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4-Hydroxynonenal-Induced MEL Cell Differentiation Involves PKC Activity Translocation

Monica Rinaldi,*,† Giuseppina Barrera,‡ Angelo Aquino,§ Paola Spinsanti,† Stefania Pizzimenti,‡ Maria Giulia Farace,¶ Mario Umberto Dianzani,‡ and Vito Michele Fazio*,‖,[1]

**Laboratory of Molecular Medicine and Biotechnology, University Campus Bio-Medico, School of Medicine, Rome, Italy; †Institute of Experimental Medicine, CNR, Rome, Italy; ‡Department of Experimental Medicine and Oncology, University of Torino, Torino, Italy; §Department of Neuroscience, and ¶Department of Experimental Medicine and Biochemical Science, University of Roma "Tor Vergata", Rome, Italy; and ‖Laboratory for Molecular Oncology and Gene Therapy, IRCCS H. Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy*

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4-Hydroxynonenal (HNE) is a highly reactive aldehyde, produced by cellular lipid peroxidation, able to inhibit proliferation and to induce differentiation in MEL cells at concentrations similar to those detected in several normal tissues. Inducer-mediated differentiation of murine erythroleukemia (MEL) cells is a multiple step process characterized by modulation of several genes as well as by a transient increase in the amount of membrane-associated protein kinase C (PKC) activity. Here we demonstrate that a rapid translocation of PKC activity from cytosol to the membranes occurs during the differentiation induced by HNE. When PKC is completely translocated by phorbol-12-myristate-13-acetate (TPA), the degree of HNE-induced MEL cells differentiation is highly decreased. However, if TPA is washed out from the culture medium before the exposition to the aldehyde, HNE gradually resumes its differentiative ability. The incubation of cells with a selective inhibitor of PKC activity, bisindolylmaleimide GF 109203X, partially prevents the HNE-induced differentiation in MEL cells. In conclusion, our results demonstrate that HNE-induced MEL cell differentiation is preceded by a rapid translocation of PKC activity, and that the inhibition of this phenomenon prevents the onset of terminal differentiation.

Lipid peroxidation of cellular membranes produces several carbonyls, including α-β unsaturated aldehydes (1). The most important class of them is the 4-hydroxy-2,3-trans alkenal group, quantitatively the main component being 4-hydroxynonenal (HNE) (2). In the recent years, it became evident that HNE is a normal constituent of several mammalian cells and tissues at concentrations ranging from 0.2 to 2.8 μM (3, 4). A negative correlation exists between proliferative activity and/or anaplastic grade, and the ability of neoplastic cells to peroxidize (5). In fact, highly undifferentiated anaplastic cells show undetectable levels of both basal or inducible lipid peroxidation (6). Leukemic cells, in particular HL-60, K562 and MEL cells, do not show any detectable level of lipid peroxidation even if exposed to prooxidant compounds (7–9), thus their endogenous production of HNE is negligible. For this reason, these cells have been used, since several years, as models for the study of the exogenous HNE effects (7–11). When HNE is added to the leukemic cell suspension, it shortly disappears (within 45 min), since it rapidly reacts with the proteins and it is also metabolized by the cells (12). When maintained for a longer time in cell culture, through repeated treatments, 1 μM HNE can induce the block of proliferation and the onset of differentiation in HL-60 (8) and in MEL cells (9). Biological and toxic effects of HNE largely depend on its ability to react with thiol and amino groups of low molecular weight compounds and proteins (13, 14) affecting, by this mechanism, several cellular functions and enzymatic activities (15–17).

