saturation binding curve analyses and Scatchard plots were performed using GraphPad Prism version 4.00.

Digestion of HMGB1 with trypsin

HMGB1 (10 µg in 50 mM sodium borate buffer, pH 8.0) was incubated with 0.5 µg of trypsin (Sigma–Aldrich) in a final volume of 100 µl. After 1 h at 37°C, the reaction was stopped with 1 µg of soybean trypsin inhibitor (Sigma–Aldrich), and

Figure 1 Localization and export of endogenous HMGB1 in A431 cells

(A) Starved cells were fixed with 10% (v/v) TCA and permeabilized in 0.1% (v/v) Triton X-100. HMGB1 and chromatin staining were carried out as specified in the Experimental section. (B) Starved cells (2×10^6) were cultured for 16 h in the presence of the indicated stimuli. The extracellular media were then analysed for HMGB1 and LDH activity. Numbers refer to a densitometric quantification of each signal expressed as percent of total cell amounts. Top row, levels of extracellular HMGB1 in the conditioned media of control cells (CTRL) and of cells stimulated with 15 nM EGF, 5 nM TNF α , 100 ng/ml PMA and 20 ng/ml LPS. Middle row, LDH activity, measured on cell culture media, is expressed as a percentage of total cell activity recovered following cell stimulation, as indicated in the top row. Bottom row, cells were stimulated as in the top row in the presence of 500 pM HMGB1. The leftmost lane contains 100 ng of HMGB1.

aliquots of 0.125 μ l were added to 1 ml cultures of wounded A431 cells. As a control, a parallel reaction was carried out in the absence of HMGB1, and aliquots were used in the wound-repair assay.

RESULTS

Localization of HMGB1 in A431 cells

HMGB1 expressed by A431 cells was almost completely confined to the nuclear compartment (Figure 1A). To determine whether these cells were able to release HMGB1 extracellularly, they were stimulated for 16 h with activators of protein export, and the amount of HMGB1 recovered in the cell culture media was evaluated by Western blotting. As shown in Figure 1(B) (top row), the amount of HMGB1 detected outside cells coincided with a non-specific cell leakage, since it proved to be proportional to LDH activity recovered in the conditioned media. Parallel control experiments were also carried out to determine whether extracellular HMGB1 could be lowered by cell internalization or proteolytic degradation in these conditions. Purified HMGB1 was therefore added to each cell culture, together with a specific stimulus, and the amounts of the protein molecule recovered extracellularly after 16 h of culture were measured. As reported in Figure 1(B) (bottom row), exogenously supplied HMGB1 was almost completely recovered as an intact 28 kDa protein molecule. Thus A431 cells express nuclear HMGB1, but are not able to release

Figure 2 Binding of 125 I-labelled HMGB1 to A431 cells

Starved subconfluent cells, cultured in 24-well plates, were incubated for 15 min at 5 $^{\circ}$ C in the presence of the indicated concentrations of 125 I-labelled HMGB1 (A) or 125 I-labelled GST-HMGB1 (B). Total and non-specific protein binding were calculated as detailed in the Experimental section. The radiolabelled protein was added untreated ($\bullet$) or following pre-incubation with heparin ($\circ$). Values represent the means $\pm$ S.D. for three separate assays. Insets show Scatchard plot analyses of the saturation binding data.

this protein molecule outside when stimulated with EGF, LPS (lipopolysaccharide), PMA or TNF α (tumour necrosis factor α).

Binding of HMGB1 to A431 cells

To determine whether A431 cells interact with extracellularly added HMGB1, we performed a radioligand binding assay, using a 125 I-labelled protein. Both eukaryotic recombinant HMGB1 (Figure 2A) and prokaryotic fusion protein GST-HMGB1 (Figure 2B) interacted with low-affinity non-saturable binding sites and high-affinity saturable binding sites. The latter interaction showed a K_d of 0.65 ± 0.13 nM for HMGB1 and 0.95 ± 0.19 nM for GST-HMGB1, and indicated the presence of approx. 20000 high-affinity HMGB1-binding sites/cell. In all cases HMGB1 and GST-HMGB1 interactions with A431 cells were displaced by pre-treating the radiolabelled proteins with an excess of heparin. Similar results were obtained on measuring the binding of radiolabelled HMGB1 to immobilized plasma membranes isolated from A431 cells (results not shown). These findings indicate that, in these cells, both high- and low-affinity binding sites for HMGB1 are represented almost exclusively by HS (heparan sulphate) proteoglycans.

