RESEARCH COMMUNICATION

Inhibition of the differentiation of human myeloid cell lines by redox changes induced through glutathione depletion

Franca ESPOSITO,* Valter AGOSTI,* Giovanni MORRONE,*† Fernando MORRA,* Cristina CUOMO,* Tommaso RUSSO,* Salvatore VENUTA† and Filiberto CIMINO*‡

*Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli 'Federico II', Via S. Pansini, 5, I-80131 Napoli, and †Dipartimento di Medicina Sperimentale e Clinica, Università degli Studi di Reggio Calabria, Via T. Campanella, I-88100 Catanzaro, Italy

We have investigated the effect of redox changes *in vivo* on the differentiation of two human myeloid cell lines, HL-60 and KG-1. The glutathione-depleting agent diethyl maleate (DEM) prevented the development of differentiated features in response to phorbol esters, including adherence of the cells to plastic surfaces

and repression of the myeloperoxidase and CD34 genes. Moreover, DEM abolished phorbol 12-myristate 13-acetate-induced activation of the transcription factors AP-1 and Egr-1, suggesting that inhibition of differentiation may be due, at least in part, to redox modifications of these proteins.

INTRODUCTION

The maintenance of the haematopoietic system is ensured by differentiation of multipotent progenitors along one or multiple lineages, generating the different mature cell populations of the peripheral blood [1]. Binding of haematopoietic cytokines to specific cell surface receptors triggers intracellular transduction pathways that in turn induce co-ordinate expression of regulatory genes through activation of specific transcription factors. This results in the commitment of multipotent progenitors to terminal differentiation.

A number of myeloid cell lines, which resemble to some extent haematopoietic progenitors, which may be induced to differentiate *in vitro* and whose maturation can be accurately monitored, have been employed in biochemical and molecular studies of myeloid differentiation [2]. Among the best known myeloid cell lines are the promyelocytic HL-60 and the myeloblastic KG-1. The HL-60 line was derived from the cells of a patient with acute myeloid leukaemia type M2, and exhibits several features of normal human promyelocytes. When exposed to a variety of chemicals, vitamins or cytokines, these cells differentiate towards granulocytes or monocytes [3,4]. Suitable markers for evaluating HL-60 maturation include the expression of two genes, those for myeloperoxidase (MPO), which is down-regulated in differentiated cells [5], and lysozyme, which is greatly increased during granulocytic differentiation [6]. The KG-1 cell line, established from the bone marrow cells of a patient with erythroid leukaemia [7], shows immature characteristics, such as expression of the progenitor-related CD34 antigen. However, the differentiation potential of this cell line is restricted to the monocyte/macrophage lineage. Such differentiation can be achieved by phorbol ester treatment, and is accompanied by a drastic decline in the level of the CD34 transcript [8].

The study of myeloid cell lines has led to the discovery of a number of DNA-binding factors involved in the regulation of haematopoietic differentiation [9–11]. A zinc finger transcription factor, Egr-1, has been discovered to be of crucial importance in the commitment of myeloid progenitors towards monocyte-

restricted differentiation. Egr-1 is strictly required for maturation of HL-60 cells to monocytes following phorbol 12-myristate 13-acetate (PMA) treatment, as well as for differentiation of bone marrow monocyte precursors in response to macrophage colony-stimulating factor. An HL-60 subclone constitutively expressing Egr-1 became incapable of granulocytic differentiation [12]. This factor is therefore a useful tool for the study of the monocytic pathway.

Following a number of reports on the involvement of redox modifications of transcription factors in the modulation of their DNA-binding activity *in vitro*, we became interested in testing the *in vivo* effect of agents that may affect the redox status of such factors, using differentiation-inducible myeloid cell lines as a suitable experimental model. Here we show that glutathione depletion induced by diethyl maleate (DEM) counteracts the PMA-stimulated monocytic differentiation of HL-60 and KG-1 cells, as judged by molecular as well as morphological criteria. This inhibitory effect appears to be reversible upon withdrawal of the oxidizing agent, and is associated with a lack of activation of the transcription factors AP-1 and Egr-1 in response to PMA.

