Biochemical Pharmacology, Vol. 42, No. 2, pp. 403–407, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EFFECTS OF HEXAMETHYLENE BISACETAMIDE ON INDUCTION OF MONOCYTIC DIFFERENTIATION OF HUMAN U-937 MYELOID LEUKEMIA CELLS

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(*Received* 7 *September* 1990; *accepted* 14 *February* 1991)

Abstract—The present studies have examined the effects of hexamethylene bisacetamide (HMBA) on the human U-937 monocytic cell line. HMBA treatment was associated with: (1) decreases in U-937 cell proliferation, (2) increases in nonspecific esterase activity and cell surface antigen expression consistent with monocytic differentiation, (3) decreases in c-*myc* gene expression, and (4) induction of tumor necrosis factor (TNF) transcripts. Treatment of U-937 cells with HMBA was also associated with increases in phospholipase A_2 activity and increases in the release of arachidonic acid and its metabolites. Dexamethasone, an agent previously shown to inhibit monocytic differentiation, had no detectable effect on the down-regulation of c-*myc*, but blocked the induction of TNF expression. Taken together, the results demonstrate that HMBA induces monocytic differentiation of U-937 cells and that this effect is sensitive, in part, to dexamethasone.

Hexamethylene bisacetamide (HMBA) is a polar planar agent which induces granulocytic differentiation of the human HL-60 promyelocytic cell line [1]. This agent also induces differentiation of Friend murine erythroleukemia (MEL) cells along the erythroid lineage [2]. The mechanism by which HMBA induces terminal differentiation is not clear. Related polar planar compounds, such as dimethyl sulfoxide (DMSO), inhibit phosphatidylinositol turnover in MEL cells with a rapid decrease in inositol trisphosphate (IP_3) and diacylglycerol (DAG) levels [3]. Furthermore, other studies have shown that HMBA-mediated MEL cell differentiation involves protein kinase C (PKC) and its proteolytic fragment PKM [4]. Other early changes seen during MEL cell differentiation include alterations in membrane fluidity [5], ion permeability [6, 7] and cell volume [8].

The present studies have examined the effects of HMBA on U-937 myeloid leukemia cells. The results demonstrate that, in contrast to the effects of HMBA on granulocytic differentiation of HL-60 cells, this agent induces U-937 cells to differentiate along a monocytic lineage. The results also demonstrate that this effect is sensitive, in part, to dexamethasone.

MATERIALS AND METHODS

Cell culture. U-937 cells were grown in RPMI 1640 medium (Hazelton Laboratories, Vienna, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Viable cells were assayed by trypan blue exclusion. HMBA was dissolved in RPMI 1640 medium at a stock concentration of 80 mM. The final concentration of HMBA in all experiments was 4 mM. Dexamethasone was dissolved in 100% ethanol and used at a concentration of 10 μM. All chemicals were obtained from Sigma. The final concentration of ethanol was 0.1% in all experiments.

Induction of monocytic differentiation. Cytocentrifuge smears of cultured cells were examined for nonspecific esterase (NSE) staining [9]. The monoclonal antibody (MAb) LM2/1 anti-human Mac-1 was provided by Dr. T. Springer (Center for Blood Research, Boston, MA). The W6/32 framework HLA-A,B MAbs were used as positive antibodies. The P3X63 myeloma supernatant was used as a negative control. Cells were incubated with the MAbs for 30 min at 4°, and then washed with 5% FBS in Hanks' balanced salt solution with 10 mM HEPES. Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG for 30 min at 4°, washed three times, and fixed in 1% paraformaldehyde/phosphate-buffered saline [10, 11]. Fluorescence was determined by analysis on an EPICS V fluorescein-activated cell sorter (Coulter Electronics, Hialeah, FL).

