DNA double-stranded breaks (dsb) activate surveillance systems that identify DNA damage and either initiate repair or signal cell death. Failure of cells to undergo appropriate death in response to DNA damage leads to misrepair, mutations, and neoplastic transformation. Pathways linking DNA dsb to reproductive or apoptotic death are virtually unknown. Here we report that metabolic incorporation of \(^{125}\text{I}\)-labeled 5-iodo-2'-deoxyuridine, which produces DNA dsb, signaled de novo ceramide synthesis by post-translational activation of ceramide synthase (CS) and apoptosis. CS activation was obligatory, since fumonisin B1, a fungal pathogen that acts as a specific CS inhibitor, abrogated DNA damage-induced death. X-irradiation yielded similar results. Furthermore, inhibition of apoptosis using the peptide caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp fluoromethylketone did not affect CS activation, indicating this event is not a consequence of induction of apoptosis. ATM, the gene mutated in ataxia telangiectasia, is a member of the phosphatidylinositol 3-kinase family that constitutes the DNA damage surveillance/repair system. Epstein-Barr virus-immortalized B cell lines from six ataxia telangiectasia patients with different mutations exhibited radiation-induced CS activation, ceramide generation, and apoptosis, whereas three lines from normal patients failed to manifest these responses. Stable transfection of wild type ATM cDNA reversed these events, whereas antisense inactivation of ataxia telangiectasia-mutated gene product in normal B cells conferred the ataxia telangiectasia phenotype. We propose that one of the functions of ataxia telangiectasia-mutated gene product is to constrain activation of CS, thereby regulating DNA damage-induced apoptosis.

The predominant form of death induced in mammalian cells by ionizing radiation is reproductive (also known as clonogenic) cell death. The target for radiation is the DNA, and double-stranded breaks (dsb)\(^1\) are regarded as the specific lesions that initiate this lethal pathway (1, 2). Although most radiation-induced DNA dsb are rapidly repaired by constitutively expressed repair mechanisms, residual unrepaird or misrepaired breaks lead to genetic instability, increased frequency of mutations, and chromosomal aberrations (1–3). Lethal mutations or dysfunctional chromosomal aberrations eventually lead to progeny cell death (4, 5), usually after several mitotic cycles (3, 6).

Although this mechanism has been extensively investigated, the signaling pathways involved are only partially known. Once generated, DNA damage activates a coordinate network of signal transduction pathways that detect DNA breaks, arrest the cells temporarily at G\(_1\), S, and G\(_2\) checkpoints, and activate DNA repair (7–15). This signaling network is regulated by a family of phosphatidylinositol 3-kinases (PI3-K) which in the human includes the ataxia telangiectasia-mutated gene product (ATM), the catalytic unit of DNA-dependent protein kinase (DNA-PK), the AT- and RAD3-related kinase (Atr), and Frap (FKBP12 and rapamycin-binding protein kinase) (8, 10, 11, 13–16). Repair of DNA damage is believed to be carried out, at least in part, by non-homologous recombination and ligase IV-mediated end joining (17).

Radiation-induced DNA dsb can also signal apoptosis, albeit significantly less frequently than reproductive cell death (18). Definitive evidence for the involvement of radiation-induced DNA dsb in apoptosis was provided by experiments utilizing metabolic incorporation of \(^{125}\text{I}\)-labeled 5-iodo-2'-deoxyuridine (\(^{125}\text{I}\)dURd). IdURd replaces thymidine in DNA without affecting cell viability, although it sensitizes mammalian cells to the lethal effects of radiation (4, 19). \(^{125}\text{I}\) decays by electron capture, emitting cascades of low energy Auger electrons that deposit radiation energy within less than 40 Å from the decay site (20, 21). When targeted to DNA via \(^{125}\text{I}\)IdURd, the radioactive iodine produces dsb within a range of 6 base pairs from the site of \(^{125}\text{I}\) incorporation (22, 23). \(^{125}\text{I}\)IdURd incorporation into the DNA was reported to induce apoptotic responses in murine lymphoid and myeloid cell lines (5, 24), whereas targeting of \(^{125}\text{I}\) to mitochondria (25), lysosomes (26), or the cytosol (27) was found to be non-toxic. The apoptotic response in cells treated with \(^{125}\text{I}\)IdURd was shown to correlate with the number of \(^{125}\text{I}\)-induced DNA dsb (5, 24).

Of the PI-3 kinases associated with DNA damage surveil-

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\(^1\) The abbreviations used are: dsb, double-stranded breaks; DURd, 5-iodo-2'-deoxyuridine; CS, ceramide synthase; PI3-K, phosphatidylinositol 3-kinases; BAEc, bovine aortic endothelial cell; SMase, sphingomyelinase; FB1, fumonisin B1; EBv, Epstein-Barr virus; ATM, ataxia telangiectasia-mutated gene product; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; Gy, gray; AT, ataxia telangiectasia; TPA, 12-O-tetradecanoylphorbol-13-acetate DAG, diacylglycerol; DNA-PK, DNA-dependent protein kinase; Z, benzyloxyxycarbonyl; AFC, 7-amino-4-trifluoromethylcoumarin, ASMase, acid sphingomyelinase.

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lance, only ATM has been found to be involved in the apoptotic response to radiation. Hypersensitivity to radiation-induced apoptosis was reported recently in ATM mutant fibroblast lines (28), ATM knock-out mouse intestinal and dermal epithelial cells (29), and in M059J human glioma cells.\(^2\) ATM mutations produce the genetic disorder ataxia telangiectasia (AT), a multi-system disease manifested by abnormalities of the immune, nervous, cutaneous, and endocrine systems (16, 30). At the cellular level the AT phenotype is characterized by chromosomal instability (31), increased frequency of malignant transformation (16), defective G1, S, and G2/M cell cycle checkpoints (32, 33), and hypersensitivity to ionizing radiation (34, 35). The hypersensitivity of some AT cells to radiation-induced apoptosis suggests that concomitant with its role in the regulation of DNA repair and recovery of the cell from potentially lethal DNA damage, ATM may function as inhibitor of an apoptotic pathway activated by DNA damage.

