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Ebselen Inhibition of Apoptosis by Reduction of Peroxides

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ABSTRACT. We investigated the capacity of ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one], a glutathione peroxidase mimic, to protect cells from radiation-induced apoptosis. Incubating mouse thymocytes with 25 μM ebselen immediately after ^{60}Co gamma-radiation exposure (5 Gy) inhibited morphological changes associated with apoptosis. Treatment of thymocytes with ebselen before, during, or after irradiation completely blocked internucleosomal DNA fragmentation, a biochemical marker for apoptosis. We measured peroxides formed in cells during and after irradiation, using the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate. By 2 min postirradiation, levels of peroxides in irradiated thymocytes were approximately 10–11 times greater than those in the same cells before irradiation, and levels continued to increase with time. We also measured membrane lipid peroxidation using *cis*-parinaric acid, a naturally fluorescent polyunsaturated fatty acid that readily incorporates into cell membranes. The oxidation of *cis*-parinaric acid also began soon after irradiation and increased with time. Peroxide generation and membrane lipid peroxidation preceded both internucleosomal DNA fragmentation and morphological changes characteristic of apoptosis. Treatment of cells with ebselen reduced peroxide levels and appeared to protect thymocytes from radiation-induced apoptosis by scavenging peroxides generated during and after irradiation. The results suggest that peroxide generation and membrane lipid peroxidation may be important signaling events that trigger apoptosis in irradiated cells. BIOCHEM PHARMACOL 51;11:1443–1451, 1996.

KEY WORDS. apoptosis; ebselen; peroxides; lipid peroxidation; radiation; lymphocytes

Apoptosis is a distinct mode of cell death that has been shown to play a critical role in many aspects of biology and medicine. A wide range of diseases are now thought to be associated with the inappropriate induction of apoptosis, including acquired immune deficiency syndrome and a variety of neurodegenerative disorders [1]. Apoptosis can be triggered experimentally by a variety of extra- and intracellular stimuli, one of which is ionizing radiation [2, 3]. Radiation-induced apoptosis has been characterized primarily in thymocytes, splenocytes, and other cells of hematopoietic lineage that constitute the immune system [4]. Radiation doses as low as 0.05 Gy induce apoptosis in lymphocytes [5].

Most of the cellular damage produced by ionizing radiation is a consequence of the production of free radicals. These highly reactive intermediates can directly react with and alter biological molecules or can produce secondary species that then react. The plasma membrane is especially susceptible to free radical damage because it contains significant quantities of easily peroxidizable lipids and proteins [6, 7]. We have shown previously that membrane lipid peroxidation is an important, early lesion that plays a role in radiation-induced apoptosis [8, 9]. Free radical activity initiated by ionizing radiation in the aqueous extra- and intracellular media adjacent to membranes can be propagated and amplified by lipid peroxidation chain reactions within the membrane [10, 11]. Membrane lipid peroxidation generates hydroperoxides, lipid hydroperoxides, and aldehydes of lipid hydroperoxides, all of which have been shown to be mediators of apoptosis [12, 13].

The cell possesses a number of defense systems that are involved in the protection from free-radical damage. One is the selenium-dependent enzyme glutathione peroxidase, which functions to detoxify peroxides. This protective system, however, can become overwhelmed after exposure to damaging doses of radiation. Ebselen, a relatively non-toxic selenoorganic compound, has been shown to exhibit glutathione peroxidase-like activity that can reduce peroxide levels in many physiological and pathological conditions [14, 15]. Because our earlier work suggested that peroxide production plays an important role in radiation-induced apoptosis, we sought to test if ebselen might prove effective at blocking apoptosis in irradiated cells.

MATERIALS AND METHODS

Thymocyte Incubation Medium

Thymocytes were incubated in either an RPMI-based medium (hereafter referred to as RPMI) or Na^+ Hanks' buffer.

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RPMI consisted of RPMI 1640 medium containing 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (all GIBCO/BRL, Grand Island, NY, U.S.A.), and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.). Na^+ Hanks' buffer, pH 7.4, consisted of 10 mM HEPES, 145 mM NaCl, 1.3 mM $MgCl_2$, 1.6 mM $CaCl_2$, 4.5 mM KCl, 0.5 mg/mL bovine serum albumin, and 1.8 mg/mL glucose.

Thymocyte Isolation

CD2F1 male mice, 6 to 7-weeks-old, were euthanized with CO_2. Thymuses were removed aseptically, rinsed in RPMI, and homogenized by pressing in a Stomacher device (Techman Co., Cincinnati, OH, U.S.A.) for 1 min. Single-cell suspensions were prepared by filtering the homogenate through 100 μm nylon mesh. Suspensions were washed once and resuspended in RPMI. Cell numbers were obtained using a Coulter Counter (model ZM, Coulter Industries, Luton, Beds, England), and cell viability was estimated by the capacity of the cells to exclude trypan blue [16].

Ebselen Treatment

Stock solutions (10 mM) of ebselen (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) were prepared in DMSO. Cells were incubated in RPMI containing 25 μM ebselen at 37° in an atmosphere of 5% CO_2 in air before, during, or after irradiation, depending on the experiment. All cell suspensions not treated with ebselen were brought to a concentration of DMSO equivalent to that delivered with ebselen.

