Stimulation of erythroleukaemia cell differentiation by extracellular high-mobility group-box protein 1 is independent of the receptor for advanced glycation end-products

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In several cell types the binding of extracellular high-mobility group-box protein 1 (HMGB1) with the receptor for advanced glycation end-products (RAGE) induces cytoskeletal reorganization and cell motility. To establish whether RAGE is also involved in murine erythroleukaemia (MEL) cell differentiation stimulated by HMGB1, we have demonstrated that these cells express a 51 kDa protein identified as RAGE, and then we have produced stable transfectants overexpressing wild-type (wt) RAGE or a dominant negative (dn) RAGE mutant lacking the cytoplasmic domain to analyse the differentiation process in these cells. Several experimental findings indicated that RAGE

was not involved in the MEL cell differentiation programme. This was also supported by the identical stimulatory effect exerted by HMGB1 on both wt- or dn-RAGE transfectants. We have also observed that HMGB1 binds a 65 kDa protein on the surface of MEL cells, supporting the hypothesis that alternative targets of HMGB1 are expressed on the MEL cell membrane and may be involved as mediators of its signalling.

Key words: amphoterin, hexamethylenebisacetamide, protein cross-linking.

INTRODUCTION

High-mobility group-box protein 1 (HMGB1) [1] was originally identified as a chromosomal DNA-binding protein [2], and later shown to also be localized in the extracellular medium of different cell types as a matrix-bound or soluble molecule [3,4]. The export of HMGB1 occurs spontaneously in neurons from the developing central nervous system and in tumour cell lines of nerve origin [5,6], whereas specific stimuli are required to trigger the release of HMGB1 in cells of the haematopoietic system [4,7], in pituitary cells [8] or in type 1 astrocytes [9]. An increased level of HMGB1, attributed to a release from activated macrophages, has been measured in serum of mice treated with endotoxin as well as from septic patients [10], and evidence has been provided to indicate that HMGB1 operates as a late mediator of endotoxin lethality at a systemic level [10]. However, when added to target cell cultures, HMGB1 behaves as a potent signalling molecule stimulating specific responses, such as differentiation, cytoskeletal reorganization, migration or release of proinflammatory cytokines [11–14].

The extracellular release of HMGB1 is an early event in the multi-step process of murine erythroleukaemia (MEL) cell differentiation [4] induced by polar/apolar compounds, such as hexamethylenebisacetamide (HMBA) [15]. The export of HMGB1 and its subsequent interaction with the cell membrane are crucial events in the MEL cell differentiation programme. In fact, it has been shown that the specific removal of HMGB1 from the cell medium of induced cells prevents the acquirement of the committed state [16]. A receptor for HMGB1, named receptor for advanced glycation end-products (RAGE), has been identified in different cells [17]. This multi-ligand protein, belonging to the

immunoglobulin superfamily [18], co-localizes with HMGB1 and mediates its extracellular signals in developing brain, smooth muscle cells and tumour cell lines of nerve origin [14,19,20].

In the present study, we report that MEL cells also express RAGE. In order to evaluate the possible involvement of this receptor in the MEL cell differentiation process triggered by extracellular HMGB1, we have produced cells expressing wild-type (wt) RAGE or a mutated dominant negative (dn) RAGE and measured their sensitivity to the chemical inducer HMBA and extracellular HMGB1. Several experimental findings support the conclusion that RAGE is not a mediator of the cell response to HMGB1 in the MEL cell differentiation pathway. To identify alternative binding sites for HMGB1 on the MEL cell plasma membrane, we have used a chemical cross-linking approach. Covalent binding of HMGB1 to the cleavable photoreactive heterobifunctional cross-linker sulphosuccinimidyl-2-(*p*-azidosalicylamido)-1,3'-dithiopropionate (SASD), labelled with ¹²⁵I, demonstrates that a new putative receptor protein is recognized by extracellular HMGB1 in intact cells. A MEL cell membrane protein having the same molecular mass is also recognized by the HMGB1_{129–138} peptide, shown previously to maintain the differentiation-stimulating activity characteristic of native HMGB1 [21].

