Ionizing Radiation Acts on Cellular Membranes to Generate Ceramide and Initiate Apoptosis

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Summary

Recent investigations provided evidence that the sphingomyelin signal transduction pathway mediates apoptosis for tumor necrosis factor α (TNF-α) in several hematopoietic and nonhematopoietic cells. In this pathway, TNF-receptor interaction initiates sphingomyelin hydrolysis to ceramide by a sphingomyelinase. Ceramide acts as a second messenger stimulating a ceramide-activated serine/threonine protein kinase. The present studies show that ionizing radiation, like TNF, induces rapid sphingomyelin hydrolysis to ceramide and apoptosis in bovine aortic endothelial cells. Elevation of ceramide with exogenous ceramide analogues was sufficient for induction of apoptosis. Protein kinase C activation blocked both radiation-induced sphingomyelin hydrolysis and apoptosis, and apoptosis was restored by ceramide analogues added exogenously. Ionizing radiation acted directly on membrane preparations devoid of nuclei, stimulating sphingomyelin hydrolysis enzymatically through a neutral sphingomyelinase. These studies provide the first conclusive evidence that apoptotic signaling can be generated by interaction of ionizing radiation with cellular membranes and suggest an alternative to the hypothesis that direct DNA damage mediates radiation-induced cell kill.

The biochemical regulation of programmed cell death (apoptosis) has recently attracted a great deal of attention because of its role as a physiological mechanism of cell death. Apoptosis represents a major regulatory mechanism in embryonal development, growth and differentiation, and in the wear and tear maintenance of adult mammalian tissues (1–3). In addition, programmed cell death serves as one of the pleiotropic mechanisms of cell kill by cytokines (4, 5), chemical agents (6, 7), radiation (8, 9), and heat (10). Apoptosis is conceptualized as a pre-programmed pathway of sequential biochemical events that are only partially known, eventually leading to activation of a calcium-magnesium–dependent endonuclease that cleaves the nuclear chromatin at selective internucleosomal linker sites (1). Signals generated at the membrane of the affected cell activate neighboring intact cells and infiltrating macrophages to phagocytize the dying cell and its disintegrating nucleus (11). Presently, there is little information on the signaling mechanisms that initiate the apoptotic response. Recently, however, the sphingomyelin pathway has been shown to constitute the early events in the apoptotic cascade of TNF-α–induced programmed cell death (12, 13).

The sphingomyelin pathway is a signal transduction pathway (14) that mediates the signaling of several cytokines, such as TNF-α (12, 13, 15) and IL-1β (16). In these systems, stimulation of cell surface receptors activates a plasma membrane neutral sphingomyelinase that hydrolyzes sphingomyelin to generate ceramide and phosphocholine. Ceramide then serves as a second messenger, activating a proline-directed serine/threonine kinase, termed ceramide-activated protein kinase (17, 18). The further downstream signaling events are only partially known, but phosphorylation of the p42 isoform of microtubule-associated protein (MAP) kinase (19) and nuclear translocation of NF-κB (20, 21) have been reported in Jurkat T and HL-60 cells. Activation of the sphingomyelin pathway by TNF-α is mediated via the 55-kD low affinity receptor (22, 23) and is tightly coupled to receptor activity, as activation of the sphingomyelin cascade was induced by TNF or by a stimulatory antibody for the 55-kD receptor in isolated membranes of HL-60 cells (23, 24). Obeid et al. (12) and Jarvis et al. (13) have recently provided evidence that TNF-α–induced apoptosis is mediated via activation of sphingomyelin degradation and generation of ceramide in U937 monoblastic and HL-60 promyelocytic leukemia cell lines, and in L929/LM and WEHI-164/13 human fibrosarcoma cell lines. In these studies, TNF stimulated sphingomyelin degradation to ceramide, and elevation of cellular ceramide levels by addition of synthetic ceramide analogues or bacterial sphingomyelinase mimicked TNF action to induce apoptosis. In contrast, activation of other messenger systems with

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different phospholipases or synthetic lipid analogues failed to induce apoptosis. In fact, activation of protein kinase C (PKC)[1] by phorbol esters, 1,2-diacylglycerols, or phospholipase C antagonized apoptosis through the sphingomyelin pathway (12, 13).

The activating signals for other modes of apoptosis are less well established. In some forms of chemotherapy- and radiation-induced apoptosis, primary lesions to the DNA have been considered as signals that trigger the apoptotic response (6, 9). The prevailing hypothesis on the nature of the lethal damage produced by ionizing radiation identifies heterologous double strand breaks in the DNA as the most common type of lesions that lead to mammalian cell death (25, 26). Such lesions are produced in the DNA by direct interaction with x rays, or with reactive oxygen intermediates generated within the cell by the radiation (27). Whereas mammalian cells are proficient in the capacity to repair most DNA double strand breaks (27, 28), not all such lesions are repairable (26). Residual unrepaired DNA lesions are known to lead to postmitotic cell death, associated with chromosomal aberrations and DNA dysfunction (28). Whether such breaks also trigger the intermitotic (interphase) mode of radiation-induced cell kill, associated with programmed cell death (29), is unknown. Both the interphase and postmitotic mechanisms contribute to cell killing after exposure of mammalian cells to ionizing irradiation. The relative contribution from each mode of cell death may differ with dose and from one cell type to another, relative to their inherent and inducible capacities to overcome each of these types of lethal radiation damage (30).

Experiments to explore the role of signals originating from radiation-damaged DNA in triggering the apoptotic pathway were performed using pretreatment of cells with bromo deoxyuridine (BrdUrd). This halogenated pyrimidine is known to sensitize mammalian cells to radiation killing, because it replaces thymidine during DNA synthesis, thus creating defective DNA with increased propensity for DNA breaks upon radiation exposure (31). Warters (32) reported that pretreatment of murine T-hybridoma cells with BrdUrd enhanced both x-ray–induced DNA strand breaks and apoptosis to a similar extent. In contrast, Akagi et al. (33) reported that pretreatment of Molt-4 cells with BrdUrd had no effect on the incidence of apoptotic cell death, although it did sensitize the cells to the postmitotic mode of radiation-induced cell kill. Macklis et al. (34) reported that binding of ^{212}Bi-radiolabeled anti-Thy 1.2 mAb to cell surface receptors in EL-4 cells resulted in significant radiation-induced cell kill via activation of the apoptotic mechanism. Dosimetry evaluations of the range of migration of the particle irradiation emitted from the ^{212}Bi-radionucleide indicated, however, that the majority of the radiation was deposited in the cell membrane. Thus, the question of whether radiation-induced apoptosis is initiated by signals generated at the cell membrane, nucleus, or both, remains open.