Irradiation

Thymocytes were suspended in either RPMI or Na^+ Hanks' buffer (approximately 10^7 cells/mL) and irradiated at room temperature with doses of 5 or 10 Gy at a dose rate of 0.5 Gy/min using the Armed Forces Radiobiology Research Institute's ^{60}Co Facility.

Microscopy

Cells (0.5 to 1 × 10^7) were pelleted by centrifugation (750 *g*, 10 min) and fixed by resuspension in 1 mL of freshly prepared 3% formaldehyde in ice-cold HBSS.* Fixed cells could be maintained under refrigeration for several weeks without any apparent degradation. Cells were concentrated for microscopy by allowing them to settle by gravity to the bottom of a test tube at 4°. Centrifugation to pellet cells was avoided because centrifugation of the fixed cells appeared to contribute to distorted cell morphology. For fluorescence microscopy, all but about 0.1 mL of the fixing buffer overlaying the cells was removed, and the cells were resuspended gently in the remaining buffer. A 20-μL aliquot was removed and mixed with 20 μL of 0.1 mg/mL of ethidium bromide in HBSS (final concentration, 50 μg/mL). The stained suspension was kept in the dark on ice until used. Ten microliters of suspension was placed on a microscope slide and gently covered with a 20-mm square cover slip. The cover slip was sealed with cement to prevent drying. Cells were allowed to settle and adhere to the surface of the slide for 5–10 min before beginning observation. Photomicroscopy was performed with an Olympus AHBT3 Research Microscope with Nomarski-type differential interference contrast and reflected-light fluorescence. Images were preserved on high-speed Polaroid Type 57 film.

DNA Agarose Gel Electrophoresis

Electrophoresis of DNA was performed according to the method of Gong *et al.* [17], which is particularly applicable to the qualitative detection of internucleosomal DNA fragments typical of apoptosis. Briefly, 1–2 × 10^6 cells were pelleted from the medium, washed once with HBSS, resuspended in 1 mL of HBSS, diluted with 10 mL of ice-cold 70% ethanol, and stored at −20° for 24 hr. The cells were then pelleted by centrifugation (800 *g* for 10 min) and ethanol was removed completely. The pellet was resuspended and the cells were lysed in 40 μL of phosphate-citrate buffer (192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8). After incubation at room temperature for 30 min, the cell lysate was centrifuged (1000 *g* for 5 min) and the supernatant concentrated to about 20 μL using a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.). A 3-μL aliquot of 0.25% Nonidet P-40 in distilled water was added to each sample followed by 3 μL of RNase A (1 mg/mL in water, Sigma Chemical Co., St. Louis, MO, U.S.A.) and the suspension incubated at 37° for 30 min. A 3-μL aliquot of proteinase K (1 mg/mL in water, Boehringer Mannheim, Indianapolis, IN, U.S.A.) was added and the sample incubated for an additional 30 min at 37°. Each sample was then mixed with an appropriate volume of 6× sample loading buffer (0.25% bromophenol blue/40% sucrose in water) and the entire mixture loaded onto an 0.8% agarose gel containing 0.5 μg ethidium bromide/mL. Electrophoresis was performed at 1.5 V/cm of gel length for about 16 hr. DNA bands were visualized using UV transillumination, and photographs of gels were obtained using Polaroid Type 665 positive/negative film.

DNA Fragmentation Assay

DNA fragmentation was assayed as previously described [18]. Briefly, cells (5 × 10^6) were collected by centrifugation (800 g for 10 min), lysed with 0.2 mL of ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and

* *Abbreviations:* HBSS, Hanks' balanced salt solution; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DCFH, 2',7'-dichlorofluorescin; and DCF, dichlorofluorescein.

0.2% Triton X-100, and pelleted by centrifugation (13,000 *g* for 20 min) to separate intact from fragmented DNA. The supernatant was conserved and the pellet resuspended in 0.2 mL of lysis buffer and sonicated for 10 sec at 4°. DNA concentration in the pellet and supernatant fractions was determined by an automated fluorometric protocol that we designed using AutoAnalyzer II components (Technicon, Tarrytown, NY, U.S.A.) and the DNA-specific fluorochrome Hoechst 33258 (Calbiochem-Behring, La Jolla, CA, U.S.A.). The percentage of DNA fragmentation refers to the ratio of the amount of DNA present in the 13,000 *g* supernatant (fragmented) to the total of the DNA in the pellet (unfragmented) and 13,000 *g* supernatant.

Peroxide Measurements

A sensitive fluorometric method was used to quantitate intracellular peroxides to determine whether ebselen affected peroxide levels in irradiated thymocytes. This method has been used previously to quantitate picomolar concentrations of hydrogen peroxide and lipid hydroperoxides [19, 20]. The assay is based on the fact that the nonpolar, non-fluorescent DCFH-DA can diffuse through the cell membrane and be deacetylated by cytosolic esterases to form the polar, non-fluorescent DCFH. DCFH is trapped within the cytoplasm where it is available to react with peroxides to form the fluorescent compound DCF, a reaction that can be monitored fluorimetrically [21].