Recent data demonstrate that HNE is able to affect PKC activity in isolated rat hepatocytes (18). The term PKC includes a family of closely related serine/threonine protein kinases that function in cell surface signal transduction for a variety of external stimuli related to cell proliferation, metabolism and differentiation (19). PKC activity is generally found in the cell cytosol and then translocated to the plasma membranes upon activation (20). Upon exposure to hexamethylenebisacetamide (HMBA), MEL cells exhibit a rapid and transient increase in membrane-associated

[1] To whom correspondence should be addressed at CNR, Institute of Experimental Medicine, Via Fosso del Cavaliere 100, 00133 Rome, Italy. Fax: (39) (06) 49934257. E-mail: VitoM.Fazio@ims.rm.cnr.it.

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PKC activity, followed by a progressive decay of total PKC activity (21). In DMSO-induced cells the increase of PKC activity arises from the activation of a pool of inactive PKC residing inside membranes (22).

It has been reported that dexamethasone and the tumor promoter phorbol-12-myristate-13-acetate (TPA) suppress DMSO- and HMBA-induced MEL cells terminal differentiation (23, 24), probably by acting at a late step during the "latent period" that precedes the onset of terminal differentiation (25). To examine carefully the mechanisms by which HNE induces erythroid differentiation in MEL cells, we studied the distribution of PKC activity after HNE treatment and the effect exerted by TPA and by a selective inhibitor of PKC activity, bisindolylmaleimide GF 109203X (BIM), on HNE-induced MEL cell differentiation.

MATERIALS AND METHODS

Cells and culture conditions. Mouse erythroleukemic (MEL) cells (clone 86, kindly provided by Professor Pontremoli, University of Genova, Italy) were cultured at 37°C in a humidified atmosphere of 5% CO_2–air in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% fetal calf serum (Gibco BRL). Cells were maintained at a density of 1–2 $\times$ 10^5/ml. Growth rate and cell viability were determined daily by conventional techniques.

Induction of differentiation. 4-Hydroxynonenal (HNE, gift from Professor J. Schaur, University of Graz, Austria) was prepared as previously reported (11). Exponentially growing cells were resuspended at a concentration of 2 $\times$ 10^5/ml in fresh complete medium. Since HNE rapidly disappears from the culture medium, its repeated addition was necessary to induce terminal differentiation of MEL cells (9). Addition of HNE (1 μM) was performed according to a protocol previously established (9). Briefly, HNE (1 μM) was added at regular intervals of time (45 min) up to 14 treatments at day 1, and moreover 14 treatments with 1 μM HNE were performed at day 2.

Since the susceptibility of MEL cells to undergo terminal differentiation was not identical in different experiments, the degree of HNE-induced differentiation was compared with that observed in DMSO-treated cells. DMSO (Sigma Chemical Co.) was added to the culture medium at the beginning of experiments, at concentration of 1.5%. The degree of HNE-induced differentiation was compared with that induced by DMSO also in experiments with TPA and BIM treatments (data not shown).

TPA and BIM treatments. TPA (Sigma Chemical Co.) was prepared as a concentrated stock in DMSO and used at final concentration of 0.1 μM. In the experiments in which the distribution of PKC activity between cytosol and membranes was analyzed, TPA was used as a positive control of PKC activity translocation. In these cases, TPA was added to the cell suspension for 15 min. After this time cells were collected for the membrane separation. In experiments performed in order to evaluate the TPA effects on MEL cells differentiation, TPA treatments were performed according to two experimental procedures:

(1) MEL cells were preincubated in the presence of TPA (0.1 μM) for 30 min, then the cells were washed, resuspended in fresh medium and treated with HNE as above described.

(2) TPA (0.1 μM) was added directly to culture medium together with HNE.

Bisindolylmaleimide GF 109203X (BIM) (Boehringer Mannheim), a potent and selective inhibitor of PKC, was prepared according to manufacturer's protocol. The inhibitor was added to the culture at the concentration of 1 μM, 15 min before induction with HNE.