MATERIALS AND METHODS

Cell culture and stimulation

HL-60 and KG-1 cells were cultured in RPMI 1640 medium (Flow Laboratories) containing 10% foetal calf serum (Hyclone, Logan, UT, U.S.A.), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (Flow). PMA was purchased from Sigma (St. Louis, MO, U.S.A.). For stimulation of differentiation, KG-1 cells were maintained for the indicated times in the presence of 100 nM PMA, whereas HL-60 cells were treated with 10 nM PMA. DEM (Sigma) was used at 1 mM final concentration unless otherwise indicated. Intracellular GSH depletion was measured by an h.p.l.c. procedure as described [13]; cell viability after the treatments was assessed by Trypan Blue exclusion. Cell proliferation was evaluated by measuring [³H]thymidine incorporation into the cellular DNA.

Abbreviations used: DEM, diethyl maleate; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; MPO, myeloperoxidase; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; RT-PCR, reverse transcription-PCR; TRE, PMA (TPA)-responsive element.

‡ To whom correspondence should be addressed.

Northern blot analysis

For RNA preparation, cells were lysed in 3 ml of guanidinium thiocyanate buffer and subjected to phenol/chloroform extraction as described by Chomczynsky and Sacchi [14]. Samples of 10–15 μ g of total cellular RNA were electrophoresed on 1.5% agarose/formaldehyde gels and transferred on to nitrocellulose membranes (Nytran; Schleicher and Schuell, Dassel, Germany). Filters were incubated at 80 °C for 2 h under vacuum, hybridized for 16 h at 65 °C with 10–30 μ Ci of radiolabelled probes, extensively washed and autoradiographed with X-ray film (Kodak, Rochester, NY, U.S.A.).

Molecular probes were generated by reverse transcription-PCR (RT-PCR): cDNA was synthesized from approx. 2 μ g of cellular RNA using Superscript reverse transcriptase (BRL Life Technologies G.m.b.H., Berlin, Germany) and random hexamers (0.5–2.5 μ M; Boehringer Mannheim) as primers. cDNA was subjected to 30 cycles of PCR amplification using the oligonucleotides described below. These were designed in order to obtain similar melting temperature (T_m) and PCR products of comparable size. After analytical digestion with specific restriction enzymes to verify the faithfulness of amplification, PCR products were purified (Qiaex; Qiagen Inc., Chatsworth, CA, U.S.A.) from agarose gels and amplified by 10 PCR cycles in the presence of 40 μ Ci of [α -³²P]dATP (Amersham, Arlington Heights, IL, U.S.A.). Labelled probes were separated from non-incorporated [α -³²P]dATP on G-50 columns (Pharmacia Biotech Inc., Piscataway, NJ, U.S.A.). Taq polymerase was from Perkin Elmer (Vaterstetten, Germany). The PCR primers were as follows: MPO: forward (nucleotides 1069–1086), 5' CCCA-ATGACCCCCGCATC 3'; reverse (nucleotides 1387–1369), 5' TGTCCCCTGCCAGGAAGCA 3'; CD34: forward (nucleotides 811–830), 5' TGA CTCAGGGCATCTGCCTG 3'; reverse (nucleotides 1206–1225), 5' CTTTCTCCTGTGGGGCTCCA 3'.

Preparation of nuclear extracts

Nuclear extracts were prepared from HL-60 cells according to Schreiber et al. [15] with slight modifications. Briefly, HL-60 cells (1×10^7 /100 mm plastic dish) were washed twice with 10 ml of TBS (58.44 mM Tris, pH 7.5, 85.55 mM NaCl) and pelleted by centrifugation at 1500 *g* for 5 min at 0 °C. The pellets were gently resuspended in 400 μ l of ice-cold buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethanesulphonyl fluoride (PMSF), freshly prepared, were added at the last moment]. After swelling on ice for 15 min, 25 μ l of 10% Nonidet P40 (Sigma) was added; the samples were vortexed for 10 s and centrifuged for 30 s at 4 °C. Nuclear pellets were subsequently resuspended in 30 μ l of ice-cold buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF), rocked for 15 min at 4 °C and centrifuged at 15000 *g* for 5 min at 4 °C. Supernatants were collected and stored in aliquots at –80 °C.