[1] *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cell; bFGF, basic fibroblast growth factor; BCS, bovine calf serum; PKC, protein kinase C; TDT, terminal deoxynucleotidyl transferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; XRT, radiation.

Apoptosis as a mechanism of radiation-induced cell death is common in thymic, lymphoid, and other cells of hematopoietic lineage, but has been infrequently documented in other adult mammalian cell types (8, 9). Recent studies have, however, reported that the apoptotic pathway is induced in G_0-G_1 phase bovine aortic endothelial cells (BAEC) upon radiation exposure, and that basic fibroblast growth factor (bFGF) inhibits the apoptotic response in these cells (30). bFGF did not affect the initial yield or rate of repair of DNA breaks produced directly by the interaction with radiation or with reactive oxygen intermediates (30), and the antiapoptotic effect of bFGF was mediated via activation of PKC (35). In this context, it is of interest to note that TNF was reported to induce in BAEC a similar apoptotic response (36). Because of the similarity between the radiation and TNF induction of apoptosis in BAEC, we postulated that the triggering of apoptosis by radiation in these cells may be generated by primary cell membrane signals and may be mediated via the sphingomyelin signaling pathway.

These studies demonstrate that ionizing radiation triggers a rapid hydrolysis of sphingomyelin to produce ceramide, and that ceramide activates the apoptotic pathway in cultures of BAEC. The effect of ionizing radiation to induce sphingomyelin hydrolysis to ceramide occurred in cellular preparations devoid of nuclei and hence, appears to be independent of direct DNA damage. Furthermore, activation of PKC blocked both radiation-induced ceramide generation and apoptosis, but addition of exogenous ceramide overcame the blockage to restore apoptosis. These experiments provide the first conclusive evidence that apoptotic signaling can be generated by interaction of ionizing irradiation with the cell membrane. These studies suggest an alternative to the notion that direct DNA damage mediates radiation-induced cell kill, and therefore primary membrane signals as well as nuclear signals must be considered in the evaluation of the lethal effects of ionizing irradiation.

Materials and Methods

Reagents. Ceramide (type III), sphingomyelin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), pentosan polysulfate (PPS), and *N*-octyl-D-glucopyranoside were from Sigma Chemical Co. (St. Louis, MO). Cardiolipin (bovine heart) was from Avanti Polar Lipids, Inc. (Birmingham, AL). *sn*-1,2-Diacylglycerol kinase was from Lipidex, Inc. (Westfield, NJ) or Calbiochem-Novabiochem Corp. (La Jolla, CA). [Methyl-^{3}H]Choline chloride (86.7 Ci/mmol) and ^{32}[γ-P]ATP (3,000 Ci/mmol) were from Du Pont-New England Nuclear (Boston, MA). Liquid scintillation solution (CytoScint) was from ICN Radiochemicals (Irvine, CA). Silica gel TLC plates (LK60D) were from Whatman (Clifton, NJ). Solvents were from Fisher Scientific Co. (Springfield, NJ) and were HPLC grade.

Preparation of Cells and Radiation Procedures. Cloned populations of BAEC were established from the intima of bovine aorta as previously described (37). To expand the cell population, stock cultures were split at a ratio of 1:10 (up to a maximum of 10 subcultures). The monolayers were first dissociated by 2–3-min incubation at 22°C with 0.05% trypsin-0.02% EDTA in HBSS, washed twice with DMEM, and seeded in DMEM supplemented with glucose (1 g/liter), 10% heat-inactivated bovine calf serum (BCS), peni-

cillin (50 U/ml), and streptomycin (50 μg/ml). Human recombinant bFGF (R&D Systems, Inc., Minneapolis, MN) was added (0.75 ng/ml) every other day during the phase of exponential growth. After 8–10 d, the cells reached confluence and exhibited features of contact inhibited monolayers. These plateau phase cells were either used for experiments, or further subcultured to expand the cell population for other experiments. 24 h before irradiation, the culture medium was changed to fresh 10% BCS-DMEM. Irradiation of intact cells or membrane fractions was carried out in a Gamma-cell 40 chamber containing two sources of ^{137}Cs (Atomic Energy of Canada Ltd., Kanata, Ont., Canada) at a dose rate of 100 cGy/min.

Lipid Studies. For quantitative measurements of sphingomyelin levels, cultures of BAEC were incubated with [^{3}H]choline chloride (0.25 mCi/ml) for 7 d until isotopic equilibrium was reached (24). The cells were then washed and transferred into fresh bFGF-, choline-, and serum-free medium containing PPS (50 mM). After 2 h, the cells were detached by trypsinization, washed twice in serum-free medium, and resuspended in PBS at 12 × 10^6/ml. In some experiments, TPA (50 ng/ml) was added to the serum-free medium for 5 min or 3 h before irradiation. In experiments using nuclei-free membranes, cells were resuspended into homogenization buffer (50 mM NaF, 5 mM EGTA, and 25 mM Hepes, pH 7.4), disrupted at 4°C with 150 strokes of a tight-fitting Dounce homogenizer (Fisher Scientific, Co.), centrifuged for 5 min at 500 *g*, and the postnuclear supernatants were used for experiments. After irradiation at 4°C, cells were incubated at 37°C in DMEM and nuclei-free supernatants were incubated at 37°C in a mixture of 3 mM ATP/homogenization buffer/deionized water/sphingomyelinase assay buffer (50 mM Hepes, pH 7.4, and 20 mM $MgCl_2$) (30:30:90:300, vol/vol). At the indicated times, cells or membrane fractions were extracted with chloroform/methanol/hydrochloric acid (1 N) (500:500:5, vol/vol) and buffered saline solution containing 15 mM EDTA, as previously described (24). The lipids in the organic phase extract were then dried under N_2 and subjected to mild alkaline hydrolysis (1 N methanolic KOH for 1 h at 37°C) to remove glycerophospholipids. Sphingomyelin was resolved by TLC using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:30:8:4, vol/vol) as solvent, identified by iodine vapor staining, and quantified by liquid scintillation spectrometry, as previously described (24). The use of [^{3}H]choline as a measure of sphingomyelin content was validated by phospholipid phosphorous measurements as previously described (38).

