Brief Communication

EFFECT OF 4-HYDROXYNONENAL ON CELL CYCLE PROGRESSION AND EXPRESSION OF DIFFERENTIATION-ASSOCIATED ANTIGENS IN HL-60 CELLS

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Abstract—4-Hydroxynonenal (HNE) is a highly reactive aldehyde produced by lipid peroxidation of cellular membranes that inhibits growth and induces differentiation in HL-60 cells. Its mechanisms of action were investigated by analyzing the cell cycle distribution and the appearance of differentiated phenotypes in HL-60 cells. Data obtained by exposing cells to DMSO for 7.5 h (same time as for HNE treatment) or for the whole length of the experiments (5 d) were used for comparison. HNE induced a marked increase in the proportion of G0/G1 cells after 1 and 2 d. The brief DMSO treatment did not affect the distribution, whereas continuous exposure led to a progressive accumulation of cells in G0/G1 (maximal at day 5). The proportion of phagocytic cells gradually increased in HNE-treated and DMSO long-exposed cultures from day 2 and peaked at day 5 (35 and 63%, respectively), whereas the effect of the brief DMSO treatment was negligible. The expression of CD11b and CD67 increased in cells treated with HNE or continuously exposed to DMSO, whereas CD36 was expressed at low levels on both treatments. These results indicate that the pathway of the granulocytic differentiation induced by HNE in HL-60 cells differs from that of DMSO: with HNE, growth inhibition precedes the onset of differentiation, whereas in DMSO-treated cells the two processes are chronologically associated.

Keywords—Lipid peroxidation, HNE, DMSO, HL-60 cells, Cell cycle, Differentiation, CD antigens, Phagocytosis, Free radicals

INTRODUCTION

Lipid peroxidation produces several toxic carbonyls, including alpha-beta unsaturated aldehydes. HNE (one of the major end products of membrane peroxidation) exerts many biological effects.1 Lipid peroxidation also occurs under physiological conditions, particularly in cells that are not rapidly proliferating.2 It has recently become evident that HNE is a normal constituent of many mammalian cells and tissues at concentrations ranging from 0.28 to 2.8 μM.3 By contrast, proliferating neoplastic cells, such as K562 and HL-60, do not show detectable levels of lipid peroxidation even if exposed to prooxidant compounds,2,4,5 and their endogenous production of HNE can, therefore, be ruled out.

The inverse relationship between lipid peroxidation levels and cell proliferation6 suggests that some lipid peroxidation products, particularly those with the highest biological activity, are involved in the control of cell proliferation. Aliphatic aldehydes inhibit the proliferative response of human peripheral blood lymphocytes to phytohemagglutinin and alloantigens,7 and studies on leukemic cell lines have demonstrated that at concentrations similar to those found in normal cells, HNE inhibits ornithine decarboxylase and c-myc expression in K562 and HL-60 cells.5,8,9 Moreover, blockade of cell proliferation and onset of differentiation have both been observed10 in HL-60 cells maintained for several hours in the presence of micromolar aldehyde concentrations.
Our previous observations on HL-60 cells treated with HNE\(^{10}\) suggest that growth inhibition precedes the onset of differentiation, whereas in DMSO-treated cells the two processes are temporally associated.\(^{10,11}\) However, because HL-60 cells can differentiate along the monocytic or granulocytic pathway, no information about the phenotypic characteristics of HNE-treated cells was provided by these studies. To further explore the action of HNE in the control of cell proliferation and differentiation, we examined for 5 d the cell cycle distribution and appearance of the differentiated phenotype in the HL-60 line treated with HNE or DMSO. DMSO is one of the most studied differentiation inducers of leukemic cells,\(^{12,13}\) and the first compound to demonstrate a differentiative effect on the HL-60 cell line.\(^{11}\) It also induces HL-60 maturation in granulocytic-like cells,\(^{13}\) and is good reference for studies of HNE action in this line. Unlike other differentiating agents, such as DMSO and retinoids, HNE is highly unstable in the culture medium (it disappears within 45 min) and must be repeatedly added to activate the differentiation program.\(^{10}\) Differentiation was evaluated both as acquisition of phagocytic capability and as expression of the differentiation-associated surface antigens CD11b, CD67, and CD36. CD11b is a leukocyte integrin subunit that occurs on the surface of both human granulocytes and monocyte-macrophages, and mediates their multifaceted adherence reactivity.\(^{14,15}\) CD67, a granulocyte-specific antigen, is upregulated during the intermediate stages of maturation,\(^{16}\) and occurs within specific granules\(^{17}\) attached to membranes via a glycosyl-phosphatidyl inositol anchor.\(^{18}\) CD36, identified as the platelet glycoprotein gp IV, is induced during the monocytic differentiation of myeloid cells.\(^{19}\)

**MATERIALS AND METHODS**

**Cells and culture conditions**

HL-60 cells (DSM, German collection of microorganisms and cell cultures, Braunschweig, Germany) were cultured at 37°C in a humidified atmosphere of 5% CO\(_2\) in air in RPMI 1640 medium (Biochrom KG, Berlin) supplemented with 2 mM glutamine, antibiotics, and 10% FCS (Biochrom KG, Berlin). Growth rate and cell viability were monitored daily by the trypan blue exclusion test.