DCFH-DA was purchased from Molecular Probes (Junction City, OR, U.S.A.). Stock solutions of DCFH-DA (50 mM) were prepared in DMSO and stored in 100-μL aliquots in the dark at −20°. Thymocytes (1×10^7 cells/mL) were resuspended in Na^+ Hanks' buffer containing 25 μM DCFH-DA and incubated at 37° for 30 min prior to irradiation. Fluorescence measurements were made at room temperature at selected times postirradiation with an SLM 8000 Spectrofluorometer (SLM Instruments Inc., Urbana, IL, U.S.A.) using a stirred cuvette and excitation and emission wavelenghts set at 485 and 530 nm, respectively (band widths 4 nm). Cell viability was not affected by incubation with 25 μM DCFH-DA.

Lipid Peroxidation Measurements

Stock solutions of *cis*-parinaric acid (1 mM) were prepared in 100% ethanol, through which nitrogen had been bubbled for 15 min to remove oxygen. Stocks were stored in 1-mL aliquots in screw-top vials at −20°. For experiments, cells (1×10^7 cells/mL) were incubated in RPMI containing 5 μM *cis*-parinaric acid (Molecular Probes) for 1 hr before irradiation, and then were transferred to Na^+ Hanks' buffer without *cis*-parinaric acid immediately before irradiation. Fluorescence measurements were carried out at room temperature using a stirred cuvette with excitation and emission wavelengths set at 324 and 425 nm, respectively (band widths 4 nm).

RESULTS

The effect of ebselen on morphological changes associated with apoptosis in irradiated (5 Gy, 24 hr post-irradiation) thymocytes is shown in Fig. 1. Panels A and B are bright-field and fluorescence photomicrographs of unirradiated thymocytes, demonstrating the smoothly contoured, similarly sized cells containing evenly distributed DNA that is typical of normal cells. Irradiated thymocytes (Fig. 1, C and D) exhibited morphological changes typical of apoptosis, including cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation, and formation of DNA-containing apoptotic bodies. These morphological changes typically began to appear in thymocytes 16–24 hr after radiation exposure. Incubation of irradiated cells with ebselen for 24 hr beginning immediately after radiation exposure resulted in cells with normal morphology (Fig. 1, E and F), indicating that ebselen completely blocks the morphological changes characteristic of radiation-induced apoptosis. The morphology of unirradiated cells incubated with ebselen for 24 hr was not affected by incubation with the drug (data not shown).

Figure 2 shows the effect of different concentrations of ebselen on radiation-induced internucleosomal DNA fragmentation, a biochemical marker for apoptosis. DNA fragmentation was measured using a sensitive, quantitative fluorometric method developed in our laboratory [18]. Thymocytes were treated with different concentrations of ebselen immediately after irradiation, and DNA fragmentation was measured 8 hr later. As shown, maximal inhibition of DNA fragmentation occurred with ebselen concentrations of about 10 μM and greater.

The effect of ebselen on the viability of unirradiated and irradiated thymocytes was determined by measuring the capacity of these cells to exclude trypan blue. Unirradiated cells incubated with 25 μM ebselen for 8 hr remained 94% viable, a value not different from DMSO-treated (vehicle-control) cells (Table 1). Radiation decreased the viability of otherwise untreated cells to 81%, but irradiated cells incubated with 25 or 50 μM ebselen remained 89 and 86% viable, respectively (Table 1). The results indicate that ebselen is not significantly toxic to cells in this range of concentrations. Based on these results and the effect of ebselen on DNA fragmentation (Fig. 2), we decided to use 25 μM ebselen for the remainder of our studies.

Figure 3 shows the effect of ebselen on the progression of DNA fragmentation in irradiated thymocytes. In these experiments, ebselen was added immediately after radiation exposure and DNA fragmentation was monitored for 8 hr. Fragmentation began 2–4 hr postirradiation (5 Gy) and increased thereafter almost linearly with time. Ebselen treatment not only blocked the radiation-induced fragmentation of DNA but reduced it to levels below the background of fragmentation observed in thymocytes treated with DMSO alone. Figure 4 shows agarose gels of fragmented DNA isolated from thymocytes after various treatments. Gel electrophoresis was used to confirm the pres-