The interaction of extracellular HMGB1 with A431 cells was also evaluated by means of an immunofluorescence approach

Figure 3 Immunofluorescence analysis of HMGB1–A431 cells interaction

Cells were cultured and starved on coverslips, treated for 5 min at 5 °C with 10 nM GST–HMGB1 in the absence (**A**) or the presence (**B**) of 1.5 nM EGF, or with 10 nM GST–HMGB1 pre-incubated with 10 µg/ml heparin (**C**). Cells were fixed with 10% (v/v) TCA and immunostained with a monoclonal anti-HMGB1 antibody. Nuclei were stained with propidium iodide. Scale bar, 10 µm. The micrographs are representative of data obtained on analysing at least 20 random non-overlapping fields.

using GST–HMGB1. As shown in Figure 3, apical cell surfaces displayed HMGB1 immunoreactivity in both unstimulated (Figure 3A) and EGF-stimulated (Figure 3B) cells. Under the latter conditions, the protruding lamellipodia also proved positive to HMGB1 staining. Alternative cell fixations with TCA or paraformaldehyde methods yielded similar results. This immunostaining was eliminated by pre-incubation of the protein molecule with heparin (Figure 3C).

These observations indicate that HMGB1 and GST–HMGB1 fusion protein possess comparable A431 cell-binding properties and that high- and low-affinity binding of both radiolabelled and native HMGB1 protein involves HS moieties of A431 cell-surface molecules.

Activation of cell responses to extracellular HMGB1

A431 cells overexpress the EGFR and are highly sensitive to EGF [18]. In order to ascertain whether HMGB1 and EGF activate distinct signal transduction cascades, we analysed the effect of the single or combined addition of the two proteins on the phosphorylation of EGFR, and of different members of the MAPK (mitogen-activated protein kinase) family.

As shown in Figure 4(A), HMGB1 itself did not induce any detectable phosphorylation of EGFR and ERK1/2. In the presence of 0.15 nM EGF, HMGB1 did not significantly modify the extent of phosphorylation of EGFR, but produced a dose-dependent increase in the extent of activation of ERK1/2, with a maximal efficiency at 500 pM HMGB1. A time-course analysis (Figure 4B) indicated that the combined HMGB1/EGF stimulus resulted in a 2–3-fold increased in the amount of phospho-ERK1/2 in the first 1 h, in comparison with cells stimulated with EGF alone.

Conversely (Figure 5), HMGB1 in combination with EGF did not modify either the level of phosphorylation or the total levels of c-Jun and p38 MAPK, which proved similar to those measured in the absence of HMGB1. These findings indicate that extracellular HMGB1 promotes a specific activation of the ERK1/2 pathway in A431 cells induced with EGF.

The effect of HMGB1 was studied further by analysing possible modifications in the influx of Ca^{2+} across the plasma membrane, which is known to occur as an early event in these cells when stimulated with appropriate concentrations of EGF. We observed that HMGB1 alone, when added to A431 cell cultures at concentrations up to 20 nM, did not affect the rate of Ca^{2+} influx (results not shown). We then evaluated the rate and extent of Ca^{2+} influx in

cells treated with increasing concentrations of EGF in the absence or presence of sub-nanomolar concentrations of HMGB1. As shown in Table 1, no increase in Ca^{2+} influx was observed at a concentration of 0.15 nM EGF, which is sufficient to induce a mitogenic effect. However, the addition of sub-nanomolar concentrations of HMGB1 evoked a significant increase in Ca^{2+} influx, which reached an extent comparable with that obtained by adding EGF alone at concentrations two orders of magnitude higher. Potentiation of this cell response to EGF by HMGB1 was dose-dependent and saturation was reached at a concentration of 500 pM HMGB1, which was also found to be optimal in stimulating ERK1/2 activation. Thus extracellularly supplied HMGB1 promotes a significant increase in Ca^{2+} influx in the presence of low concentrations of EGF, which are ineffective in increasing Ca^{2+} influx.

Functional roles of extracellular HMGB1

The finding that HMGB1 promotes ERK1/2 phosphorylation and cell calcium influx suggested that these events could be correlated with the activation of downstream effectors that control cell motility [30]. We therefore explored the effect of HMGB1 on A431 cell wound-repair capacity. As shown in Figure 6(A), recombinant HMGB1 obtained from prokaryotic or eukaryotic sources did not stimulate A431 cell motility. However, in combination with EGF, it enhanced wound-repair ability in comparison with cells stimulated with the same concentration of EGF alone. In this assay too, the amount of recombinant HMGB1 able to promote the highest wound-repair activity was 400–500 pM (results not shown), and the activating effect was lost following pre-treatment of HMGB1 with an excess of heparin. Moreover, the MEK (MAPK/ERK kinase) inhibitor PD98059 blocked cell motility, even in the presence of optimal concentrations of HMGB1/EGF, indicating that A431 cell migration is strictly dependent on ERK1/2 activation. The possible involvement of non-protein HMGB1-contaminating agents in this cell response was ruled out because the stimulating activity of purified HMGB1 preparations was lost when samples were subjected to trypsin digestion or immunoprecipitated with an anti-HMGB1 monoclonal antibody (Figure 6B). Moreover, in separate experiments, we established that the rapid wound closure obtained in the presence of HMGB1 was not due to an increase in the rate of cell proliferation, which proved to be unaffected in these conditions.

