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Lipid hydroperoxide-induced apoptosis: lack of inhibition by Bcl-2 over-expression

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Abstract Increased membrane lipid peroxidation has recently been implicated as being associated with apoptosis. In the present study the addition of 15-hydroperoxyeicosatetraenoic acid (15-HPETE) or 13-hydroperoxydodecadienoic acid (13-HPODE) to A3.01 T cells is shown to induce marked chromatin condensation coincident with DNA fragmentation, indicative of apoptosis. 15-HPETE also evoked an immediate and sustained rise in cytoplasmic calcium which was required for the induction of apoptosis. A3.01 cells transfected with the *bcl-2* proto-oncogene were 6- to 8-fold more resistant to apoptotic killing by tumor necrosis factor-α, but only 0.4-fold more resistant to 15-HPETE. Thus, Bcl-2 is not capable of protecting cells from undergoing apoptosis following the direct addition of lipid hydroperoxides.

Key words: Cell death; Membrane lipid; Antioxidant; Oxygen radical; Lymphocyte

1. Introduction

Apoptosis is a form of cell death which is defined by, and is the result of, a temporal program of cellular events [1,2]. Numerous experimental model systems of inducing apoptosis have been reported, and although the stimuli which induce the apoptotic death program vary widely, the morphological features of cells undergoing apoptotic death appear to be highly conserved, suggesting that a single common death program may exist. Apoptosis-defining morphological changes occur at several sites within the cell [3]. Within the nucleus, wherein the chromatin of the dying cell condenses, first into a distinctive crescent-shaped pattern along the inner margins of the nuclear membrane, and then into a dense body which eventually breaks apart into a number of nuclear fragments. Coincident with these changes taking place in gross nuclear morphology, although possibly via a different pathway [4], the DNA is cleaved by a Ca^{2+}-dependent endonuclease at internucleosomal sites, generating a distinctive 'ladder' of 180-bp repeats often considered to be pathognomonic of apoptotic death.

Collective evidence has led to the suggestion that oxidative stress is a common mediator of apoptosis, perhaps via the formation of lipid hydroperoxides [5–7]. Indeed, lipid hydroperoxides are known to be toxic [7,8], to increase cytosolic Ca^{2+} [9,10], and to promote DNA fragmentation [7,11,12]. Nevertheless, it is a subject of debate as to whether something as seemingly nonspecific and uncontrolled as membrane peroxidation could result in a structured form of cell death such as that which occurs during apoptosis [13]. Central to this debate are the recent reports that the *bcl-2* oncogene, which has apoptosis-mitigating abilities, may similarly prevent lipid peroxidation [14,15].

We now demonstrate that hydroperoxy fatty acids of the type common to membrane peroxidation are fully capable of eliciting all the major morphological and chromatin changes associated with apoptosis. When considered in the context of the recent reports that Bcl-2 expression may suppress lipid peroxidation, these observations support the thesis that the inhibition of apoptosis by *bcl-2* may be entirely attributable to this capacity.

2. Materials and methods

2.1. Cell lines and culture conditions

The A3.01 human T cell line [16] and *bcl-2* transfectants derived from it (see below) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and glutamine. Cultures were incubated at 37°C in a humidified incubator containing 6% CO_2/94% air.

2.2. Transfection with bcl-2

A3.01 cells were transfected by electroporation with either an SFFV vector containing human *bcl-2* cDNA under the control of a constitutive CMV promotor or the same vector in which the *bcl-2* cDNA had been deleted that were obtained from Dr. S. Korsmeyer. Prior to transfection both plasmids were linearized by digestion with *kpnI*. Stable transfectants were selected based on their resistance to geneticin (600 μg/ml) and cloned at 0.3 cells per well in the presence of the same. Clones were screened for Bcl-2 expression by immunofluorescence assay using a purified 6C8 hamster monoclonal antibody specific for the human Bcl-2 protein [17] and developed with a fluorescein isothiocyanate labeled goat anti-hamster antibody. Two separate clones each of Bcl-2 expressing and nonexpressing (vector only) were used.

2.3. Viability assays

The effects of lipid hydroperoxides and tumor necrosis factor-α (TNF-α) on cell viability were assayed using either [^{3}H]thymidine ([^{3}H]Tdr) or MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-

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Abbreviations: BAPTA-AM, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxydodecadienoic acid; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt); ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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phenyl)-2-(4-sulfophe nyl)-2*H*-tetrazolium, inner salt) as described previously [18,19].