EXPERIMENTAL

Materials

Anti-[p44/42 mitogen-activated protein kinase (MAPK)] and anti-(phospho-p44/42 MAPK) antibodies were from Cell Signalling Technology (Beverly, MA, U.S.A.). Anti-[protein kinase C (PKC)- δ] antibody was from Santa Cruz Biotechnology (Santa

Abbreviations used: dn, dominant negative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBA, hexamethylenebisacetamide; HMGB1, high-mobility group-box protein 1; MEL, murine erythroleukaemia; MAPK, mitogen-activated protein kinase; PBST, PBS containing 0.1% Tween 20; PKC, protein kinase C; RAGE, receptor for advanced glycation end-products; RT, reverse transcriptase; SASD, sulphosuccinimidyl-2-(*p*-azidosalicylamido)-1,3'-dithiopropionate; sRAGE, soluble extracellular domain of RAGE; wt, wild-type.

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Cruz, CA, U.S.A.). Human wt-RAGE and dn-RAGE cDNA cloned into the pcDNA3 expression vector [20], the soluble extracellular domain of RAGE (sRAGE; 7.1 $\mu\text{g}/\mu\text{l}$) [19] and an anti-(RAGE) antibody (6.5 $\mu\text{g}/\mu\text{l}$) were generously given by Dr David Stern (Columbia University, New York, NY, U.S.A.). In the dn-RAGE construct, the cDNA fragment spanning nucleotides 1099–1340 in the sequence of wt-RAGE (GenBank® accession number M91211) was deleted to produce a transmembrane RAGE form lacking the cytoplasmic domain (corresponding to amino acids 367–404). BSA (fraction V; Sigma–Aldrich, St. Louis, MO, U.S.A.) was glycosylated as described previously [20]. SASD was from Pierce (New York, NY, U.S.A.). The HMGB1_{129–138} peptide was obtained from Affina Immunotechnik (Berlin, Germany).

Cell culture, reverse transcriptase (RT)-PCR and cell transfection

N23 MEL cells and human neuroblastoma LAN-5 cells were cultured and induced as described previously [9,22]. All cell cultures were free of mycoplasma contamination, as established by a routine assay with a Mycoplasma Detection Kit (Roche Diagnostics, Basel, Switzerland). Total cell RNA was obtained and reverse-transcribed as described previously [23]. N23 MEL and LAN-5 cells were transfected with the pcDNA3 vector coding for wt-RAGE or dn-RAGE by using LIPOFECTAMINE® (Invitrogen, San Diego, CA, U.S.A.), according to the manufacturer's instructions. G-418 (0.8 mg/ml; Invitrogen) was added to the culture medium 48 h after transfection to select stably transfected clones. Cells were then grown in the presence of 0.1 mg/ml G-418. Expression of RAGE was assayed by RT-PCR on a PerkinElmer Thermal Cycler (Indianapolis, IN, U.S.A.). Primers used were 5'-GCCTAATGAGAAGGAGT-ATC-3', corresponding to nucleotides 495–516 in the human RAGE cDNA sequence (GenBank® accession number M91211), and 5'-GCTCTAGCATTTAGGTGAC-3', complementary to nucleotides 1006–1024 in the pcDNA vector (Invitrogen), or 5'-TGCTCTATGGGGAGCTGTAGC-3' and 5'-CTTGAGGCA-CTTCCTTCTCCA-3', corresponding to nucleotides 47–67 and complementary to nucleotides 1284–1304 respectively, in the mouse RAGE cDNA sequence (GenBank® accession number L33412). Quantitative estimation of the transcripts was carried out by co-amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as an internal control, by using the following pair of primers: 5'-ACGACCCCTTCATTCATTGACC-3' and 5'-TGCTTCACCACCTTCTTG-3' [24]. PCR was carried out using *Taq* DNA polymerase (Invitrogen) and 25 cycles of denaturation (45 s at 94 °C), annealing (45 s at 56 °C for human RAGE and GAPDH cDNAs; 45 s at 66 °C for mouse RAGE cDNA) and extension (45 s at 72 °C).

Purification of HMGB1 and induction of MEL cell differentiation

Eukaryotic recombinant HMGB1 was obtained and purified as described previously [16]. MEL cell differentiation was induced by adding 5 mM HMBA (Sigma–Aldrich) to cell cultures containing 1×10^5 cells/ml. Erythroid differentiation was assayed by benzidine staining [25].

