Extracellular high-mobility group 1 protein is essential for murine erythroleukaemia cell differentiation

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A high-mobility group 1 (HMG1) protein type isolated from murine erythroleukaemia (MEL) cells promotes acceleration of the differentiation process when added to a MEL cell culture together with the inducer hexamethylene bisacetamide. We now provide direct evidence that the presence of HMG1 protein in the extracellular medium is essential for terminal erythroid differentiation. An extracellular function for HMG1 protein in MEL cell is further supported by a demonstration that this protein is released from MEL cells exposed to the chemical inducer and that the addition of an anti-(HMG1 protein) monoclonal antibody to the cell culture inhibits the differentiation process almost completely. The release of HMG1 protein from MEL cells is modulated by compounds affecting cell calcium homoeostasis, such as a calcium ionophore or verapamil. In fact, in the presence of the ionophore an increased rate of differentiation is accompanied by an enhanced extracellular release of HMG1 protein, whereas in the presence of verapamil both phenomena are significantly decreased.

INTRODUCTION

MEL cells contain a protein, originally defined by us as 'differentiation-enhancing factor' (DEF) [1], retained intracellularly in resting cells and released into the extracellular medium after induction of the cells to terminal erythroid differentiation [2]. The release of this protein factor is almost completed within the time required for cell commitment, which is much earlier than the appearance of differentiated cells [2]. The protein factor has been successively demonstrated [3] to correspond to high-mobility group 1 (HMG1) protein [4]. At a biochemical level the addition of purified HMG1 protein to hexamethylene bisacetamide (HMBA)-induced cells resulted in a significant acceleration in the down-regulation of the protein kinase C δ isoform crucially involved in the modulation of the responsiveness of murine erythroleukaemia (MEL) cells to the inducer [5]. Once released from MEL cells, or added externally, the protein has been shown to reduce the time required for the onset of the differentiated state [3]. The amino acid sequence of this protein factor, isolated either from the soluble fraction of MEL cells or from the extracellular medium, revealed its identity with the type 1 of the HMG protein family [3], generally considered to be a DNA-binding protein but whose precise function(s) have yet to be defined; they have been suggested to include the stimulation of neurite outgrowth in neuronal precursor cells, an effect presumably involving membrane localization preceded by extracellular release [6].

The hypothesis that HMG1 protein can exert a biological activity from outside the cell is consistent with several reports indicating a pronounced enhancing effect on MEL cell differentiation induced by the addition of the purified protein factor to the culture medium [3,7]. In this study we have characterized the extracellular function of HMG1 protein by using a monoclonal antibody raised against this protein type. On the basis of the results obtained we suggest an extracellular localization, occurring at a very early stage of MEL cell induction to differentiation, as the site of action of HMG1 protein in the promotion of the differentiation process of these cells.

EXPERIMENTAL

Cell culture and differentiation

MEL cell N23 slowly differentiating clone and V3.17 [44], named C44 in this study, fast differentiating clone were obtained and cultured as described [8,9]. Cell differentiation was induced by addition of 5 mM HMBA to a culture containing 10^5 cells/ml and at the indicated times the percentage of differentiated cells was assayed by benzidine reaction [10].

Production and purification of eukaryotic recombinant HMG1 protein

Total RNA was isolated from C44 MEL cells and reverse-transcribed as described [3]. Recombinant pBlueBacIII vector (Invitrogen) was constructed with the PCR primers and the procedure previously described [11]. This vector, containing the entire coding region of HMG1 cDNA as demonstrated by sequencing performed with the Sequenase 2.0 kit (Amersham), was co-transfected with wild-type baculovirus to *Spodoptera frugiperda* (Sf9) cells (Invitrogen) and the recombinant virus was isolated in accordance with the manufacturer's instructions. Sf9 cells were grown at 27 °C in 75 cm^2 flasks in TC100 insect medium (Biochrom KG) containing 10 % (v/v) fetal calf serum and infected with high-titre recombinant viral stock (5 plaque-forming units per cell). After 72 h of culture, cells were lysed and the cells' soluble fraction was prepared [11]. The recombinant HMG1 protein was purified by the procedure previously described for MEL cell HMG1 protein [3]. The N-terminal sequence of purified recombinant HMG1 protein was evaluated by automated Edman degradation with a gas-phase sequencer on line with a phenylthiohydantoin-amino acid analyser (Beckman LF 3000) and was found to correspond to that of mouse HMG1 protein [6]. The specific activity of purified recombinant HMG1 protein was 2 units/ng, corresponding to that of HMG1 protein purified from MEL cells; one unit of HMG1 activity is defined as the amount that doubles the proportion of N23 benzidine-positive cells after 72 h of exposure to HMBA.