RNA extraction and hybridization. Total cellular RNA was purified by the guanidine isothiocyanate-cesium chloride method [12], analyzed by gel electrophoresis through 1% agarose-formaldehyde gels, and transferred to nitrocellulose filters [13]. Hybridization reactions were performed as described [14] using the following ^{32}P-labeled probes: (1) the 1.8-kb ClaI/EcoRI fragment of the human c-*myc* 3′ exon purified from the pM C41-3 RC plasmid [15]; (2) the 1.9-kb BamHI fragment of the human tumor necrosis factor (TNF) gene [16]; and (3) the 2.0-kb PstI fragment of the chicken β-actin gene purified from the pA1 plasmid [17]. Autoradiograms were

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scanned by laser densitometry using an LKB UltroScan XL densitometer and analyzed with the Gelscan XL software.

Measurement of phospholipase A_2 (PLA_2) activity. PLA_2 activity was assayed using a modification of a described method [18]. HMBA-treated cells ($2–4 \times 10^7$) were washed in phosphate-buffered saline and resuspended at a concentration of 10^7 cells/mL in lysis buffer (0.34 M sucrose, 10 mM HEPES). The membrane fraction was obtained by repeatedly passing the cell suspension through a 26-gauge needle. Membranes were obtained by centrifugation, washed, and resuspended in lysis buffer. Aliquots of the membrane fraction were incubated for 2 hr at 37° in 70% glycerol, 5 mM $CaCl_2$, 20 mM glycine buffer, pH 9.0, 200 μM Triton X-100, 10 μM phosphatidylcholine (L-3-phosphatidylcholine, 1,2-dipalmitoyl), and 50,000 cpm of L-3-phosphatidylcholine 1-palmitoyl 2-[1-^{14}C]palmitoyl (58 mCi/mmol; Amersham Corp., Arlington Heights, IL). The reaction was terminated by adding 250 μL of chloroform:methanol:acetic acid (4:2:1), 500 μL of deionized water and 250 μL of chloroform. After centrifugation, the chloroform phase was removed and lyophilized, and the lipids were dissolved in 30 μL of chloroform:methanol (2:1) for separation on 250 μm Whatman LK 6 silica gel thin-layer chromatography (TLC) plates. The solvent system was chloroform:methanol:acetic acid (300:20:1). Authentic palmitic acid was cochromatographed and visualized by exposing to iodine vapor. The palmitic acid fraction was scraped from the plates and monitored for ^{14}C. Protein concentrations were determined using the Bio-Rad Protein Assay (Richmond, CA).

Measurement of the release of arachidonic acid and its metabolites. The release of arachidonic acid and its metabolites was monitored as described [19]. Cells in logarithmic growth phase were incubated for 16 hr in 0.3 μCi/mL [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (209 Ci/mmol; Amersham). Cells were resuspended in 1× Hanks' solution supplemented with 1 mg/mL fatty acid free bovine serum albumin and 4 mM $CaCl_2$, and then exposed to HMBA. Release of tritium into cell-free supernatants was determined by scintillation counting.

RESULTS

Differentiation of myeloid leukemia cell lines is associated with a loss of proliferative capacity and phenotypic changes. The growth of U-937 cells was similarly inhibited following exposure of 4 mM HMBA (Fig. 1A). This inhibition was apparent after 2 days of HMBA treatment and continued through 3 days (Fig. 1A). The effects of HMBA on U-937 cell differentiation were also determined by monitoring changes in histochemical staining and cell surface antigen expression. HMBA treatment was associated with a progressive increase in the percentage of cells staining positively for NSE activity (Fig. 1B). For example, nearly 70% of these cells were NSE positive after 3 days of HMBA exposure, while only 12% of the uninduced control cells had detectable staining at this time (Fig. 1B).

