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# 4-Hydroxynonenal, an End-Product of Lipid Peroxidation, Induces Apoptosis in Human Leukemic T- and B-Cell Lines

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4-Hydroxynonenal (HNE) is the major aldehydic product resulting from lipid peroxidation and has been implicated as involved in several pathological conditions. In our continuing studies on the role of membranes and lipid peroxidation in the induction of apoptosis, we investigated the effect of HNE on cultured human malignant immune system cells. Two cell lines were utilized; MOLT-4, a human T-cell leukemia cell line, and Reh, a human B-cell lymphoma cell line. A 10 min treatment with 0.01 mM HNE resulted in the apoptotic death, as determined by flow cytometric and morphological analyses, of both cell lines within 24 h. MOLT-4 cells exhibited the manifestations of impending apoptotic death much sooner than did Reh cells, indicating that MOLT-4 cells were more sensitive or not as efficient at detoxifying HNE than were Reh cells. These results suggest that peroxidative damage to cellular membranes resulting in the production of HNE may be a trigger for the induction of apoptosis in immune system cells.

*Keywords:* Apoptosis, hydroxynonenal, lipid peroxidation, T-cell, B-cell

## INTRODUCTION

An important component of the signaling process in apoptosis induced by ionizing radiation or oxidative damage appears to be the early generation of free radicals in the plasma membrane. The plasma membrane is especially susceptible to oxidative damage because it contains significant quantities of easily peroxidizable lipids. These reactions can be self-propagating, by a process known as lipid peroxidation chain reaction.[1] The resulting radicals and toxic metabolites are thought to be the main cause of damage in the cell.[2] One of the major aldehydic end-products of the oxidation of $\omega$-6 polyunsaturated fatty acids such as linoleic and arachidonic acids is 4-hydroxynonenal (HNE, Figure 1). HNE has been postulated to play a role in several disease states including atherosclerosis,[3,4] alcohol-induced

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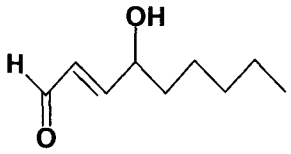


FIGURE 1 Structure of HNE.

liver disease,[5] and neurodegenerative disorders.[6–8] Because this compound has been shown to be both cytotoxic and mutagenic, as well as have an effect on cell proliferation and gene expression, it has been categorized as a second toxic messenger, a compound that disseminates and augments initial free radical events.[9]

Normal cellular concentrations of HNE range from 0.2 to 0.8 μM[10,11] but can reach 25 to 50 μM during periods of severe oxidative stress.[12] It has been proposed that localized concentrations of HNE in peroxidized membranes can reach as high as 4.5 mM.[13] HNE exhibits a wide range of biological effects, depending upon its concentration and the model system used. At low concentrations (< 1 μM) it induces adenylate cyclase[14] and phospholipase C activity,[15–18] disrupts calcium homeostasis,[19] activates the transcription factor AP-1,[20] blocks expression of c-myc and c-myb,[21–23] and induces differentiation in HL-60 cells.[24,25] Higher concentrations of HNE result in the inhibition of plasma membrane ($Ca^{2+}$–$Mg^{2+}$)-ATPase,[26] ($Na^{+}$–$K^{+}$)-ATPase,[27,28] 5′-nucleotidase,[29] adenylate cyclase,[30] α-ketoglutarate dehydrogenase,[31] pyruvate dehydrogenase,[32] and glutathione reductase.[33] However, within the same concentration range, it induces glutathione S-transferase[34] and aldose reductase,[35] as well as increases passive membrane permeability to $Ca^{2+}$.[19,27] Although the exact mechanism of action of HNE on these biological systems is not known, HNE has been demonstrated to chemically react with –SH and $-NH_2$ groups[9] and could therefore react with amino acids such as cysteine, lysine, and histidine,[36] as well as the metabolites glutathione and lipoic acid.[32,37] Based on our earlier work showing that the plasma membrane is a critical site for radiation- and oxidant-induced apoptosis,[38–40] we extended our investigations to the role of HNE in apoptotic death using cultured immune system cells.