Electrophoretic mobility shift assays (EMSAs)

The PMA-responsive element (TRE) and Egr-1 probes used for EMSA were prepared by labelling 1 pmol of double-stranded oligonucleotides, using 16 units of T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.) and 40 μ Ci of [γ -³²P]ATP (Amersham) at 37 °C for 30 min. The labelled oligonucleotides were purified from non-denaturing 12% polyacrylamide gels. A sample containing (20–40) $\times 10^3$ c.p.m. of the eluted radioactivity (corresponding to approx. 1 fmol of DNA) was used for each

reaction. The binding reactions were performed as follows. Nuclear extracts (5 μ g) were incubated for 15 min on ice in 18 μ l of a buffer containing 10 mM Tris/HCl, pH 7.5, 100 mM KCl, 2% Ficoll, 6% glycerol, 1 mM DTT, 2 μ g of poly(dI-dC) and, where indicated, a 100-fold excess of either non-specific or specific unlabelled oligonucleotide. In a second step, 2 μ l of radiolabelled probe was added to each sample, and incubated for 15 min at room temperature. The DNA–protein complexes were separated from the unbound DNA probe on a 4% native polyacrylamide gel in 0.5 $\times$ Tris/borate/EDTA buffer (44 mM Tris/HCl, 44 mM boric acid, 12.5 mM EDTA). The electrophoreses were run at room temperature (150 V/20 mA). The oligonucleotides used were: Egr-1, 5' ATCCCGGCGCGGGG-GCGAGGGCGT 3' and 3' TAGGGCCGCGCCCCGCTCC-CGCA 5'; AP-1, 5' TTCCGGCTGACTCATCAAGCG 3' and 3' AAGGCCGACTGAGTAGTTCGC 5'.

RESULTS

DEM treatment of HL-60 and KG-1 cells prevents the appearance of the differentiated phenotype induced by PMA

We were interested in testing the effects of redox changes on the PMA-induced differentiation of the myeloid cell lines HL-60 and KG-1 that can be monitored by both morphological and molecular approaches. To modify the intracellular redox conditions, the cells were exposed to the oxidizing agent DEM. This molecule induces GSH depletion by acting as a substrate for glutathione S-transferase, which catalyses the formation of covalently linked GSH–DEM complexes [16]. GSH depletion causes an increase in reactive oxygen intermediates due to the impairment of antioxidant enzyme systems that use GSH as a reducing agent [17].

In our system, measurement of intracellular GSH by an h.p.l.c.-based procedure [13] revealed a rapid and drastic decrease in the GSH levels in response to DEM (approx. 70% decrease after 1 h of treatment). Cell viability was assessed by measuring Trypan Blue exclusion at the end of the treatment, and was found to be greater than 80% regardless of the stimulant used. Under our experimental conditions, a 6 h incubation with PMA did not significantly affect HL-60 proliferation (measured as [³H]thymidine incorporation); after treatment with DEM or DEM + PMA, a significant fraction (approximately one-third) of the cells was still actively proliferating.

One of the clearest effects of PMA treatment was the rapid attachment of the majority (> 70–80%) of the HL-60 and KG-1 cells, which normally grow in suspension, to the surface of culture dishes. Stimulation of adherence by PMA was already evident after 2–3 h of treatment, and persisted for at least 18–24 h. Addition of 1 mM DEM completely prevented the appearance of the adherent phenotype induced by PMA. Lower concentrations of DEM were progressively less effective in counteracting the effect of PMA (results not shown).

A well established molecular marker of HL-60 and KG-1 differentiation is the repression of the MPO [5] and CD34 [8] genes respectively. Northern blot analyses of these transcripts were carried out using RNA from HL-60 and KG-1 cells treated with PMA alone or in combination with different combinations of DEM. A drastic decrease in both the MPO and CD34 mRNAs (Figure 1, lanes b and b') was evident after 6 h of exposure to PMA. DEM (1 mM) antagonized the effect of PMA: in cells treated with both agents (lanes c and c'), the MPO and CD34 mRNA levels were similar to those of untreated cells (lanes a and a'). Again, this effect of DEM appeared to be dose-dependent and lower concentrations of DEM (0.6 mM, lanes d

Figure 1 Effect of PMA and DEM on the expression of the MPO and CD34 genes

Northern blot analysis was performed as described in the Materials and methods section. RNAs of HL-60 (lanes a–e) and KG-1 (lanes a'–d') cells were analysed by hybridization with radiolabelled cDNA probes for MPO and CD34 respectively, as shown. HL-60 cells were treated for 6 h with control medium (lane a), 10 nM PMA (lane b), or PMA in combination with DEM at 1 mM (lane c), 0.6 mM (lane d) or 0.15 mM (lane e). DEM treatment of KG-1 cells (lanes a'–d') was at a concentration of 1 mM and 0.6 mM for 6 h (lanes c' and d' respectively), but the PMA concentration was always 100 nM. A photograph of each gel (bottom panels) is shown to confirm that equivalent amounts of RNA were used.

and d'; 0.15 mM, lane e) resulted in a marked reduction in the amounts of MPO and CD34 mRNAs.

