Resistance to Radiation-induced Apoptosis in Burkitt's Lymphoma Cells Is Associated with Defective Ceramide Signaling

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ABSTRACT

Increased sensitivity to ionizing radiation has been shown to be due to defects in double-strand break repair and mutations in the proteins that detect DNA damage. However, it is now recognized that the cellular radiation response is complex and that radiosensitivity/radioresistance may also be regulated at different levels in the radiation signal transduction pathway. Here, we describe a direct relationship between resistance to radiation-induced apoptosis and defective ceramide signaling. Radiation sensitivity in human tumor cells correlated with the immediate accumulation of the second messenger ceramide. In the BL30A Burkitt's lymphoma line, ceramide increased 4-fold by 10 min postirradiation (10 Gy), and in the moderately sensitive HL-60 leukemia cells, ceramide accumulated 2.5-fold above basal levels. In contrast, in all radioresistant tumor cells examined, including several Burkitt's lymphoma lines (BL30K, BL29, and BL36) and the M059K glioma cell line, ceramide did not accumulate postirradiation. The ability to abrogate ceramide production by pretreatment with the tumor promoter, 12-O-tetradecanoylphorbol 13-acetate, conferred resistance to radiation-induced apoptosis in the sensitive BL30A cells. An isogenic subline of BL30A, BL30K, was resistant to both C2-ceramide (20 μM) and ionizing radiation-induced apoptosis. Bypassing the block in radiation-induced ceramide production by the addition of exogenous ceramide was not sufficient to induce apoptosis; this suggests the existence of a second ceramide-associated signaling defect in these radioresistant cells that confers resistance to ceramide-induced apoptosis. Thus, these results provide compelling evidence that ceramide is an essential mediator of radiation-induced apoptosis and that defective ceramide signaling confers an apoptosis-resistant phenotype in tumor cells.

INTRODUCTION

Inhibition of apoptosis is important in the development of human malignancy. Cells from a wide variety of tumors exhibit a decreased ability to undergo apoptosis in response to a number of physiological and therapeutic agents, including ionizing radiation (1, 2). Notwithstanding the significant advances of recent years, major gaps still exist in our understanding of the molecular and biochemical changes associated with apoptosis. In particular, the initiation steps responsible for radiation-induced apoptotic signaling are not well defined.

Ionizing radiation gives rise to a variety of cellular lesions, including both DNA and membrane damage. In the nucleus, primary lesions to DNA in the form of unrepaired double-strand breaks contribute directly to radiation-induced cell death (3). The cell membrane is also a primary target for radiation damage, with ionizing radiation stimulating several protein tyrosine kinases, including the Src family tyrosine kinases p59fyn, p55 lyn, and the recently identified Bruton's tyrosine kinase (4). The kinase domain of Bruton's tyrosine kinase is indispensable for the radiation-induced apoptotic response (5). Ionizing radiation also initiates other signaling events critical for the apoptotic response, notably, the cleavage of membrane sphingomyelin resulting in the formation of ceramide, a lipid second messenger. Because ceramide production is triggered by ionizing radiation in cytoplasmic extracts (6), this precludes the possibility that the radiation signal is transduced first via the nucleus and subsequently to the cell membrane to initiate sphingomyelin hydrolysis.

The sphingomyelin cycle and regulated sphingomyelin hydrolysis have been observed in a number of cell types, including lymphocytes, myelocytes, fibroblasts, and glioma cells (7). Ceramide generation via the activation of sphingomyelinases precedes apoptosis in response to an expanding list of diverse stimuli, including TNF-α (8). The cytotoxicity of exogenous ceramide analogues mimics the action of TNF-α, thus providing further evidence that endogenously generated ceramide transduces the effects of TNF-α and other inducers of cell death (7).

Defective ceramide signaling has been implicated in the mediation of resistance to TNF-α, UV radiation, Fas receptor ligation, and anti-IgM-induced apoptosis (12–14). Hence, in the context of radiation resistance, it is important to determine whether malignant cells that escape radiation-induced apoptosis are still responsive to ceramide signaling or whether they are unable to produce ceramide in response to ionizing radiation. In an attempt to answer this question, we compared the integrity of the ceramide signaling pathway in a variety of sensitive and resistant human tumor cells. In particular, we focused our studies on an isogenic pair of EBV-negative Burkitt's lymphoma lines, one of which (BL30K) has acquired resistance to radiation-induced apoptosis, whereas the other (BL30A) is intrinsically sensitive to ionizing radiation-induced apoptosis (15). Here, we present evidence that radioresistant tumor cells do not accumulate ceramide in response to ionizing radiation.