After 16 h of exposure of wounded cell monolayers to different stimuli, actin cytoskeleton organization of cells localized at the

Figure 4 Effect of extracellular HMGB1 and EGF on activation of EGFR and ERK1/2

Western blotting was carried out using 5×10^4 cells. (A) Starved A431 cells were untreated (CTRL) or cultured for 15 min in the presence of the indicated stimuli. EGF was 0.15 nM. Phospho-EGFR (p-EGFR) and phospho-ERK1/2 (p-ERK1/2) signals were quantified as specified in the Experimental section, and numbers under the immunoreactive bands refer to their intensity in relation to that detected in the presence of EGF alone. Blots are representative of three separate experiments. (B) Time course analysis and quantification of p-ERK1/2 immunoreactive signals from control cells (CTRL), cells treated with 0.15 nM EGF plus 500 pM HMGB1, 0.15 nM EGF or 500 pM HMGB1. Values represent the means $\pm$ S.D. for three separate assays. The inset shows a representative immunoblot obtained with EGF and HMGB1/EGF stimulated cells. The total level of ERK1/2 is also shown.

Figure 5 Effect of HMGB1 on phosphorylation of members of MAPK pathways

Starved A431 cells were stimulated with 0.15 nM EGF in the absence or the presence of 500 pM HMGB1. At the indicated times, Western blotting was carried out using 5×10^4 cells and the levels of phospho-c-Jun (p-c-Jun), c-Jun, phospho-p38 (p-p38) and p38 were evaluated. Similar results were obtained in three separate experiments.

Table 1 Effect of HMGB1 and EGF on cell Ca^{2+} influx

Starved A431 cells were incubated for 5 min with $^{45}\text{Ca}^{2+}$ in the presence of the indicated additions. The amount of Ca^{2+} accumulated in cells was determined as detailed in the Experimental section. Results shown are the means $\pm$ S.D. for three experiments.

Addition (nM)		Ca^{2+} influx (pmol/ 10^6 cells)
EGF	HMGB1	
–	–	2.2 ± 0.2
–	0.1	2.0 ± 0.3
–	0.5	2.2 ± 0.1
–	1.0	2.0 ± 0.2
0.15	–	2.1 ± 0.2
1.50	–	2.2 ± 0.3
15.00	–	3.8 ± 0.5
0.15	0.1	2.5 ± 0.4
0.15	0.5	3.5 ± 0.4
0.15	1.0	3.4 ± 0.3

wound edge was also evaluated by means of phalloidin–TRITC staining. As shown in Figure 7, in the absence of any addition or in the presence of HMGB1 alone, cells maintained polygonal shapes, with peripheral actin particularly polarized at the apical cell surfaces. Cell treatment with 0.15 nM EGF produced the appearance of short stress fibres, but most cells still retained peripheral actin. In cells exposed to HMGB1/EGF coupled stimuli, the cytoskeletal network showed a more profound reorganization, with an almost complete loss of cortical actin filaments. This result indicates that HMGB1 operates as a stimulator of EGF-induced cell migration, activating intracellular cascades that control the assembly of the cytoskeletal architecture and the turnover of stress fibres.

DISCUSSION

One of the cytokine functions described for HMGB1 consists of the activation of cell motility in normal and pathological conditions, thereby promoting tissue development, regeneration and remodelling [11,31,32]. Moreover, the signal released by extracellular HMGB1 has been postulated to increase the invasiveness of some mammalian transformed phenotypes both *in vitro* and *in vivo* [13]. In the present study, we investigated whether similar effects were induced by HMGB1 on A431 human epidermoid carcinoma cell line. We observed that, in these cells, HMGB1 does not operate as an autocrine factor, since it is not exported outside the cell either in resting conditions or upon exposure to different factors which activate cell protein release. However, when supplied extracellularly, HMGB1 potentiates short- and long-term cell responses to EGF. No direct interaction between HMGB1 and EGFR is likely to occur, since this receptor has not been found to be co-localized with extracellularly bound HMGB1 on the plasma membrane (results not shown). Moreover, although A431 cells express low levels of the putative HMGB1 receptor, termed RAGE, its involvement in the signalling activity of HMGB1 identified in the present study can be excluded on the basis of preliminary results indicating that transfected A431 cells, which overexpress dominant-negative or dominant-positive RAGE, become insensitive to the co-addition of HMGB1 and EGF, probably owing to an unproductive binding of HMGB1 to RAGE (M. Passalacqua and S. Ledda, unpublished work). The involvement of high-affinity binding sites for extracellular HMGB1, other than RAGE, has been shown to be responsible for the HMGB1-differentiation-stimulating activity identified in a murine erythroleukaemia cell line [17] and has also been