Inhibition of HL-60 cells differentiation by DEM is associated with abrogation of PMA-induced activation of AP-1 and Egr-1

It is well documented that PMA treatment induces activation of protein kinase C [18], which initiates a number of metabolic events leading to the activation of gene expression programs. One of the early steps in this process is the appearance of the transcription factor AP-1, which consists of a heterodimeric complex of the Jun and Fos proteins [19]. Since the monocytic differentiation of HL-60 and KG-1 cells is triggered by PMA, we studied whether DEM treatment could affect the DNA-binding capacity of AP-1. Figure 2(a) shows the EMSA of an oligonucleotide containing the TRE, which represents the DNA *cis*-element interacting with AP-1. When this oligonucleotide was incubated with nuclear extracts from HL-60 cells, a specific DNA–protein complex appeared following PMA treatment (lanes b and c). Exposure to DEM + PMA did not result in the appearance of this complex (lane d).

It is likely that additional steps of the HL-60 and KG-1 cell differentiation pathways may be compromised by GSH depletion. It was recently demonstrated that the transcription factor Egr-1 is essential for HL-60 differentiation along the monocytic lineage [12]. The appearance of Egr-1 is an early event, and therefore it could be independent from AP-1 activation. EMSA of Egr-1 oligonucleotides, incubated with the same extracts used for AP-1 evaluation, demonstrated an Egr-1-specific band present in PMA-treated HL-60 cells (Figure 2b, lane c), but this was absent from cells treated with both DEM and PMA (lane d). The same extracts were analysed for the presence of the ubiquitous transcription factor C/EBP, which is known to remain unaffected by

Figure 2 Effects of DEM on DNA binding of AP-1 and Egr-1 in HL-60 cells

(a) AP-1 EMSA. Lane a, labelled TRE oligonucleotide without extracts; lanes b–e, labelled TRE oligonucleotide incubated with nuclear extracts from untreated HL-60 cells (lane b), or HL-60 cells treated for 6 h with 10 nM PMA (lane c), 10 nM PMA + 1 mM DEM (lane d) or 1 mM DEM alone (lane e). In lanes f and g, a nuclear extract from untreated HL-60 cells was preincubated with a 100-fold molar excess of unlabelled TRE oligonucleotide or of unlabelled non-specific competitor oligonucleotide respectively. (b) Egr-1 EMSA. Lane a, labelled Egr-1 oligonucleotide without extracts; lanes b–e, as in (a). In lanes f and g, a nuclear extract from untreated HL-60 cells was preincubated with a 100-fold molar excess of unlabelled Egr-1 oligonucleotide or of unlabelled non-specific competitor oligonucleotide respectively.

Figure 3 Effects of H₂O₂ treatment on the DNA binding of Egr-1

Labelled Egr-1 oligonucleotide was electrophoresed after a 30 min incubation on ice with: lane a, no extracts; lane b, nuclear extracts from PMA-treated HL-60 cells; lane c, nuclear extracts from PMA-treated HL-60 cells in the presence of 20 mM H₂O₂.

redox changes [20]; identical amounts of C/EBP were detected in all samples (results not shown).

The impairment of the DNA binding of Egr-1 could be the direct result of oxidation of this factor; we tested this possibility by treating *in vitro* the same extracts used for the experiment shown in Figures 2(a) and 2(b) (lane e) with 20 mM H₂O₂. The ability of Egr-1 to bind to its target DNA sequence was abolished following incubation of the nuclear extracts with H₂O₂ (Figure 3), whereas the DNA binding of C/EBP, used as a negative control, remained unaffected (results not shown).