For morphological assessment, cytospin slide preparations of cell suspensions were prepared using a Cytospin 2 (Shandon) centrifuge and stained with May-Grunwald-Giemsa.

**Cell treatments**

HL-60 cells were seeded at 100,000/ml and cultured for 5 d. HNE, kindly provided by Prof. H. Esterbauer, University of Graz, Austria, was prepared as previously described.\(^{10}\) At the beginning of each experiment, 1 µM HNE was added 10 times to the cultures at 45-min intervals. HNE concentrations were monitored by HPLC after each addition, and never exceeded 1 µM (data not shown). HNE was no longer detectable in the culture medium 45 min after the last addition. Cells were exposed to 1.25% DMSO (Sigma Chemical Co.) in the culture medium for the initial 7.5 h only, corresponding to the length of the HNE treatment, after which they were washed and resuspended in DMSO-free medium.

**Cell cycle analysis**

Cells (10^6) were pelleted, washed twice with Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS (GIBCO), fixed in 70% ethanol, and stored at 4°C. Prior to analysis, they were centrifuged and resuspended in PBS containing DNase-free RNase (type 1-A, Sigma Chemical Co.) and PI (Sigma Chemical Co.) at the final concentrations of 0.4 and 0.18 mg/ml PBS, respectively. After 30 min at room temperature, their DNA content was analyzed in duplicate with a FacScan flow cytometer (Becton & Dickinson, Mountain View, CA) equipped with a 488 nm argon laser. Two filters were used to collect the red fluorescence due to PI staining of the DNA, one transmitting at 585 nm with a bandwidth of 42 nm (FL2), the other above 620 nm (FL3). FL2 and FL3 were registered on a linear and a logarithmic scale, respectively. Forward and side light scatter were simultaneously measured. The flow rate was about 200 cells/s and at least 10^4 cells were analyzed from each sample. Data were recorded in a Hewlett-Packard computer (HP 9000, model 300), using CellFit software (Becton & Dickinson).

**Phagocytosis assay**

Phagocytosis was evaluated by counting the number of HL-60 cells that engulfed opsonized zymosan (Sigma Chemical Co.) as previously described.\(^{10}\)

**Detection of differentiation-associated surface antigens**

Expression of cell surface antigens CD11b, CD67 and CD36 was tested by immunofluorescence and detected by both fluorescence microscopy and flow cytometry.

Cells were washed twice with PBS, then incubated with mouse monoclonal antibodies (Serotec, Oxford, UK) directed against CD11b (clone 44), CD67 (80H3), and CD36 (SMO). CD36 expression was evaluated by direct immunofluorescence because its antibody is FITC-conjugated; double immunofluores-
cence with a secondary FITC-conjugated antimouse IgG (Vector, Burlingame, CA) was needed to detect CD11b and CD67.

**Fluorescence microscopy**

After incubation with the antibodies, 3 × 10⁶ cells per sample were pelleted, resuspended in 1 ml of 0.1% sodium azide in PBS layered onto a slide, covered with a coverslip, and scored for fluorescence in an epilluminated microscope (Leitz, Dialux 20). Results were expressed as percentage of fluorescent cells.

**Flow cytometric analysis**

After incubation with the antibodies, 10⁶ cells per sample were washed twice with PBS. Background fluorescence was determined with unstained cells. A filter transmitting at 530 nm (30 nm bandwith) was used to collect the green fluorescence. A minimum of 10,000 cells per sample were analyzed; data were acquired in list mode and analyzed with Lysis software (Becton & Dickinson).

**RESULTS**

**Growth and cell cycle analysis**

HNE (10 additions of 1 μM HNE in the initial 7.5 h) arrested growth for 2 d, followed by a slight recovery on days 4 and 5 (Fig. 1). Viability was not affected. By contrast, an incubation of the same length with 1.25% DMSO did not significantly affect growth. When cultures were continuously exposed to DMSO, a significant reduction of cell growth was observed after 4 and 5 d with no effect on viability.

