Minireview

Ceramide and the Induction of Apoptosis

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Introduction

Widespread attention recently has focused on the process of cellular self-destruction known as programmed cell death or apoptosis (1-4). Apoptosis represents the subtractive component of the physiological regulation of tissue mass [originally referred to as regressive or shrinkage necrosis (5)], and thus is viewed as a natural counterpart of mitosis. Apoptosis results from activation of a preprogrammed pathway of biochemical events that lead to cell death. The biochemical apparatus required to carry out apoptosis is constitutively expressed, albeit in an inactive form, in most cells. In the activated state the apoptotic pathway consists of a dynamic, energy-dependent cascade of events resulting in extensive degradation of genomic DNA and striking alterations in cytoarchitecture. Apoptotic cells classically exhibit condensed nucleoplasm and cytoplasm, degeneration of the nucleus into membrane-bound apoptotic bodies, formation of membrane blebs, and a pronounced decrease in cell volume (2).

Apoptotic cell death is initiated by a growing variety of physiological stimuli such as inflammatory cytokines (6), glucocorticoids (7), and excitatory amino acids (8). In addition, induction of apoptosis underlies the cytotoxic activities of many therapeutic agents such as antineoplastic nucleoside analogues (9), topoisomerase inhibitors (10), platinum-based organometallic compounds (11), and ionizing radiation (12). Considerable effort has therefore been focused on gaining a better understanding of the biological regulation of apoptosis with the intent of developing novel therapeutic strategies that exploit this process. Recent studies have established that the generation of the lipid messenger ceramide through the sphingomyelin-signaling pathway represents a stimulus involved in the induction of apoptosis by cytotoxic humoral factors of the TNF? superfamily and a variety of therapeutic interventions. The purpose of this review is to provide a summary of the current understanding of ceramide-dependent signaling processes as they pertain to the induction of apoptotic cell death, and to relate these to other signaling pathways that regulate apoptosis.

Signaling through Ceramide

Ceramide generation is associated with the postreceptor actions of numerous cytokines, hormones, and growth factors. Many of the receptors that couple to the sphingomyelin pathway are members of the TNF receptor superfamily including the Mr 55,000 TNF receptor (13, 14), the Apo-1 or CD-95 receptor, also known as Fas, which is activated by the TNF homologue Fas ligand (FasL; Ref. 15), and the low-affinity nerve growth factor receptor (16). In addition, ceramide has been implicated in the actions of other unrelated humoral factors such as interleukin 1 (17) and 1,25-dihydroxyvitamin D3 (18). Activation of these receptors stimulates multiple forms of SMase, which is a form of PLC that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphorylcholine (Fig. 1). In the human myeloid leukemia cell lines HL-60 and U937, for example, activation of the Mr 55,000 TNF receptor promotes rapid breakdown of sphingomyelin, increasing intracellular free ceramide levels within seconds (13, 14). A more recent study (15) has demonstrated that the sphingomyelin pathway is engaged with similar kinetics following specific ligation of Fas in Jurkat T cells.

At least two forms of SMase, distinguishable on the basis of subcellular location and distinct pH optima, may couple to the sphingomyelin pathway in mammalian cells (Fig. 2; Refs. 19 and 20). A neutral SMase has been characterized that is Mg2+-dependent, has a physiological pH optimum, and may signal in the plasma membrane. A cytoplasmic Mg2+-independent variant has also been reported (18). Alternatively, an acidic sphingomyelinase with a pH optimum of 5 has been defined which may represent a processed form of the lysosomal enzyme and signal in an endosomal compartment (21). It has been proposed that this latter enzyme is activated indirectly via cell surface receptors perhaps through 1,2-diradylglycerol generated subsequent to enzymatic hydrolysis of phosphatidylycholine (21). Both sphingomyelinase isoforms may signal in response to engagement of the Mr 55,000 TNF receptor via interaction with specific functional domains of the receptor cytoplasmic tail (22). Activation of two different sphingomyelinases may result in the generation of ceramide in different subcellular compartments and permit signaling of distinct biological processes. In this regard, preliminary evidence suggests that the neutral sphingomyelinase may link the TNF receptor to the inflammatory cascade whereas acidic sphingomyelinase may link to apoptosis (22). Numerous questions remain regarding the mechanism of sphingomyelinase activation through cell surface receptors. In particular, how these enzymes link to these receptors is unknown. Cloning of the neutral sphingomyelinase and development of better reagents would appear to be required for further progress of this field.