## MATERIALS AND METHODS

### Cells

MOLT-4 and Reh cells (American Type Culture Collection, Rockville, MD) were grown in RPMI-1640 medium, containing 10% fetal bovine serum (FBS), 3 mM glutamine, and 100 U/ml penicillin/streptomycin (all from Gibco/BRL, Grand Island, NY) in an atmosphere of 5% $CO_2$ in air at 37°C. Cell cultures were maintained at 1–1.5 × $10^6$ cells/ml. Only cells with viabilities greater than 95% (trypan blue dye exclusion) were used for experiments.

### 4-Hydroxynonenal Treatment

Cells were removed from growth medium by centrifugation, washed twice with RPMI-1640 without FBS, and resuspended at 1 × $10^6$ cells/ml in RPMI-1640 without FBS. HNE (Cayman Chemical Co., Ann Arbor, MI) was added to a final concentration of 1, 10, or 100 μM and the cell suspension incubated at room temperature for 10 min. The reaction was terminated by the addition of FBS to 10% and the cells returned to the incubator (37°C/5% $CO_2$ in air) until the appropriate time.

### Viability

Viability was determined by the trypan blue dye exclusion method or with a modified version of the 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium (MTT, Sigma Chemical Company, St. Louis, MO) assay.[41]

### Flow Cytometric Analysis

At 4 and 24 h post-treatment, cells were incubated with fluorescein isothiocyanate (FITC)-labeled

annexin V and propidium iodide using the ApoAlert Annexin-V Apoptosis kit (Clontech, Palo Alto, CA). Briefly, $1 \times 10^6$ cells were washed with Hanks' Balanced Salt Solution (HBSS) and resuspended in 200 µl of 1× binding buffer (supplied in the assay kit). The cell suspension was incubated with annexin V-FITC (final concentration 1 µg/ml) or propidium iodide (final concentration 1 µg/ml) at room temperature for 5–15 min in the dark. The samples were analyzed by flow cytometry using a Becton Dickinson FACSCalibur.

### Photomicroscopy

Cells ($0.5–1.5 \times 10^7$) were pelleted by centrifugation (750× *g*, 5 min), gently resuspended in 1 ml of freshly prepared 3% formaldehyde in HBSS, and refrigerated (4°C) for a sufficient time to allow the cells to settle to the bottom of the tube. Once fixed, the cells could be stored at 4°C for several weeks without any apparent degradation.

For bright-field and fluorescence microscopy, all but about 0.1 ml of the overlaying fixative buffer was carefully removed and the cells gently resuspended in the remaining buffer. A 20 µl aliquot of the cell suspension was removed and mixed with 20 µl of a 0.1 mg/ml solution of ethidium bromide. The stained cell suspension was kept on ice in the dark until needed. For microscopic examination, 10 µl of the stained suspension was placed on a microscope slide and covered with a 20 mm square coverslip. The cells were allowed to settle onto the surface of the microscope slide for 5–10 min before examination. Photomicroscopy was performed with an Olympus AHBT3 Research microscope with Nomarski-type differential interference contrast and reflected-light fluorescence.

## RESULTS

HNE is an extremely reactive molecule. In fact, the presence of 10% FBS in the cell-culture medium during treatment with HNE greatly decreased the deleterious effects of the aldehyde, probably through the reaction of HNE with various amino acids of the serum proteins. Therefore, HNE treatments were run in serum-free medium for 10 min at room temperature. Longer treatment times decreased the viability of the control cells as well as the treated cells (data not shown). It appeared that neither MOLT-4 nor Reh cells tolerated serum-free medium for periods longer than 15 min. For times less than 15 min there was no difference between the viability of cells incubated in serum-free medium versus cells incubated in medium with 10% FBS.