The best known apoptotic response to radiation-induced DNA damage is p53-mediated (32, 36, 37). In murine thymic cells, p53-mediated apoptosis was not affected by ATM (29, 38), although in spermatagonia undergoing meiosis ATM suppressed p53-dependent apoptosis (39). Furthermore, the ATM/DNA-PK doubly mutated M059J glioma cell line was shown to respond to radiation with apoptosis, whereas its sister line M059K, isolated from a different region of the same tumor, was shown to have intact ATM/DNA-PK and to be apoptosis-resistant.\(^2\) Both cell lines are p53-mutated (40). These studies indicate that in some cells p53 and ATM may function coordinately, whereas in others they may regulate apoptosis independently.

Similar to the lack of effect on thymic apoptosis, ATM does not appear to regulate sphingomyelinase (SMase)-mediated apoptosis, known to be activated at the plasma membrane rather than the nucleus. Haimovitz-Friedman et al. (41) reported that radiation induced in nuclei-free membrane preparations of endothelial cells a rapid hydrolysis of the phospholipid sphingomyelin to generate ceramide via activation of SMase. The same response was observed in intact cells, and the ceramide generated served as a second messenger in initiating apoptotic signaling. Definitive evidence for the role of SMase in initiating radiation-induced apoptosis was provided by the use of genetic models. ASMase knock-out mice failed to generate ceramide and to develop typical apoptotic lesions in the pulmonary endothelium after whole body irradiation, although the apoptotic response in the thymus was preserved (42). The converse pattern was observed in whole body irradiated p53 knock-out mice, which failed to exhibit thymic apoptosis but showed a normal apoptotic response in the lung. These data indicated that ASMase-mediated apoptosis, expressed in pulmonary endothelial cells, is distinct from the p53-dependent pathway that operates in thymocytes. Barlow et al. (38) reported that both thymic and pulmonary endothelial apoptosis remained unaltered in whole body irradiated ATM knock-out mice, indicating that ATM may regulate yet another, currently undefined, apoptotic pathway.

Whereas ASMase activation represents a general mechanism for pro-apoptotic generation of ceramide, there is an alternative mechanism that involves de novo synthesis of ceramide and apoptosis via activation of the enzyme ceramide synthase (CS). De novo synthesis of ceramide occurs in the endoplasmic reticulum and mitochondria (43, 44) via CS-mediated condensation of the sphingoid base sphinganine and fatty acyl-CoA to form dihydroceramide, which is rapidly oxidized to ceramide (45). Bose et al. (46) have recently reported that daunorubicin activated CS and induced ceramide generation and apoptosis in P388 and U937 cells. Furthermore, the fungal toxin fumonisin B1 (FB1), a natural specific inhibitor of CS (47), blocked daunorubicin-induced ceramide generation and apoptosis in both cell types. FB1 was also reported to abrogate daunorubicin-induced apoptosis in hen granulosa cells (48) and CPT-11-induced apoptosis in L929 cells (49). Whether CS activation induced by daunorubicin and CPT-11 is associated with DNA damage remains unknown. Since FB1 did not block tumor necrosis factor-induced ceramide generation nor tumor necrosis factor-induced apoptosis, both of which involve SMase activation in U937 cells (46), it appears that CS-mediated apoptosis is distinct and independent of the SMase-mediated mechanism.

In the present study, we investigated whether DNA damage and ATM regulate the pro-apoptotic function of CS. The data indicate that radiation-induced DNA damage signals post-translational activation of CS, ceramide generation, and apoptosis, whereas ATM down-modulates this response. CS activation appeared obligatory for DNA damage-induced death since FB1 blocked \(^{125}\text{I}\)dURd-induced ceramide elevation and apoptosis. Irradiated AT cells exhibited increased CS activation and apoptosis, reversed by transfection with wild type ATM, whereas antisense inactivation of ATM in normal cells recapitulated the AT phenotype. Thus, signals from DNA damage and ATM appear to reciprocally regulate the activity of CS and consequently apoptosis after radiation exposure.

**MATERIALS AND METHODS**

**Cell Cultures, Mice, and Irradiation**—Cloned populations of BAEC were established from the intima of bovine aorta and grown as described previously in DMEM supplemented with 10% calf serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml) at 37 °C in a 10% CO\(_2\) atmosphere (50). During the phase of exponential growth, purified human recombinant basic fibroblast growth factor (1 ng/ml) was added every day. After 5–7 days in culture, the cells reached confluence and exhibited features of contact-inhibited monolayers. The culture medium was then changed to fresh DMEM supplemented with 5% heat-inactivated calf serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml). These plateau phase cells were maintained in culture medium for another 3–4 days before experiments.

AT lines are EBV-transformed lymphoblastoid cells derived from the peripheral blood of adult AT patients and have been previously characterized, whereas control lymphoblastoid lines are derived from normal donors (35). Cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) at 37 °C in a 5% CO\(_2\) atmosphere. Stably transfected cell lines containing ATM or antisense ATM cDNA expression vectors were generated by Lipofectin (Life Technologies, Inc.) transfection of pMAT1 and pMAT2 vectors, respectively (51, 52), and grown in medium containing 0.2 \(\mu\)g/ml hygromycin (Roche Molecular Biochemicals). For experiments, expression of ATM or antisense ATM cDNA constructs in C3ABR and AT1ABR cells was achieved by treating cells with 3–5 \(\mu\)M CdCl\(_2\) (Sigma) for 8–16 h and was evaluated by Western blotting using ATM-4BA antibody as described (51). Cell numbers were determined using a Coulter counter, model ZM (Coulter Electronics), and cell viability was assessed by trypan blue exclusion.