Ceramide was quantified by the diacylglycerol kinase assay as ^{32}P incorporated upon phosphorylation of ceramide to ceramide-1-phosphate by diacylglycerol (DG) kinase (15). Treatment of the cells was identical to that described above for the sphingomyelin determination, except that the [^{3}H]choline labeling was omitted. After irradiation and extraction of lipids, ceramide contained within the organic phase extract was resuspended into 20 μl of 7.5% *n*-octyl-β-D-glucopyranoside/5 mM cardiolipin/1 mM DTPA (Sigma Chemical Co.). Thereafter, 40 μl of purified DG kinase in enzyme buffer (20 mM Tris-HCl, 10 mM dithiothreital, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol, pH 7.4) was added to the organic phase extract. γ-[^{32}P]ATP (20 μl 10 mM; 1,000 dpm/pmol) in enzyme buffer was added to start the reaction. After 30 min at 22°C, the reaction was stopped by extraction of lipids with 1 ml of $CHCl_3/CH_3OH/HCl$ (100:100:1), 170 μl buffered saline solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM Hepes, pH 7.2), and 30 μl of 100 mM EDTA. The lower organic phase was dried under N_2. Ceramide-1-phosphate was resolved by TLC using $CHCl_3/CH_3OH/HAc$ (65:15:5, vol/vol) as solvent, detected by autoradiography, and the incorporated ^{32}P was quantified by Cherenkov counting. The level of ceramide was determined by comparison to a concomitantly run standard curve comprised of known amounts of ceramide.

The Terminal Transferase Nick Translation Assay. The presence of DNA breaks was evaluated by a modification of the nick translation method for in situ labeling of DNA breaks as previously described (30, 39, 40). Briefly, 10^6 cells were removed from the culture dishes by 2-min incubation in 0.05% trypsin and 0.02% EDTA in HBSS, washed twice in HBSS, and collected in 10% BCS-DMEM. The cells were fixed with 4% buffered formaldehyde for 15 min on ice and stored in 70% ethanol overnight at −20°C. The cells were then rehydrated by double washing in PBS and incubated in terminal deoxynucleotidyl transferase (TDT) buffer (25 mM Tris-HCl, 200 mM potassium cacodylate, 5 mM cobalt chloride at pH 6.6), 0.2 U/μl TDT and 2 nM biotin-11-dUTP (both purchased from Boehringer Mannheim Biochemica, Indianapolis, IN) at 37°C for 30 min. The reaction was stopped by addition of ice-cold PBS and the cells were incubated for 30 min at room temperature in the dark in a solution containing 4X SSC buffer (Sigma Chemical Co.), 15 μg/ml fluorescinated avidin, 0.1% Triton X-100 (vol/vol), and 0.5% nonfat milk. After one additional wash in ice-cold PBS containing 0.1% Triton X-100 (vol/vol), the cells were suspended at a concentration of 10^4 cells/ml in PBS containing propidium iodide (5 μg/ml) and 0.1% RNase. The red (propidium iodide) and green (fluorescein) fluorescence were measured using a FACScan® flow cytometer equipped with a doublet discriminating module (Becton Dickinson & Co., San Jose, CA). The data were analyzed using the LYSYS II software (Hewlett-Packard Co., Palo Alto, CA). An analysis region was set to exclude cell aggregates, and the green channel was set to score <1 % of the signals from unirradiated control cells.

Agarose Gel Electrophoresis of DNA. The presence of apoptosis was evaluated by agarose gel electrophoresis of DNA extracted from BAEC, as previously described (1). Briefly, 5 × 10^6 cells were washed twice with cold PBS and lysed with Nicoletti lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% SDS and 50 μg/ml proteinase K) at 43°C overnight. The DNA was extracted with an equal volume of phenol, after a chloroform/isoamyl wash. The DNA solution was then incubated for 1 h at 37°C with 50 μg/ml RNase, followed by a chloroform/isoamyl wash. The DNA was then precipitated with 0.5 M NaCl and 95% ethanol at −20°C overnight. The pellets were resuspended in 50 μl H_2O, and the DNA concentration was determined from the absorbance at 260 nm. Each DNA sample (10 μg) was electrophoresed at 80 V for 4 h through a 2.0% agarose gel in Tris-borate buffer. DNA bands were visualized under UV light after staining with ethidium bromide. Haelll digests of double-stranded fX174 DNA were used as molecular size markers.

Cytochemical Staining of Apoptotic Cells. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome *bis*benzimide trihydrochloride (Hoechst-33258) as previously described (5). Briefly, confluent monolayers of BAEC were washed with cold PBS and irradiated as described above. Before staining, the cells were trypsinized with 0.05% trypsin and 0.02% EDTA in HBSS, collected in 10% BCS-DMEM, resuspended for fixation in 200 μl of 3% paraformaldehyde, and incubated for 10 min at room temperature. The fixative was then removed, the cells were washed with PBS, resuspended in 20 μl of PBS containing 8 μg/ml of Hoechst-33258, and incubated at room temperature for 15 min. Aliquots of 10 μl were then placed on glass slides coated with 3-aminopropyl-triethoxysilane and 500 cells were counted and scored for the incidence of apoptotic chromatin changes under an Olympus

BH-2 fluorescence microscope using a BH2-DMU2UV Dich Mirror Cube filter.

Statistics. Statistical analysis was performed by Student's *t* test.

Results

Ceramide Induces Apoptotic DNA Degradation in BAEC. To ascertain whether ceramide initiates an apoptotic response in endothelial cell, experiments were carried out using the synthetic ceramide analogue *N*-acetyl sphingosine (C2-ceramide). Treatment with synthetic ceramide analogues has been shown to trigger the apoptotic pathway in several types of cells (12, 13). Fig. 1 shows that incubation of plateau phase BAEC for 24 h with 50 μM C2-ceramide produced typical apoptotic changes of nuclear condensation, segmentation, and apoptotic bodies, detected by staining with *bis*benzimide trihydrochloride (Hoechst-33258). Similar changes were observed when endogenous ceramide was generated by treatment with 1.0 U/ml of *Staphylococcus aureus* sphingomyelinase, or in cells treated with a single radiation dose of 500 cGy. In addition to the typical morphological changes, ceramide-induced apoptosis in BAEC was also demonstrated by neutral agarose gel electrophoresis of DNA extracted from treated cells. Fig. 2 shows that treatment with 50 μM C2-ceramide produced a typical ladder pattern of oligonucleosomal fragments, similar to that induced by exposure to a radiation does of 500 cGy. Apoptotic DNA fragmentation was also observed after treatment of the cells with 1.0 U/ml *Staphylococcus aureus* sphingomyelinase (data not shown).