2.4. Apoptosis assay

Apoptosis was assayed by confirming the presence of ~180-bp DNA fragmentation by gel electrophoresis [20,21].

2.5. Measurement of cytoplasmic calcium

A3.01 cells were collected by centrifugation, washed once in Ca^{2+}-free buffer, and resuspended in the same buffer to yield a density of 1×10^7 cells/ml. Fura-2/AM was added from a stock solution in DMSO to achieve a final concentration of 5 μM, and the cells were incubated at 30°C for 30 min. The Fura-2-loaded cells were washed twice with Ca^{2+}-free phosphate-buffered saline, followed by resupension in PBS containing Ca^{2+}. The cells were transferred to a cuvette containing 2 ml of PBS and maintained at 37°C for the duration of the assay. Fluorescence measurements were performed using a Perkin Elmer LS 50 spectrometer as described previously [22].

2.6. Electron microscopy

Cultured cells were pelleted and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h at 4°C, rinsed with phosphate buffer, and post-fixed with 1% osmium tetroxide for 1 h. Cells were then dehydrated through a series of graded alcohols, followed by propylene oxide. Cells were embedded in Epon/Araldite resin. Ultrathin tissue sections were obtained and counterstained with both lead citrate and uranyl acetate and viewed with a JEOL 100CX electron microscope.

2.7. Reagents

15-HPETE and 13-HPODE were prepared from arachidonic and linoleic acids (Sigma), respectively, and purified by HPLC [23]. MTS was provided by Dr. T.C. Owen [19] or purchased from Promega (Madison, WI). Fura-2 and BAPTA-AM were purchased from Molecular Probes (Eugene, OR). Geneticin was obtained from Boehringer Mannheim.

3. Results

As shown in Fig. 1, both 15-HPETE and 13-HPODE, hydroperoxy derivatives of arachidonic and linoleic acids, respec-

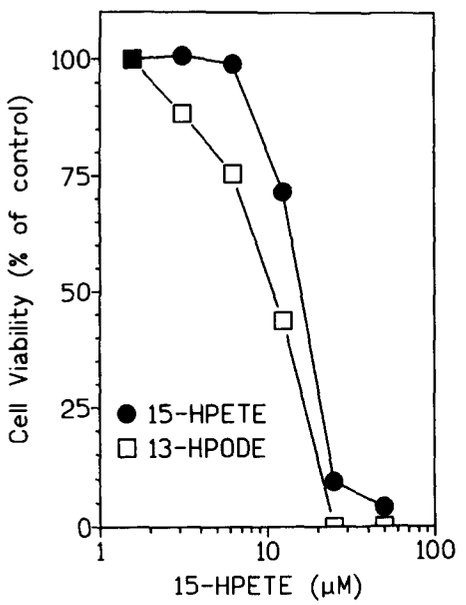

Fig. 1. Susceptibility of A3.01 cells to killing by 15-HPETE and 13-HPODE. A3.01 cells were exposed to the indicated doses of either 15-HPETE, 13-HPODE, or were left untreated. After 16 h, cell viabilities were assayed by measuring the incorporation of [^{3}H]Tdr. The viabilities observed for each fatty acid treatment are expressed as the percentage of untreated control cells.

Fig. 2. Nuclear changes induced by exposure to 15-HPETE. (A) A3.01 cells exposed to 40 μM 15-HPETE for 3 h (7,699× magnification); (B) untreated A3.01 cells (8,166× magnification).

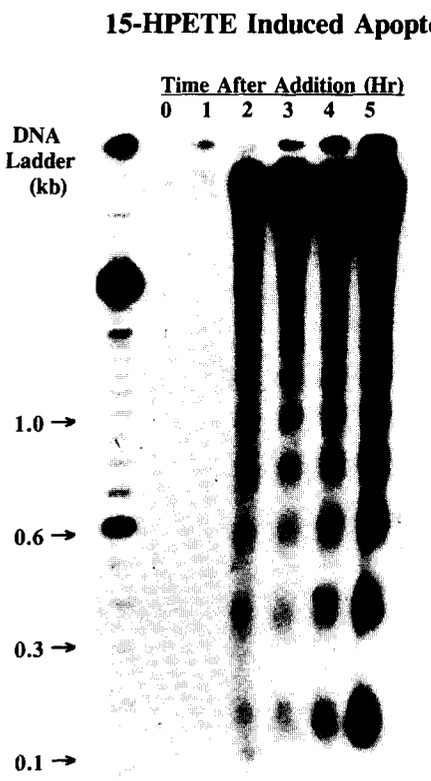

Fig. 3. 15-HPETE-induced DNA fragmentation. A3.01 cells were exposed to 40 μM 15-HPETE for the indicated periods of time, after which the cells were harvested and low-molecular-weight DNA was isolated and radiolabeled using Klenow fragment and [^{32}P]dCTP. The labeled DNA fragments were subsequently separated by gel electrophoresis and visualized by autoradiography. The extreme left lane corresponds to a 100-bp DNA ladder.