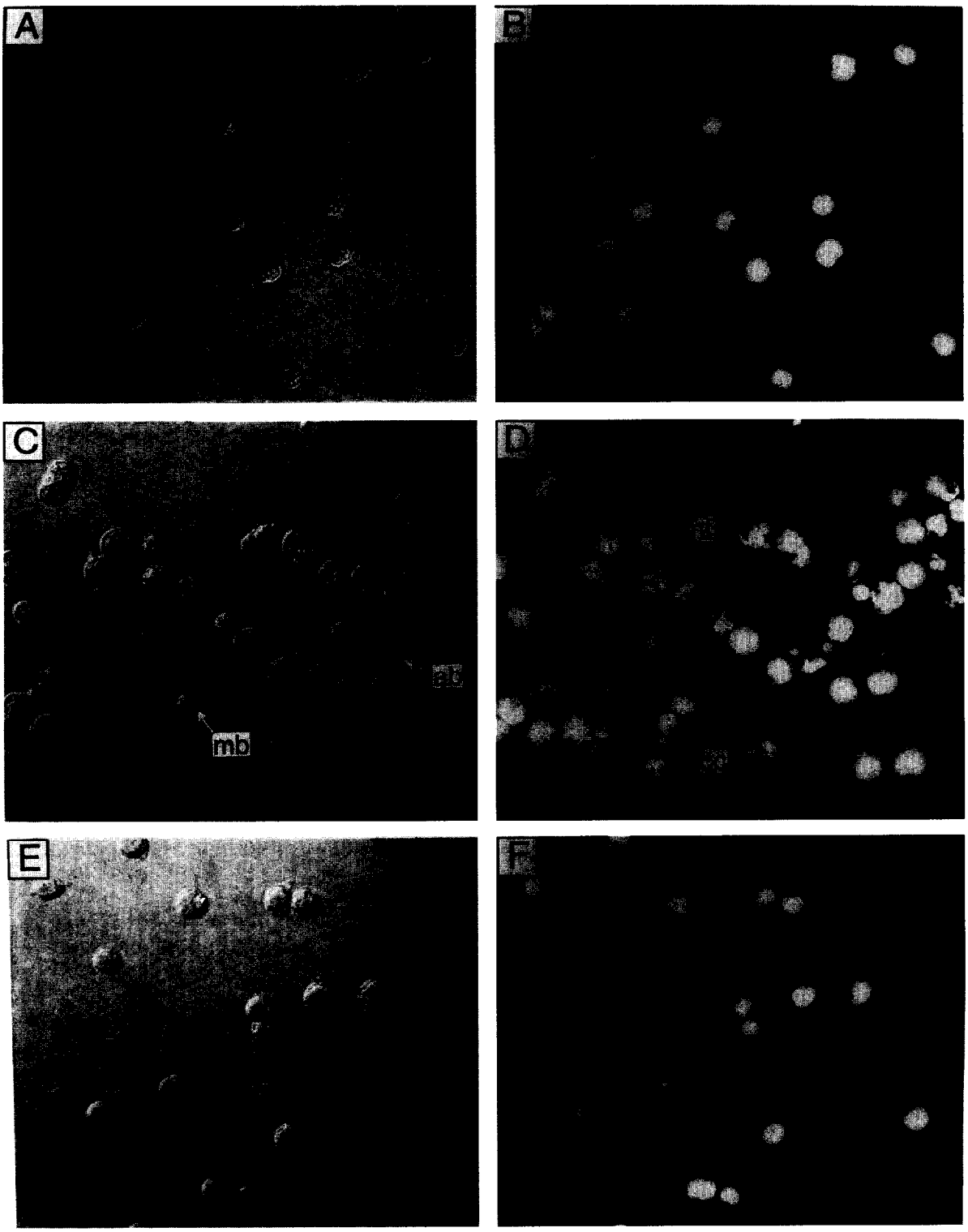

FIG. 1. Effect of ebselen on morphological changes associated with apoptosis in irradiated thymocytes. Thymocytes were prepared for microscopy as described in Materials and Methods. Panels A and B show, respectively, bright-field and DNA-fluorescence images of unirradiated thymocytes, indicating normal morphology. Panels C and D show analogous images 24 hr after radiation exposure (5 Gy, 0.5 Gy/min). Radiation induces morphological changes characteristic of apoptosis, including cell shrinkage, plasma membrane blebbing (mb), and formation of apoptotic bodies (ab). DNA staining shows pronounced chromatin condensation (cc), nuclear fragmentation (nf), and presence of DNA fragments in apoptotic bodies. Panels E and F demonstrate the effect of ebselen on the morphology of irradiated cells. Cells in these images were irradiated and then incubated with 25 μM ebselen for 24 hr postirradiation. Neither bright-field nor fluorescence images exhibited apoptotic morphology. Unirradiated cells treated with ebselen were no different than unirradiated cells not treated with ebselen (data not shown).

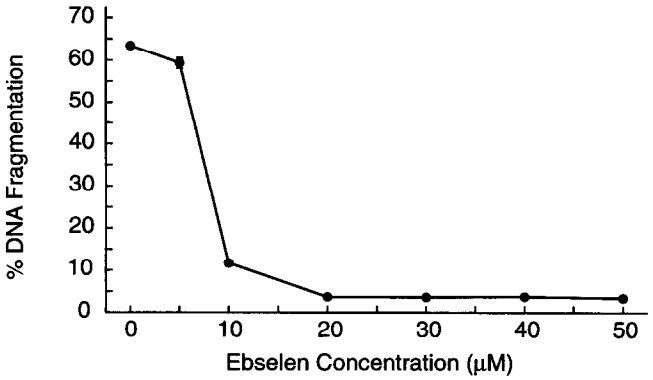

FIG. 2. Influence of different concentrations of ebselen on radiation-induced DNA fragmentation. Thymocytes were incubated with indicated concentrations of ebselen immediately after radiation exposures (5 Gy, 0.5 Gy/min). Fragmented DNA was measured 8 hr after irradiation as described in Materials and Methods. Data represent means ± SEM of 3 experiments.