The intracellular signaling functions of ceramide are mediated through physical interaction with specific target proteins in a manner similar to that established for diglyceride-mediated...
activation of PKC. In fact, multiple ceramide-sensitive intracellular targets have now been identified in mammalian cells (Fig. 2). The most well-defined of these targets is a Mg²⁺-dependent, proline-directed serine/threonine protein kinase referred to as CAPK (23–26). CAPK activity is associated with a Mr 97,000 protein that appears to be exclusively localized to the plasma membrane. Analysis of the substrate recognition site for this enzyme revealed that CAPK prefers the sequence X-Ser/Thr-Pro-Leu-Pro-X in which the phosphoacceptor site (Ser/Thr) is amino terminal to a proline residue, and X may be any amino acid. This recognition sequence is unusual because the activity of other proline-directed protein kinases is generally reduced by insertion of amino acids between Thr and Pro. Substrate phosphorylation by CAPK and CAPK autophosphorylation are stimulated nearly 10-fold upon elevation of cellular ceramide levels by treatment with either ceramide analogues or TNF-α (23, 25).

Early studies provided evidence that TNF-induced ceramide generation mediated activation of the Mr 42,000 form of the serine/threonine protein kinase MAP kinase (also known as ERK) (27, 28). MAP kinase participates in a protein kinase cascade from the cell surface involving sequential phosphorylation and activation of Raf-1 or MEKK and MEK. Recent investigations provide evidence that ceramide modulates this signaling cascade by activating Raf-1 (29). In this context, ceramide stimulates CAPK which in turn phosphorylates Raf-1 on Thr269, increasing its kinase activity. Hence, CAPK functions as a Raf-1 kinase. Although the role of MAP kinase activation in ceramide signaling is not fully understood, preliminary observations (30) suggest that activated MAP kinase phosphorylates Ser505 of cytosolic phospholipase A2, resulting in the release of arachidonic acid. Thus, this cascade may play a crucial role in the inflammatory response to TNF.

The role in this process of the proto-oncogene Ras, which docks Raf-1 to membrane during activation via tyrosine kinases, is unknown. However, it is conceivable that ceramide might contribute to the regulation of Raf-1 activation by a novel mechanism of modulation of the guanine nucleotide-binding activity of Ras. Ras proteins, like other G proteins, contain bound GDP in the inactive state and GTP in the active state, and ras activation involves rapid exchange of GTP for bound GDP. This process is facilitated by numerous guanine nucleotide exchange factors (28). The putative GEF VAV, which is uniquely expressed in cells of hematopoietic origin, contains a lipid-binding site. Gulbins et al. (31) have argued that VAV might serve as a target for ceramide stimulation, contributing to activation of Ras-1 studies.

A functional relationship between ceramide and Ras is also supported by other studies. Generation of ceramide and activation of Ras were demonstrated after ligation of Fas in Jurkat cells and in murine P815 cells stably transfected with human Fas cDNA (15). Moreover, Fas-induced apoptosis in both of these cell lines was abolished by electroporation of neutralizing anti-Fas antibodies or by transfection with the dominant-negative ras mutant Ras-Asn-17 (15). Collectively, these findings suggest that ras may be necessary for ceramide signaling of apoptosis.

Among other potential intracellular targets for ceramide is a cytosolic CAPP (32–35). CAPP is a member of the PP2A class of serine/threonine protein phosphatases which are expressed as
heterotrimers with distinct functional domains (33). Catalytic activity resides within the C subunit, while sensitivity to ceramide is conferred by the B subunit. Increased availability of ceramide may trigger translocation of CAPP from the cytoplasm to the membrane, where dephosphorylation of multiple phosphoprotein substrates would presumably occur. CAPP activity is stimulated by ceramide, but not by a closely related analogue dihydroceramide (34), indicating that this response is stereospecific, and CAPP is inhibited by okadaic acid (33, 34).