tively, evoked dose-dependent toxicity when added to A3.01 cells. Similar doses of either arachidonic acid or 15-hydroxyeicosatetraenoic acid (15-HETE), the reduced form of 15-HPETE had no effect on cell viability (data not shown), clearly associating cytotoxicity with the hydroperoxy moiety. Within 2 to 3 h after addition of 40 μM 15-HPETE, nuclear condensation and fragmentation was obvious by phase-contrast microscopy (data not shown). Electron microscopy revealed the presence of condensed, crescent-shaped chromatin associated with the nuclear membrane (Fig. 2A). Throughout these early stages of cell death, the plasma membrane and cellular organelles such as the mitochondria remained intact, consistent with the cells undergoing apoptosis as opposed to necrosis. Nevertheless, some morphological changes were observed within the cytoplasm of 15-HPETE-treated cells. Compared with control cells (Fig. 2B), the mitochondria of apoptotic cells were considerably more electron dense and there was a disappearance of cytoplasmic granularity that could be indicative of a loss of polyribosomes from the rough endoplasmic reticulum. These gross morphological changes coincided with the occurrence of DNA fragmentation, as demonstrated by the appearance of a 180-bp DNA ladder that was detectable within 2 h after the addition of 15-HPETE (Fig. 3). Collectively, these data clearly establish the ability of lipid hydroperoxides to elicit the major morphological changes associated with apoptosis.

Elevation of cytoplasmic calcium ($[Ca^{2+}]_i$) has been reported to coincide with the initiation of apoptosis and in several models is a requisite event for the apoptotic response [24–26]. Lipid peroxidation has previously been reported to result in the elevation of $[Ca^{2+}]_i$ [9,10], and consistent with this, A3.01 cells exposed to 40 μM 15-HPETE displayed an immediate and sustained increase in $[Ca^{2+}]_i$ (Fig. 4). To determine if the rise in $[Ca^{2+}]_i$ was required for lipid hydroperoxide-mediated apoptosis, A3.01 cells were preloaded with the cell-permeable calcium chelator, BAPTA-AM [27], followed by exposure to various concentrations of 15-HPETE. As shown in Fig. 5, pretreatment with 6 μM BAPTA-AM resulted in a 4-fold increase in the amount of 15-HPETE required to kill 50% of A3.01 cells. Thus, the rise in $[Ca^{2+}]_i$ is required for the induction of apoptotic death.

It has been reported that Bcl-2 overexpression prevents apoptosis either by preventing the formation of lipid hydroperoxides, or more directly, by protecting cells from the toxic effects of lipid hydroperoxides [14,15]. To distinguish between these two possibilities, A3.01 cells were stably transfected with human Bcl-2 and subsequently tested for their sensitivity to killing by TNF-α and 15-HPETE. TNF-α was chosen due to the well-established role of reactive oxygen species (ROS) in mediating TNF-α cytotoxicity [28–30], and the ability of Bcl-2 to protect cells from TNF-α-induced cell death [31]. Compared with A3.01 cells transfected with the control (empty) vector, the Bcl-2 transfectants were found to be 6–8 times more resistant to killing by TNF-α (Fig. 6A), thereby confirming that the over-expressed Bcl-2 protein was functioning to inhibit apoptosis. By contrast, when the Bcl-2 transfectants were induced to undergo apoptosis with various doses of 15-HPETE, they displayed only a 25–50% increase in resistance over cells transfected with the vector alone (Fig. 6B).