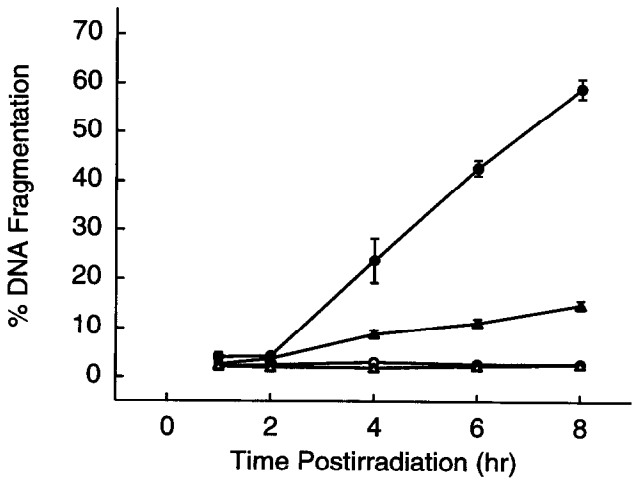

FIG. 3. Effect of ebselen on the time-course of DNA fragmentation in irradiated and unirradiated thymocytes. Unirradiated and irradiated thymocytes were incubated with 25 μM ebselen beginning immediately after irradiation (5 Gy, 0.5 Gy/min). Cell suspensions not treated with ebselen were brought to the same concentration of DMSO equivalent to that delivered with ebselen. Fragmented DNA was measured as described in Materials and Methods at indicated times. Key: (●) 5 Gy; (○) 5 Gy + ebselen; (▲) 0 Gy; and (△) 0 Gy + ebselen. Data represent means ± SEM of three experiments.

ence of apoptotic DNA. The internucleosomal cleavage of DNA during apoptosis produced a "ladder" pattern of DNA fragments on the gel that were multiples of approximately 200 base pairs. There was a small amount of DNA fragmentation detected in unirradiated thymocytes that represented the background fragmentation in untreated cells (lane 2). DNA isolated from irradiated thymocytes showed a typical, pronounced fragmentation pattern (lane 4). Consistent with the data presented in Fig. 3, ebselen completely blocked the appearance of the ladder pattern in both unirradiated and irradiated cells (lanes 3 and 5, respectively). Note that even background fragmentation was no longer apparent.

In all of the studies described above, thymocytes were incubated with ebselen immediately after irradiation. To determine when ebselen treatment of irradiated cells was most effective, thymocytes were treated with ebselen for either 30 min before or during irradiation, after which cells were transferred to medium without ebselen. The results of these experiments (Fig. 5) indicate that ebselen treatment before or during irradiation inhibits DNA fragmentation almost as effectively as treatment after radiation exposure.

A very sensitive fluorimetric method employing DCFH-DA was used to quantitate intracellular peroxides. Incubation of the cells with DCFH-DA began 30 min before irradiation, and the cells were maintained in the presence of the drug throughout the course of the experiments. Fluorescence was then monitored various times after irradiation. Figure 6 summarizes the results of those experiments. Fluorescence intensity in irradiated thymocytes not treated with ebselen increased in a biphasic manner, quickly reaching levels 10–11 times higher than preirradiation levels within the time of the first measurement (2 min postirradiation), followed by a slower rise to 16 times initial levels by 2 hr postirradiation. The early increase in fluorescence intensity after irradiation in ebselen-treated cells was 50% that measured in irradiated cells not treated with ebselen, indicating that ebselen inhibits the formation of radiation-induced peroxides. Ebselen appeared to block completely the second, slower rise in fluorescence that was measured between 2 min and 2 hr postirradiation in irradiated cells not treated with ebselen. Note that there was a "spontaneous" generation of peroxides in unirradiated thymocytes not exposed to ebselen. The level of peroxides in these cells after a 2-hr incubation was about three times that present in the cells before incubation. Our data show that ebselen inhibited this peroxide increase as well.

Peroxides and free radicals produced during irradiation can react with polyunsaturated fatty acids in the membrane to initiate lipid peroxidation reactions. The kinetics of lipid peroxidation during apoptosis in irradiated thymocytes were analyzed using *cis*-parinaric acid, a naturally fluorescent polyunsaturated fatty acid that readily incorporates into cell membranes [22]. Peroxidation of this fatty acid results in loss of fluorescence, which can be monitored over

TABLE 1. Effect of ebselen on the viability of unirradiated and irradiated thymocytes

	Viable cells (%)	
Incubation conditions	**O Gy**	**5 GY**
DMSO	95.0 ± 0.8	81.3 ± 0.3
Ebselen (25 μM)	93.6 ± 1.0	88.8 ± 0.1
Ebselen (50 μM)	86.1 ± 1.0	85.6 ± 1.2

Unirradiated and irradiated thymocytes were incubated with indicated concentrations of ebselen until viability was determined 8 hr after irradiation. Cells excluding trypan blue were considered viable. Initial viability was 92.4 ± 0.6%. Data represent means ± SEM of three independent measurements.