LNCaP, MS1418, GM0988, and A431 cells were grown as described previously (42, 53). Irradiation of cultured cells was carried out at 25 °C in a Gamma-cell 40 chamber containing two sources of \(^{60}\)Co (Atomic Energy of Canada) at a dose rate of 100 cGy/min. One hour prior to irradiation, the culture medium was changed to RPMI 1640 medium containing 0.1% human serum albumin (Alpine Biologics). For irradiation of mice, 4–6-week-old male C3H/HeJ or homozygous acid sphingomyelinase knock-out mice (54) received whole body irradiation, delivered using a Cs-137 Irradiator (Shepherd Arch-L, model 68, SN6453) at a dose rate of 270 cGy/min, as previously reported (42). Our animal housing facility is approved by the American Association for Accreditation of Laboratory Animal Care and is maintained in accordance with the regulations and standards of the United States Department of Agriculture and the Department of Health and Human Services, National Institutes of Health.

**Treatment of BAEC with \(^{125}\text{I}\)dURd**—Incorporation of \(^{125}\text{I}\)dURd was carried out using pre-confluent BAEC, pulsed with \(^{125}\text{I}\)dURd
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(0.25 nm IdUrd; 0.5 μCi [125I]IdUrd) for 16 h at 37 °C. The cells were then washed twice with Hank’s buffered saline solution and incubated in fresh DMEM supplemented with 10% calf serum. After 3 h, cells were detached from the monolayer by 2 min incubation with 0.05% trypsin, 0.02% EDTA at 25 °C, pelleted, and resuspended in the freezing medium (5% calf serum, 10% Me2SO, 65% DMEM). The cell pellet was frozen at −80 °C overnight and subsequently stored at −180 °C in liquid nitrogen for 1–30 days. Prior to experiments, cells were thawed by incubation in a 38 °C water bath and subsequently plated in culture media as described above. Endothelial cells appear resistant to the freeze and thaw procedure employed, since after thawing >95% of the cells were recovered, and the viability according to the trypan blue exclusion test was >98%.

Quantification of Apoptosis—Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bisbenzimide trihydrochloride ( Hoechst 33258; Sigma) as described previously (46). A minimum of 500 cells was scored for the incidence of apoptosis for each data point.

Caspase activity was measured using the fluorogenic caspase substrate Z-DEVD- AFC (55). For these studies, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) and nuclei and cell debris pelleted by centrifugation at 11,000 × g. Reactions were performed using 20 μg of cell extract in 0.2 ml of reaction buffer containing 8 μM Z-DEVD-AFC according to the manufacturer’s instructions (Kamiya Biomedical Company, Seattle, WA).

Lipid Studies—At the indicated times after irradiation, monolayers of BAEC or A431 cells were washed once with cold phosphate-buffered saline (PBS), and lipids were extracted with two incubations of methanol for 10 min at 4 °C, followed by an equal volume of chloroform and 0.6 volume of buffered saline solution/EDTA (135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, 10 mM HEPES, pH 7.2, 10 mM EDTA). For cells in suspension culture, cells were pelleted at 800 × g for 5 min, washed twice with cold PBS, resuspended in 0.6 ml of cold PBS, and extracted with 1 ml of chloroform/methanol, 1 N HCl (100:100:1, v/v/v), as described (46).

Lipids in the organic (lower) phase were dried under N2 and subjected to mild alkaline hydrolysis (1 N NaOH) for 1 h at 37 °C) to remove glycerophospholipids. Ceramide was quantified by the diacylglycerol kinase (DAG) assay as described previously (46). Sphingomyelin content was measured using cells labeled to isotopic equilibrium with [3H]choline and verified by phospholipid phosphorus as described (42).

Evaluation de Novo Synthesis of Ceramide in Intact Cells—[3H]IdUrd-labeled BAEC were thawed, plated in culture medium, and incubated at 37 °C for 12 h as described above. Cells were then placed on ice followed by the addition of [9,10-3H]palmitic acid (60 Ci/mmol; American Radiolabeled Chemicals Inc.) and incubation at 37 °C to start incorporation. At the indicated times, lipids were extracted and subjected to mild alkaline hydrolysis as described above. [3H]-Labeled ceramide was resolved by thin layer chromatography on silica gel 60 plates. Dihydroceramide was resolved using a solvent system of chloroform/methanol, 3.5N ammonium hydroxide (85:15:1), detected by comigration with ceramide standards, and quantified by liquid scintillation counting (46). Nonspecific incorporation, which represented less than 10% of the total incorporation, was determined at 4 °C and subtracted from each data point.

Ceramide Synthase Assay—Microsomal membranes were prepared as described previously (46). Briefly, cells were washed with cold PBS, scraped off plates, pelleted, and resuspended in 300 μl of homogenization buffer (25 mM HEPES, pH 7.4, 5 mM EGTA, 50 mM NaF, and 10 μg/ml each of leupeptin and soybean trypsin inhibitor). Cells were disrupted using a motor-driven 7-ml Tenbroeck tissue homogenizer. The postnuclear supernatant was centrifuged at 250,000 × g for 35 min. The microsomal membrane pellet was resuspended in 1.0 ml of homogenization buffer. Membranes were prepared fresh daily.

Assays of ceramide synthase activity were performed as described previously (46). Briefly, microsomal membrane protein (75 μg) was incubated with 2 mM MgCl2, 50 mM HEPES, pH 7.4, 20 mM fatty acid-free bovine serum albumin (Sigma), escalating concentrations (0–20 μM) of sphinganine (Biomol), 70 μM unlabeled palmitoyl-coenzyme A (Sigma), 1.5 μM [1-14C]palmitoyl-coenzyme A (55 mCi/mmol) (Amersham Pharmacia Biotech). Sphinganine was dried under N2 from a stock solution in 100% methanol and dissolved by sonication in reaction mixture prior to addition of microsomal membranes. The reaction mixture (1 ml) was incubated at 37 °C for 1 h and then stopped by extraction of lipids using 2 ml of chloroform/methanol (1:2). The lower phase (500 μl) was re-moved, dried under N2, and applied to a silica gel 60 thin layer chromatography plate. Dihydroceramide was resolved using a solvent system of chloroform/methanol, 3.5 N ammonium hydroxide (85:15:1), detected by comigration with ceramide standards, and quantified by liquid scintillation counting. Under the conditions used, the substrate was not rate-limiting, and the reaction was linear for enzyme concentration and time.