To identify the subpopulation of endothelial cells undergoing apoptosis, flow cytometry was performed in cells stained for apoptotic DNA breaks by the in situ terminal transferase nick translation assay. This method is based on nick-end translation of 3′-OH DNA termini by the enzyme TDT and biotin-conjugated dUTP, and subsequent binding of fluorescinated avidin to the incorporated biotin (30, 39, 40). Concomitant staining with propidium iodide makes it possible to correlate the presence of fluorescing DNA breaks in individual cells with their DNA ploidy and position in the cell cycle.

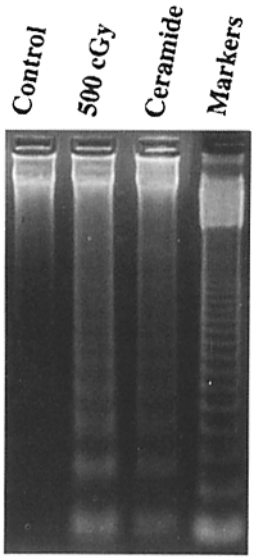

Figure 2. Agarose gel electrophoresis of DNA from cells treated for 24 h with 50 μM C2-ceramide, or exposed to a single radiation dose of 500 cGy. After 24 h, the cells were lysed, and the DNA was extracted and submitted to agarose gel electrophoresis as previously described (1). Similar results were observed in three consecutive experiments.

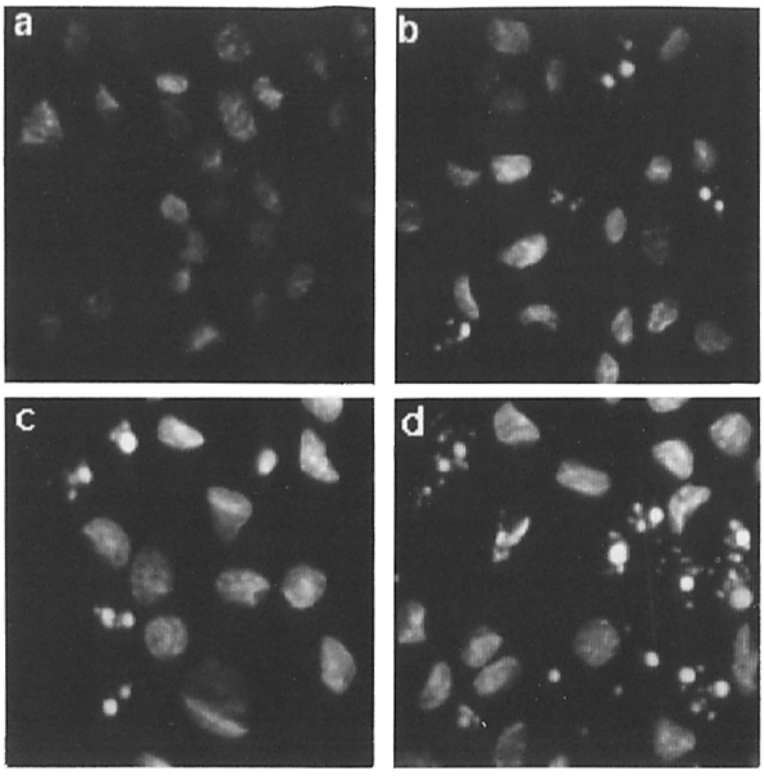

Figure 1. Morphological changes after treatment of plateau phase BAEC with C2-ceramide, sphingomyelinase, or ionizing radiation. Untreated cells (*a*), or cells incubated with 50 μM C2-ceramide (*b*), 1.0 U/ml bacterial sphingomyelinase (*c*), or exposed to a single radiation dose of 500 cGy (*d*), were fixed after 24 h in culture with paraformaldehyde and stained with the DNA-specific fluorochrome Hoechst-33258 (5). Note the typical morphological features of apoptosis, including nucleoplasmic condensation, chromatin segmentation, and apoptotic bodies in *b–d*.

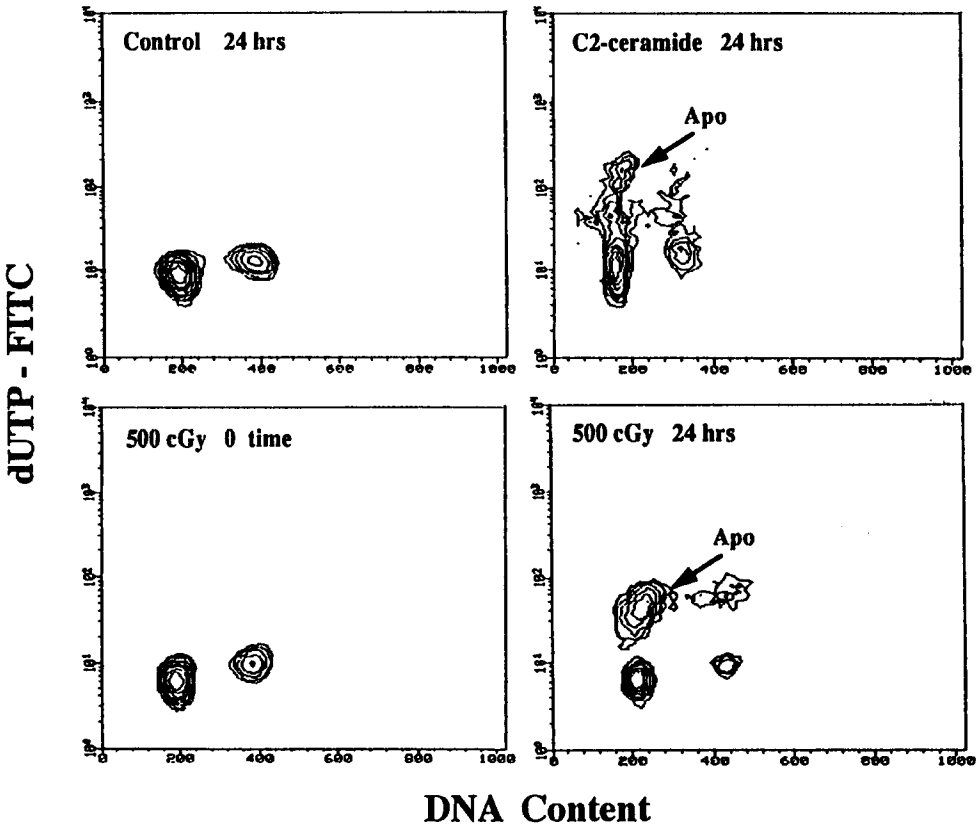

Figure 3. Detection of ceramide- and radiation-induced apoptotic DNA breaks in plateau phase BAEC by the in situ terminal transferase nick translation assay. Plateau phase BAEC were incubated with 50 μM C2-ceramide or exposed to a single radiation dose of 500 cGy. After 24 h, flow cytometry was performed using concomitant terminal transferase staining of apoptotic DNA breaks and propidium iodide staining of total cellular DNA content, as previously described (40). Contour plots are shown of the frequency of cells exhibiting fluorescence emitted from the dUTP-FITC (dUTP-biotin-avidin-fluorscein isothiocyanate) complexes versus the cellular DNA. Note that the majority of the fluorescing apoptotic cells (*Apo*) have DNA ploidy of G_0-G_1 cells. Similar results were observed in five consecutive experiments.