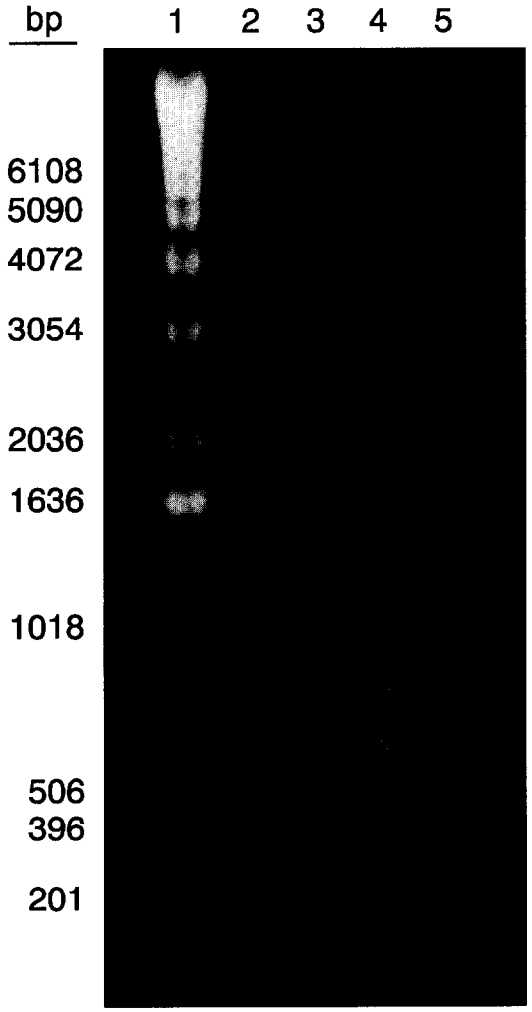

FIG. 4. Effect of ebselen on internucleosomal DNA fragmentation. Fragmented DNA fraction was extracted from irradiated cells (5 Gy, 0.5 Gy/min) and electrophoresed as described in Materials and Methods. Lane 1, standard 1 kb DNA ladder. Lane 2, unirradiated cells after an 8-hr incubation at 37° under 5% CO_2 in air. Lane 3, unirradiated cells incubated with 25 μM ebselen for 8 hr. Lane 4, irradiated thymocytes 8 hr postirradiation. Lane 5, irradiated thymocytes after an 8-hr incubation with 25 μM ebselen.

time [23]. The measurements show (Fig. 7) that the loss of fluorescence began soon after irradiation, with 5% of *cis*-parinaric acid becoming oxidized within 2 min, and the oxidation increased with time. A small amount of oxidation also occurred in unirradiated cells, probably caused by the oxidation of *cis*-parinaric acid by "spontaneous" intracellular peroxides produced as a consequence of incubation conditions, a result consistent with the data in Fig. 6.

DISCUSSION

Apoptosis plays a major role in development, homeostasis, and many diseases including cancer, acquired immune deficiency syndrome, and neurodegenerative disorders [1]. It has been shown that apoptosis induced by a variety of stimuli is associated with increased oxidation levels in the cell. These increases may result from either an overproduction of reactive oxygen species or an attenuation of cellular antioxidant defense systems [12, 24]. The antioxidant defense

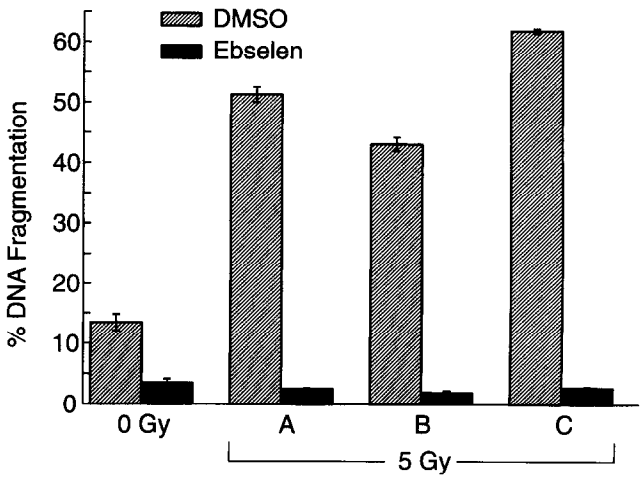

FIG. 5. Optimal timing of ebselen treatment relative to radiation exposure. (A) Preirradiation treatment. Thymocytes were incubated with 25 μM ebselen 30 min prior to irradiation (5 Gy, 0.5 Gy/min). Cells were pelleted and resuspended in fresh medium without ebselen immediately before irradiation. (B) Ebselen during exposure. Ebselen was added to the cell suspension immediately before irradiation. Cells were pelleted and resuspended in fresh medium without ebselen immediately after irradiation. (C) Postirradiation treatment. Cells were incubated with ebselen beginning immediately after irradiation. DNA fragmentation was measured in all samples 8 hr after irradiation. Data represent means ± SEM of three experiments.