Three concentrations of HNE were tested (1, 10, and 100 µM) with both cell lines. Higher concentrations of HNE (e.g., 250 µM) resulted in the rapid (< 30 min) necrotic death of both cell lines (data not shown). Viability, as assessed by the trypan blue dye exclusion method, is shown in Figure 2 (Panel A: MOLT-4; Panel B: Reh). Treatment with 1 µM HNE did not affect the viability of MOLT-4 or Reh cells, as compared to controls, at either 4 or 24 h. When the concentration of

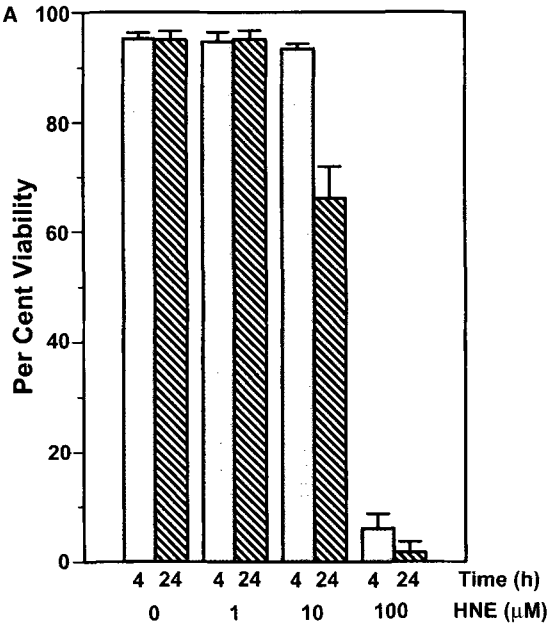


FIGURE 2A

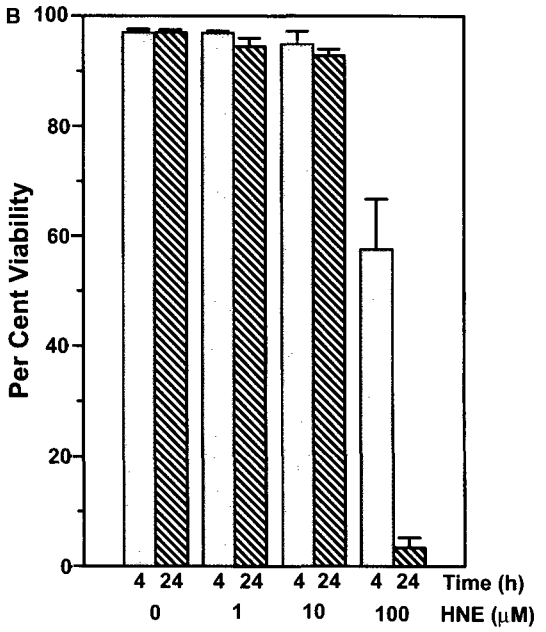


FIGURE 2B

FIGURE 2 Cell viability determined by trypan blue dye exclusion. MOLT-4 (Panel A) and Reh (Panel B) cells were treated with 0, 1, 10 or 100 μM HNE in serum-free RPMI-1640. After 10 min at room temperature, FBS was added to final concentration of 10% and cells incubated at 37°C. Cell viability was determined 4 h (solid shading) and 24 h (cross-hatch shading) post-treatment by trypan blue dye exclusion and direct microscopic observation. Data are expressed as percentage of viable cells in culture and are average of four independent experiments. Error bars represent standard deviation.

HNE was raised to 10 μM, Reh cells again showed no change in viability. However, MOLT-4 cell viability decreased to 66% 24 h post-treatment with 10 μM HNE, although at 4 h post-treatment viability was not greatly affected. With 100 μM HNE, viabilities of both MOLT-4 and Reh cells were greatly affected. Less than 10% of MOLT-4 cells were viable 4 h after a 100 μM HNE treatment. Viability dropped to less than 2% by 24 h. Reh cell viability 24 h after treatment with 100 μM HNE was equally low (3%), although at 4 h post-treatment viability was 57%. These results indicated that Reh cells were slightly more resistant to the deleterious effects of HNE than are MOLT-4 cells. Although, if the concentration of HNE is high enough, both cell lines are sensitive and will eventually die.

Viability determination using trypan blue relies upon the plasma membrane of the badly damaged, dying, or dead cell becoming permeable to the dye molecule. However, cells can be metabolically dead yet still exclude trypan blue and thus appear viable. A more sensitive method to determine cell viability is the reduction of MTT to an insoluble formazan compound by mitochondrially located enzymes. Figure 3 shows the ability of MOLT-4 (Panel A) and Reh (Panel B) cells to reduce MTT at various times after being treated with 1, 10, or 100 μM HNE. For both MOLT-4 and Reh cells, treatment with 10 or 100 μM HNE resulted in a time- and dose-dependent decrease in the ability to reduce MTT, as compared to controls. Treatment with 1 μM HNE resulted in only a slight decrease in viability. These results showed that even a short (10 min) treatment with low concentrations of HNE affected the viability of MOLT-4 and Reh cells.