Fig. 3 shows that control cells incubated for 24 h under the same culture conditions used for the experimental groups had DNA ploidy of G_0-G_1 phase in 82% of the cells. Treatment with 50 μM C2-ceramide for 24 h resulted in the appearance of cells exhibiting intensive fluorescence, indicating the development of apoptotic DNA fragmentation in these cells (30, 40). DNA fragmentation was observed mostly in G_0-G_1 cells, comprising 23% of these cells. BAEC treated in parallel with a radiation dose of 500 cGy showed similar apoptotic changes in 49% of the G_0-G_1 cells. Treatment with both radiation (500 cGy) and 50 μM of C2-ceramide did not result in an additive response, with an overall 52% of the G_0-G_1 cells exhibiting apoptotic fluorescence (data not shown). These data demonstrate that ceramide mimics radiation to activate apoptotic DNA degradation in BAEC, with both affecting predominantly G_0-G_1 phase cells.

Generation of Ceramide by Exposure to Ionizing Irradiation. To determine whether exposure of BAEC to ionizing irradia-

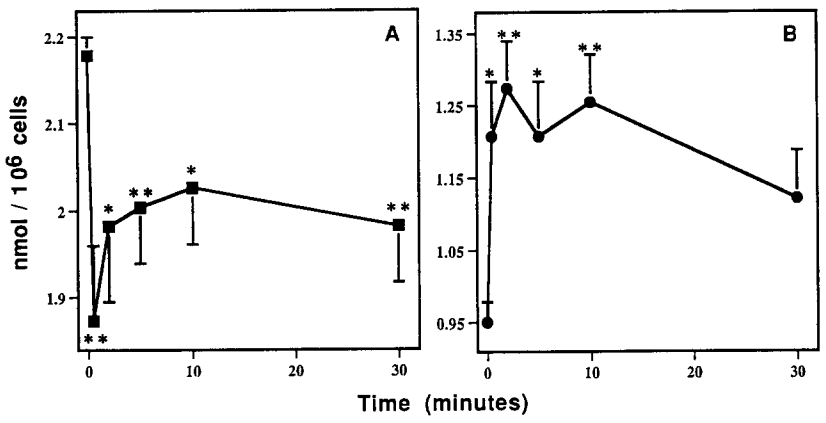

Figure 4. Effect of ionizing radiation on sphingomyelin and ceramide levels in plateau phase BAEC. (*A*) Sphingomyelin time course. For sphingomyelin measurements, cells were labeled to iostopic equilibrium with [^{3}H]choline (1 μCi/ml) for 7 d before an experiment, and on the day of an experiment were resuspended into PBS at 12 × 10^6 cells per ml. Cells (3.6 × 10^6) were irradiated with a single dose of 1,000 cGy on ice and then incubated at 37°C. At the indicated times, lipids were extracted in chloroform/methanol/hydrochloric acid (1 M) (100:100:1, vol/vol) and subjected to mild alkaline hydrolysis (1 h at 37°C in 0.1 N KOH in methanol) (24). Sphingomyelin was resolved by TLC using chloroform/methanol/glacial acetic acid/water (50:30:8:4, vol/vol) and visualized by iodine vapor staining. The use of [^{3}H]choline as a measure of sphingomyelin content was validated by simultaneous phospholipid phosphorus measurements. Each value represents the mean ± SEM of triplicate determinations from five experiments. (*B*) Ceramide time course. For ceramide measurements, unlabeled cells were handled as above and ceramide contained in the organic phase extract was quantified by the diacylglycerol kinase assay as described (15). Each value represents the mean ± SEM of triplicate determinations from five experiments. (*) $p < 0.025$; (**) $p < 0.005$.

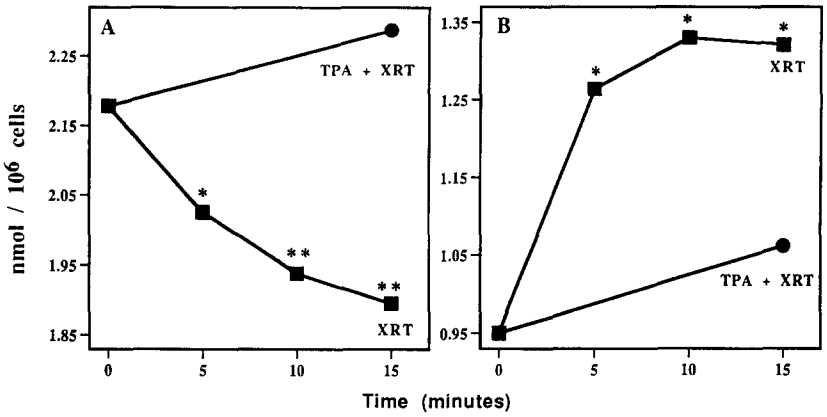

Figure 5. Phorbol esters inhibit sphingomyelin degradation to ceramide by ionizing radiation. Cells were cultured as described in Fig. 4. except that TPA (50 ng/ml) or the diluent DMSO was added for 5 min before the cells were processed. Sphingomyelin and ceramide levels were quantified as described in Fig. 4. (*A*) Sphingomyelin in irradiated (*XRT*) and TPA-treated and irradiated (*TPA + XRT*) cells. (*B*) Time course for ceramide. Values are derived from triplicate determinations from two experiments. The mean range of values for sphingomyelin and ceramide was 2 and 7%, respectively. (*) $p < 0.025$; (**) $p < 0.001$.