Immunoblot analyses

Parental or transfected MEL cells were subjected to SDS/PAGE on 10 % (w/v) polyacrylamide gels under reducing conditions [26]. Proteins were then transferred on to a nitrocellulose membrane and blots were incubated with anti-(p44/42 MAPK), anti-(phospho-p44/42 MAPK), anti-(PKC- δ) or anti-(RAGE) antibodies [27] diluted 1:4000 in PBS (pH 7.6)

containing 0.1 % Tween 20 (PBST) for 1 h at 22 °C. After five washes with PBST, the blots were incubated with a horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in PBST) or ¹²⁵I-labelled Protein A (Amersham Biosciences, Little Chalfont, Bucks., U.K.). After 1 h at 20 °C, membranes were washed four times with PBST and developed using the ECL® (Amersham Biosciences), or autoradiography, followed by excision of the radioactive bands and quantification of the signal in a γ -counter.

Photoaffinity labelling of MEL cells

SASD was labelled with Na¹²⁵I (Amersham Biosciences) and linked to HMGB1 or the HMGB1_{129–138} peptide [21], following the procedure described by Sorensen et al. [28]. MEL cells were washed with buffer A [10 mM Hepes (pH 7.4) containing 10 mM glucose, 0.14 M NaCl, 5 mM KCl and 1 % (w/v) polyvinylpyrrolidone]. Finally, 5×10^7 cells were diluted in 1 ml of buffer A containing 0.3 mg of bacitracin (Sigma–Aldrich), 50 pmol of ¹²⁵I-SASD-linked HMGB1 or HMGB1_{129–138} peptide (1.8×10^6 c.p.m.) and, where indicated, 5 nmol of HMGB1. After 90 min at 37 °C in the dark, cells were exposed to UV irradiation [28] and then washed twice with 1 ml of buffer A. Samples of 5×10^5 cells were diluted in sample buffer in the absence or presence of the reducing agent 2-mercaptoethanol (0.2 %), and subjected to SDS/PAGE [8 % (w/v) gels]. The presence of radioactive bands was evaluated on gels with a Cyclone Storage Phosphor System equipped with OpiQuant image analysis software (Canberra Packard, Meriden, CT, U.S.A.). Where indicated, the gel lanes were subjected to immunoblot analysis to reveal the electrophoretic mobility of RAGE.

Evaluation of MEL cell membrane proteins bound by immobilized HMGB1_{129–138} peptide

The HMGB1_{129–138} peptide (11 mg) was coupled to 1 ml of CNBr-activated Sepharose 4B (Amersham Biosciences), according to the manufacturer's instructions. MEL cell plasma membranes were isolated from 2×10^8 cells collected after 18 h of induction with HMBA. Cells were suspended in 2.5 ml of 0.25 M sucrose containing 2 mM PMSF and 100 $\mu\text{g}/\text{ml}$ leupeptin, and homogenized at 0 °C in a 10 ml Potter homogenizer with 20 down-and-up strokes. Unbroken cells were pelleted by centrifugation for 5 min at 800 *g* and discarded. The supernatant was fractionated as described previously [29]. On the basis of the specific activity of 5'-nucleotidase [30], the purity of plasma membrane preparation was 94–96 %. Plasma membranes were then solubilized in PBS containing 0.1 % octyl glucoside, and 200 μg of protein was precleared by incubation with 50 μl of Sepharose 4B at 20 °C for 15 min, followed by incubation with 50 μl of Sepharose-immobilized HMGB1_{129–138} peptide. After 1 h at 20 °C, the beads were washed extensively with PBST and bound proteins were labelled with Na¹²⁵I [31]. Free iodine was removed by dialysis against PBS, and the beads were boiled in the SDS/PAGE sample buffer.