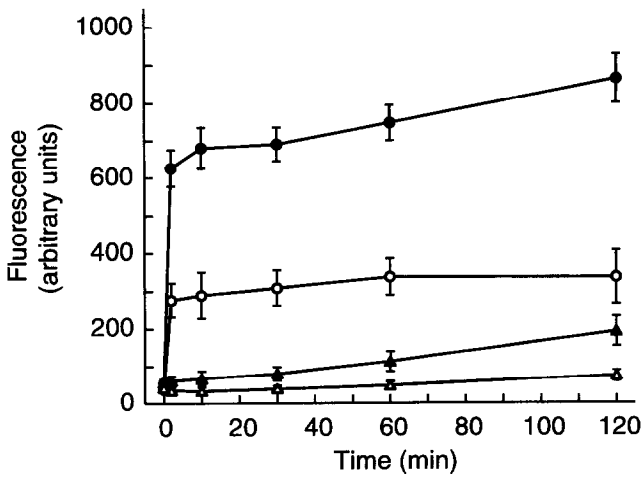

FIG. 6. Peroxide levels in irradiated thymocytes treated or not treated with ebselen prior to radiation exposure. Cell suspensions in Na^+ Hanks' buffer were incubated with 25 μM DCFH-DA at 37° for 30 min prior to irradiation (10 Gy, 0.5 Gy/min) and maintained in the same buffer during measurements. Ebselen-treated cells were exposed continuously to 25 μM ebselen beginning 30 min before irradiation. Key: (●) 10 Gy; (○) 10 Gy + ebselen; (▲) 0 Gy; and (△) 0 Gy + ebselen. Data represent means ± SEM of four experiments.

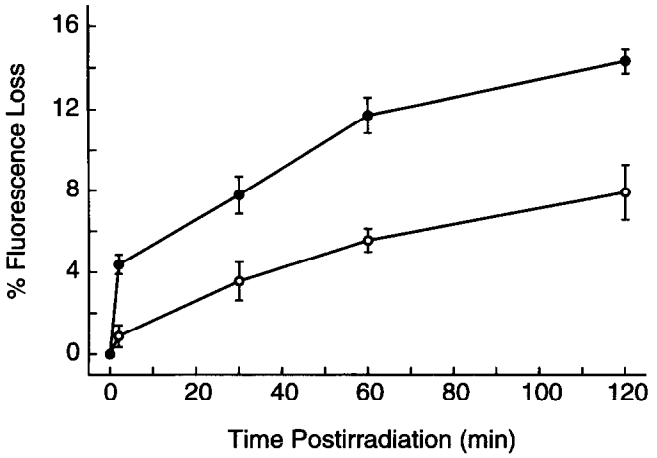

FIG. 7. Lipid peroxidation in irradiated thymocytes. Cell suspensions in medium were incubated with 5 μM *cis*-parinaric acid for 1 hr before irradiation. Cells were resuspended in Na^+ Hanks' buffer without *cis*-parinaric acid prior to irradiation (10 Gy, 0.5 Gy/min) and maintained in the same buffer during measurements. Key: (●) 10 Gy; and (○) 0 Gy. Data represent means ± SEM of three experiments.

system consists of two components: (1) radical scavengers, which quench free radicals, and (2) peroxidases, which prevent radical formation by reducing hydroperoxides to their corresponding, harmless, hydroxy derivatives. There are pharmacological agents that inhibit apoptosis by blocking the production of free radicals, including *N*-acetylcysteine [25], *N*-(2-mercaptoethyl)-1,3-propanediamine [18], and trolox [8, 9, 26]. There have been no previous reports of agents that protect cells from apoptosis by enhancing peroxidase activity. Several studies indicate that the drug ebselen exhibits glutathione peroxidase-like activity similar to that of phospholipid hydroperoxide glutathione peroxidase [15, 27–32]. This report presents results of experiments that are the first to show that ebselen protects cells from radiation-induced apoptosis. These findings also provide circumstantial evidence to support a role for peroxides in signaling apoptosis in the irradiated cell.

The results of these experiments clearly demonstrate that ebselen is an effective inhibitor of the indicators of apoptosis in irradiated thymocytes. First, ebselen treatment preserved normal cell morphology; the plasma membrane appeared normal, chromatin condensation and nuclear fragmentation were absent, and apoptotic bodies were scarce in irradiated cell preparations (Fig. 1). Second, ebselen treatment reduced DNA fragmentation to levels even below those of unirradiated controls (Figs. 3 and 4). Third, the viability of ebselen-treated cells was improved (Table 1).

The inhibition of apoptosis mediated by ebselen, along with the fact that the biological effectiveness of ebselen lies in its capacity to detoxify peroxides, implies that peroxides play a role in radiation-induced apoptosis, but it is not known whether peroxides actually signal (cause) apoptosis. However, our data showing the potent capacity of ebselen to block radiation-induced peroxide generation, combined with the time course of peroxide production in irradiated thymocytes relative to that of apoptosis, suggest that such a causal relationship might exist.