tion induced hydrolysis of membrane sphingomyelin to ceramide, experiments were performed to examine the effect of a dose of 1,000 cGy on sphingomyelin and ceramide levels in confluent monolayers of BAEC. Fig. 4 shows that the level of sphingomyelin decreased to 86% of control, from 2.2 to 1.9 pmol/10^6 cells ($p < 0.001$), within 30 s of radiation exposure and remained reduced for at least 30 min. The kinetics and magnitude of this effect were similar to those reported for TNF-α in human leukemia HL-60 and U937 cells (12, 13). Concomitantly, the ceramide level increased from 0.95 to 1.3 pmol/10^6 cells ($p < 0.025$) within 30 s of radiation exposure and remained elevated for 30 min. Studies to evaluate the relationship of the radiation dose to the generation of ceramide revealed an increase in ceramide to 115% of control with as low a dose as 50 cGy, with a maximal effect observed at 1,000 cGy, increasing the ceramide level to 155% of control ($n = 3$) (data not shown). The calculated ED_{50} for radiation-induced generation of ceramide was ~100–200 cGy. These results show that exposure of endothelial cells to ionizing radiation generates an elevation of ceramide levels to the same extent and with similar kinetics as previously observed with TNF in other cell systems (13, 24).

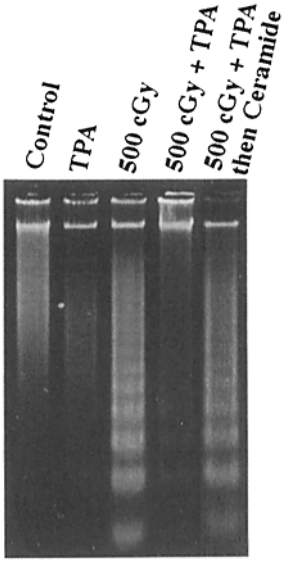

Figure 6. Restoration of apoptotic DNA fragmentation by C2-ceramide in endothelial cells treated with ionizing irradiation and TPA, detected by agarose gel electrophoresis. Plateau phase BAEC were incubated for 5 min with or without 50 ng/ml of TPA. The cells were then irradiated with a single dose of 500 cGy and incubated for an additional 30 min at 37°C. The cells were then washed and incubated for an additional 24 h at 37°C in DMEM with or without 50 μM C2-ceramide. The cells were subsequently lysed, and the DNA was extracted and submitted to agarose gel electrophoresis. Similar results were observed in three consecutive experiments.

The Critical Role of Ceramide Generation in Radiation-induced Apoptosis. To evaluate whether there is a cause-and-effect relationship between radiation-induced ceramide generation and the subsequent progression of the radiation-induced apoptotic cascade, the effect of TPA on ceramide generation was examined. Previous studies have reported that PKC activation by phorbol esters abolished programmed cell death in response to various agents that induce apoptosis (12, 13, 41–44). Similarly, phorbol ester stimulation of PKC was found to abolish radiation-induced apoptosis in BAEC and to protect the cells from clonogenic cell death (35). Fig. 5 shows that treatment of plateau phase BAEC with TPA (50 ng/ml) abolished radiation-induced sphingomyelin hydrolysis to ceramide. Radiation induced a progressive reduction in sphingomyelin levels, decreasing from 2.17 to 1.9 nmol/10^6 cells ($p < 0.001$) and a concomitant elevation in ceramide levels from 0.95 to 1.33 nmol/10^6 cells ($p < 0.025$). TPA treatment abolished these effects (for sphingomyelin, TPA plus radiation (XRT) versus radiation, $p < 0.001$; for ceramide $p < 0.025$). Similar results were obtained with a 5-min or 3-h pretreatment with TPA. Fig. 6 shows that TPA also inhibited apoptotic DNA degradation after exposure to a radiation dose of 500 cGy. Hence, activation of PKC appears to block both the generation of ceramide and apoptosis after radiation exposure. Whereas these experiments suggested a correlation between radiation induction of ceramide and apoptosis, they did not necessarily indicate a cause-and-effect relationship. Hypothetically, PKC activation could abolish apoptosis at another step. To rigorously prove that ceramide is the critical second messenger in the radiation-induced apoptotic cascade inhibited by TPA, experiments were performed to examine whether selective restoration of ceramide would overcome the inhibition. BAEC were treated for 30 min with 50 ng/ml TPA and irradiated with a dose of 500 cGy. Subsequent incubation with 50 μM C2-ceramide restored the apoptotic response, as demonstrated by the reappearance of the ladder pattern in agarose gel electrophoresis (Fig. 6), and by the terminal transferase nick translation assay (Fig. 7). These data provide compelling evidence that radiation-induced generation of ceramide is a critical and obligatory event in the radiation induction of the apoptotic cascade in endothelial cells.