RESULTS

Identification of RAGE in MEL cells

Following RT-PCR and immunoblotting, we have demonstrated the presence of both RAGE transcript and protein in MEL cells. As shown in Figure 1(A), an amplification product, having the expected size of approx. 1250 bp, was identified in the cDNA obtained from these cells by using two primers localized between nucleotides 47 and 1304 of the mouse RAGE translated sequence. The identity of this DNA fragment with RAGE was also

Figure 1 Expression of RAGE in MEL cells

(A) Lane 1, molecular-size markers (50–2000 bp); lane 2, expression of RAGE transcript in MEL cells, evaluated by RT-PCR using 0.4 μ g of total RNA. (B) Expression of RAGE protein determined by Western blotting using 100 μ g of total MEL cell protein. The membrane was probed with the anti-(RAGE) antibody in the absence (lane 1) or the presence (lane 2) of 20 μ g/ml sRAGE added during the incubation with primary antibody.

Figure 2 MEL cell transfection with wt- and dn-RAGE constructs

Parental MEL cells (lanes 1 and 4), two wt-RAGE stable transfectants (lanes 2 and 3) and two dn-RAGE stable transfectants (lanes 5 and 6) were analysed for the presence of RAGE transcript (top panel; RT-PCR) and protein (bottom panel; WB, Western blotting). RT-PCR was carried out with primers specific for the human pcDNA3-RAGE construct using 0.4 μ g of total RNA. Levels of GAPDH (middle panel) were used as an internal control to assess loading. Lane M, molecular-size markers (100–3000 bp). Total cell proteins (50 μ g) were used for immunoblotting with the anti-(RAGE) antibody.

confirmed by sequence analysis (results not shown). Western blotting of MEL cell lysates with an anti-(RAGE) antibody identified a single protein, with a molecular mass of 51 000 Da (Figure 1B, lane 1). The band was not detected when the primary antibody was preincubated with an excess of sRAGE for 2 h at 4 °C (Figure 1B, lane 2).

Effect of wt- and dn-RAGE on MEL cell differentiation

In order to determine if RAGE plays a role in erythroid differentiation, MEL cells were transfected with the pcDNA3 vector containing the coding sequence for wt- or dn-RAGE. Stably transfected cell clones expressing different amounts of wt- or dn-RAGE transcript and protein were obtained (Figure 2)

Figure 3 Effect of wt- and dn-RAGE expression on MEL cell differentiation

Parental MEL cells ($\blacktriangledown$), a wt-RAGE stable transfectant ($\blacklozenge$), corresponding to that shown in Figure 2, lane 3, and a dn-RAGE stable transfectant ($\blacksquare$), corresponding to that shown in Figure 2, lane 6, were induced with 5 mM HMBA. At the indicated times, cells were stained with benzidine. Data are the means $\pm$ S.D. of four independent experiments.

Figure 4 Effect of wt- and dn-RAGE expression on neuroblastoma cells

LAN-5 cells were stably transfected with wt-RAGE (A and B) or dn-RAGE (C and D). Cells were then cultured on uncoated (A and C) or 50 pmol/cm² of HMGB1-coated (B and D) wells. After 72 h, cells were fixed and stained with 0.1% Toluidine Blue. Scale, 1 cm = 18 μ m.

and showed a cell growth rate similar to that of parental cells (results not shown).

Two of these transfectants, expressing the highest level of wt- and dn-RAGE respectively, were induced with HMBA. As shown in Figure 3, the only difference observed in the time course of differentiation was a slight increase in the latent period preceding the appearance of the differentiated phenotype in the wt-RAGE-expressing cell clone, as compared with parental cells. Hence, overexpression of a functionally active (wt-RAGE) or

Figure 5 Inactivation of MAPK in parental, wt- and dn-RAGE-transfected MEL cells induced with HMBA

MEL cells were induced and, at the indicated times, samples (2×10^5 cells) were subjected to SDS/PAGE on 10% (w/v) polyacrylamide gels. Proteins were then transferred on to nitrocellulose membranes, which were probed with anti-(p44/42 MAPK) antibody and reprobed with anti-(phospho-p44/42 MAPK) antibody.

inactive RAGE (dn-RAGE), still able to bind extracellular HMGB1, but lacking the ability to mediate signal transduction, does not modify the kinetics of erythroid differentiation in MEL cells induced with HMBA. To establish if the dn-RAGE construct blocked the neurite extension triggered by extracellular HMGB1 in neuroblastoma cells [9], human LAN-5 cells were stably transfected with wt- or dn-RAGE constructs. The expression of recombinant RAGE was confirmed by Western blotting (results not shown), and both wt- and dn-RAGE neuroblastoma transfectants were cultured on uncoated or HMGB1-coated wells. As shown in Figure 4, the outgrowth of neurite processes was stimulated by HMGB1 in wt-RAGE transfected cells, but was largely reduced in dn-RAGE transfectants, confirming that this construct is able to block RAGE signalling in a neuroblastoma cell model which responds to HMGB1 in a RAGE-dependent manner.

