Commentary

Ceramide: A Signal for Apoptosis or Mitogenesis?

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In this issue of the *The Journal of Experimental Medicine*, Boucher et al. (1) report on the role of the sphingomyelin pathway in CD28 signaling of NF-κB activation and mitogenesis in human Jurkat T cells and in primary cultures of mouse splenic T cells. In contrast, two recent articles (2, 3) have reported that ceramide, the second messenger of the sphingomyelin pathway, triggers programmed cell death (apoptosis) in response to exposure to ionizing radiation or activation of Fas. Ceramide thus emerges as a pleiotropic biologic activator capable of inducing two mutually exclusive cellular functions, cell proliferation and cell death. This surprising finding provides tantalizing insight into the signaling capabilities of ceramide specifically and second messengers in general. This commentary highlights several recent discoveries on signaling through the sphingomyelin pathway and speculates on how ceramide might signal both proliferation and apoptosis.

Signaling through the Sphingomyelin Pathway

The sphingomyelin pathway is an ubiquitous signaling system linking a specific set of cell surface receptors to the nucleus. This pathway is initiated by hydrolysis of the phospholipid sphingomyelin (*N*-acylsphingosin-1-phosphocholine), which is preferentially concentrated in the plasma membrane of mammalian cells (4). Sphingomyelin hydrolysis occurs via that action of a sphingomyelin-specific form of phospholipase C, a sphingomyelinase, to generate ceramide. Ceramide serves as a second messenger in this system, transmitting the message through to the cellular interior. Of note are the recent reports that have expanded the number of potential targets for ceramide stimulation and have questioned which sphingomyelinase—a neutral or an acidic form—might initiate signaling. Comprehensive reviews on the sphingomyelin pathway have been published during the past year (5–8).

Sphingomyelinase is known to exist in two forms, a Mg^{2+}-dependent membrane-bound enzyme with a neutral pH optimum and a lysosomal acidic form (4). Mutations in the acidic sphingomyelinase gene are responsible for the inherited childhood disorder known as Niemann-Pick disease (9). Originally, it was believed that signaling was confined to the plasma membrane, involving only the neutral form, whereas the sole known function for the acidic form was for membrane turnover in the lysosomal compartment. However, Kronke and colleagues (10) have suggested that at least a portion of the acidic form of sphingomyelinase may be activated at the plasma membrane by 1,2-diacylglycerol, implicating it in signal transduction. Recent studies by Cifone et al. (3) show that activation of Fas results in stimulation of acidic sphingomyelinase in U937 cells and tends to substantiate this notion. The involvement of a lysosomal hydrolase in signaling is unprecedented and it is unclear how it might function in this capacity. It has been speculated that receptor internalization and subsequent acidification of endosomes might allow such a process to proceed. Although this is an intriguing hypothesis, it still implies that a portion of the acid sphingomyelinase must somehow be compartmentalized separately from the remainder of the enzyme contained within the lysosome. The cloning of the acidic sphingomyelinase gene by Schuchman and co-workers (11) should allow these questions to be addressed.

In addition to the membrane-associated enzyme, a cytosolic variant of the neutral sphingomyelinase that is Mg^{2+}-independent has been reported and partially purified by Hannun and co-workers (12). The activity of this enzyme is enhanced within 1 h of vitamin D stimulation of HL-60 cells (13) and it has been suggested that this enzyme uses intracellular sphingomyelin stores to initiate signaling (14). This attractive hypothesis configures the generation of ceramide at sites other than the plasma membrane. Purification of this enzyme to homogeneity and determination of its amino acid sequence would appear essential for further progress in this field.