Assays for Neutral and Acidic Sphingomyelinase—BAEC were irradiated on ice and subsequently incubated at 37 °C for various periods. The cells were then harvested by scraping, washed twice with PBS at 4 °C, and pelleted. Neutral and acidic sphingomyelinase activities were measured in total cell and lysates as described (42).

Statistical Analysis—Statistical analysis was performed by Student’s t test and t test for correlation coefficient. Linear regression analysis was by the method of least squares.

RESULTS

DNA Damage Signals Ceramide Elevation and Apoptosis in Endothelial Cells—Direct DNA damage was produced in bovine aortic endothelial cell (BAEC) by metabolic incorporation of [125I]dUrd. Since the half-life time for decay of [125I] was 60.14 days, the accumulation of [125I] decays and DNA dsb is slow. In contrast, the repair of DNA dsb and transit through the cell cycle is rapid, attenuating the effectiveness of [125I]dUrd treatment. It is, therefore, necessary to slow down cellular metabolism for extended periods to permit sufficient generation of DNA dsb to affect survival (23, 56). Prior studies showed that freezing over liquid nitrogen optimized the formation of biologically relevant DNA dsb in [125I]dUrd-treated cells. In mouse L cells and Chinese hamster V79 cells, Radford and Hodgson (23) demonstrated a linear relationship between [125I] decays per cell, DNA dsb, and the lethal lesions per cell using this procedure. Story et al. (24) showed that DNA dsb, but not single-stranded DNA breaks, signaled apoptosis in [125I]dUrd-treated LY-TH mouse lymphoma cells. Freezing and thawing per se did not affect the level of DNA dsb and, if anything, attenuated the lethal effects of radiation, perhaps by reducing the indirect effect of radiation via free radicals, such as reactive oxygen species (23).

For the present studies, pre-confluent BAEC were pulsed for 16 h (approximately 1.5 doubling times) with [125I]dUrd (0.25 nm IdUrd; 0.5 μCi of [125I]IdUrd). Excess [125I]dUrd not incorporated into the DNA was chased by multiple washings with 10% calf serum in DMEM and a 3-h incubation in the same medium. The cells were resuspended in 10% calf serum in DMEM and stored at −180 °C under liquid nitrogen for 1–30 days. The cells were then thawed, washed, and plated in Petri dishes in 10% calf serum in DMEM and incubated at 37 °C for 24 h. BAEC appear resistant to freeze and thaw as more than 95% of control cells (either untreated or those treated with non-radioactive IdUrd) were recovered after thawing, and their viability by trypan blue exclusion was >98%. Control cells cultured after thawing produced monolayers that appeared normal by morphology and growth kinetics (data not shown).

Fig. 1A shows that treatment of BAEC with [125I]dUrd for various periods resulted in an apoptotic response 24 h after thawing, whereas apoptosis was not detected in control IdUrd-untreated cells, or in cells treated with non-radioactive IdUrd (data not shown). In [125I]dUrd-treated cells, apoptosis became apparent at approximately 12 h after thawing and reached a maximal level after 18–24 h (data not shown). The extent of apoptosis was proportional to the duration of [125I]dUrd treatment. Low levels of apoptosis were detected as early as after 5 days of [125I]dUrd exposure, peaking at approximately 30% after 12 days.

Fig. 1B shows that [125I]dUrd-treated cells also exhibited an increase in cellular ceramide. The freeze-thaw procedures used in these experiments did not affect the base-line levels of ceramide. In control and [125I]dUrd-treated cells incubated at...
−180 °C for 15 days, the concentration of ceramide immediately after thawing was 225 ± 0.2 pmol/106 cells, a value similar to that reported in BAEC under standard growth conditions (41). Small elevations of ceramide were noted in [125I]dURd-treated cells as early as 4–5 h after thawing, peaking at 16 h (data not shown). The peak level of ceramide was proportional to the duration of [125I]dURd treatment. At 16 h after thawing, there was a 6.5-fold increase in the ceramide level over control in cells treated with [125I]dURd for 15 days, and for cells treated for 24 days, the fold increase was 7.7.

**DNA Damage Induces Apoptosis via Activation of Ceramide Synthase**—In previous studies in BAEC, we demonstrated that radiation induced ceramide generation via activation of plasma membrane SMase (41). To test whether SMase was also activated in response to DNA damage, BAEC were treated with [125I]dURd for 17 days and tested for neutral sphingomyelinase and ASMase activity at time points ranging from 10 min to 24 h after thawing. Control cells displayed detectable neutral sphingomyelinase and ASMase activities with maximal velocities of 302 ± 63 and 4051 ± 631 pmol/min/mg protein, respectively. There was no increase in either SMase activity in untreated or irradiated BAEC within the first 24 h after thawing (n = 3).