Apoptotic cell death is accompanied by the loss of membrane phospholipid symmetry, characterized by the translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface. Annexin V is a protein that has a strong affinity for phosphatidylserine. When fluorescently labeled, annexin V can be used in conjunction with flow cytometry to detect apoptotic cells. MOLT-4 and Reh cells were treated with either 0 or 10 μM HNE and analyzed 4 and 24 h post-treatment. The cells were stained with FITC–annexin V and propidium iodide and analyzed by flow cytometry. Figure 4 shows the histogram analyses for annexin V-stained MOLT-4 (Panel A) and Reh (Panel B) cells. The untreated controls of both cell lines showed a small amount of annexin V binding. However, 4 h after HNE treatment (10 μM), the percentage of annexinV-positive MOLT-4 cells increased from 8.7% to 16.2%, while annexin V-positive Reh cells increased from 7.5% to 21.9%. The percentages are even higher 24 h post-treatment. These data indicate that loss of membrane phospholipid

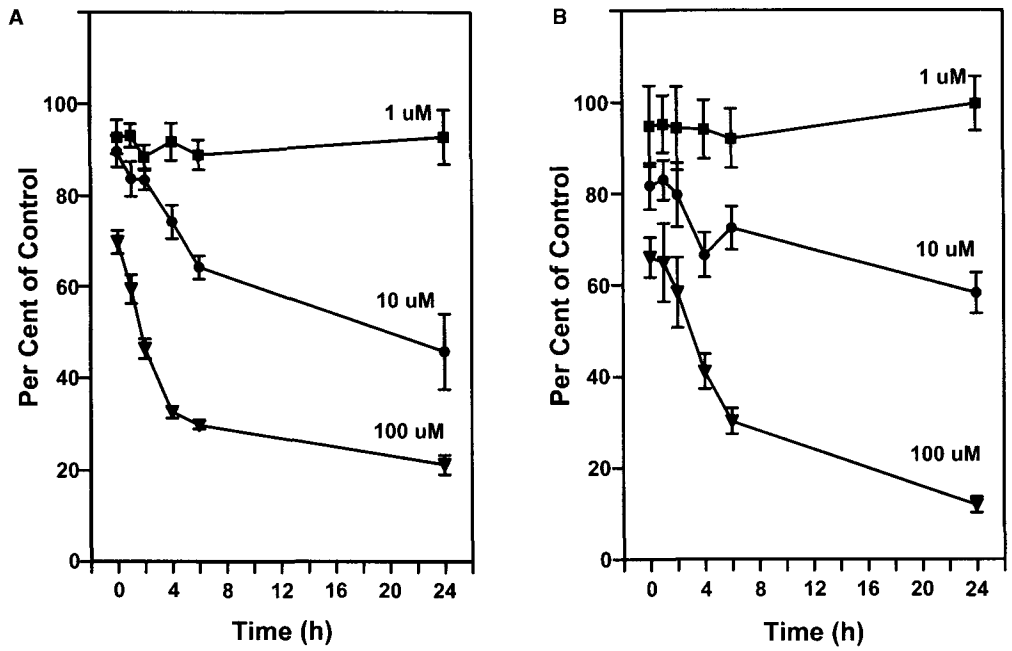


FIGURE 3 Cell viability determined by MTT assay. Cells were treated as previously described, but, at various times post-treatment, cells ($5 \times 10^4$) were incubated with MTT (125 μg/ml) at 37°C. After 2 h cells were lysed and absorbance measured at 570 nm. Data are normalized to control values (0 μM HNE) for same time point and represent average of six independent experiments. Panel A: MOLT-4 cells. Panel B: Reh cells. Error bars represent standard deviation.