Abbreviations used: DEF, 'differentiation-enhancing factor'; HMBA, hexamethylene bisacetamide; HMG, high-mobility group; mAb, monoclonal antibody; MEL, murine erythroleukaemia; PKC, protein kinase C.

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Table 1 Inhibition of MEL cell differentiation by an anti-HMG1 antibody

MEL cells were induced in the absence or in the presence of the indicated amounts of purified recombinant HMG1 protein and 20 μg/ml (N23 cells) or 60 μg/ml (C44 cells) anti-HMG1 mAb 23.38. After 72 h for N23 cells or 24 h for C44 cells, the proportion of benzidine-positive cells was measured as described in the Experimental section. Each experiment was done in triplicate; the values are given as means $\pm$ S.D. Abbreviation: n.d., not detected.

	N23 clone		C44 clone	
Addition	Benzidine-positive cells (%)	Modification of differentiation compared with control	Benzidine-positive cells (%)	Modification of differentiation compared with control
None	9 $\pm$ 3	1.0	18 $\pm$ 2	1.0
20 pM HMG1	24 $\pm$ 4	2.7	26 $\pm$ 2	1.4
mAb 23.38	1 $\pm$ 1	0.1	n.d.	n.d.
20 pM HMG1 + mAb 23.38	3 $\pm$ 2	0.3	1 $\pm$ 1	0.05
40 pM HMG1 + mAb 23.38	12 $\pm$ 4	1.3	21 $\pm$ 3	1.2
60 pM HMG1 + mAb 23.38	21 $\pm$ 3	2.3	24 $\pm$ 4	1.3

Production and characterization of an anti-HMG1 protein monoclonal antibody (mAb)

Recombinant glutathione S-transferase–HMG1 fusion protein was obtained from *Escherichia coli* TOP 10 F′ cells transfected with pGEX2T expression vector containing the coding region of mouse HMG1 cDNA and purified as described [12]. This protein was dispersed in complete Freund's adjuvant, and mice were immunized by intraperitoneal injection of 50 μg of protein at 10-day intervals. Anti-(HMG1 protein) mAbs were obtained as previously described [13,14] and tested for their anti-HMG1 protein activity by a solid-phase binding assay [13,15] with 50 μl (1 μg) aliquots of both native or eukaryotic recombinant HMG1 proteins by the procedure previously described [16]. An ^{125}I-labelled [17] goat anti-mouse Ig (Southern Biotechnology) was used as secondary antibody. A cell clone (clone 23.38) showing the highest anti-(HMG1 protein) activity was selected and injected into BALB/c mice pretreated with 2,6,10,14-tetramethylpentadecane. The ascitic fluids (10 ml) were collected, treated with 50 %-satd. $(NH_4)_2SO_4$ and the precipitated proteins were solubilized in PBS, pH 7.4. After gel chromatography on a Sephadex G-200 column (Pharmacia) (1.5 cm $\times$ 120 cm), previously equilibrated with PBS, the peak of protein containing anti-HMG1 protein activity was shown to belong to the IgM class by its molecular mass and by sandwich ELISA [18]. The anti-HMG1 mAb (named mAb 23.38 in the text) recognized both native and recombinant HMG1 proteins as a single band with a mass of 30 kDa in Western blot analysis and did not cross-react with other proteins present in cell lysates.

Electrophoretic methods

SDS/PAGE was performed on 12 % (w/v) polyacrylamide slab gels [19] and, where indicated, proteins were transferred to a nitrocellulose membrane (Bio-Rad) for Western blot analysis [20]. Immunostaining was performed with 2 μg/ml purified anti-HMG1 mAb 23.38 followed by localization of antibody bound to protein bands by using an ^{125}I-labelled goat anti-mouse Ig [21].

Affinity chromatography of the soluble fraction of MEL cells or extracellular medium on heparin-immobilized column

Serum-free culture medium (10 ml), obtained after incubation of 5×10^6 C44 MEL cells in the presence of different additions as specified elsewhere, was diluted with one vol of distilled water and loaded on a 1 ml heparin-immobilized column (Econo-Pac, Bio-Rad) previously equilibrated with 50 mM sodium phosphate buffer, pH 7.0 (buffer A). The column was washed with the same buffer and the adsorbed proteins were eluted with buffer A containing 0.8 M NaCl. The same chromatographic step was carried out on the soluble fraction of MEL cells prepared as described [3].