MATERIALS AND METHODS

Reagents. C2-Ceramide (n-octanoylsphingosine) and ceramide-1-phosphate were purchased from Calbiochem (La Jolla, CA). Escherichia coli diacylglycerol kinase and C12-dihydrorceramide were from Sapphire Bioscience (Alexandria, NSW, Australia). TPA, natural ceramide (type III), cardiolipin (bovine heart), n-octanoyl-β-D-glucopyranoside, and diethylenetriamine-pentaacetic acid were from Sigma Chemical Co. (St. Louis, MO). Silica gel TLC plates (LK6D) were purchased from Whatman, Inc. (Clifton, NJ). All other reagents were analytical grade.

Cell Culture. The cell lines referred to here, BL30A (radioresistant) and BL30K (radioresistant), are an isogenic pair of EBV-negative Burkitt's lymphoma lines, previously referred to as BL30A and BL30(s), respectively (15). BL29 and BL36 are both EBV-positive radioresistant Burkitt's lymphoma cell lines (15). HL-60 promyelocytic leukemia cells were obtained from the American Type Culture Collection (Bethesda, MD), and the M059K glioma cell line was a kind gift from Dr. Joan Turner (Gross Cancer Institute, Edmonton, Canada). Cells were maintained at 37°C in RPMI 1640 supplemented with 10% heat-inactivated FCS (except for BL30A cells, which were maintained in 20% serum), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 atmosphere. For the induction of apoptosis, confluent cells (5 × 106/ml) were exposed to 10 Gy of γ-rays from a 137Cs source (3).

Received 3/17/97; accepted 6/16/97.

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1 This project was supported by the Queensland Cancer Fund.

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3 The abbreviations used are: TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol 13-acetate; PKC, protein kinase C.
Defective Ceramide Production Correlates with Radiation Resistance. To evaluate the role of ceramide in radiation resistance, we used an isogenic pair of EBV-negative Burkitt's lymphoma cell lines, which were originally isolated from the same donor. The BL3OA line is radiation sensitive, whereas its subline, BL3OK, is resistant to the induction of apoptosis by ionizing radiation. BL3OK cells have progressed to a group III lymphoblastoid-like phenotype, characterized by tight clumping in culture. Both cell lines contain a mutated form of the p53 tumor suppressor gene; hence, radiation-induced apoptosis in these lines is p53 independent (15). Further characteristics of BL30A and BL30K cells, initially termed BL30 and BL30(s), respectively, have been described previously (15). After 10 Gy of ionizing radiation, apoptosis was rapidly induced in BL30A but not BL30K cells (Fig. 1A). By 8 h postirradiation, approximately 50% of BL30A cells contain apoptotic nuclei, characterized by chromatin margination, condensation, and apoptotic body formation, whereas the BL30K nuclei remain intact (Fig. 1B), as determined by fluorescent staining with bis-benzimide trihydrochloride (Hoechst-33258).

Defective Ceramide Production Correlates with Radiation Resistance. Ceramide production is thought to be essential for the induction of apoptosis, which is mediated by a diverse array of physiological and pharmacological agents (7). Defective ceramide production correlates with tumor cell resistance to several apoptotic stimuli, including TNF and UV radiation (12), Fas receptor ligation (13), and anti-IgM cross-linking (14). To determine whether resistance to radiation-induced apoptosis also correlates with a defect in the production of the second messenger ceramide, we measured ceramide production in the isogenic BL30A and BL30K cell lines immediately after they were exposed to 10 Gy of ionizing radiation. In the sensitive BL30A cells, ceramide increased 4-fold above basal levels and was maximal at 10 min, remaining elevated for 30 min (Fig. 2) and declining slowly thereafter toward basal levels, which were seen at 6 h (data not shown). In contrast, there was no associated increase in ceramide production in the resistant BL30K cells. Thus, BL30K cells do contain a signaling defect upstream of ceramide that appears to be responsible, at least in part, for the radioresistant phenotype.