Inactivation of p44/42 MAPK in induced MEL cells

The Ras/Raf/MEK ('MAPK/extracellular signal-regulated kinase kinase') pathway is involved in the control of cell proliferation as well as of survival or terminal differentiation induced by extracellular stimuli [21]. This kinase cascade has also been described as a downstream effector of RAGE signalling. Hence, we have initially evaluated whether MEL cell induction with HMBA promoted changes in this pathway. As shown in Figure 5, the level of p44/42 MAPK was unchanged over the first 6 h following induction of parental cells. Conversely, phosphorylated p44/42 MAPK, the active form of the enzyme, which undergoes a nuclear translocation [32], was rapidly down-regulated within the first 2 h of stimulation. These results demonstrate for the first time that HMBA stimulates a rapid inactivation of MAPK during the latent period of the differentiation process, before cell acquirement of the committed phenotype. An identical change in the extent and kinetics of MAPK dephosphorylation was detectable in wt- and dn-RAGE transfect-

Figure 6 Effect of extracellular HMGB1 on differentiation of wt- and dn-RAGE-transfected cells

Transfectants overexpressing wt-RAGE (■, □) or dn-RAGE (▲, △) were induced with 5 mM HMBA in the absence (■, ▲) or the presence (□, △) of 500 pM recombinant HMGB1. At the indicated times, cells were stained with benzidine. Data are the means $\pm$ S.D. of four independent experiments.

tants, also indicating that this early step of erythroid differentiation is not under RAGE modulation.

RAGE–ligand interaction and MEL cell differentiation

In our experimental conditions, the only source of extracellular HMGB1 was that released by induced cells. The amount of HMGB1 detectable in the culture medium of parental and wt- or dn-RAGE-transfected cells was very similar, reaching a concentration of 10 pM after 12 h of incubation with the inducer. The extracellular amount of HMGB1 then remained almost constant for an additional 24 h. To establish if this low concentration of extracellular HMGB1 was the limiting factor in preventing the formation of the HMGB1–RAGE complex, we induced wt- and dn-RAGE transfectants by adding a 50 times higher amount of HMGB1 (500 pM) to the incubation mixture. As shown in Figure 6, the high amount of HMGB1 added to the induction mixture stimulated cell differentiation of both wt- and dn-RAGE transfectants to a similar extent, indicating that conditions favouring the formation of the HMGB1–RAGE complex do not alter the cell response to extracellular HMGB1 independently of the type of RAGE expressed.

We have tested if glycated albumin, known as an alternative RAGE-activating ligand, added to cultures of wt- and dn-RAGE transfectants affected their chemical-induced erythroid differentiation. As shown in Figure 7, the kinetics of differentiation were not modified significantly, as compared with cultures induced in the absence of the glycated protein. Similar results were obtained even at concentrations up to 100 μ M (results not shown).

Effect of RAGE ligands on down-regulation of PKC- δ

Down-regulation of the PKC- δ isoenzyme is an early event in the process of MEL cell differentiation. It has been shown that it is completed within the time of cell commitment and is stimulated

Figure 7 Effect of glycated albumin on differentiation of wt- and dn-RAGE-transfected cells

Transfectants overexpressing wt-RAGE (■, □) or dn-RAGE (▲, △) were induced with 5 mM HMBA in the absence (■, ▲) or the presence (□, △) of 1 μ M glycated albumin. At the indicated times, cells were stained with benzidine. Data are the means $\pm$ S.D. of four independent experiments.