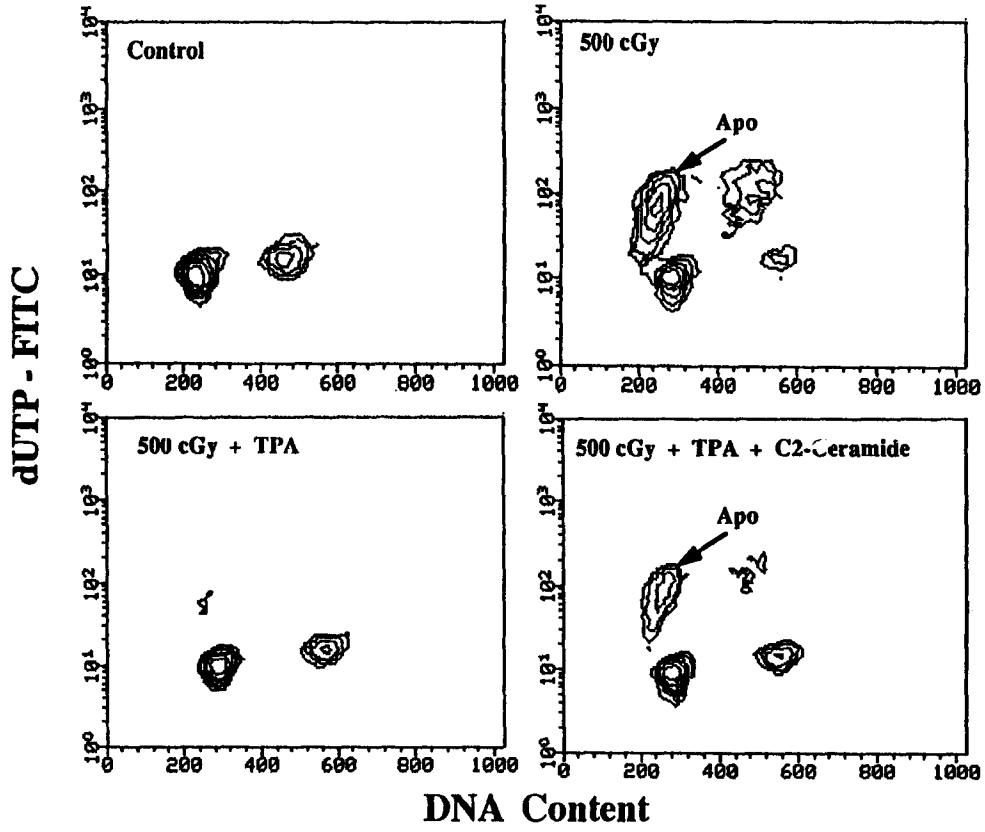

Figure 7. Restoration of apoptotic DNA fragmentation by C2-ceramide in endothelial cells treated with ionizing irradiation and TPA detected by flow cytometry. Plateau phase BAEC were incubated for 5 min with or without 50 ng/ml of TPA. The cells were then irradiated with a single dose of 500 cGy and incubated for an additional 30 min at 37°C. The cells were then washed and incubated for an additional 24 h at 37°C in DMEM with or without 50 μM C2-ceramide. Flow cytometry was then performed, using concomitant terminal transferase staining of apoptotic DNA breaks and propidium iodide staining of total cellular DNA content. Similar results were obtained in four experiments.

Radiation-induced Generation of Ceramide in Cell Membrane Preparations. Because TNF and IL-1β–induced generation of ceramide from sphingomyelin has previously been shown to be associated with the cell membrane (16, 24), we assumed that irradiation-induction of ceramide generation could also be associated with membrane signaling. However, a very rapid transfer of a nuclear-generated signal to the plasma membrane could not be excluded. To address this issue, fresh supernatants of nuclei-free membranes were prepared from BAEC and irradiated with a dose of 1,000 cGy at 4°C. The membranes were then incubated at 37°C in an assay buffer optimized for neutral sphingomyelinase activity. Under these conditions, the level of ceramide increased within 30 s from a basal level of 2.25 to 2.50 nmol/mg supernate protein (Fig. 8; $p < 0.001$). The maximal level of ceramide of 2.75 nmol/mg supernatant protein was achieved 4 min after irradiation ($p < 0.001$). The magnitude of this effect was similar to that observed after irradiation of intact BAEC (Fig. 4). A concomitant reduction in sphingomyelin levels was observed (data not shown). If, however, irradiated membranes were incubated at 4°C ($n = 4$), or at 37°C in a buffer that did not contain magnesium ($n = 3$) which is required for neutral sphingomye-

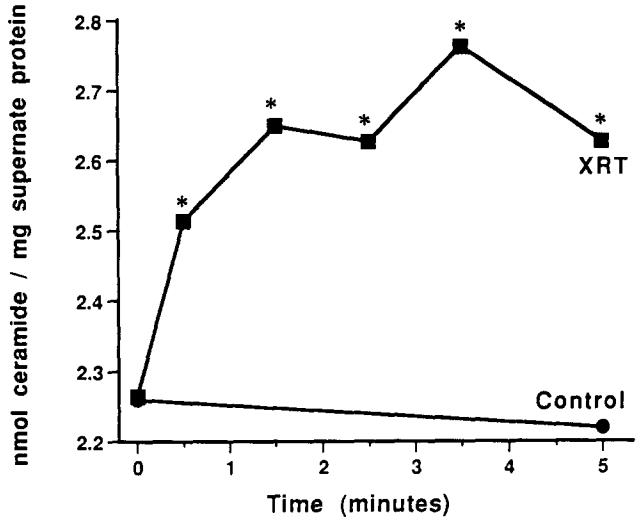

Figure 8. Effect of ionizing radiation (*XRT*) on the ceramide level of a nuclei-free membrane fraction. Detached cells were resuspended (45×10^6/ml) in homogenization buffer (50 mM NaF, 5 mM EGTA, and 25 mM Hepes, pH 7.4), disrupted at 4°C with 150 strokes of a tight-fitting Dounce homogenizer and centrifuged for 5 min at 500 *g*. The nuclei-free membrane fraction was irradiated at 4°C as described in Fig. 4. To measure the effects on ceramide levels, 150 μl of irradiated membrane (1.65 mg/ml) were incubated at 37°C in a reaction mixture containing 30 μl of 3 mM ATP, 30 μl of homogenization buffer, 90 μl deionized water, and 300 μl sphingomyelinase assay buffer (50 mM Hepes, pH 7.4, and 20 mM $MgCl_2$). The reaction was terminated and ceramide quantified as described in Fig. 4. The values are derived from triplicate determinations from two experiments. The mean range of ceramide values for control and irradiated membrane preparations was 4 and 2%, respectively. (*) $p < 0.001$.

linase activity, the ceramide elevation was not observed at any time up to 30 min after irradiation. Further, if membrane preparations were boiled at 100°C for 5 min, ceramide elevation after irradiation was not observed (n = 3). These studies suggest that the effect of ionizing irradiation to generate ceramide, like the effects of TNF and IL-1β (16, 24), is mediated via activation of neutral sphingomyelinase located at the cell membrane. Furthermore, this set of experiments indicates that a direct effect of ionizing radiation on the membrane is sufficient to produce the critical lipid ceramide that transduces the apoptotic signals without requiring the presence or involvement of the cell nucleus.

Discussion

The present studies demonstrate that ionizing radiation induces apoptotic signaling at the cell membrane of cultured BAEC. The proximal events in this pathway involve hydrolysis of sphingomyelin to ceramide by the action of a neutral sphingomyelinase. The generation of ceramide was maximal within seconds after irradiation, and was sensitive to low dose irradiation. This effect of radiation mimicked the effects of the cytokines TNF-α and IL1-β1 (12, 13, 16, 24). The notion that ceramide acts as a second messenger in the pathway of radiation-induced apoptosis is supported by the fact that the C2-ceramide analogue was capable of mimicking radiation as an inducer of the apoptotic response, as has previously been shown in TNF-induced apoptosis (12, 13). Additional support for this hypothesis is derived from the studies with phorbol esters. Phorbol ester has been shown to block apoptosis induced by TNF (12, 13), the chemotherapeutic agent ara-C (45) and by ionizing radiation (41, 46). In BAEC, phorbol esters similarly blocked radiation-induced apoptosis and abolished sphingomyelin hydrolysis to ceramide. However, when ceramide elevation was restored by addition of exogenous C2-ceramide, the phorbol ester effect to inhibit apoptosis was overcome. Hence, the current experiments suggest that ceramide is a critical and obligatory element of the apoptotic cascade when induced by radiation in endothelial cells.