An alternative pathway for generation of ceramide is by de novo synthesis via CS activation (46). Neo-synthesis was assessed by the rate of [9,10-3H]palmitic acid incorporation into ceramide. Fig. 2A shows that BAEC treated with [125I]dURd for 17 days and then labeled with [9,10-3H]palmitic acid at 12 h after thawing manifested enhanced incorporation of radiolabeled palmitic acid into ceramide over controls (p < 0.05). In contrast, incorporation of [9,10-3H]palmitic acid into other cellular lipids was not enhanced (data not shown). To assess the activity of CS, microsomal membranes were prepared from the [125I]dURd-treated cells at 12 h after thawing, and CS activity was assayed under conditions determined as linear for time and enzyme concentration and where substrate was not rate-limiting (data not shown). Fig. 2B shows the kinetics of CS activity using increasing concentrations of sphinganine. [125I]dURd enhanced the reaction velocity (p < 0.05 versus control, n = 6). Eadie-Hofstee transformation of the data revealed that CS from control cells manifested a maximal velocity of 102 pmol/min/mg protein and a [Km] of 1.01 μM. In [125I]dURd-treated cells, an increase of 91% in the Vmax was observed with no significant change in [Km] (Fig. 2C). A small increase in CS activity was detectable in [125I]dURd-treated cells as early as 4 h after thawing, and a maximal effect was observed at 12 h (data not shown). To test whether activation of CS by [125I]dURd was pre- or post-translational, the effect of the protein synthesis inhibitor cycloheximide (1 μg/ml) was tested. This concentration of cycloheximide inhibited in BAEC >90% of protein synthesis, evaluated by [35S]methionine incorporation into total cellular protein (data not shown). Fig. 3, A and B, shows that cycloheximide did not significantly affect the pattern of CS activation by [125I]dURd. There was a 94% increase in the Vmax of CS from cycloheximide-untreated cells, compared with an 89% increase in CS from cells treated with cycloheximide (n = 3).

To assess further the role of CS in [125I]dURd-induced apoptosis, experiments were performed using fumonisin B1 (FB1), a natural product of the fungus Fusarium moniliforme which acts as a specific inhibitor of CS (47). Treatment of BAEC with 1–25 μM FB1 for as long as 24 h did not affect cell proliferation or viability nor did it affect basal level of cellular ceramide (data not shown). Concentrations of 50 μM or higher were cytotoxic to BAEC, causing rapid cell lysis (data not shown). This toxicity is well defined, occurring in cells with high basal metabolism of sphingolipids (57, 58). Toxicity results from accumulation of the sphingoid bases sphinganine and sphingosine, the precursors for ceramide synthesis, which are cationic sphingolipids with detergent-like properties. Fig. 3C shows that 10 μM FB1 and 25 μM FB1, a maximally tolerated dose, significantly inhibited [125I]dURd-induced elevation of ceramide (p < 0.05 as compared with control) and apoptosis (p < 0.05) (Fig. 3D). These data provide proof-in-principle of a role for CS
Ceramide Synthase Signals DNA Damage-induced Apoptosis

**Fig. 3.** Effects of cycloheximide or FB1 on $^{125}$IIdURd-induced CS activation, ceramide elevation, and apoptosis. A and B, effect of cycloheximide on $^{125}$IIdURd-induced CS activation. BAEC were treated with $^{125}$IIdURd for 17 days as described under Materials and Methods. At thawed, plated on Petri dishes, and incubated at 37°C for 2 h to allow attachment of the cells to the culture dish. There was no increase in base-line ceramide synthase activity during this incubation period (data not shown). Distilled water (diluent) (A) or 1 μg/ml (final concentration) of cycloheximide (B) was then added. After 12 h at 37°C, microsomal membranes were prepared and subjected to the CS assay as described in Fig. 2B. Similar data were observed when cycloheximide was added immediately upon thawing (data not shown). Values represent mean ± S.E. of determinations from three separate experiments. C shows the effect of FB1 on $^{125}$IIdURd-induced ceramide generation. $^{125}$IIdURd-treated BAEC were prepared as described above. FB1 (10–55 μM) was added to the cells immediately after plating. After 16 h, cellular lipids were extracted and subjected to the DAG kinase assay to quantify the ceramide level as described in Fig. 1B. Each value represents the mean ± S.E. of triplicate determinations from one of five separate experiments. D shows the effect of FB1 on apoptosis. Apoptosis was determined as in Fig. 1A at 24 h after thawing. Each value represents the mean ± S.E. of duplicate determinations from one of five separate studies.

**Fig. 4.** Measurement of CS activity in response to external x-irradiation of BAEC. A, dose response. BAEC were irradiated with the indicated doses of ionizing radiation. 12 h after irradiation, microsomal membranes were prepared and subjected to the CS assay using 10 μM sphinganine as substrate, as in Fig. 2B. Each value represents the mean ± S.E. of triplicate determinations from three separate experiments. B, time course. BAEC were exposed to 10 Gy of external x-irradiation. At the indicated times after irradiation, microsomal membranes were prepared and assayed for CS activity as described in Fig. 2B. Similar results were obtained in five separate experiments. C, determination of Km and Vmax from B at 12 h utilizing Eadie-Hofstee analysis.

**Fig. 5.** The elevations of cellular ceramide were followed by apoptotic responses. Fig. 5B shows the evolution of apoptosis as a function of time after exposure to a dose of 5 Gy. Apoptosis was initially detected at 4 h and increased at a constant rate reaching a plateau of 40% at about 16 h after irradiation. These data were corroborated by measuring caspase activity using the fluorimetric caspase substrate Z-DEVD-AFC. Irradiation with 2.5, 5, and 10 Gy resulted in $p < 0.025$ versus control (inset in Fig. 5A). Ceramide subsequently decreased, returning to the base-line level at approximately 120 min after radiation (data not shown). This delay wave of ceramide generation, which occurred well before CS activation became detectable (Fig. 4B), was not affected by treatment with 25 μM FB1 (data not shown). Further follow-up of ceramide levels revealed that at 4 h after irradiation a second wave of ceramide elevation became apparent, which reached a maximal 4.2-fold increase in cellular ceramide levels ($p < 0.025$ versus control) (inset in Fig. 5A). This late phase of ceramide generation coincided with the pattern of CS activation shown in Fig. 4B. Consistent with this notion, treatment with 25 μM FB1 attenuated the late phase of ceramide generation, shutting it off after 12 h (Fig. 5A).

in the mechanism of the apoptotic response induced by DNA damage.