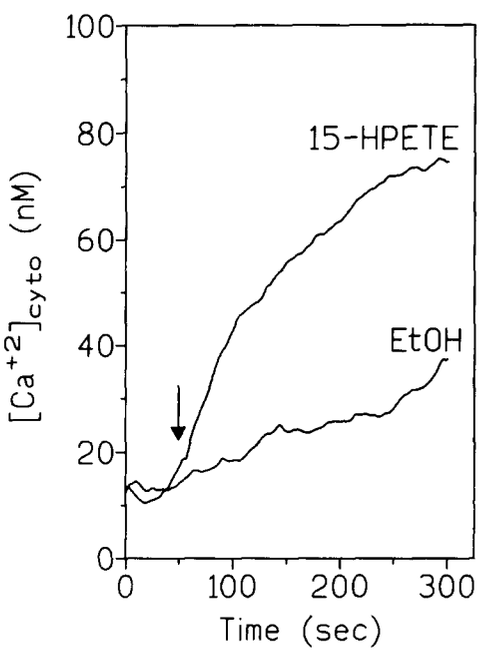

Fig. 4. 15-HPETE-induced increase in cytosolic calcium. A3.01 cells loaded with Fura-2 were exposed to either 40 μM 15-HPETE or 0.2% EtOH as a vehicle control. Changes in $[Ca^{2+}]_i$ were determined as described in section 2.

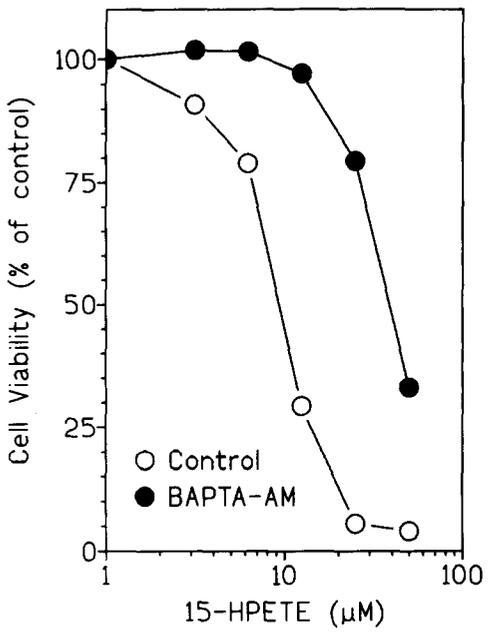

Fig. 5. Protection of A3.01 cells from 15-HPETE-induced apoptosis by the intracellular calcium chelator, BAPTA-AM. A3.01 cells were harvested by centrifugation and resuspended in Hanks balanced salt solution lacking Ca^{2+} but containing 6 μM BAPTA-AM. After 45 min, the cells were harvested, washed once in PBS, and resuspended in media containing 0 or 40 μM 15-HPETE. Three hours later, [^{3}H]Tdr was added and incorporation into cellular DNA was measured after 3 h.

4. Discussion

While there is considerable evidence implicating oxidative stress as a mediator of apoptosis [5,6,14,15,23,32–36], the underlying mechanisms have yet to be fully defined. The present study shows that at least one likely product of oxidative stress, lipid hydroperoxide, is capable of eliciting many cellular changes definitive of apoptosis, including DNA fragmentation and chromatin condensation, with the characteristic preservation of plasma membrane and organelle integrity. These results clearly demonstrate that lipid peroxides can act as mediators of apoptosis, and despite their notorious reputation, do not necessarily initiate an uncontrolled autocatalytic reaction culminating in massive cellular damage. Indeed, the selective destruction of reticulocyte mitochondria during erythrocyte maturation may represent one case where lipid hydroperoxides serve as physiological mediators of apoptosis [37].

The mechanism by which lipid hydroperoxides elicit apoptotic death is unclear. As demonstrated herein and in previous studies [9,10], the addition of lipid hydroperoxides results in a marked increase in $[Ca^{2+}]_i$ that is essential for the induction of apoptosis. The origin of the calcium increase is not known. Lipid hydroperoxides may open calcium channels or act directly as ionophores to facilitate entry of extracellular calcium. Alternatively, they may stimulate the release of calcium from mitochondrial and endoplasmic stores [10,11,37,38]. A rise in $[Ca^{2+}]_i$ could lead to the activation of various signal transduction pathways that culminate in cell death [40]. In this regard it is interesting that Bcl-2 expression has been reported to inhibit a calcium pump that is necessary for the capacitative release of Ca^{2+} from the endoplasmic reticulum [41]. A HPETE-mediated Ca^{2+} influx would also be expected to result in the activation of the Ca^{2+}-dependent endonuclease responsible for cleavage of DNA at internucleosomal sites. It has recently been suggested that the form of cleavage which leads to the DNA fragmentation seen in apoptosis results from an accumulation of single-strand breaks [42]. While ROS, and in particular lipid hydroperoxides, are capable of generating single-strand DNA breaks [11], the addition of 15-HPETE to isolated A3.01 nuclei did not elicit DNA fragmentation (P.A. Sandstrom, unpublished observations). Such observations seem to argue against a direct effect of lipid hydroperoxides on either the endonuclease or DNA. However, we cannot exclude the possibility that HPETE-generated single-strand DNA breaks elicit the activation of poly-ADP ribose transferase similar to what has recently been reported for nitric oxide-initiated neurotoxicity [43]. The activation of this enzyme by single-strand DNA breaks results in the depletion of cellular energy supplies, thus augmenting cell death.