These results were confirmed by flow cytometric analysis of the cell cycle distribution (Fig. 2). Percentages of cells in G0/G1, S, and G2/M phases were estimated at t₀, after 7.5 h (end of the HNE treatment and the brief exposure to DMSO) and every 24 h for 5 d. Control cells did not display significant differences in cycle distribution. Similarly, no changes were observed after the brief DMSO exposure; however, HNE induced a marked accumulation (up to 80% of the total) of cells in G0/G1 at 24 and 48 h, followed by a progressive decrease. The G0/G1 increase was accompanied by a corresponding reduction of S phase cells. Continuous exposure to DMSO led to a progressive increase of G0/G1 cells accompanied by a decrease of S-phase cells (more evident on days 4 and 5).

Flow cytometry did not detect the emergence of a population characterized by a subdiploid DNA fluorescence (slightly lower than that in the G0/G1 peak (A₀ region)) generally correlated with the internucleosomal DNA fragmentation typical of apoptosis.

**Phagocytic activity**

The proportion of HNE-treated phagocytic cells increased from day 2 up to 35% on the fifth day (Fig. 3). The brief exposure to DMSO did not induce any significant change, whereas continuous exposure resulted in an increase that was fully comparable with that observed in HNE-treated cells until day 3, and then rose on day 4 to reach 63% by day 5.

**Immunofluorescence analysis of differentiation-associated antigens**

As shown by the phagocytosis assay, the degree of differentiation induced by the short exposure to DMSO was negligible. Expression of differentiation-associated surface antigens was, therefore, only examined in the cultures treated with HNE or continuously exposed to DMSO (Fig. 4). CD11b expression was closely correlated with the onset of phagocytosis. The differences between DMSO- and HNE-treated cells became maximally evident at day 5, when the percentages of cells expressing CD11b were about 50 and 25%, respectively.

It is well known that DMSO induces granulocytic
differentiation of HL-60 cells, \(^\text{13}\) whereas that induced by HNE is less clear. \(^\text{10}\) The expression of CD67 (granulocytic lineage antigen) and CD36 (monocytic antigen) was, therefore, compared after both treatments. The number of cells expressing CD67 increased by 20 and 30% after HNE and DMSO, respectively. These figures, however, were lower than those obtained with anti-CD11b antibodies.

CD36 was always expressed in a very small percentage of cells, and only a slight increase was observed at days 4 and 5.

**Flow cytometric analysis of differentiation-associated antigens in HNE-treated cells**

Five days after the HNE treatment, cells were analyzed by quantitative flow cytometry for CD11b, CD67, and CD36 antigens (Fig. 5). With the anti-CD11b and anti-CD67 antibodies, the HNE-treated cells exhibited 2.5- and 3.5-fold increase in fluorescence intensity. By contrast, the anti-CD36 profiles indicated a very low fluorescence in both control and treated cells, although a small increase was observed after HNE. These results are, thus, in good agreement with the fluorescence microscopy data.

**Morphology of HL-60 cells induced by DMSO or HNE**

HL-60 untreated cells are predominantly promyelocytes (Fig. 6A) with large round nuclei each containing two to four nucleoli and scattered chromatin. The nuclear/cytoplasmic ratio is relatively high. Cells continuously exposed to 1.25% DMSO (Fig. 6B) or treated with HNE (Fig. 6C), after 5 d exhibited the following changes: smaller size, decreased nuclear/cytoplasmic ratio, marked reduction of nucleoli and indentation, convolution, and segmentation of the nuclei. This pattern is analogous to that seen in HL-60 cells induced to granulocytic differentiation.\(^\text{13}\)

**DISCUSSION**

These results demonstrate that 7.5 h treatment with HNE inhibits HL-60 cell growth, and that this substan-

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Fig. 2. Effect of HNE and DMSO on cell cycle distribution. HL-60 cells stained with propidium iodide as described in Materials and Methods and analyzed by flow cytometry. (A) controls. (B) cells exposed to DMSO for 7.5 h. (C) cells treated with 1 \(\mu\)M HNE at 45-min intervals in the initial 7.5 h. (D) cells exposed to DMSO for 5 d. Results expressed as percentages of cells in the different phases of the cell cycle: ▲ G0/G1, • S, ■ G2/M. Values are the mean ± SD of four separate experiments.
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Fig. 3. Phagocytosis assay on HL-60 cells. ▲ controls 1 μM HNE, ○ 1.25% DMSO for 7.5 h, ■ 1.25% DMSO for 5 d. Results, expressed as percentages of cells that showed phagocytic activity (see Materials and Methods for details), are the mean ± SD of six separate experiments.