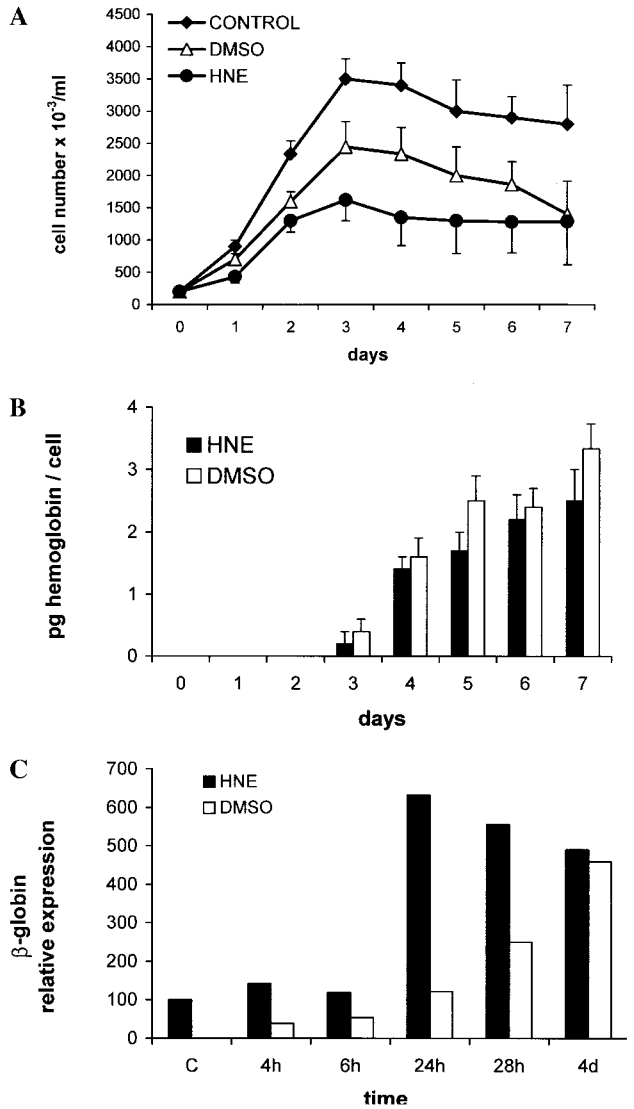

FIG. 1. (A) Growth of MEL cells repeatedly treated with 1 μM HNE (see Materials and Methods) or exposed to 1.5% DMSO for overall time of experiment. Data are the mean $\pm$ SD of four separate experiments. (B) Hemoglobin content in MEL cells repeatedly treated with 1 μM HNE (see Materials and Methods) or exposed to 1.5% DMSO for overall time of experiment. Data are the mean $\pm$ SD of four separate experiments. (C) Densitometric analysis of β-globin gene expression.

Determination of hemoglobin production. Hemoglobin concentration of MEL cells was determined as follows: 10 $\times$ 10^6 cells were lysed in 1 ml of lysing buffer (10 mM Tris, pH 7.5, 137 mM NaCl, 1.4 mM Mg-acetate and 0.5% Nonidet P-40), and incubated on ice for 15 min. The lysate was then centrifuged at 1500 rpm for 15 min (ALC, 4236 centrifuge). Hemoglobin concentration were quantified by optical density (OD) at 414 nm (OD 1000 corresponds to 0.13 mg/ml Hb).

RNA isolation. Total cytoplasmic RNA was isolated from MEL cells as described by Sambrook *et al.* (26) with minor modification: cells (40 $\times$ 10^6) were resuspended in 0.4 ml of lysis buffer containing