Table 1 Effect of HMGB1 and glycated albumin on down-regulation of PKC- δ induced by HMBA

Parental MEL cells were cultured in the presence of the specified additions (5 mM HMBA, 100 pM HMGB1 and 1 μ M glycated albumin respectively). At the indicated times, 2×10^6 cells were subjected to SDS/PAGE followed by Western blotting to identify PKC- δ . The 125 I-labelled protein bands were excised from the gel and the signal was quantified in a γ -counter. Data are means $\pm$ S.D. of three independent experiments.

Addition	Amount of PKC- δ (c.p.m./ 10^6 cells)		
	0 h	2 h	4 h
HMBA	4500 $\pm$ 380	2800 $\pm$ 320	1950 $\pm$ 220
HMBA + HMGB1	4600 $\pm$ 400	1600 $\pm$ 150	950 $\pm$ 190
HMBA + glycated albumin	4300 $\pm$ 350	2600 $\pm$ 240	2200 $\pm$ 250
Glycated albumin	4500 $\pm$ 350	4700 $\pm$ 380	4400 $\pm$ 400

by extracellular addition of HMGB1 [33]. We have compared the rate of PKC- δ disappearance as a function of addition of two RAGE ligands, HMGB1 and glycated albumin, to the cell-culture-induction medium. As shown in Table 1, addition of HMGB1 promoted a more than 2-fold increase in the down-regulation of PKC- δ . On the contrary, glycated albumin did not increase the extent of this down-regulation process when added together with HMBA, and was completely ineffective in the absence of the inducer. Together, these findings support the idea that, in MEL cells, the intracellular cascades of events leading to the generation of the differentiated phenotype do not involve RAGE activation.

HMGB1-binding proteins on MEL cell plasma membrane

We have considered the possibility that alternative binding sites for HMGB1 are present on MEL cells. To determine if membrane proteins that are able to recognize HMGB1 are available on the

Figure 8 Identification of a HMGB1-binding protein on the MEL cell plasma membrane

Parental MEL cells (5×10^5 cells/ml) were cultured for 18 h in the presence of 5 mM HMBA. Cells were then collected and incubated with the 125 I-SASD-linked HMGB1 (lanes 1, 2, 4 and 5) or 125 I-SASD-linked HMGB1_{129–138} peptide (lanes 3 and 6), as specified in the Experimental section, in the absence (–) or the presence (+) of a 100-fold excess of unlabelled HMGB1. Cells were then exposed to UV irradiation, washed and subjected to SDS/PAGE in the absence (–) or the presence (+) of 2-mercaptoethanol (MSH). The radioactive bands were detected on gels by a Cyclone Storage Phosphor System. Lane 7, detection of RAGE by Western-blot analysis of the proteins from lane 6. Lane 8, 125 I-labelled MEL cell membrane proteins recognized by the immobilized HMGB1_{129–138} peptide.

outer surface of MEL cells, we have covalently bound HMGB1 to a labelled photoreactive heterofunctional cross-linker and then evaluated its interaction with intact MEL cells. As shown in Figure 8, the formation of a labelled 57 kDa HMGB1–membrane protein complex was detected by SDS/PAGE under non-reducing conditions (Figure 8, lane 1). Following cleavage of the disulphide bridge linking the two interacting proteins, the labelled HMGB1-binding protein had a molecular mass of approx. 65 kDa (Figure 8, lane 4). The specificity of this identification was confirmed by control experiments carried out in the presence of an excess of unlabelled HMGB1 (Figure 8, lanes 2 and 5). The HMGB1 fragment spanning from amino acids 129–138 has been shown previously to be active as a stimulator of MEL cell differentiation [21]. This peptide was covalently bound to the same labelled cross-linker and used to entrap proteins on the MEL cell surface. In this case, the labelled protein–peptide complex detected under non-reducing conditions (Figure 8, lane 3) had a lower molecular mass when compared with the protein–HMGB1 complex, but, under reducing conditions, the molecular mass of the peptide-binding protein (Figure 8, lane 6) was identical with that entrapped by HMGB1 (Figure 8, compare lanes 4 and 6). To determine whether the labelled 65 kDa protein was recognized by the anti-(RAGE) antibody, proteins from lane 6 in Figure 8 were subjected to Western blotting. Figure 8 (lane 7) shows that the electrophoretic migration of RAGE was not superimposable with that of the radioactive protein. As a different approach, we used the immobilized HMGB1_{129–138} peptide to entrap proteins from solubilized MEL cell plasma membranes. As shown in Figure 8 (lane 8), a 65 kDa protein was also the major protein band detectable.