The ability of Bcl-2 to protect cells from oxidative stress-mediated cytotoxicity, implies that the protein somehow functions to bolster cellular antioxidant defenses [14,15,44]. One notable consequence of Bcl-2 overexpression in mammalian cells was decreased membrane lipid peroxidation [14]. Our data suggest that the ability of the *bcl-2* proto-oncogene to block the formation of lipid peroxidation could account entirely for its capacity to inhibit oxidative stress-induced apoptosis. Such an effect could result from either a decreased formation or an increased detoxification of lipid peroxides, consistent with the protein's obligatory association with cellular membranes to inhibit apoptosis [45]. However, the results obtained in this study implicate the former alternative as being the case. As expected, based on previous studies [46], overexpression of Bcl-2 in A3.01 cells afforded substantial protection from TNF-α, the cytotoxicity of which has been extensively linked to the formation of ROS [28–30] as well as either the availability or oxidation of arachidonic acid [46]. By contrast, the *bcl-2* transfectants were found to be only slightly more resistant to 15-HPETE-induced apoptosis than the control-transfected

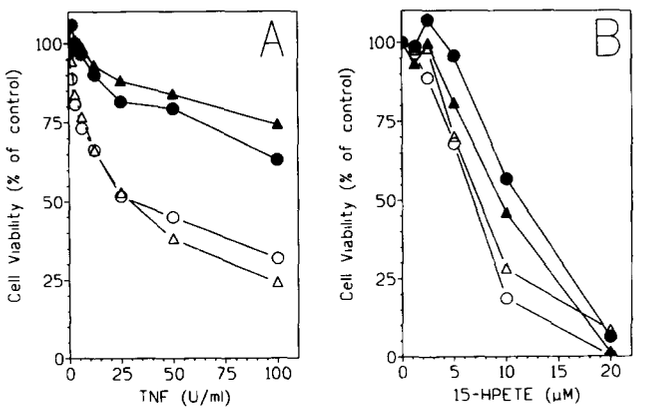

Fig. 6. Effect of Bcl-2 overexpression on TNF-α- and 15-HPETE-mediated cytotoxicity. Two *bcl-2*-transfected clones (closed symbols) and two control clones transfected with the empty vector (open symbols) were plated at 4×10^5 cells/ml in 2.5% FBS/RPMI and exposed to the indicated dose of either TNF-α or 15-HPETE. After 48 h cell viabilities were assayed using MTS. The viabilities are expressed as the percentage of untreated control cells.

A3.01 cells, implying that Bcl-2 overexpression did not endow the cells with a greater ability to survive exogenously derived lipid hydroperoxides. If Bcl-2 were acting directly to detoxify lipid hydroperoxides it would be expected that the fold increase in resistance to exogenous 15-HPETE would be more reflective of what is seen with TNFα cytotoxicity. The marginal resistance to 15-HPETE may indirectly result from a general shift in the basal redox status of the cells, incidentally allowing lipid hydroperoxides to be better tolerated. Alternatively, the decomposition of 15-HPETE may generate ROS which although present in minor amounts contribute to the observed cytotoxicity, and are detoxified by a Bcl-2 associated mechanism. It therefore seems likely that Bcl-2 can effectively prevent the formation of lipid peroxides, but neither facilitates their metabolic detoxification nor blocks their effects on the cell.

In summary, we report here that hydroperoxy fatty acids are capable of mediating all of the major morphological and chromatin changes associated with what is accepted as an apoptotic phenotype, and so supports the proposed role of lipid peroxide intermediates in programmed cell death. When considered in the context of recent reports asserting that Bcl-2 mitigates apoptosis though the regulation of lipid peroxidation, our results suggest that it may be at the level of lipid peroxide formation rather than detoxification.

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