Intracellular peroxides can be detected in thymocytes very early after irradiation—before the morphological and biochemical indicators of apoptosis. A rapid increase in peroxide levels occurred within 2 min postirradiation, leading to levels 16 times the preirradiation values by 2 hr (Fig. 6). This time course of peroxide production preceded DNA fragmentation, which began 2–4 hr after irradiation (Fig. 3), and morphological changes, which became evident 16–24 hr postirradiation (Fig. 1). Ebselen treatment greatly reduced the net production of peroxides in irradiated cells. Unirradiated cells treated with ebselen also generated lower peroxide levels than unirradiated cells not treated, an indication that ebselen reduces even the spontaneous generation of peroxides that occurs during incubation of the cells (Fig. 6).

The mechanism by which radiation-induced peroxides might induce apoptosis remains a mystery, but a variety of observations made by us and others allow us to propose a hypothetical pathway. Evidence seems to indicate that lipid peroxidation is a key step in the process. Lipid peroxidation can be initiated in the irradiated cell by reaction of hydrogen peroxide with polyunsaturated fatty acids in the membrane. Our results indicate that lipid peroxidation chain reactions begin soon after irradiation and increase with time (Fig. 7). These lipid peroxidation reactions appear to play an important role in radiation-induced apoptosis, because the lipid peroxyl radical scavenger trolox effectively inhibits apoptosis in thymocytes and human MOLT-4 cells induced by either ionizing radiation [8, 9] or hydrogen peroxide [26]. Other reports have also implicated lipid peroxidation as playing a role in apoptosis [12, 25, 33]. Oxidized lipids can subsequently be acted upon by phospholipases, which can lead to the release of fatty acid hydroperoxides from the membrane. It is known that lipid hydroperoxides can induce apoptosis when added to cells, an activity that may be related to their capacity to increase cytoplasmic calcium [34]. Little is known of the link between elevated cytoplasmic calcium and apoptosis, but a number of studies have shown that an increase in cytosolic calcium occurs during apoptosis [8, 9, 35], an event that may trigger signal transduction pathways.

The observation that ebselen was effective if added postirradiation is consistent with the proposal that lipid hydroperoxides may be key intermediates in radiation-induced apoptosis. They are formed predominantly postirradiation (as indicated by the time course of membrane lipid peroxidation), so ebselen need be present only after exposure to exert its effect. Ebselen, however, also inhibited apoptosis when cells were incubated with the drug only before and during irradiation (followed by transfer of the cells to ebselen-free medium). Such results do not necessarily diminish the importance of the postirradiation reactions. It is possible that effective concentrations of ebselen may persist in the cell even after a short incubation if ebselen quickly enters and is retained in the cytosolic and

membrane compartments. Even small amounts of ebselen may provide effective antioxidant activity, because ebselen behaves as a catalyst and as such is not consumed during detoxification reactions [15].

In our experiments, we measured significant levels of DNA fragmentation not only in irradiated but also in unirradiated cells, though at much lower levels [approximately 60 vs 15% fragmentation, respectively, at 8 hr postirradiation (Fig. 3)]. This "spontaneous" DNA fragmentation was also correlated with the increases in peroxides (Fig. 6) and lipid peroxidation (Fig. 7) in untreated cells. We believe these events are a consequence of *in vitro* incubation conditions. After thymocytes were removed from the animal, the cells were incubated in standard cell culture incubators in an atmosphere containing just under 20% oxygen, a much higher pO_2 level than *in vivo*. This relatively high O_2 level could lead to oxidative stress in the cells. The fact that ebselen decreased both peroxide production and DNA fragmentation (apoptosis) in unirradiated cells probably results from the capacity of ebselen to block oxidative damage that occurs in these cells as a result of the incubation conditions.

Ebselen is not toxic to thymocytes under the conditions of our experiments; viability of unirradiated cells treated with ebselen was not appreciably different than that of untreated cells (Table 1). *In vivo* studies with human volunteers and animals also indicate that ebselen has no toxicity [29, 36, 37]. The absence of selenium toxicity probably results from the fact that selenium is covalently bound to an organic matrix from which it is not released [31, 36, 37]. Its biochemical activity and its low toxicity have led to its therapeutic use in humans. Ebselen is now in Phase I clinical trials as an antiinflammatory drug [38], based on its glutathione peroxidase-like action in scavenging peroxides produced during various pathological inflammatory conditions [14, 39]. It may prove rewarding to explore the use of ebselen as a therapeutic agent for protecting cells from apoptosis induced by peroxides released during development [12, 40], acquired immune deficiency syndrome [12, 24], radiation exposure, and other pathological conditions [1].

To summarize, our experiments indicated that ebselen is an effective inhibitor of radiation-induced apoptosis in thymocytes. Ebselen was not significantly toxic to the cells and could be added before, during, or after irradiation to exert its effect. The data suggest that ebselen exerts its effect by detoxifying primary and/or secondary peroxides produced during and after irradiation. The results also provide new insights into the general role of lipid peroxidation in radiation-induced apoptosis. Ebeselen or drugs with similar activity may prove to be useful tools in treating diseases associated with the inappropriate triggering of apoptosis.

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