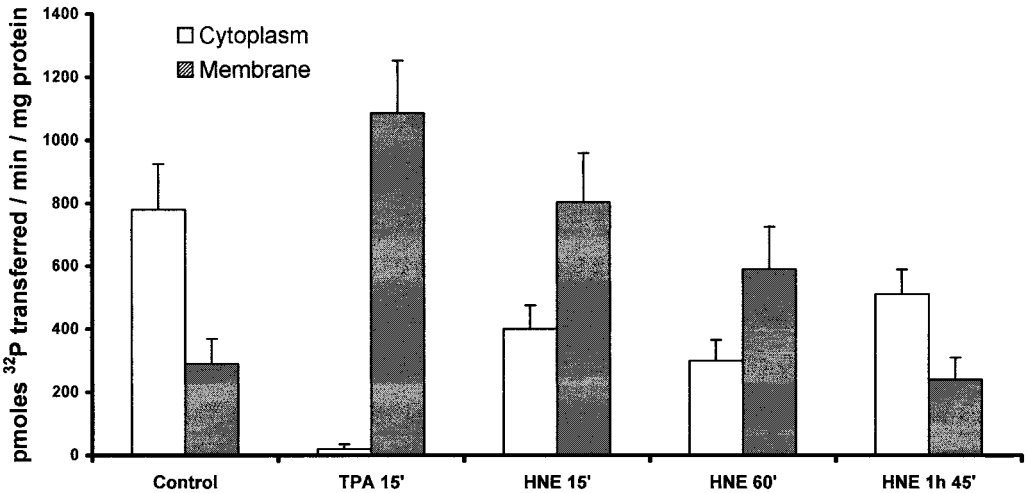

FIG. 2. PKC activity in cytosol and membranes of control MEL cells and of cells treated with TPA (0.1 μM) for 15 min or with HNE (1 μM) for 15 min, 1 h, and 1 h and 45 min. Results are the mean ± SD of three separate experiments.

10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl, 0.5% NP-40. The cytoplasmic extract was then treated 2 h at 45°C with 500 μg/ml Proteinase K (Sigma Chemical Co.) in 0.2 M LiCl, 10 mM Tris, pH 8, 10 mM EDTA, 0.5% SDS, 1% β-mercaptoethanol to denature proteins. The cytoplasmic RNA was then extracted twice with phenol/chloroform/isoamyl alcohol, once with chloroform/isoamyl alcohol, and recovered by ethanol precipitation. Each sample was examined for purity and degradation on a nondenaturing agarose gel prior to Northern blot analysis. Total RNA was isolated at different times after addition of inducers, HNE or DMSO. Uninduced cells were used as time 0.

Northern blot analysis. Equal amounts of RNA (15–30 μg), as determined by UV-absorption at 260 nm, were separated on 0.9% agarose–2.2 M formaldehyde gel and transferred to nitrocellulose filters (Hybond-C, Amersham). Hybridizations were carried out as previously described (7) with probes labeled by random priming method (Megaprime DNA labeling system RPN 1607, Amersham; 1.5×10^6 cpm/ml). Each filter was hybridized with mouse β-globin gene cDNA.

Preparation of cytosolic and membrane fractions. MEL cells were washed twice with PBS without Ca^{2+} and Mg^{2+}. The cell pellets (50×10^6 cells) were suspended in 0.5 ml of extraction buffer (25 mM Hepes, pH 7.5, 0.25 M sucrose, 2.5 mM MgCl, 2.5 mM EGTA, 50 mM 2-mercaptoethanol, 20 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), sonicated for 5 s at 4°C (Heat Systems-Ultrasonics, Inc.), and then centrifuged at 100,000*g* for 1 h at 4°C. The supernatant fractions were removed and used as the cytosolic fraction. The pellets were resuspended in 0.5 ml extraction buffer containing 1% Triton X-100, sonicated for 5 s and centrifuged at 15,000*g* for 10 min at 4°C in an Eppendorf microcentrifuge. The supernatants were used for the determination of membrane-associated enzyme activity. The times of incubation with HNE and TPA are indicated in the figure legends.

DEAE-Sepharose chromatography. The cytosolic and membrane fractions were adsorbed in a 1.5 ml microcentrifuge tube to a 0.2 ml of DEAE-Sepharose fast flow (Pharmacia) previously equilibrated with buffer A (20 mM Tris, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol). The column was washed three times with the same buffer and then eluted with buffer A containing 0.2 M NaCl. Protein concentrations were determined with the Bio-Rad protein reagent using BSA as the standard.