Other exciting developments include the identification of multiple potential targets for ceramide stimulation. The first reported target for this activity was a 97-kD proline-directed serine/threonine protein kinase termed ceramide-activated protein kinase (15). This exclusively membrane-bound enzyme is tightly coupled to both TNF and IL-1 receptors based on reconstitution experiments performed in cell-free systems (16, 17). This kinase is distinguished from other proline-directed protein kinases by its preference for the minimal substrate sequence -T-L-P (18). Sequences similar to this are found in a variety of potential substrates including the epidermal growth factor receptor and the protooncogene Raf. In fact, preliminary data show that Raf is phosphorylated and thus activated by purified ceramide-activated protein kinase (19).

Another potential membrane-bound target for ceramide in B and T cells is the protooncogene Vav. This protein has sequence homology to members of the family of guanine-nucleotide exchange proteins, which activate Ras by enhancing exchange of GDP for GTP. Vav contains, downstream of its putative catalytic domain, a cysteine-rich motif similar to the protein kinase C (PKC) lipid-binding domain that recognizes phorbol ester and 1,2-diacylglycerol (DG). Recently, Gulbins et al. (20) demonstrated that ceramide enhanced Vav exchange activity in vitro and in Vav preparations immunoprecipitated

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from ceramide-stimulated cells. Concomitant stimulation of ceramide-activated protein kinase and Vav might hypothetically allow for coordinate activation of multiple elements of the MAP kinase cascade within the plasma membrane. Other guanine nucleotide exchange proteins would have to serve this function in nonhematopoietic cell systems for this to represent an ubiquitous signaling mechanism in response to ceramide.

Hannun and co-workers have published extensively on a cytosolic ceramide-activated protein phosphatase (CAPP) that is activated in vitro by ceramide (21). This protein is a cation-independent heterotrimetic protein phosphatase of the class PP2A, and it is inhibited by low concentrations of okadaic acid. Phosphatase activity was enhanced specifically by ceramide but not by dihydroceramide. The ceramide binding site appears to be contained within the B subunit. In resting cells, CAPP is found in the cytosol, but it may translocate to membranes within a few minutes of cellular stimulation by TNF. This activity was found in *Saccharomyces cerevisiae* and was closely correlated to inhibition of proliferation by ceramide. The latter studies suggest that the sphingomyelin-signaling pathway may be evolutionarily conserved.

PKC ζ may also be a direct target for ceramide stimulation (22). Originally cloned by Nishizuka and co-workers, this kinase contains a lipid-binding domain and is stimulated by phospholipid (23). However, in contrast to other members of the PKC family, neither phorbol ester nor DG activate PKC ζ. Recently, Moscat and colleagues showed that ceramide enhanced the activity of purified PKC ζ in vitro (22). This group also showed that treatment of quiescent NIH-3T3 cells with a sphingomyelinase to elevate cellular ceramide levels enhanced the I-κB phosphorylating activity of a PKC ζ immunoprecipitate.

The designation of numerous potential targets for ceramide action leads to the conclusion that a complex pattern of integrated signals may be generated in response to acute elevation of cellular ceramide content. The fact that distinct end results are observed in response to ceramide raises the possibility that the targets for ceramide stimulation are differentially expressed in different cell types. Alternatively, the pattern of signal transmission and the ultimate outcome may be subject to transmodulatory regulation through other signaling systems. Thus, the readiness of the system to respond may be determined not only by genetics, but may also be affected by external stimuli in a dynamic manner.

Coordinate Signaling through Ceramide and DG

The simplest form of transmodulation would involve the interaction of only one signaling system with the sphingomyelin pathway. Such an interaction might lead to induction of a set of events that neither system would otherwise express. Costimulated activation of T helper cells through the IL-1 and T cell receptors may represent an example of this mode of coordinate signaling involving lipid second messengers (24–26). Specifically, as discussed below, this event may be reduced to its simplest form, the generation of ceramide and DG, respectively.