Two groups have now reported that ceramide selectively activates one member of the Ca\(^{2+}\)-insensitive family of protein kinase C isoenzymes, PKC-\(\xi\) (36, 37). This is consistent with the previous observation that PKC-\(\xi\) contained a lipid-binding motif and was activated by an undefined mixture of lipids but not by 1,2-diacylglycerol or phorbol esters, the traditional activators of many PKC isoforms. Although it was suggested that PKC-\(\xi\) activation might represent a mechanism by which ceramide stimulates the transcription factor NF-\(\kappa\)B (21), it is our opinion that these results must still be regarded as preliminary.

Ceramide and Cytokine-induced Apoptotic Cell Death

Apoptosis is a well-established consequence of exposure of many different cell types to TNF-\(\alpha\) (38–41). Characterization of the receptor subtype mediating this response in U937 and HL-60 cells revealed that apoptotic DNA damage is elicited by specific antibodies directed against the low-affinity \(M_f 55,000\) (type B) receptor, but not the high-affinity \(M_f 75,000\) (type A) species (40). The functional association of the sphingomyelin pathway with this receptor system prompted investigation into the role of ceramide in the induction of DNA damage in response to TNF. Attempts to link ceramide functionally to TNF-related cell death revealed that experimental manipulations that increase the availability of intracellular free ceramide (e.g., treatment with bacterial preparations of SMase or acute exposure to various synthetic analogues of ceramide) also promoted extensive double-stranded DNA damage (42–44). Ceramide-related DNA damage has now been confirmed by multiple biochemical analyses, including (a) resolution of excised 50-kbp DNA fragments (which correspond to loop structures in static chromatim) by pulsed field electrophoresis (44); (b) generation of the 0.2–1.2-kbp DNA fragment ladders classically associated with apoptotic cell death by static field gel electrophoresis (42–44); (c) measurement of the formation and release from cells of small double-stranded DNA fragments and a corresponding loss of integrity of bulk DNA by quantitative spectrofluorophotometry (43, 44) or radiochemical assay (42); and (d) end labeling of apoptotic DNA fragments by terminal transferase (44). Furthermore, neutral and alkaline elution analyses showed that ceramide-induced DNA damage is restricted to the introduction of double-stranded breaks into mature DNA, but does not entail damage of nascent DNA (43). Quantitative biochemical analyses of ceramide-dependent DNA strand breakage demonstrated a close correlation between the extent of DNA damage and other biological sequelae of apoptosis such as reduced clonogenicity and expression of apoptotic morphology (43, 44). Collectively, these findings have established the intrinsic apoptotic capacity of ceramide, and have led to the contention that generation of this lipid messenger underlies the lethal effects of TNF in mammalian cell lines.

Fas/Apo-1 is a cell surface receptor that initiates apoptosis in activated lymphocytes and many transformed hematopoietic cells (45). Based on the strong sequence homology between Fas and the TNF receptor, Cifone \textit{et al.} (46) examined the role of the sphingomyelin pathway in this response. In numerous distinct Fas\(^+\) cell lines, including Jurkat T, U937 monoblastic and a murine lymphocytic cell line transfected with Fas, cross-linking of Fas appeared to activate the sphingomyelin pathway and initiate apoptosis. This event appeared to occur specifically via activation of acidic sphingomyelinase. Furthermore, Gulbins \textit{et al.} (15) showed that Fas-induced apoptosis required activation of \(ras\), and that \(ras\) activation occurred via ceramide stimulation. These studies provided strong support for the notion that ceramide was a second messenger for induction of apoptosis in some systems.