Fig. 4. CD11b, CD67, and CD36 expression, detected by fluorescence microscopy, on the surface of HL-60 cells treated with 1 μM HNE or exposed for 5 d to 1.25% DMSO. Results, expressed as percentages of positive (fluorescent) cells, are the mean ± SD of five separate experiments.

tially reflects impairment of the progression from the G0/G1 to S phase, as indicated by the progressive accumulation and depletion of cells in these two phases. These effects are most evident after 24 and 48 h. The proportion of G0/G1 cells also increases after DMSO, though 5 d are needed to reach a comparable number of cells in G0/G1 (about 80%), and the 7.5 h exposure has no effect.

This growth arrest and G0/G1 accumulation is not accompanied by cell death, as demonstrated by trypan blue exclusion testing and by flow cytometry, nor by morphological evidence of cell necrosis or apoptosis. The latter was also ruled out by the lack of evidence of internucleosomal DNA fragmentation, as evaluated by agarose gel electrophoresis (data not shown).

Reversibility of G0/G1 accumulation after HNE treatment may be a result of the shortness of the treatment as reported for other inducers. 11,22

In HNE-treated cultures phagocytosis begins when ceases the G0/G1 arrest, whereas in those continuously exposed to DMSO it is initiated in a situation of progressive cell cycle arrest. The highest increase in phagocytosis occurs from the fourth to the fifth day, whereas the number of phagocytosing cells induced by HNE remains virtually unchanged during this period.

Several reports indicate that the highest degree of HL-60 cell differentiation by DMSO occurs after 5 d and that its extent depends on the number of HL-60 passages. 11,23 The number of passages of the HL-60 cells used in our experiments is unknown: about 60% display differentiation after 5 d of exposition to DMSO, whereas this figure was far smaller after 7.5 h only. HNE is, thus, more effective than DMSO, because approximately 35% of cells develop differentiated characteristics after an exposition of only 7.5 h. On the other hand, the fact that the percentage of differentiated cells remains unchanged from the fourth to the fifth day after HNE treatment may indicate that the inductive effect is almost exhausted, and that the proliferation of uninduced cells is resumed.

The time course of the appearance of phagocytosing cells is closely comparable with that of CD11b surface antigen expression. This molecule functions as the C3bi receptor and has been proposed as a marker for in vitro models of myeloid differentiation. 24

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of cells expressing CD11b. During myelocytic maturation, CD67, which is not expressed in the progenitor cells, is transiently upregulated during the intermediate stages, and is almost absent on the surface of mature granulocytes. This difference in CD11b and CD67 expression may, thus, reflect the stages of HL-60 cell maturation. By contrast, the CD36 expression is very low after induction with HNE and DMSO.

These results, together with the morphological findings, indicate that HNE, like DMSO, induces granulocytic differentiation of HL-60 cells. However, with HNE, differentiation is preceded by accumulation of G0/G1 cells. Thus, HNE differs from DMSO in its inhibition of the cell cycle over the same cell exposure time.

The molecular basis for growth arrest and induction of the cell cycle over the same cell exposure time.

The molecular basis for growth arrest and induction...
of differentiation are not completely known, and several intracellular targets may be involved. Modulations of oncogene expression and enzyme activities by the inducing agents have been reported. HNE strongly inhibits c-myc expression as well as the induction of ornithine decarboxylase activity in HL-60 cells. It also inhibits DNA polymerase α and β activities in both rat liver and Yoshida ascites hepatoma, though only at concentrations higher than 1 μM. All these effects can be related to the accumulation of cells in the G0/G1 phase. Even so other mechanisms may be involved.

In conclusion, our results demonstrate that although HNE can induce the granulocytic differentiation of HL-60 cells with a potency comparable to that exhibited by DMSO, its induction pathway is at least partly different. With DMSO, growth inhibition and induction of differentiation are chronologically associated, and it has been proposed that induction includes some replication cycles prior to growth arrest and terminal differentiation. With HNE, however, growth inhibition directly precedes the switch to differentiation; these effects, thus, resemble those of TPA’s induction of the macrophage-monocytic differentiation of these cells. This supports the hypothesis that cell replication cycles are not essential for the granulocytic differentiation program in HL-60 cells.

Finally, it is noteworthy that HNE differs from the majority of differentiation inducers, because it is endogenously produced from the peroxidative process in a number of nonproliferating cells at concentrations similar to that used in this study. This suggests that it may play a role in the control of such processes in normal cells and tissues as well.

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REFERENCES


ABBREVIATIONS

DMSO—dimethyl sulfoxide
FCS—fetal calf serum
FITC—fluorescein isothiocyanate
HNE—4-hydroxynonenal
PBS—phosphate-buffered saline
PI—propidium iodide