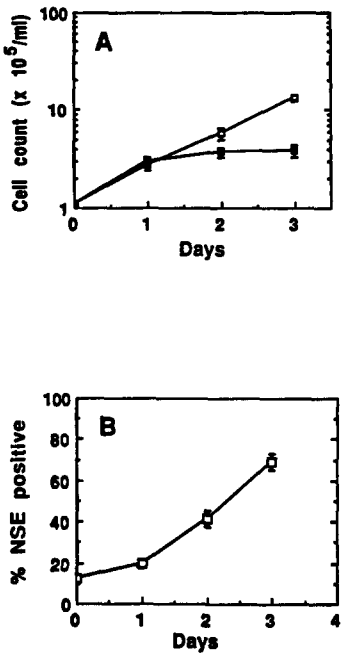

Fig. 1. Effects of HMBA on U-937 cell growth and NSE staining. (A) Viable cells were determined by trypan blue exclusion for untreated U-937 cells (□) and cells treated with 4 mM HMBA (■). Values are means ± SD for three separate experiments. (B) Cytocentrifuge smears of cells were examined for NSE staining after 1, 2, and 3 days of treatment with 4 mM HMBA. Data are expressed as the mean percent ± SD of cells with ≥ 3 positively stained granules (200 cells counted). Data were obtained from two separate experiments, each performed in duplicate. There was no significant change in NSE staining for untreated U-937 cells during the 3 days.

In addition to NSE activity, expression of the Mac-1 cell surface antigen is increased during 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced monocytic differentiation of U-937 cells [10]. HMBA treatment of U-937 cells was also associated with increases in Mac-1 surface antigen expression. For example, flow cytometry of untreated cells demonstrated a mean fluorescence of 29 (arbitrary units) for Mac-1 expression; this value was increased to 69 after exposure to HMBA for 96 hr. Similar increases were obtained in two separate experiments. Taken together, these findings indicated that HMBA induces U-937 cells to differentiate along a monocytic lineage.

Previous studies have demonstrated that induction of monocytic differentiation by phorbol esters or DMSO is associated with activation of PLA_2 and eicosanoid release [18, 19]. Cell-free extracts of HMBA-treated U-937 cells were monitored for their ability to hydrolyze dipalmitoyl phosphatidylcholine to free palmitic acid by PLA_2. PLA_2 activity was 67 pmol/mg protein/hr in uninduced cells (Table 1); treatment with HMBA was associated with stimulation of this enzyme. For example, the activity of this enzyme at 6 hr of HMBA exposure was approximately 75% greater than that detected in

Table 1. Effects of HMBA on membrane-bound phospholipase A_2 activity in U-937 cells

HMBA treatment (hr)	Specific activity (pmol/mg protein/hr)
0	67 ± 7
1	96 ± 9
3	103 ± 13
6	115 ± 13
24	96 ± 4

U-937 cells were treated with HMBA for the indicated times. The cells were then harvested and assayed for PLA_2 activity. The results (means ± SEM for two separate experiments each with six replicates) are expressed as pmol [^{14}C]phosphatidylcholine hydrolyzed/mg protein/hr.

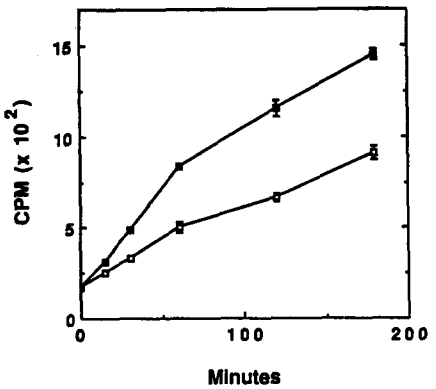

Fig. 2. Effect of HMBA on the release of arachidonic acid and its metabolites from U-937 cells. U-937 cells were prelabeled with [^{3}H]arachidonic acid for 16 hr, washed and exposed to 4 mM HMBA. Release of tritium into the supernatant was monitored at the indicated times for the untreated (□) and HMBA-treated cells (■). The determinations were performed in triplicate for each time point. Values are means ± SEM. Four other independent experiments demonstrated similar results.