Ceramide and Radiation-induced Apoptotic Cell Death

Fuks \textit{et al.} (47) first noted that bFGF substantially reduced apoptosis in response to TNF and ionizing radiation in primary cultures of BAECs. This form of apoptosis occurred within hours of cellular stimulation primarily in cells in \(G_1\). Since TNF-induced apoptosis appeared to be mediated via the sphingomyelin pathway in these cells, the question was raised as to whether radiation-induced apoptosis similarly engaged this system. Initial studies noted that therapeutically relevant doses of ionizing radiation induced dose-dependent elevation of cellular ceramide levels accompanied by quantitative reduction in sphingomyelin content (48). Furthermore, the dose dependencies for ceramide generation and apoptosis correlated closely. Manipulations that inhibited apoptosis also appeared to block sphingomyelinase activation by radiation in these cells. Hence, pretreatment of BAECs with phorbol ester or bFGF blocked ceramide generation and apoptosis, and selective restoration of the ceramide elevation by treatment of these cells with ceramide analogues also restored apoptosis. These studies indicated that inhibition of apoptosis by phorbol ester and bFGF occurred at the level of ceramide generation because there was no apparent defect in the cellular machinery required for ceramide-induced cell death. Furthermore, these studies confirmed ceramide as a mediator of radiation-induced apoptotic cell death. In subsequent studies performed with membrane fractions devoid of nuclei, it was established that ionizing radiation activated a membrane-bound sphingomyelinase to generate ceramide (48). Hence, at least for this form of radiation-induced cell kill, direct DNA damage was not required. Although the mechanism by which radiation activated the sphingomyelinase was not investigated, the working hypothesis for this phenomenon is that energy transfer to membranes disrupted normal membrane architecture, allowing substrates and enzymes that are compartmentalized to mix. Other potential explanations exist. It should be noted that these studies in no way contradict the generally accepted notion that double-stranded DNA breaks mediate a delayed or postmitotic form of radiation-induced cell death. Whether therapeutically relevant doses of radiation kill cells by either or both mechanisms in particular cell types is a topic of
ongoing investigation. The role of ceramide in radiation-induced cell death has also been reported by Quintans et al. (49).

**Ceramide in Daunorubicin-induced Apoptotic Cell Death**

In an attempt to generalize this response, the effect of daunorubicin was assessed in P388 lymphocytic and U937 monoblastic leukemia cells. In both of these lines, ceramide analogues induced apoptosis that was indistinguishable from that induced by daunorubicin (50). Furthermore, daunorubicin induced selective ceramide elevation because the level of the second messenger 1,2-diaclylglycerol was unaffected. In contrast to cytokine and radiation effects, however, ceramide levels did not increase until 4 h after incubation with drug. On closer inspection, it was determined that sphingomyelin levels were not reduced but in fact were increased in parallel with ceramide. This implicated the ceramide synthetic cascade in daunorubicin-induced ceramide generation (Fig. 2). Additional studies showed that the maximum enzyme velocity of ceramide synthase, the synthetic enzyme mediating conversion of sphingoid base and fatty acyl CoA to dihydroceramide, was enhanced. There exists a natural specific inhibitor of ceramide synthase, fumonisin B1, which is a mycotoxin produced by the fungus *Fusarium moniliforme* (51). Pretreatment of P388 and U937 cells with fumonisin B1 defined an obligatory role for ceramide synthase in daunorubicin-induced apoptosis, since this inhibitor blocked daunorubicin-induced ceramide generation and cell death. These studies delineate an alternative mechanism for ceramide generation that appears required for apoptotic cell death. It should be noted that activation of the synthase may be specific for daunorubicin since the deoxyctydine analogue ara-C reportedly stimulated neutral SMase activity and increased ceramide in myeloid leukemia cells (52). Interestingly, treatment of leukemia cells with ara-C has also been shown to increase intracellular levels of diglyceride, presumably through reversal of phosphatidylycholine synthase activity (53), and to stimulate leukemic cell PKC activity (54). It is conceivable, therefore, that the lethal effects of ara-C may depend, at least in part, on the relative contribution of ceramide- and diglyceride-dependent signaling pathways.