Figure 6 Effect of HMGB1 on wound repair

Confluent starved A431 cells were wounded and cultured for 16 h in the presence of the indicated stimuli. Cells were then fixed with 4% (w/v) paraformaldehyde and stained with Toluidine Blue. EGF was at 0.15 nM, and recombinant eukaryotic HMGB1 (eHMGB1) or prokaryotic GST-HMGB1 (pHMGB1) were at 500 pM. Images are representative of three independent experiments. **(A)** PD98059 was 25 μ M; where indicated, HMGB1 was pre-treated with heparin at a 100-fold molar excess. **(B)** To rule out the interference of non-protein contaminants in HMGB1 preparations, samples of eHMGB1 were alternatively digested with trypsin (digested HMGB1), immunoprecipitated with an anti-HMGB1 monoclonal antibody (IP antiHMGB1) or treated with an irrelevant anti-calpastatin monoclonal antibody (IP antiCST) as specified in the Experimental section. EGF + digestion buffer and EGF + trypsin refer to control assays carried out in the absence of HMGB1.

suggested by a phage display approach [33]. Moreover, HMGB1 is a HS-binding protein that is able to interact with the trans-membrane HS-proteoglycans, termed syndecans, expressed on all adherent cells [34]. This observation suggests that HMGB1/syndecan-regulated receptor signalling [35] might be involved in the A431 cell response, as also indicated by the loss of HMGB1 effects following pre-treatment with heparin. Thus we have provided evidence for an interaction between HMGB1 and A431 cells, although the nature of this interaction is as yet unclear.

Figure 7 Changes in the cytoskeletal organization of A431 cells stimulated with EGF and HMGB1

Monolayers of starved A431 cells were wounded and left unstimulated (CTRL) or stimulated with 500 pM HMGB1, 0.15 nM EGF or 0.15 nM EGF plus 500 pM HMGB1. After 16 h of culture, cells were fixed and stained with phalloidin-TRITC. Micrographs are representative of three independent experiments. Scale bar, 10 μ m.

We observed that, in spite of the lack of a direct effect, HMGB1 binding results in a significant enhancement of the signal released by the single addition of EGF. Thus, in the presence of EGF and HMGB1, both at sub-nanomolar concentrations, the sequential pathway activated by downstream effectors of EGFR and involving ERK1/2, is more markedly activated than in the presence of EGF alone. Activation accounts for a 3-fold increase in the extent of phosphorylation of this kinase. Since the combined action of HMGB1/EGF does not affect the level of phosphorylation of other MAPKs, which have also been shown to be stimulated following EGFR activation [36,37], it can be concluded that the stimulation of a single downstream pathway is indicative of a rather strict specificity in the action of HMGB1.

Previous reports have indicated that activation of phospholipase A₂, followed by the generation of second messengers involved in the activation of voltage-dependent and -independent Ca²⁺ channels, is promoted by downstream effectors of the EGFR, such as ERK1/2 [38,39]. In agreement with these reports, we observed that sub-nanomolar concentrations of EGF and HMGB1 induced a significant rise in Ca²⁺ influx across the plasma membrane of A431 cells. Similar influxes can be obtained by EGF alone only if its concentration is increased by two orders of magnitude.

Moreover, in accordance with the known functional properties of ERK1/2 as a feedback modulator of EGFR activity in the regulation of actin cytoskeleton remodelling and cell migration [30], under our experimental conditions, the increased phosphorylation of ERK1/2 resulted in a more extensive rearrangement of the actin network and more rapid cell locomotion.

The hypothesis of a specific role of HMGB1 in EGF signalling is supported by preliminary observations that indicate that both

short- and long-term effects elicited by TNF α on A431 cells are not affected by HMGB1 (M. Passalacqua and S. Ledda, unpublished work).

Taken together, these results suggest that, by potentiating the effects induced by low concentrations of EGF, extracellular HMGB1 may facilitate the metastatic invasiveness of human tumour cells that overexpress EGFR.

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