Protein kinase C assay. PKC activity was determined by measuring the transfer of ^{32}P from -P^{32} ATP (Amersham) to a substrate (histone H1). DEAE-Sepharose fractions (5 μl) were incubated in a 50 μl reaction mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM MgCl, 40 μg of histone H1, 100 μg/ml of phosphatidylserine (PS), 500 μM $CaCl_2$ or 200 μM EGTA and PS without $CaCl_2$. Assay was carried out as described by Aquino *et al.* (27).

RESULTS

The differentiation induction of MEL cells by HNE is demonstrated by modulation of growth, hemoglobin content and β-globin mRNA level in MEL cells (Fig. 1). HNE effects were compared to those obtained in DMSO-treated cells. Growth of MEL cells was highly reduced by HNE treatments (Fig. 1A). Hemoglobin content (Fig. 1B) was significantly increased in HNE treated cells starting from day 4, whereas β-globin expression (Fig. 1C) increased as soon as 24 h after the beginning of treatments and remained at high level even at 4 days.

DMSO induced the same modulations observed in HNE-treated cells, but with some important differences: DMSO inhibitory effect on cell growth was lower than that displayed by HNE; β-globin accumulation in HNE-treated cells preceded that induced by DMSO, but the two expressions became similar after 4 days. The subcellular distribution of PKC activity between cytosol and membrane fraction was compared in uninduced and induced cells at early times during incubation with HNE. TPA-treated cells were used as a positive control (Fig. 2). After 15 min from the addition of TPA, a total translocation of PKC activity to the membrane fraction occurred. Equally rapid translocation, even if lower than in TPA-treated cells, was demonstrated also in HNE-treated cells 15 min after the treatment and it persisted up to 1 h, whereas, after 1 h and 45 min, the membrane-associated PKC activity became lower than that detected in the cytosol. In the