To determine whether defective ceramide production is a general phenomenon attributable to a radioresistant phenotype, we then measured the ceramide status of several other radioresistant human tumor cell lines (data not shown). In contrast, there was no associated increase in ceramide production in the resistant BL30K cells. Thus, BL30K cells do contain a signaling defect upstream of ceramide that appears to be responsible, at least in part, for the radioresistant phenotype.
TPA Confers Radiation Resistance on BL30A Cells by Abrogating Ceramide Production. The tumor promoter and PKC activator TPA delays apoptosis initiated by Fas ligand (13) and various stress stimuli, including TNF (8), UV radiation (18), growth factor withdrawal (19), and ionizing radiation (6). We also found that TPA delayed radiation-induced apoptosis in BL30A cells (Fig. 4A). Following 30 min of pretreatment with TPA, BL30A cells that were exposed to 10 Gy of ionizing radiation demonstrated a similar kinetics of resistance to apoptosis as did the BL30K subline, up to 24 h postirradiation. This was evidenced by the absence of apoptotic nuclei (Fig. 4A) and a failure to observe DNA fragmentation at 8 h postirradiation (Fig. 4B), both of which are hallmarks of cells undergoing apoptosis (20).

Because TPA delayed radiation-induced apoptosis and ceramide production is linked to this process, we predicted that this compound might act by preventing the radiation-associated increase in intracellular ceramide. Abrogated ceramide production was observed when BL30A cells were pretreated with TPA (100 nM) before exposure to...
46% apoptosis evident at 8 h postirradiation (Fig. 6). This response of apoptosis, as did 10 Gy of ionizing radiation in BL3OA cells, with the absence of serum, was sufficient to induce a similar kinetic profile cell lines (21). Therefore, we investigated the effect of adding exogenous ceramide to the radiation-resistant Burkitt’s Lymphoma Cells Are Also Resistant to Ceramide-induced Apoptosis. If the radioresistant BL3OK phenotype is due solely to defective ceramide production, the addition of exogenous ceramide should bypass this defect and induce apoptosis. The synthetic cell-permeable analogue C8-ceramide (n-octanoylsphingosine) has been shown previously to induce apoptosis in a variety of cell types, including hematopoietic and nonhematopoietic cell lines (21). Therefore, we investigated the effect of adding exogenous ceramide to BL30A and BL30K cells. C8-ceramide (20 μM), in the absence of serum, was sufficient to induce a similar kinetic profile of apoptosis, as did 10 Gy of ionizing radiation in BL30A cells, with 46% apoptosis evident at 8 h postirradiation (Fig. 6). This response was dose dependent, and exposure to the inactive analogue C8-dihydroceramide was without effect (data not shown). When BL30A cells were pretreated with TPA (100 nm) for 30 min prior to the addition of C8-ceramide (20 μM), TPA was not able to delay ceramide-induced apoptosis (Fig. 6). This was in contrast to the TPA-protective effect observed postirradiation. Treatment of BL30K cells with C8-ceramide did not lead to a significant amount of ceramide-induced apoptosis (Fig. 6), and pretreatment with C8-ceramide did not override the radiosensitivity of BL30K cells (data not shown). Thus, these radioresistant cells must also contain a second signaling defect that confers resistance to ceramide-induced apoptosis.

**DISCUSSION***

The lipid second messenger ceramide has emerged as an important player in the initiation of most apoptotic signaling pathways, including radiation-induced apoptosis (7). Here, we have shown that ionizing radiation promotes the immediate accumulation of ceramide in radiosensitive Burkitt’s lymphoma cells in a time-dependent manner. These findings are consistent with a previous report in endothelial cells, in which sphingomyelin is rapidly cleaved to generate ceramide in response to ionizing radiation (6). The observed kinetics of ceramide generation in BL30A cells postirradiation are consistent with the hydrolysis of sphingomyelin to ceramide and phosphorylcholine by the action of sphingomyelinases (6, 22). In BL30A cells, ceramide levels peaked significantly (4-fold) at 10 min postirradiation and remained elevated for 30 min, gradually declining toward baseline levels thereafter. We did not observe another later peak of ceramide accumulation before the onset of apoptosis (data not shown), as has been observed during serum deprivation, TNF-α and Fas-induced apoptosis (23). Therefore, in BL30A cells, late accumulation of ceramide is not necessary for the apoptotic process, and no ceramide accumulation was observed as a consequence of radiation-induced apoptosis. Furthermore, we found that, although radiation-associated ceramide accumulation correlated with radiosensitivity in both BL30A and HL-60 cells, in BL30K, BL29, BL36, and MO59K cells, the absence of radiation-associated ceramide generation correlated with radiation resistance. Hence, the immediate accumulation of ceramide appears to an essential trigger for radiation-induced apoptosis, and the magnitude of ceramide production paralleled sensitivity to radiation-induced apoptosis in all of the human tumor cell lines tested.