DISCUSSION

The growth of uncommitted haematopoietic progenitor cells can be controlled by inducing their commitment to terminal differentiation. This strategy has led to the development of chemothera-

peutic agents able to induce differentiation of transformed cells thus preventing the progression of cancers. MEL cells respond to hybrid polar inducers [15] by exporting HMGB1, and the interaction of this protein with the plasma membrane has been shown to be essential for the acquirement of the erythroid phenotype [11]. It has been reported that the multi-ligand RAGE represents a binding site for extracellular HMGB1 [19]. As RAGE–HMGB1 interaction activates a cascade of events triggering changes in cell motility [34], neurite outgrowth and activation of nuclear factor κ B ('NF- κ B') [20] in different cell types, we have investigated whether extracellular HMGB1 promotes MEL cell differentiation through a RAGE-activating mechanism. Firstly, on the basis of RT-PCR and Western-blot analysis, we have demonstrated that MEL cells express RAGE. However, the requirement for extracellular HMGB1 in MEL cell differentiation is two orders of magnitude lower than the K_d of purified RAGE for HMGB1 [19]. This discrepancy could be ascribed to the lack of extracellular-matrix components [35] facilitating the presentation of HMGB1 to RAGE in the purified system, as demonstrated for other heparin-binding factors [36]. However, all attempts to demonstrate an involvement of RAGE in the MEL cell differentiation process were unsuccessful, leading to the conclusion that, in this cell type, the HMGB1 signalling is not mediated by RAGE. This conclusion is supported by the fact that the kinetics of cell growth, the rate of MAPK inactivation and the appearance and accumulation of the erythroid phenotype were unchanged in MEL cell clones overexpressing an active (wt-RAGE) or inactive (dn-RAGE) RAGE. Taken together, these observations indicate that the possible formation of the RAGE–HMGB1 complex does not participate in the MEL cell differentiation process. Induction of MEL cells in the presence of glycated albumin as a RAGE stimulator [17] was also ineffective in promoting the rapid down-regulation of PKC- δ , which occurs in the presence of extracellularly added HMGB1. This suggests that the biochemical steps underlying terminal differentiation of MEL cells and RAGE activation are totally unrelated.

In an attempt to identify different HMGB1-binding sites on MEL cells, we have obtained evidence for the presence of a 65 kDa protein that can be covalently cross-linked to extracellular HMGB1 using intact cells. The cross-linked protein does not react with the anti-(RAGE) antibody and hence could represent an alternative binding site recognizing extracellular HMGB1. The slower electrophoretic migration observed for the isolated HMGB1-binding protein, with respect to the cross-linked protein complex, is not an unusual phenomenon and has been reported for other protein receptors, such as the interleukin-3 receptor [28]. This behaviour of the protein can be ascribed to the presence of intrachain disulphide bridges, which, following reduction, can promote large changes in the molecular shape. The HMGB1_{129–138} peptide, a fragment derived by extracellular processing of the native protein [21] and having a stimulatory activity on MEL cell differentiation, entraps a cell membrane protein with a molecular mass identical with that of the protein cross-linked by native HMGB1. This result has also been confirmed using this peptide covalently bound to Sepharose, suggesting that this membrane protein, recognizing a HMGB1 segment spanning from residues 129–138, could be functionally linked to the cell response elicited by extracellular HMGB1. Furthermore, the presence of receptor-type protein-tyrosine phosphatase β/ζ ('RPTP β/ζ '), a cell membrane receptor described as a HMGB1-binding protein [37], has also been considered as a possible HMGB1-binding protein that could be responsible for the high-molecular-mass labelled material detected in cross-linking experiments. However, at present, we can

exclude this receptor from being involved in the MEL cell differentiation process, as receptor-type protein-tyrosine phosphatase β/ζ was not found to be expressed in detectable amounts in MEL cells, as determined by both by RT-PCR and Western-blot analysis (results not shown). Further experiments are in progress to characterize the 65 kDa MEL cell membrane protein interacting with extracellular HMGB1.

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