Radiation-induced hydrolysis of sphingomyelin to ceramide occurred in cellular extracts devoid of nuclei and hence appears to be independent of direct radiation-induced DNA damage. These data serve as compelling evidence that radiation generates apoptotic signaling at the cell membrane. This concept represents an alternative to the prevailing paradigm on the mechanisms of the lethal effects of radiation, which identifies the nucleus as the primary and direct target for the lethal effects of ionizing irradiation (25, 26, 28, 47, 48). Apoptosis triggered by membrane signals may occur after exposure of mammalian cells to ionizing irradiation more frequently than has been appreciated in the past. This mechanism may prevail at the clinically relevant low dose range, in which unrepaired lethal damage to the DNA may be less prevalent than at the higher doses rarely used in clinical practice (30).

Whereas radiation is known to activate a variety of kinases, transcription factors and stress response genes (for a review see reference 49), there is at present limited information on signaling molecules downstream of the generation of ceramide that are in common with the radiation response. NF-κB does, however, represent one such common element. Schutze et al. (20) reported that exogenous sphingomyelinases and ceramide induced nuclear translocation of NF-κB using permeabilized preparations of Jurkat T cells. In intact HL-60 cells, sphingomyelinase and cell-permeable ceramide analogues mimicked the effect of TNF and increased nuclear NF-κB binding within 30 s (21). As for radiation effects, Brach et al. (50, 51) reported that nuclear binding of NF-κB occurs in KG-1 human myeloid leukemia cells and in human lung fibroblasts after exposure to x ray doses ranging between 200 and 2,000 cGy. NF-κB binding to DNA occurred in the presence of cycloheximide, indicating that ionizing radiation activated preexisting NF-κB protein. Similarly, as low radiation doses as 100 cGy were shown to activate NF-κB in B lymphocyte precursor cells (52), in which radiation also resulted in apoptosis and decreased clonogenic survival (53). This study also reported that both NF-κB activation and apoptosis were effectively prevented by the protein tyrosine kinase inhibitors genistein and herbimycin A. In this context, it should be noted that the sphingomyelin pathway has recently been shown to rapidly integrate the activity of classical serine-threonine and tyrosine kinase pathways (19).

Another event common to the activation of the sphingomyelin pathway by TNF and the radiation response is the generation of TNF itself. TNF has been shown to increase its own synthesis, initiating a mechanism of feed-forward stimulation (54, 55). Rivas et al. (56) have recently reported that treatment of J_{22}-HL-60 cells with bacterial sphingomyelinase similarly stimulated TNF production. Hallahan et al. (57) reported that conditioned media from irradiated human sarcoma cell lines were cytotoxic to other cells and that this cell killing was inhibited by neutralizing mAbs against TNF-α. Nuclear run-on and Northern blot analysis in HL-60 cells confirmed that the TNF-α gene was induced by x rays, and was regulated at the level of transcription (57). Whether sphingomyelinase and radiation induction of the TNF gene activation are mediated via the same signaling pathway remains to be established.

The mechanism by which radiation triggers sphingomyelin hydrolysis to ceramide is unknown, but there is increasing evidence that reactive oxygen intermediates play a major role in this signaling. Oxidative stress due to production of reactive oxygen intermediates occurs frequently in cells exposed to ionizing or UV irradiation, low concentrations of H_2O_2, stimulation of cells with cytokines and other natural ligands for cell surface receptors, or as result of the interaction with neutrophils during the inflammatory response (for a review see reference 58). The notion that reactive oxygen intermediates may mediate radiation-induced signaling is supported by recent reports that radiation-induced NF-κB activation was blocked by the antioxidant N-acetyl-L-cysteine (50, 52, 59). Whether reactive oxygen intermediates are involved in the mechanism of radiation-induced hydrolysis of sphingomyelin requires further investigations.

The inhibition of radiation-induced sphingomyelin hydrolysis to ceramide by phorbol esters and the associated inhibi-

tion of apoptotic DNA degradation in endothelial cells may have important implications for the understanding of mechanisms of radiation resistance in vitro and in vivo. Several hematopoietic cytokines were found to confer protection against radiation-induced apoptosis via PKC in normal and malignant hematopoietic cells (42–44). Thymic and lymphoid cells are similarly protected by PKC activation against radiation-induced apoptosis (46, 60, 61). Recent studies reported that PKC activation by bFGF or TPA protected endothelial cells against apoptotic DNA degration and radiation-induced cell kill in vitro (30, 35). Furthermore, intravenous bFGF rescued whole lung–irradiated mice from lethal radiation pneumonitis. The latter effect resulted from inhibition of apoptotic cell death induced selectively in the capillary endothelial cells of the lung by the radiation exposure (30). Hence, PKC activation may provide an antiapoptotic mechanism in vitro and in vivo and may constitute a generic mechanism of radiation resistance. Some cells activate PKC spontaneously after irradiation (52, 62, 63), whereas other cells, such as endothelial cells, lack this property and require cytokine or growth factor stimulation to elicit this response (35). In this context, it is of interest to note that recent studies have reported that radiation directly activated membrane PKC without apparent stimulation by cytokines or growth factors in human sarcoma, small cell lung carcinoma, and B lymphocyte precursor cells (52, 62, 63), and that PKC inhibitors sensitized the radiation killing of such cells (63, 64). This sensitization was not associated with direct radiation damage to the DNA or its repair (63, 64). Taken together, these observations provide a basis for a hypothesis on the existence of a balance between radiation induction of programmed cell death via the sphingomyelin pathway and its downregulation by natural suppressor mechanisms through PKC. According to this model, spontaneous radiation activation of membrane PKC or its activation by growth factors and cytokines may play an important role in the homeostatic control of radiation resistance in many types of cells. The evolution of radiation injury in normal and tumor tissues is thus placed within a cellular and molecular context that provides a background for testable experimental hypotheses with relevance to clinical radiotherapy. As mechanisms of radiation-induced apoptosis in normal and tumor tissues are unraveled, it may become possible to actively intervene in specific pathways to favorably alter the therapeutic ratio in the radiation treatments of some human cancers.

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