Ceramide functions as an intracellular messenger in a way that is somewhat analogous to the role of diacylglycerol in the PKC signal transduction pathway of mitogenesis (24). The current signaling paradigm suggests that ceramide and PKC have opposing roles in governing the regulation of apoptosis (8). Thus, there appears to be a balance between apoptosis triggered via activation of the sphingomyelin pathway and the down-regulation of apoptosis by natural suppressor mechanisms that function through PKC (6). In support of this hypothesis, activation of PKC by the tumor promoter, TPA, antagonizes apoptosis induced by TNF-α (8), Fas ligand (13), interleukin 6 withdrawal (19), and UV irradiation (18). Also, inhibition of PKC has recently been shown to induce ceramide production and apoptosis through the activation of a neutral sphingomyelinase (25, 26). The
The present findings demonstrate that TPA is able to inhibit radiation-induced DNA fragmentation and subsequent apoptosis in BL30A cells. Other activators of PKC, such as interleukin 3 and basic fibroblast growth factor, have also been shown to mediate the suppression of apoptosis induced by growth deprivation and ionizing radiation, respectively (27, 28). The exact mechanism by which TPA-induced PKC activation modulates its antiapoptotic effect is not known. However, our data indicate a specific effect of TPA in abrogating radiation-induced ceramide production in BL30A cells. This is consistent with the previous findings of Haimovitz-Friedman et al. (28), who found that TPA blocked radiation-induced ceramide generation and apoptosis in endothelial cells.

Because the effects of TPA on cells are pleiotropic, it is feasible that several PKC-mediated actions of TPA may contribute to a delayed apoptotic response. These include: growth arrest (19); increased Bcl-2 expression (29); increased sphingomyelin synthesis with subsequent ceramide decrease (30); prevention of ceramide-induced apoptosis via the increased production of sphingosine-1-phosphate (31); and activation of the nuclear transcription factor κB, which was recently shown to have an inhibitory effect on radiation-induced apoptosis (32). However, because our TPA pretreatment time was relatively short (30 min) and the radiation-associated ceramide production was very rapid (elevated within 1 min), it is not immediately obvious how any of the above mentioned effects of PKC phosphorylation might be linked to the observed abrogation in ceramide production postirradiation. Taken together, our findings that TPA blocks radiation-induced ceramide generation, DNA fragmentation, and subsequent apoptosis in BL30A cells suggest that the selective abrogation of ceramide production is alone sufficient to convey a radiation-resistant phenotype in tumor cells. This is confirmed by the recent generation of a sphingomyelinase knockout mouse, in which a defective sphingomyelinase enzyme abolished radiation-induced ceramide production and subsequent apoptosis (33). It is also noteworthy that a single signaling defect upstream of sphingomyelin hydrolysis appears to be solely responsible for the acquisition of a TNF- and UV-resistant phenotype in a U937 variant (12). The addition of Fas antibody was sufficient to bypass the TNF/UV signaling defect in these cells, with resultant sphingomyelin hydrolysis, ceramide accumulation, and subsequent apoptosis.

The observation here that TPA was unable to rescue BL30A cells from C8-ceramide-induced apoptosis provides further evidence for a defined role of TPA in abrogating ceramide production, after ionizing radiation, rather than a more general antiapoptotic effect. Two previous reports found that TPA did protect against ceramide-induced apoptosis (8, 34); however, these studies used the C2-ceramide analogue and HL-60 cells. The reason for this apparent discrepancy may be that the shorter chain C2-ceramide has access to different cellular compartments than its more hydrophobic C8-ceramide counterpart. Also, the effect of the C2-ceramide analogue seems to vary with cell type, and we have found that C2-ceramide causes differentiation, rather than apoptosis, in BL30A cells.4 In addition, because the distribution of PKC isoforms is cell type-specific, the effects of both PKC activators and inhibitors, with regard to apoptosis, are understandably varied between different cell lines (35).