RESULTS

To esyablish the role of extracellular localized HMG1 protein as an enhancer or as an essential factor in MEL cell differentiation, we have assayed the effect of an anti-HMG1 protein mAb (mAb 23.38) added to a MEL cell culture together with the chemical inducer HMBA, which also promotes the release of HMG1 protein from MEL cells. As shown in Table 1, the addition of purified HMG1 protein to cultures of MEL cell clones with high and low sensitivities to induction produced an increased accumulation of differentiated cells compared with that of cells stimulated by the addition of HMBA alone. In contrast, the addition of anti-HMG1 protein mAb 23.38 in the absence or in the presence of the same amount of HMG1 protein not only abolished the stimulatory effect of HMG1 protein but inhibited MEL cell differentiation almost completely. This effect produced by mAb 23.38 on MEL cell differentiation was not due to a cytotoxic effect or to modifications of the cell surface resulting in a lower sensitivity of cells to the chemical inducer, because the addition of increasing concentrations of HMG1 protein to the cell culture restored the original differentiation capacity. Taken together, these results indicate that the presence of extracellular HMG1 protein is crucial for the promotion of MEL cell differentiation and thereby suggest that the release of HMG1 protein is an early limiting event occurring in HMBA-induced cells.

The inhibitory effect of mAb 23.38 on cell differentiation was dose-dependent with both N23 and C44 MEL cell clones. As shown in Figure 1, the inhibition increased progressively as a function of the amount of anti-HMG1 antibody added to the cell culture, reaching a saturation point. It is interesting to note that the amount of anti-HMG1 antibody required to obtain maximal inhibition of differentiation of N23 cells was approximately one-quarter of that required by C44 cells. These results are in agreement with previous observations indicating that, after exposure to HMBA, N23 cells release significantly smaller amounts of HMG1 protein into the culture medium than do C44 cells [2].

To evaluate further the relationship between the differentiation process in MEL cells and the amount of extracellular HMG1

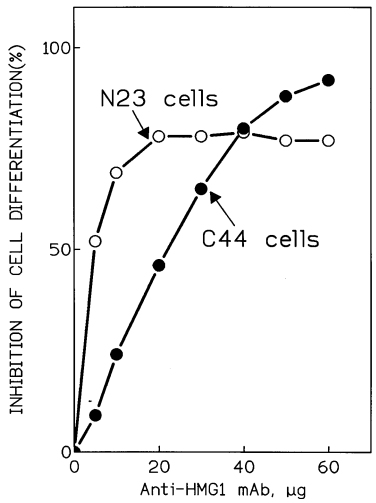

Figure 1 Dose–response inhibition of MEL cell differentiation by anti-HMG1 protein mAb 23.38

N23 and C44 MEL cells were induced as described in the Experimental section, in the presence of the indicated amounts of purified anti-HMG1 protein mAb 23.38. After 48 h (C44 cells) or 120 h (N23 cells) the percentage of benzidine-positive cells was measured.

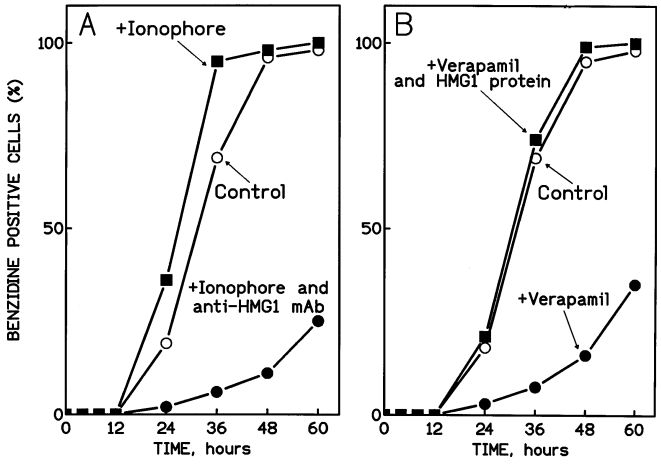

Figure 2 Anti-HMG1 protein mAb and HMG1 protein reverse the effect produced on MEL cell differentiation by a Ca^{2+} ionophore or verapamil respectively

C44 MEL cells were induced in the absence (control) or in the presence of the indicated additions. The final concentrations af each addition were: 1 μM A23187 Ca^{2+} ionophore; 60 μg/ml anti-HMG1 mAb 23.38; 10 μM verapamil; 50 pM recombinant HMG1 protein. At the indicated times the percentage of benzidine-positive cells was measured.

protein, we treated MEL cells with compounds known to affect the rate of secretion of proteins from cells [22]. As shown in Figure 2A, addition of ionophore A23187 to HMBA promoted a large increase in the rate of accumulation of differentiated cells, whereas addition of the anti-HMG1 protein antibody to the mixture produced a large inhibitory effect. As shown in Figure 2B, the addition of verapamil to HMBA-induced cells resulted in a greater than 80% decrease in the rate of accumulation of differentiated cells. Because the differentiation capacity of MEL cells treated with verapamil was fully restored by the addition of purified HMG1, these results can be related to changes in the

Table 2 Effect of Ca^{2+} ionophore A23187 and verapamil on the release of HMG1 protein by MEL cells induced with HMBA