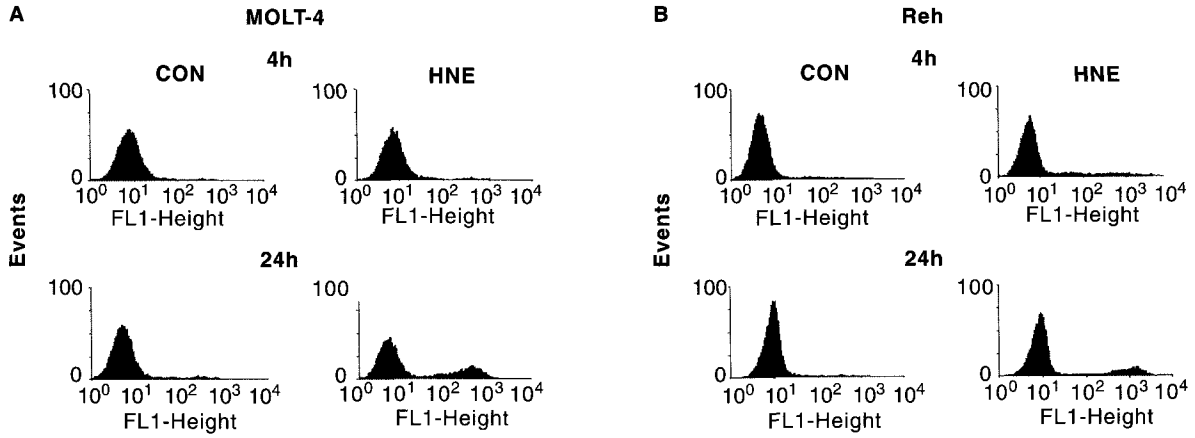


FIGURE 4 Flow cytometric analysis of control and HNE-treated MOLT-4 and Reh cells. Cells were treated with 10 μM HNE as previously described. Flow cytometric analysis was conducted as detailed in Materials and Methods. Panel A: Annexin V staining of MOLT-4 cells 4 and 24 h post-treatment with HNE; Panel B: Annexin V staining of Reh cells 4 and 24 h post-treatment with HNE.

symmetry, indicative of impending apoptotic death, occurred early after HNE treatment.

A morphological comparison of control and HNE-treated MOLT-4 and Reh cells is shown in Figure 5. As early as 4 h after treatment with 10 μM HNE, MOLT-4 cells exhibited morphological signs of apoptosis, including cell shrinkage, chromatin condensation, and apoptotic bodies.