Figure 4 Recovery of AP-1 DNA binding after removal of DEM

The labelled TRE oligonucleotide was electrophoresed after incubation with: lane 1, no extracts; lane 2, nuclear extracts from HL-60 cells treated for 6 h with 10 nM PMA; lane 3, nuclear extracts from cells treated for 6 h with 10 nM PMA + 1 mM DEM; lanes 4 and 5, nuclear extracts from cells treated for 6 h with PMA alone or PMA + DEM respectively, extensively washed to remove the stimuli from the medium and harvested 24 h later.

To test whether the effect of DEM is reversible, and to rule out the possibility of aspecific cellular toxicity, HL-60 cells were maintained in the presence of PMA alone or PMA + DEM for 6 h, washed and cultured for an additional 24 h in control medium. Under these conditions, even without re-addition of PMA, an almost complete recovery of AP-1 DNA-binding efficiency was observed in the extracts from cells treated with PMA + DEM (Figure 4; compare lanes c and e).

DISCUSSION

Many transcription factors have been reported to be sensitive to redox changes *in vitro*; this phenomenon was observed for AP-1 [21], NF- κ B [22], USF [23], HoxB5 [24], BZFL1 [20] and for the glucocorticoid receptor [25]. The relevance of these observations *in vivo* is still to be demonstrated, although several pieces of evidence support the hypothesis that *in vivo* changes in intracellular redox conditions may result in modifications of the DNA-binding activity of these transcription factors [21,26–28]. Furthermore, the identification of a complex machinery that regulates the redox status of several transcription factors [29–31], and of another enzymic apparatus that controls intracellular redox conditions [32], favours the hypothesis that oxidation or reduction of DNA-binding factors may affect the transcriptional regulation of a number of genes. However, direct evidence that redox changes may in fact affect gene expression has not yet been produced.

In this paper we demonstrate that redox modifications induced by DEM dramatically affect the PMA-driven differentiation of myeloid cell lines, as indicated by the reversal of morphological and molecular signs of differentiation, including the repression of the CD34 and MPO genes. The molecular basis of the phenomenon could reside in alterations in the behaviour of transcription factors that are involved in the PMA-dependent differentiation pathways, due to the oxidizing environment generated by GSH depletion. Two transcription factors were examined: AP-1, a well known target of the PMA-induced protein kinase C cascade [19], and Egr-1, which was recently demonstrated to be an important mediator of myeloid differentiation [12].

That AP-1 DNA-binding activity is sensitive to redox changes is a well demonstrated phenomenon [21]. The results reported

here, indicating that DEM treatment prevents the enhancement of AP-1 DNA binding caused by protein kinase C activation, further confirm and extend these previous observations. Egr-1 activation was similarly inhibited in nuclear extracts from HL-60 cells exposed to PMA and DEM, compared with those from cells treated with PMA alone. This could be a consequence either of the inhibition of the pathway(s) leading to Egr-1 activation or of a direct effect of the oxidizing environment on this transcription factor. The observation that Egr-1 DNA binding is abolished by treatment of nuclear extracts with H_2O_2 *in vitro* supports the hypothesis of a direct sensitivity of Egr-1 to redox alterations in the intact cells.

The intracellular redox conditions are the result of the equilibrium between the production of oxidizing radicals, which occurs in many metabolic reactions (mitochondrial electron transfer, arachidonate metabolism and peroxisomal reactions), and their inactivation catalysed by a complex enzyme system [32]. A number of situations are known in which intracellular concentrations of oxidizing radicals are increased. These include exposure to u.v.-B light or γ rays, binding of extracellular ligands to their membrane receptors [31] and reperfusion of hypoxic cells [33]. In some of these cases, modifications in the expression of several genes were observed, such as inhibition of tumour necrosis factor biosynthesis [34], overproduction of interleukin-1 [35,36] or interleukin-8 [37], and enhancement of HIV-1 transcription [38,39]. It has been proposed that this is a consequence of the activation of NF- κ B and/or other transcription factors, due to oxidant stress [28,40]. In the light of these data, our observation that GSH depletion in intact cells interferes with the myeloid differentiation driven by PMA suggests that this maturation pathway may be modulated by different stimuli that induce an increase in reactive oxygen radicals. To gain additional insight into this matter, it will be of considerable interest to investigate the possible alterations in myeloid differentiation associated with physiological conditions known to affect the intracellular redox status, such as ageing. In this situation an increase in oxidizing radicals is well documented [41], and we have observed a marked decrease in the DNA-binding activity of the transcription factor Sp1 [42] in old animals.

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