**X-irradiation also Induces Apoptosis via Activation of Ceramide Synthase**—Since external beam x-irradiation, like $^{125}$IIdUrd, damages DNA and produces DNA dsb (2, 23), we tested whether external radiation also signals CS activation. Confluent cultures of BAEC were exposed to increasing doses of external beam x-irradiation, and 12 h later microsomal membranes were prepared and assayed for CS activity. Fig. 4A shows that there was a dose-dependent increase in CS activity, peaking with a dose of 10 Gy. At this dose, minimal CS activation was observed at 3–4 h, and a maximal increase from a base-line of 103–158 pmol/min/mg protein ($p = 0.05$) occurred at 12 h without a significant change in the $K_m$ of the reaction (Fig. 4, B and C). There was also a dose-dependent increase in de novo synthesis of cellular ceramide, as detected by increased incorporation of $[3]$Hpalmitic acid into ceramide in BAEC exposed to 10 Gy (data not shown). Radiolabeling of other cellular lipids was, however, not increased (data not shown). Radiation-induced activation of CS was a post-transcriptional and post-translational event, since both actinomycin D (10 ng/ml) and cycloheximide (1 μg/ml) failed to inhibit the activation of the enzyme after exposure to 10 Gy (data not shown).

Consistent with this notion, treatment with 25 μM FB1 attenuated the late phase of ceramide generation, shutting it off after 12 h (Fig. 5A).

The elevations of cellular ceramide were followed by apoptotic responses. Fig. 5B shows the evolution of apoptosis as a function of time after exposure to a dose of 5 Gy. Apoptosis was initially detected at 4 h and increased at a constant rate reaching a plateau of 40% at about 16 h after irradiation. These data were corroborated by measuring caspase activity using the fluorimetric caspase substrate Z-DEVD-AFC. Irradiation with 2.5, 5, and 10 Gy resulted in $p = 0.025$, 20 ± 1, and 34 ± 1-fold increases in caspase activity over unirradiated controls at 16 h, which were maintained for as long as 24 h. This profile did not enable distinction between the apoptotic fraction ef-
The media of confluent BAEC were switched to fresh serum-free DMEM, and 25 μM FB1 was added to cells 4 h before irradiation. After incubation of cells at 37 °C for the indicated times (inset, short time course), ceramide levels were measured by DAG kinase method as in Fig. 1B. Each value represents the mean ± S.E. of triplicate determinations from five experiments. B, effect of FB1 on radiation-induced apoptosis. BAEC were exposed to ionizing radiation and treated with FB1 as in A and morphological changes of nuclear apoptosis quantified as in Fig. 1A. Each value represents the mean ± S.E. of duplicate determinations from seven independent experiments. C, survey of radiation-induced SMase and CS activation in different cell types. Cells were cultured and irradiated (10 Gy, except 20 Gy for lung endothelium in vivo) as described under “Materials and Methods.” Activation of SMase was defined by a reduction in sphingomyelin content coupled with quantitative elevation of ceramide. CS assays were performed as described under “Materials and Methods.” LNCap cells were treated with 10 ng/ml TPA for 45 min prior to irradiation.

The effect of SMase activation versus that mediated via CS. Treatment with 25 μM FB1 did not alter the apoptotic response up to 8 h after irradiation (Fig. 5B). Thereafter, apoptosis began to slow and like ceramide generation was shut off after 12 h (Fig. 5B). These data suggest the existence of a late phase apoptotic response mediated via CS activation, the beginning of which coincides with post-irradiation activation of CS (Fig. 4B).

Since these studies indicated that radiation may activate two distinct mechanisms for generation of ceramide, we investigated SMase and CS responses to ionizing radiation in other cell types, and we compiled this information with already published data from our laboratory. Evaluation of SMase activity was carried out at multiple time points between 30 s and 120 min after irradiation and of CS activity between 30 min and 24 h, as detailed under “Materials and Methods.” Fig. 5C shows that some cells activate CS selectively (AT1ABR and TPA-treated LNCap cells), whereas other cells activate only SMase (GM0988 human B cell and C3H/HeJ mice lung endothelium), neither enzyme (C3ABR, 1418 NPD, A431, and LN Cap cells), or both (BAEC). In instances where radiation stimulated ASMase selectively, such as in the wild type B cell line GM0988 or the murine lung endothelium (42), our present studies disclosed no activation of CS within the first 24 h of irradiation with doses (10–20 Gy) previously shown to produce maximal apoptotic responses in these cells (64). Furthermore, genetic inactivation of AS Mase, such as occurred in 1418 NPD cells or in the AS Mase knock-out mouse, abrogated apoptosis, whereas restoration of AS Mase activity by retroviral transduction restored radiation-induced ceramide generation and apoptosis (42). Alternatively, in cells where CS activation predominated (AT1ABR and TPA-treated LN Cap cells), increased SMase activity was not detected within 120 min of doses up to 20 Gy, whereas FB1 uniformly inhibited radiation-induced ceramide generation and apoptosis (Figs. 3D, 5B, and 6F). In C3ABR, 1418 NPD human B cells and A431 cells there was no evidence of SMase or CS activation, or apoptosis, with any dose up to 20 Gy. In human prostate LN Cap cells, radiation with doses up to 50 Gy failed to activate SMase or CS, generate ceramide, or induce apoptosis within 48 h of treatment. However, in cells pretreated with 10 ng/ml TPA, 10 Gy induced a 1.5-fold increase in CS activity from base line of 156 pmol/mg prot/min by 3 h, a 50–100% increase in ceramide levels from a base line of 650 pmol/10⁶ cells by 6 h, and induction of apoptosis beginning at 12 h. These studies indicate that signaling through CS and/or SMase is cell type-specific and suggest that these mechanisms, when activated, may play a crucial role in the induction of apoptosis in response to x-irradiation.