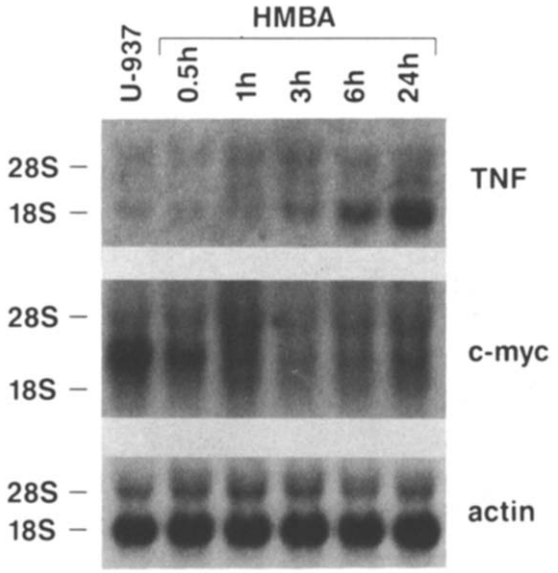

Fig. 3. Effects of HMBA on TNF, c-*myc*, and actin mRNA levels in U-937 cells. U-937 cells were treated with 4 mM HMBA. Total cellular RNA (20 μg) was isolated at the indicated times for Northern analysis with ^{32}P-labeled TNF, c-*myc* and actin probes.

uninduced cells (Table 1). The stimulation of PLA_2 activity was also associated with an increase in the release of arachidonic acid and its metabolites. The release of tritium-labeled arachidonic acid and metabolites into culture supernatants was higher by 30 min of HMBA exposure and reached levels approximately 45–70% over that of untreated control cells (Fig. 2).

Induction of monocytic differentiation in leukemic cell lines is associated with a decrease in the level of c-*myc* mRNA and induction of TNF transcripts [14, 16]. Treatment of U-937 cells with HMBA was similarly associated with a down-regulation of c-*myc* gene expression which was detectable after 30 min of drug exposure (Fig. 3). This effect of HMBA on c-*myc* mRNA levels persisted through 24 hr. In contrast to c-*myc*, TNF transcripts were constitutively expressed at a low, but detectable level. Furthermore, HMBA treatment was associated with a progressive increase in TNF mRNA levels during HMBA exposures of 3–24 hr (Fig. 3). There was no detectable effect of HMBA on actin mRNA levels (Fig. 3). We also monitored the effects of dexamethasone, an inhibitor of monocytic differentiation [18, 19], on HMBA-induced changes in gene expression. Dexamethasone had no detectable effect on the down-regulation of c-*myc* expression by HMBA (Fig. 4). However, HMBA-induced increases in TNF mRNA levels were blocked by nearly 75% (determined by densitometric scanning) after pretreatment with 10 μM dexamethasone for 2 hr (Fig. 4).

DISCUSSION

HMBA is presently undergoing clinical trials as a differentiating agent in the treatment of myelodysplastic syndromes and acute myeloid leukemia [20]. This agent induces erythroid differentiation of MEL cells [2] and granulocytic differentiation of HL-60 cells [1]. The present studies demonstrate that HMBA induces a more differentiated monocytic U-937 cell phenotype as characterized by increases in NSE staining and Mac-1 cell surface expression. HMBA treatment was also associated with growth inhibition, down-regulation of c-*myc* expression and induction of TNF transcripts. The effects of this agent on hematopoietic cell differentiation thus appear to be dependent on the prior commitment of the leukemic cells to a specific lineage.

The signaling pathways activated by HMBA in the induction of differentiation are unclear. The effects of this agent have been shown to be mediated, in part, through a PKC-dependent pathway in MEL cells [4]. The present results demonstrate that HMBA activates PLA_2 and eicosanoid release. The