Although the specific PKC inhibitor calphostin C has proved useful in many studies of PKC function, in the context of apoptosis, we found that calphostin C itself induced apoptosis to a similar degree in both the sensitive BL30A and radioresistant BL30K cells (data not shown). Similarly, Chmura et al. (25) found that calphostin C induced apoptosis in a murine B-cell lymphoma and another PKC inhibitor, safinogol, has also been shown to potentiate the apoptotic effect of the chemotherapeutic agent mitomycin C in gastric cancer cells, regardless of their drug-resistance status (36). It is important to note that, because BL30K cells are capable of undergoing apoptosis induced by both selective PKC inhibition and cycloheximide (data not shown), the ability of BL30K cells to evade radiation-induced apoptosis is not due to a defect in the execution phase of the apoptotic pathway.

Furthermore, the present study shows that, whereas the radiosensitive BL30A cells are sensitive to C8-ceramide-induced apoptosis, the radioresistant BL30K cells are also resistant to the induction of apoptosis by exogenous ceramide addition. This observation raises several possibilities: these cells contain another apoptotic signaling defect that is downstream from ceramide production (see Fig. 7); a second signaling defect exists on another apoptotic pathway, because it is possible that exogenous ceramide is not mimicking the radiation response, i.e., the ceramide analogue may well be accessing different cellular compartments to that of the longer chain natural ceramide produced by ionizing radiation because the ceramide produced in vivo by sphingomyelin hydrolysis is highly compartmentalized at the cell surface (37) and, therefore, care should be exercised when interpreting data from exogenous ceramide experiments; or a single defect is responsible for the appearance of the second defect, through signaling cross-talk or a transcriptional loop in which a defect in one gene causes the up- or down-regulation of another gene. Further studies are warranted to determine which of these possibilities is correct, particularly as BL29, another radioresistant Burkitt’s lymphoma cell line that demonstrated defective ceramide production postirradiation, was also resistant to C8-ceramide-induced apoptosis (data not shown). Thus, these defects are not peculiar to BL30K cells, but rather, the same signaling defect(s) may be commonly acquired by radioresistant cells.

The upstream defect appears to be an early membrane event that blocks ceramide production or perhaps a defective sphingomyelinase enzyme, as shown recently through the generation of an acidic sphingomyelinase knockout mouse (33). Two forms of sphingomyelinase, distinguishable by their pH optima (acidic sphingomyelinase pH optimum 4.5–5.0 and neutral sphingomyelinase pH optimum 7.4), are capable of cleaving sphingomyelin to initiate ceramide signaling. Inherited mutations of the human acidic sphingomyelinase gene lead to enzyme deficiency and the genetic disorder known as Niemann-Pick disease (38). Lymphoblasts from Niemann-Pick patients fail to generate ceramide and undergo apoptosis in response to ionizing radiation (33). Acidic sphingomyelinase knockout mice also express tissue defects in radiation-induced ceramide generation and apoptosis in vivo. In U937 cells, both the acidic and neutral sphingomyelinases are activated by ionizing radiation to generate distinct pools of intracellular ceramide (39). It is not certain whether acidic sphingomyelinase...
nase activity alone or both acidic and neutral activities are required for transmission of the ionizing radiation-induced apoptotic signal.

In summary, the present studies demonstrate a direct association between radiation resistance and defective ceramide signaling in a variety of human tumor cells. These findings contribute to our understanding of apoptosis signaling, providing further compelling evidence that ceramide is an essential mediator of radiation-induced apoptosis. The ability to abrogate ceramide production by TPA pretreatment in sensitive cells confers a radiosensitive phenotype. The radiosensitive BL3OK cells are also resistant to apoptosis induced by exogenous ceramide; therefore, a second ceramide-associated signaling defect may be responsible for the acquisition of C8-ceramide-induced apoptosis resistance in these cells. Taken together, these findings strongly suggest that loss of ceramide production and defective ceramide signaling may be a general mechanism of acquired radiation resistance in tumor cells. While this manuscript was being reviewed, similar findings were also reported by Chmura et al. (40) in murine B-lymphoma cells.

In the case of radiosensitive tumors, new approaches to radiotherapy would be possible if apoptosis resistance could be reversed, thereby enabling the use of low radiation doses to selectively activate tumor cell "suicide" by apoptosis. Because radiotherapy is still one of the most widely used treatments for cancer (41), the elucidation of the signaling pathway(s) that lead to apoptosis and the identification of defects within them is the first step toward manipulation of these pathways for therapeutic benefit.

ACKNOWLEDGMENTS

We thank Dr. Douglas Green for critical review of the manuscript and helpful discussion.

REFERENCES


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*Cancer Res* 1997;57:3600-3605.

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