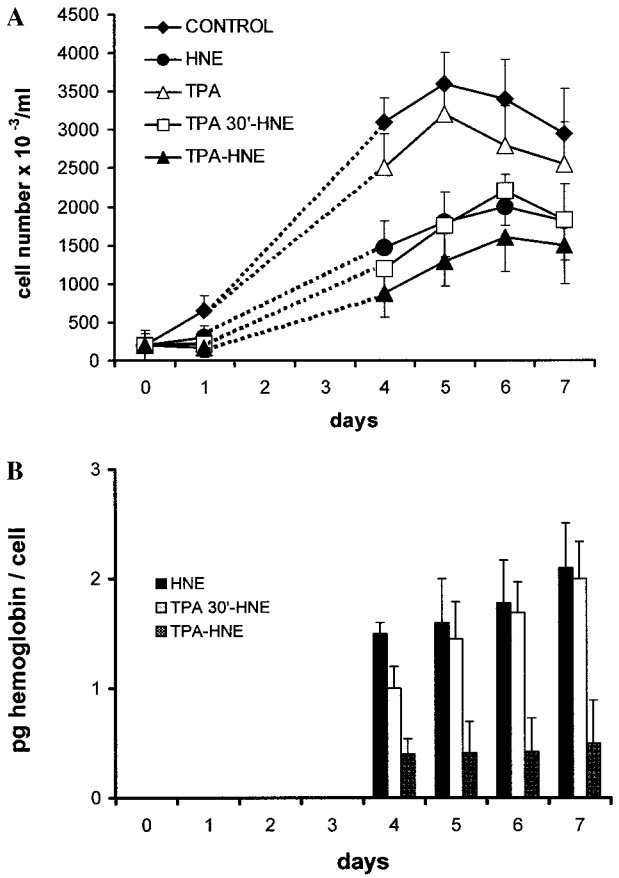

FIG. 3. (A) Growth of MEL cells treated with TPA (0.1 μM), HNE (repeated treatments over 2 days with 1 μM HNE) or preincubated with TPA for 30 min before HNE treatments or maintained in presence of TPA during the HNE treatments and for all the length of experiments (7 days). Results are the mean ± SD of three separated experiments. (B) Hemoglobin content of MEL cells treated with HNE alone (repeated treatments over 2 days with 1 μM HNE), preincubated with TPA for 30 min before HNE treatments or maintained in the presence of TPA during the HNE treatments and for all the length of experiments (7 days). Results are the mean ± SD of three separate experiments.

days following the HNE treatments, PKC activity distribution between cytosol and membranes was similar to those observed in the control cells (data not shown).

In Fig. 3A is reported the growth rate of cells treated either with HNE or TPA, or both, according to the procedures described in materials and methods. HNE strongly inhibited MEL cells proliferation, whereas TPA alone did not show any significant effect. Interestingly, a significant reduction of cell growth in HNE-treated cells persisted even after TPA treatment, not only when TPA was removed before HNE treatments, but also when the compound was maintained in cell cultures for 7 days. None of these treatments affected cell viability.

The degree of differentiation was evaluated by analyzing the hemoglobin content in MEL cells treated either with HNE or with TPA in combination with HNE (Fig. 2B). The values of hemoglobin were checked starting for 4th to 7th day, by subtracting the control values obtained at the corresponding time. TPA alone did not induce hemoglobin accumulation (data not shown), according to the results reported by others (28). When TPA was added to cell suspension for 30 min and washed out before HNE treatment, the hemoglobin content at day 4 was lower than that obtained with HNE alone. However, during the following days, hemoglobin content increased, reaching values comparable with those of HNE-treated cells. On the contrary, the simultaneous addition of TPA and HNE caused a strong decrease of inducer-mediated hemoglobin accumulation at every time it was tested.

In this study we also evaluated the effect of a selective inhibitor of PKC in HNE-induced differentiation of MEL cells. BIM is a potent inhibitor of PKC acting in the range of 1–5 μM, both *in vitro* and *in vivo* (29). Growth of MEL cells was significantly inhibited either by HNE or by HNE plus 1 μM BIM, whereas BIM alone did not affect this parameter (Fig. 4A). Hemoglobin content in MEL cells treated with both BIM and HNE

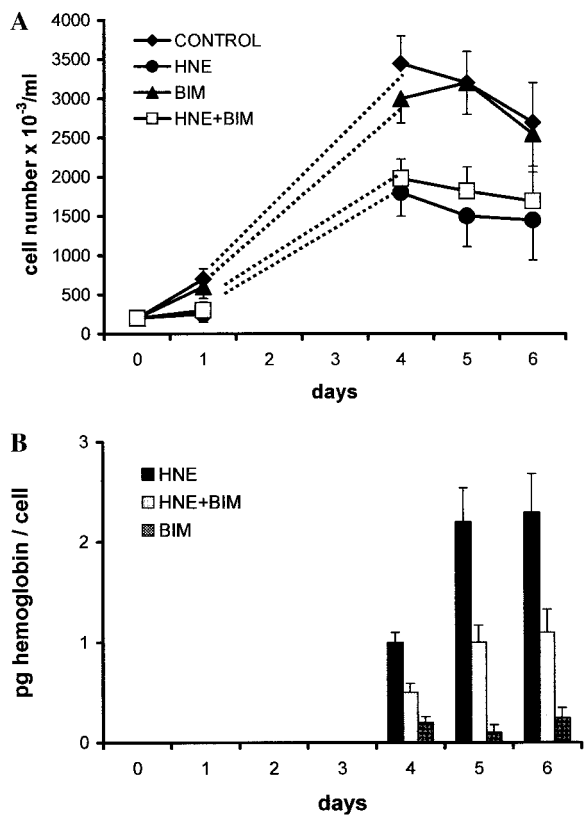

FIG. 4. (A) Growth of MEL cells treated with HNE (repeated treatments over 2 days with 1 μM HNE), BIM (1 μM) or with both HNE and BIM. Results are the mean ± S.D. of three separated experiments. (B) Hemoglobin content of MEL cells treated with HNE (repeated treatments over 2 days with 1 μM HNE), BIM (1 μM) or with both HNE and BIM. Results are the mean ± SD of three separate experiments.