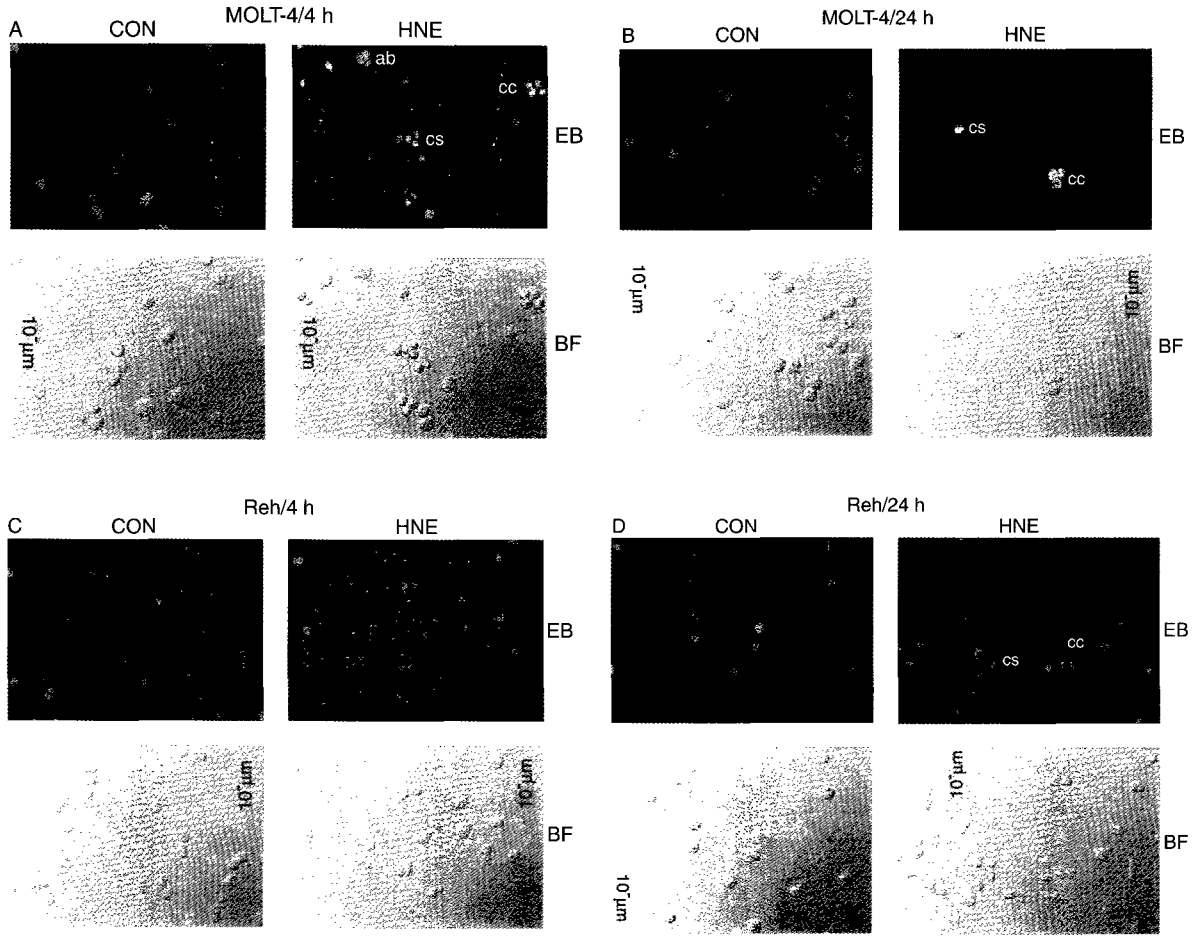


FIGURE 5 Bright-field (BF) and ethidium bromide (EB) fluorescence images of control and HNE-treated MOLT-4 and Reh cells. Cells were treated with 10 μM HNE as previously described and prepared for photomicroscopy as detailed in Material and Methods. Panel A: Control and HNE-treated MOLT-4 cells 4 h post-treatment; Panel B: Control and HNE-treated MOLT-4 cells 24 h post-treatment; Panel C: Control and HNE-treated Reh cells 4 h post-treatment; Panel D: Control and HNE-treated Reh cells 24 h post-treatment. Ab: apoptotic body; cc: condensed chromatin; cs: cell shrinkage.

These changes were even more pronounced at 24 h post-treatment. Reh cells, on the other hand, did not exhibit any of these morphological indicators of apoptosis 4 h post-treatment; however, they were readily apparent by 24 h post-treatment.

## DISCUSSION

The role of HNE in the development of oxidative stress-induced cellular injury is currently an area of extensive research. Our studies have focused on the plasma membrane as the initial site of radiation damage. We have previously shown that radiation exposure results in the rapid induction of lipid peroxidation and production of intracellular peroxides in immune system cells.[40] These results, along with the formation/damage proposal of Esterbauer and colleagues[9,42,43] stating that free radicals lead to lipid peroxidation that results in aldehyde production, have prompted us to investigate the effect of HNE, the major aldehydic product of

lipid peroxidation, on cultured immune system cells.

Using MOLT-4 as a T-cell model and Reh as a B-cell model, we have demonstrated that both cell lines exhibited a dose-dependent response to HNE. Low concentrations (1 μM) of the aldehyde had no effect on cell viability as determined by trypan blue dye exclusion and the MTT assay. However, when the HNE concentration was raised to 10 μM, both cell lines showed a time-dependent decrease in viability. While the viability of both cell lines decreased, MOLT-4 cells appeared more sensitive to 10 μM HNE than Reh cells. HNE concentrations of 100 μM resulted in the rapid loss of viability of both cell lines and by 24 h post-treatment the viability of both cell lines was less than 5%. HNE concentrations of 250 μM or greater caused rapid cell lysis.