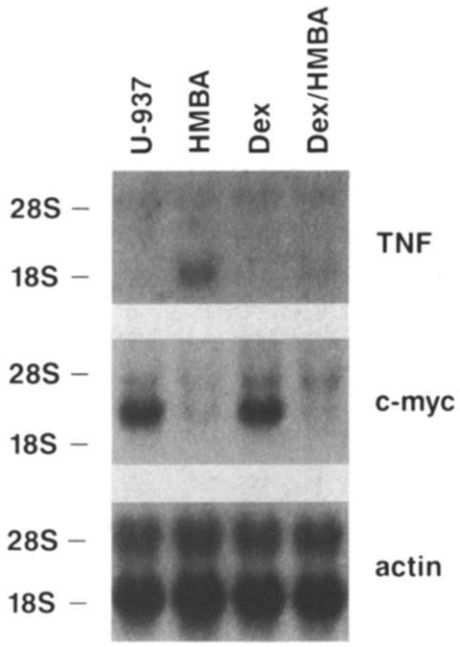

Fig. 4. Effects of dexamethasone on HMBA-induced gene expression. U-937 cells were pretreated for 2 hr with 10 μM dexamethasone. Cells were then treated with 4 mM HMBA for 24 hr. Total cellular RNA (20 μg) was isolated for Northern analysis with ^{32}P-labeled TNF, c-*myc* and actin probes.

rate-limiting step in eicosanoid synthesis is the deacylation of arachidonic acid from membrane phospholipids [21]. A major route for arachidonate liberation is the direct deacylation of arachidonate from membrane phospholipids by PLA_2 [22]. Eicosanoids have been shown to function as second messengers that regulate diverse cellular functions. Recent work has indicated a role for eicosanoids in the regulation of gene transcription during monocytic differentiation. For example, eicosanoids appear to regulate TNF, CSF-1 and c-*fms* gene expression during monocytic differentiation [19, 23, 24].

The mechanism whereby HMBA activates PLA_2 needs further study. PLA_2 has been shown to be activated by several different mechanisms. PLA_2 activation is Ca^{2+} dependent [25], and increases in Ca^{2+} flux have been shown to activate this enzyme in endothelial cells [26, 27]. Mechanisms of PLA_2 activation also include: (1) DAG-dependent PKC activation [28]; (2) DAG activation of PLA_2, independent of PKC in fibroblast cells [29]; and (3) PKC inactivation of lipocortins [30], 32–39 kD proteins which are presumed to regulate PLA_2 [31]. These mechanisms appear unlikely for HMBA since this agent has been shown to decrease PKC activity in MEL cells [4]. Furthermore, the related polar planar compound DMSO has been shown to decrease DAG levels in MEL cells [3]. Activation of a PLA_2 linked G-protein has been demonstrated in FRTL5 thyroid cells [32]. While it seems unlikely that a specific HMBA receptor exists, the membrane effects of polar planar compounds [5] could be associated with the stimulation of a membrane-bound G-protein or even direct activation of membrane-bound PLA_2.

The present studies also demonstrate that the induction of TNF transcripts by HMBA is blocked by dexamethasone. This glucocorticoid has pleiotropic effects on intracellular signaling mechanisms. For example, previous studies have demonstrated that dexamethasone inhibits activation of PLA_2 in induced U-937 cells [19, 33]. Dexamethasone also suppresses the induction of cyclooxygenase expression and inhibits activity of this enzyme [34]. Moreover, recent studies have demonstrated that the activated glucocorticoid receptor down-modulates the trans-acting function of the AP-1 transcription factor [35–37]. These findings are of potential relevance to the present results since the TNF gene promoter has an AP-1 binding site which may play a functional role in the transcriptional regulation of this gene [38]. Thus, further studies are needed to determine the dexamethasone-sensitive signaling mechanisms that are responsible for the induction of TNF expression by HMBA. In this regard, preliminary studies from this laboratory have suggested that HMBA treatment of U-937 cells is associated with increases in c-*jun* transcripts that encode for the AP-1 transcription factor (data not shown). Finally, the demonstration that HMBA down-regulates c-*myc* expression by a dexamethasone-insensitive mechanism suggests that multiple signaling pathways are activated by this agent during induction of monocytic differentiation.

Acknowledgements—This investigation was supported by PHS Grants HL 07516 (S.H.B.), CA 42802 and CA 01092 (M.L.S.), by a fellowship from the Deutsche Forschungsgemeinschaft (R.H.) and by a Burroughs Wellcome Clinical Pharmacology Scholar Award (D.W.K.).

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