ATM Inhibits Radiation-induced Ceramide Synthase Activation and Apoptosis—As discussed in the Introduction, ATM is involved in several responses to radiation-induced DNA damage, one of which appears to be the inhibition of radiation-induced apoptosis. Since the CS-mediated mechanism of radiation-induced apoptosis constitutes a response to DNA damage, we investigated whether ATM might regulate this process. EBV-transformed peripheral blood lymphoblastoid lines from a normal adult donor (C3ABR) and a homozygous AT patient (AT1ABR) (35) were used for these initial experiments. Exposure of C3ABR cells to doses of up to 10 Gy failed to induce an apoptotic response as measured by bisbenzimide staining (Fig. 6A), whereas AT1ABR cells exhibited both time- and dose-dependent apoptosis (Fig. 6B). When treated with a dose of 10 Gy, apoptosis was evident by 16 h and peaked at 36 h. Consistent with these data, irradiation of AT1ABR cells with 5 and 10 Gy induced 6-1- and 8-1-fold increases in caspase activity, respectively, over unirradiated AT1ABR cells at 24 h. Similar elevations were detected for as long as 36 h post-irradiation. In contrast, in wild type C3ABR cells, irradiation had no effect on caspase activity at any time from 24 to 36 h. Fig. 6C shows that exposure of C3ABR cells to 10 Gy did not induce CS activation. In contrast to AT1ABR cells, 10 Gy induced a significant increase of CS activity (Fig. 6D), from a base line of 86–146 pmol/min/mg protein at 20 h (p < 0.05) without a significant change in the Km of the reaction. The peptide caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp fluoromethylketone (40 μM) abolished apoptosis as measured by caspase activation or morphology but was without effect on CS activation, indicating CS stimulation is not a consequence of induction of apoptosis. Concomitant with CS activation, there was a time-dependent rise of cellular ceramide in AT1ABR but not in C3ABR cells (Fig. 6E) and was noted initially at 8 h and peaked at 24 h (data not shown). To confirm a role for CS activation in ceramide generation and apoptosis in AT1ABR cells, the effects of FB1 were explored. Fig. 6F shows that at 24 h, there was a 6-fold increase of ceramide that was almost completely blocked by 50 μM FB1. The apoptotic response was also inhibited by 50 μM FB1, which was non-toxic in these cells (Fig. 6F). There was
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no significant effect of FB1 on cellular ceramide and apoptosis in C3ABR cells. These data suggest, although do not provide definitive evidence, that ATM suppresses the CS-mediated mechanism of radiation-induced apoptosis.

To determine whether CS activation is a general characteristic of the AT phenotype of B cells, we compared three normal lines to six AT lines derived from patients with different AT mutations (Table I). All six AT lines displayed radiation-induced ceramide synthase activation and apoptosis, whereas none of the normal lines manifested CS activation or significant apoptosis. For the most part, the AT lines that displayed brisk CS responses showed greater apoptosis.

To explore further this hypothesis, we investigated whether reconstitution of ATM function would revert the CS response to radiation in AT cells. We used AT1ABR cells stably transfected with the pMAT1 vector, which contains a full-length ATM cDNA plasmid under the regulation of a metallothionein II promoter (51). Recent studies demonstrated that expression of ATM in these cells improved the survival, reduced chromosomal aberrations, and partially corrected defective cell cycle checkpoints after radiation (51). In the present studies, pMAT1-transfected AT1ABR cells were irradiated with 4 Gy, and the promoter was activated with 4 μM CdCl₂, and the cells were evaluated for apoptosis at 24 h after irradiation. Expression of ATM, which was confirmed by Western blot (Fig. 7A, inset), resulted in a 50% reduction in the apoptotic response as compared with pMAT1-transfected AT1ABR cells not stimulated with CdCl₂ or AT1ABR cells transfected with an empty vector (data not shown). To assess the effect of ATM on CS activation, pMAT1-transfected AT1ABR cells were irradiated with a dose of 10 Gy, and 16 h later microsomal membranes were prepared and subjected to the CS assay using 10 μM sphinganine as substrate. Fig. 7A shows that in the absence of induction with CdCl₂, radiation induced, as in naïve AT1ABR cells, a 1.8-fold increase in the maximal velocity of CS in cells stably transfected with empty vector or pMAT1 (p < 0.005 each versus control). 5 μM CdCl₂ alone did not significantly affect base-line CS activity in AT1ABR cells (data not shown). However, ATM expression stimulated by CdCl₂ prevented radiation-induced CS activation almost entirely (10 Gy + CdCl₂ in ATM-transfected cells p < 0.005 versus 10 Gy + CdCl₂ in cells transfected with vector only). Similar effects of the reconstitution of ATM function on radiation-induced CS activation and apoptosis were observed in pMAT1-transfected AT3ABR cells (data not shown).

A converse pattern was observed in normal C3ABR cells that were converted to an AT-like phenotype by stable transfection with an antisense construct of ATM cDNA, as recently described (52). The vector used for transfection (pMAT2) contains an antisense construct of full-length ATM cDNA under the control of a metallothionein II promoter. Induction of antisense ATM leads to loss of 70–80% of the endogenous ATM protein by 9 h (Fig. 7B, inset) and to increased radiation sensitivity (52). pMAT2-mediated inactivation of ATM with 5 μM CdCl₂ was associated with significant CS activation 16 h after exposure to 10-Gy irradiation (10 Gy + CdCl₂ in anti-ATM-transfected cells, p < 0.005, versus 10 Gy + CdCl₂ in cells transfected with vector only; Fig. 7B) and an increase in apoptosis at 24 h from the 6 ± 2% in irradiated control cells to 24 ± 1% (p < 0.05 versus control) (data not shown). In C3ABR cells, CdCl₂ alone did not affect base-line CS activity, caspase activity, or apoptosis which represented 8 ± 1% of the population as measured by bisbenzimide staining (data not shown). Together these data provide compelling genetic and biochemical evidence supporting the concept that ATM regulates CS activation, ceramide generation, and apoptosis in response to DNA damage.
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TABLE I