C44 MEL cells (5×10^6 cells) were incubated for 3 h in 10 ml of serum-free culture medium in the presence of the indicated additions. After 3 h the cells and the clear supernatant were collected separately and both the soluble fraction of the cell, obtained as described in the Experimental section, and the extracellular medium were submitted to a single affinity-chromatography step on a heparin-immobilized column as specified in the Experimental section. The peak of eluted proteins was collected and aliquots were used to determine the amount of HMG1 protein by assaying the stimulatory effect on N23 MEL cell differentiation (see the Experimental section). One unit of HMG1 protein is defined as the amount that doubles the proportion of benzidine-positive cells after 72 h of exposure to HMBA. Each experiment was done in triplicate; the values are given as means ± S.D.

Addition	Extracellular HMG1 protein (units)	Percentage of total
None	20 ± 5	6 ± 2
HMBA	192 ± 20	55 ± 6
HMBA + A23187	300 ± 28	85 ± 7
HMBA + verapamil	64 ± 12	18 ± 3

concentration of HMG1 protein available to cells in the extracellular medium. This hypothesis was tested by measuring the amount of HMG1 protein released by MEL cells in each experimental condition. As shown in Table 2, when C44 MEL cells were incubated in serum-free culture medium for 3 h, approx. 6% of total cellular HMG1 protein was recovered in the extracellular compartment, an amount probably due to the corresponding percentage of cell death during the incubation (results not shown). In the presence of HMBA more than 50% of total HMG1 protein was released from cells and this value was increased to 85% by the simultaneous addition of A23187 ionophore. In contrast, the addition of verapamil to HMBA-stimulated cells decreased the amount of HMG1 protein released to less than 20% of the total. Both ionophore and verapamil were used at concentrations shown to be ineffective on cell viability during the period of incubation (results not shown).

DISCUSSION

The results presented in this paper, together with previous results [3,6,12], indicate for HMG1 protein an additional function different from that generally proposed and considered to be confined to a mainly chromosomal localization [23]. This abundant and highly conserved protein, present in all vertebrate nuclei, shows the property of binding DNA through its N-terminal basic region, which contains two HMG boxes [24], without sequence specificity [25] but recognizing specific DNA structures such as four-way junction DNA [26], irregular or bent structures in the DNA helix [27] and DNA damaged by cisplatin [28], suggesting its involvement in DNA recombination, repair, replication and gene transcription. HMG1 protein has also been found to function as a general class II transcription factor [29], and it has been proposed to have a direct role in gene regulation, related to the different intracellular concentration of this protein found in undifferentiated and differentiated cells [4]. We now provide evidence that the release of HMG1 protein, originally designated by us as differentiation-enhancing factor [1], from MEL cells after induction with HMBA, represents a crucial step in the process of MEL cell differentiation. The relevance of the accumulation of HMG1 protein in the external cell environment during the time preceding cell commitment has been demonstrated by the inhibition of the overall differentiation process observed when a specific anti-HMG1 mAb is added to the

culture medium of HMBA-stimulated MEL cells. The present observations are thus consistent with an extracellular function for HMG1 protein and are related to a crucial role in the multistep process of MEL cell differentiation, involving localization of the protein outside cells. This conclusion is in agreement with results reported by other authors [6] indicating that, in neurons from developing rat brain, HMG1 protein promotes neurite outgrowth and that the process can be inhibited by addition of anti-HMG1 protein antibodies to the cell culture medium.

The crucial role of extracellular HMG1 protein in MEL cell differentiation is further supported by the observation that compounds (the Ca^{2+} ionophore A23187 and verapamil) that positively or negatively affect the transmembrane exchange of ions or molecules respectively [30,31] promote an increase or a decrease in the amount of HMG1 protein released from HMBA-stimulated cells and accordingly promote an increase or a decrease in the rate of MEL cell differentiation. In addition, under these conditions the rate of differentiation can be negatively or positively affected by the addition to the cell culture of a monoclonal anti-(HMG1 protein) antibody or of HMG1 protein respectively. Taken together these results suggest that HMG1 protein can be considered a multifunctional protein factor localized and active at different cell sites and generally involved in promoting events related with the control of terminal cell differentiation. This conclusion is consistent with the observation that HMG1, when added to the cell medium, promotes a significant acceleration in the rate of differentiation of human promyelocytic HL60 cells accompanied by a marked decrease in their requirement for chemical inducers [7].

A number of questions remain to be answered, particularly in connection with the mechanism of the release of HMG1 protein from cells, because the protein lacks a classical hydrophobic signal peptide for secretion. It will be also important to establish how HMG1 protein can be recognized by cells as well as the nature of the signal detected by the cell itself. Experiments in progress are aimed at clarifying these important aspects of the biological function of HMG1 protein in cell differentiation.

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