**Apoptosis and Anti-Apoptosis**

In addition to the tightly regulated proapoptotic systems described above, mammalian cells contains highly regulated antiapoptotic mechanisms. Disruption of these control systems may play a prominent role in oncogenic transformation. Recent studies show interaction between the sphingomyelin pathway and these systems. Datta et al. (55) showed that U937 cells with acquired resistance to diverse chemotherapeutic agents also demonstrated cross-resistance to ionizing radiation, TNF, and ceramide. Further biochemical characterization of this phenomenon demonstrated that these cells overexpressed the Bcl-2-related protein Bcl-xL, and that constitutive expression of this gene product conferred resistance onto these cells. Complementary studies performed by Chen et al. (56) showed that treatment of HL-60 and U-937 cells with TNF-α, ionizing radiation, or C2-ceramide induced an apoptotic response concomitant with a decrease in Bcl-2 mRNA, suggesting that Bcl-2 gene expression my, in fact, be targeted during ceramide-mediated apoptosis.

Elevation of 1,2-diacylglycerol, presumably through its target PKC, significantly impacts on ceramide-stimulated apoptosis. Phorbol esters, which are 1,2-diacylglycerol analogues, were previously shown to be antiapoptotic in diverse systems. Detailed investigation of the interaction of the PKC and sphingomyelin pathways revealed that the capacity of ceramide to trigger apoptotic DNA degradation, suppress clonogenicity, and promote expression of apoptotic morphology in HL-60 and U937 cells was virtually abolished by increasing intracellular diglyceride (43, 44). This potent antiapoptotic action of diglyceride was shared by other pharmacological activators of PKC, including the stage 1 tumor promoters phorbol dibutyrate (44) and phorbol 12-myristate 13-acetate (42, 44), the stage 2 tumor promoter mezerein (44), and the nontumor-promoting PKC activator bryostatin 1 (44). Taken together, these findings define distinct cytotoxic and cytoprotective roles for ceramide and diglyceride and, by extension, for their respective target enzymes in the regulation of leukemic cell survival.

This effect of diglyceride may represent more than a pharmacological manipulation of the apoptotic systems. In this regard, the radioprotective effect of bFGF in BAECs and in vitro may be mediated via this mechanism. Previous investigations established that PLC-γ1 binds to the bFGF receptor through its SH2 domain and is phosphorylated by the bFGF receptor tyrosine kinase (57–59). This enhances the phospholipid activity of PLC-γ1, resulting in phosphoinositide degradation, diglyceride generation, and PKC activation. In BAECs, bFGF stimulated phosphoinositide turnover and activation of PKC-α within seconds (60), resulting in inhibition of radiation-induced apoptosis. Phorbol ester treatment of intact cells mimicked bFGF and inhibited apoptosis, while down-regulation of PKC, or its inactivation by antagonists, abrogated the radioprotective effects of bFGF and enhanced apoptosis. Similarly, pretreatment of C3H mice with i.v. bFGF protected against radiation-induced apoptosis of the pulmonary vasculature and pneumonitis (60).

As stated above, in BAECs, PKC appeared to inhibit neutral sphingomyelinase (48) and block apoptosis, whereas in U937 cells additional sites downstream of sphingomyelinase appeared to be involved (44). Hence, the PKC system may comprehensively inhibit ceramide-induced apoptosis. It should be noted that other than sphingomyelinase the sites of inhibition of ceramide-induced apoptosis via the protein kinase C system are unknown. Determination of the exact site or sites is presently hindered by the lack of available information concerning the signal transduction pathways that mediate ceramide-initiated cell death.

**Summary**

An accumulating body of evidence suggests that generation of the lipid ceramide constitutes an important trigger or decision switch in the apoptotic response to some antineoplastic interventions. In this capacity, ceramide represents the first of perhaps many intracellular messengers capable of initiating apoptotic programs. Elucidation of the mechanism(s) by which ceramide and other physiological effectors regulate cell death may ultimately provide new insights into solving the problem of
drug resistance, and may constitute a basis for the development of novel therapeutic strategies in the treatment of human malignancies.

References