(Fig. 4B) was reduced by 50% with respect to that detected in HNE-treated cells at 4, 5, and 6 days. BIM alone did not induce hemoglobin accumulation. None of these treatments affected cell viability (data not shown).

DISCUSSION

Several direct and indirect evidences are consistent with the rapid translocation of PKC in the process of differentiation induction (21, 30). We demonstrated that HNE induces an early translocation to the membrane of PKC activity. Since TPA decreases drastically the proportion of cells induced to differentiate by HNE, this translocation seems to be necessary for the HNE-induction. Moreover, the reduction of differentiated cells is reversed when TPA was removed from the cell cultures before HNE treatments. Similar results have been obtained in HMBA-induced MEL cell differentiation, in which, upon removal of TPA, the restoration of MEL cells sensitivity to HMBA was correlated with reaccumulation of PKC activity (21).

Although TPA completely prevents the HNE-induced differentiation, it does not affect the reduction of proliferation induced by HNE treatments. This phenomenon may be explained by assuming that the inhibition of cell growth and the accumulation of hemoglobin are determined by HNE acting on different mechanisms. In fact, our results and other previous observations (31) demonstrate that the inhibition of growth in HNE-treated cells is stronger than that provoked by other differentiation inducers, i.e., DMSO and precedes the onset of differentiation (8, 9, 31). HNE inhibition of cell growth was observed even in the absence of terminal differentiation (7) but it is always accompanied by a strong inhibition of c-myc (7, 11) and c-myb oncogene mRNA expressions (32). This may suggest that the inhibition of proliferation is, at least at the outset, not linked to the hemoglobin accumulation. According our observations, it has been reported that inducer-mediated accumulation of hemoglobin is more sensitive to inhibition by TPA than the early inducer-mediated changes are (21). In fact, TPA does not block the early HMBA- and DMSO- induced inhibition of c-myc and c-myb expressions (21, 23).

The reduction of HNE-induced differentiation by BIM confirms the involvement of some PKC isoforms in this process. BIM shows a highly selective inhibition of PKC isoenzymes α, $\beta 1$, $\beta 2$, γ, δ and ϵ (33), some of which are highly expressed in MEL cells (α, γ, ϵ) (34). An increase in the membrane associate PKC isoforms delta and epsilon has been reported during MEL cells differentiation induced by HMBA (34). These isoforms are both inhibited by BIM. Thus, we may suppose that delta and epsilon isoforms of PKC may be involved also in HNE-induced differentiation. However, the inhibition of HNE-dependent differentiation induced by BIM is not complete. This may suggest that other PKC isoforms, not inhibited by BIM, may be involved. Further studies are in progress to clarify this observation.

The role of PKC activity during HNE-induced differentiation is by no doubt complex, and it is reasonable that it represents only one element in a cascade of signal transduction. PKC activity can be modulated in different ways by the differentiation inducers: HMBA causes, like HNE, a transient increase in PKC membrane-associated activity (30, 34), while DMSO seems to activate the enzyme residing on the membrane in a responsive but inactivated state and not by triggering the translocation of cytosolic PKC to membranes (35). Our results point out that the translocation of PKC activity is required to induce MEL cells differentiation HNE. In fact, the differentiation is completely prevented when cytosolic PKC is depleted by TPA.

In conclusion, the present results add a new evidence to the complex differentiation pathway induced in MEL cells by HNE.

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