To ascertain the mode of HNE-induced cell death, the presence of phosphatidylserine in the outer leaflet of the plasma membrane was measured. The appearance of phosphatidylserine in the outer leaflet of the plasma membrane is one of the earliest indicators of apoptotic death.[44] In normal cells, phosphatidylserine is found predominantly in the inner leaflet of the plasma membrane. As the cell begins to undergo apoptosis, phosphatidylserine is translocated to the outer leaflet, presumably as a signal for macrophages to phagocytose the apoptotic cell.[45] Significant changes in surface phosphatidylserine levels in both cell lines were apparent by 4 h post-treatment with 10 μM HNE.

Specific morphological changes, such as cell shrinkage, chromatin condensation, and formation of apoptotic bodies, are representative of apoptotic death, while cell swelling and lysis indicate necrotic death. MOLT-4 cells exhibited morphological signs of apoptosis as early as 4 h after treatment with 10 μM HNE. However, at 4 h, Reh cells did not show any morphological indicators of apoptosis, although these changes were present 24 h post-treatment. This again suggested that Reh cells were more tolerant of HNE than were MOLT-4 cells.

There have been two other reports of HNE inducing apoptotic death in primary or established cell lines. Both PC-12 cells and primary rat hippocampal neurons undergo apoptosis when treated with 10 μM HNE.[6] However, it took up to 72 h for these cells to reach the apoptotic levels (as determined by MTT and trypan blue assays) that MOLT-4 cells exhibited by 4 h post-treatment. Murine alveolar macrophages, isolated from bronchial lavage fluid, also die by apoptosis when treated with HNE at concentrations ranging from 25 to 100 μM. No effect was seen at lower concentrations.[46]

As previously mentioned, radiation exposure results in the production of free radicals in the plasma membrane as well as the induction of lipid peroxidation.[40] Both MOLT-4 and Reh cells are radiation sensitive, with MOLT-4 being more sensitive than Reh.[47] Macrophages, both primary cultures and established cell lines, are considered radioresistant.[48–50] It is tempting to speculate that the radioresistance of macrophages and differences in radiosensitivity of MOLT-4 and Reh cells might be due, in some part, to the ability to detoxify HNE (and related aldehydes) produced as a consequence of radiation exposure. Since HNE is an extremely reactive molecule capable of causing considerable damage to the cell, a variety of pathways exist to detoxify it. These pathways include conversion, via alcohol dehydrogenase activity, to 1,4-dihydroxynonene, conjugation to glutathione by glutathione S-transferase activity, and conversion, as a result of aldehyde dehydrogenase activity, to 4-hydroxynonenoic acid.[43,51–54] These compounds are then either exported from the cell or further metabolized. HNE can also react directly with proteins through lysine, histidine, and cysteine residues.[36] Clearly there are multiple detoxification pathways that must be investigated before the differences in sensitivity to HNE of MOLT-4 and Reh cells can be explained. To this end, we have found that HNE treatment (100 μM/10 min) resulted in an immediate decrease in intracellular glutathione levels in both cell lines.

However, when the cells were first treated with buthionine sulfoximine (BSO) to deplete cellular glutathione levels then exposed to HNE, MOLT-4 cells that were first treated with BSO and then HNE had lower viabilities than MOLT-4 cells treated with HNE alone. On the other hand, the BSO-pre-treatment regimen had no effect on Reh cell viability after HNE treatment (data not shown). These results suggest that Reh cells possess alternate (or more efficient) pathways to detoxify HNE than just conjugation to glutathione. We are currently investigating the differential effect of HNE on MOLT-4 and Reh cells.

To summarize, we have shown that treatment with 10 μM HNE resulted in the apoptotic death of both MOLT-4 and Reh cells. Significant changes in viability (as determined by the MTT assay) and outer plasma membrane phosphatidylserine levels were readily apparent at 4 h after HNE treatment. Morphological indices of apoptosis, were clearly visible in MOLT-4 cells by 4 h post-treatment, but were not present until 24 h in Reh cells, indicating that Reh cells may be more resistant to HNE. Taken together these results suggest that production of HNE derived from peroxidative damage to cellular membranes may be a trigger for the induction of apoptosis in immune system cells.

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