| Cell line | Mutationa | CS activityb | Apoptosisc |
|-----------|-----------|--------------|------------|
| Control cells |  |  |  |
| C2ABR | Wild type | 96 ± 8 | 4 ± 1 |
| C3ABR | Wild type | 98 ± 4 | 2 ± 1 |
| C5ABR | Wild type | 105 ± 2 | 7 ± 1 |
| AT cells |  |  |  |
| AT1ABR | Deletion 7638–7646 | 162 ± 1 | 3 ± 1 |
| AT3ABR | A8266T | 151 ± 11 | 3 ± 1 |
| AT5ABR | Deletion 4777–5673 | 149 ± 2 | 4 ± 1 |
| AT9ABR | Truncationd | 123 ± 1 | 6 ± 2 |
| GM15 | Ins 6404 TT | 126 ± 3 | 4 ± 1 |
| BMA | Group C | 135 ± 6 | 6 ± 1 |

at Nucleotide numbering based on human ATM sequence (71).
a Cells were irradiated with 10 Gy. 20 h after irradiation CS activity was assayed with 10 μM sphinganine as in Fig. 2. Unirradiated cells served as controls. Values represent mean ± S.D. of determinations from 4 of 4 similar studies.
b Apoptosis was measured at 24 h as in Fig. 1A.
c Truncation was detected by the protein truncation test (72); truncation sites are unknown.
d AT complementation group was previously described (73).

FIG. 7. Effects of sense or antisense ATM cDNA expression on irradiation-induced CS activation. A, prevention of radiation-induced CS activity in AT1ABR lymphoblasts by restoration of ATM activity. ATM was expressed in AT1ABR cells stably expressing pMAT1 (containing ATM under control of a metallothionein promoter) by stimulation with 5 μM CdCl2. After 9 h, the medium was changed to 0.1% human albumin/RPMI (5 × 106 cells/ml), and cells were irradiated (10 Gy) as described under "Materials and Methods." 16 h post-irradiation, microsomal membranes were prepared and assayed for CS activity using 10 μM sphinganine as in Fig. 2B. Values represent mean ± S.D. of duplicate determinations from one of three independent experiments. Inset, ATM levels were detected by Western blot 9 h after CdCl2 treatment as described (51). B, antisense inactivation of ATM confers radiation-induced CS activation onto C3ABR lymphoblasts. Endogenous ATM was inactivated in normal lymphoblasts stably transfected with the pMAT2 vector (containing an antisense ATM cDNA construct under control of a metallothionein promoter) by stimulation with 5 μM CdCl2 for 9 h prior to irradiation. 16 h after irradiation, microsomal membrane was prepared and assayed for CS activity as in A. Values represent mean ± S.D. of duplicate determinations from one of three independent experiments. Inset, ATM levels were detected by Western blot 9 h after CdCl2 treatment as described (51).

DISCUSSION

The present studies provide evidence that radiation-induced DNA damage signals apoptosis via post-translational activation of CS. The dose dependencies for CS activation, ceramide generation, and apoptosis correlated closely. Inhibition of apoptosis using a peptide caspase inhibitor did not affect CS activation, indicating this event is not a consequence of induction of apoptosis. An obligatory role for CS activation in this pathway was defined by the use of FB1 that blocked [125I]dUrDNA and x-radiation-induced ceramide elevation and apoptosis. The pro-apoptotic signals that activate CS are regulated by ATM. Not only do immortalized B cells from AT patients exhibit increased radiation-induced CS activation, ceramide generation, and apoptosis but stable transfection of wild type ATM reversed these events. Furthermore, antisense inactivation of ATM in normal B cells recapitulated the AT phenotype. Thus, signals from DNA damage and ATM appear to reciprocally regulate the activity of CS and apoptosis after radiation exposure.

The prevalence of the pro-apoptotic responses of CS and SMase to radiation is unknown. Our data indicate that the CS response is cell type-specific. Some cells, such as the AT1ABR and TPA-treated LNCaP cells, activate CS selectively, whereas others, such as BAEC, activate it coordinately with SMase. The data define a number of issues. We examined two pairs of EBV-immortalized human B cell lines generated in different laboratories. The C3ABR line, derived from peripheral blood of a normal adult (35), activated neither SMase nor CS and was radioresistant. Genetic inactivation of ATM in the sister line AT1ABR conferred both CS activation and radiosensitivity but did not confer SMase activation. In contrast, the wild type B cell line GM0988, derived from the peripheral blood of an 8-year-old (42), responded to ionizing radiation with SMase activation and apoptosis but not CS stimulation. Inactivation of ASMase in its sister line 1418 derived from a child with NPD did not permit radiation-induced CS activation but rather resulted in radioresistance. These studies suggest that radiation-induced ASMase activation, which occurs at the plasma membrane, and CS activation, which results from DNA damage, are independent stress response systems. Furthermore, these studies suggest that cell lines developed from the same apparent source may respond differently depending on developmental contexts or the microenvironmental milieu. Although it is possible that selection of specific B cell populations occurred due to the ages of the donors, more likely the differences reflect known clonal variations associated with strain-specific differences in EBV gene expression (59–61). Similarly, we noted differences between the radiation responses of primary cultures of bovine macrovascular endothelium in vitro and pulmonary microvascular endothelium in vivo. Whereas the former appeared to utilize both SMase and CS, the latter used only SMase. These systems also appear subject to transmutation. Whereas LN-CaP cells were noted to be radioresistant and to activate neither SMase nor CS, low doses of the phorbol ester TPA conferred radiation-induced CS activation, ceramide generation, and apoptosis onto these cells. Ultimately, it would appear
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Although it is not presently possible to molecularly order CS activation within the myriad events that encompass ATM action, the delineation of it as transcriptionally independent defines a select group of potential ATM partners, such as those involved in the system that surveys and repairs DNA damage.

...of strict ATM regulation and whether it represents a clinically relevant component of radiation resistance are presently uncertain. The present studies may also provide a target to address the mechanisms by which other DNA damage/repair proteins such as p53, DNA-protein kinase, or c-Abl affect the apoptotic response. Integration of the ATM/CS pathway into the set of events that regulate DNA damage responses holds the promise of understanding mechanisms involved in the decision of cells to repair DNA breaks or to die.

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