DG and ceramide form the backbone of the glycerophospholipids and sphingolipids, respectively, and are rapidly released by receptor-induced activation of phospholipases contained within the plasma membrane (4). These molecules possess some structural and functional similarities. Both are small molecules of ~600 D and are quite hydrophobic. Both molecules readily redistribute across a membrane bilayer, a property that may be necessary for a lipid to serve as a second messenger. Furthermore, the level of these lipids appears to be tightly regulated in most cells at ~1–3 mol % with respect to phospholipid. Perhaps the most convincing evidence for similarities between these two lipid second messengers is derived from studies that showed that a number of enzymes recognize both ceramide and DG as substrates. For instance, *Escherichia coli* DG kinase phosphorylates both ceramide and DG (27), and sphingomyelin synthase, the enzyme responsible for sphingomyelin synthesis in mammalian cells, transfers the phosphocholine headgroup from DG to ceramide, generating sphingomyelin from phosphatidylcholine. Other examples exist.

While similarities clearly exist, there are substantive structural and functional differences between DG and ceramide (28). Natural DG is comprised of two long-chain fatty acids of approximately equal chain length (C16–C20), whereas ceramide frequently contains a 24-carbon fatty acid. This confers a more asymmetric structure onto ceramide. Natural ceramide is also far more saturated than DG. Additionally, the amide-linked fatty acid at position 2, the free hydroxyl at position 3, and perhaps the *trans* double bond at positions 4 and 5 of the sphingoid base backbone of ceramide present a significantly more polar region than the complementary region of DG. This allows for greater interaction with water and intra- and intermolecular hydrogen bonding, resulting in stabilization of the lipid bilayer.

The structural similarities between DG and ceramide may be clues to those properties that may allow these lipids to serve as second messengers, while the differences are recognized by mammalian cells and must determine specificity of function. In this regard, the isoforms of PKC stimulated by DG appear unaffected by ceramide, whereas PKC ζ is recognized by ceramide but not by DG. In fact, other than Vav, the identified targets for ceramide stimulation are not affected by DG. The ability of cellular targets to distinguish between these two lipids translates into specificity of action. Stimulation of ceramide-activated protein kinase (16), NF-κB translocation to the nucleus (29), HIV replication (30) and apoptosis in HL-60 cells (31), and IL-2 secretion in EL4 cells (17) were induced by ceramide but not DG (or other potential lipid second messengers) in these cells.

Costimulation through the IL-1 and TCR may be interpreted in terms of these two second messengers. It is well-documented that the TCR uses DG as a second messenger subsequent to activation of phospholipase Cγ through one or more receptor-associated tyrosine kinases (26, 32–34). This function can be mimicked by low concentrations of phorbol ester, a DG analogue, which in combination with IL-1 is used in costimulation assays for IL-2 secretion and IL-2 receptor up-regulation. As for the participation of ceramide in this costimulatory function, Mathias et al. have recently reported

that the sphingomyelin pathway is the major signaling system for IL-1 in EL4 T helper cells and that ceramide substitutes for IL-1 to costimulate IL-2 secretion (17). Hence, costimulation of IL-2 secretion was successfully recapitulated with lipid analogues of DG and ceramide. The studies reported by Boucher et al. (1) similarly demonstrate recapitulation of costimulated signaling through CD28 and the TCR by a combination of sphingomyelinase and phorbol ester.

The concept of costimulation allows interpretation of the role of ceramide as mediator of Fas- and TNF-induced apoptosis to be viewed in the context of reciprocal regulation of cell death versus proliferation by lipid second messengers. Cellular stimulation with bacterial sphingomyelinase, ceramide analogues, or TNF induces apoptosis in sensitive cell lines concomitant with relatively large elevations in cellular ceramide content and without effect on cellular DG levels (16, 29, 31). This contrasts with the costimulation phenomenon through IL-1 or CD28 and the TCR, in which elevation of cellular ceramide content likely occurs together with DG, and proliferation and T cell activation ensue. Hence, the outcome of cellular stimulation may depend on whether single or dual second messenger stimulation is activated, and this variable may determine the ultimate outcome even within a single cell type. In this regard, two laboratories have recently demonstrated that isolated elevation of the ceramide level in Jurkat cells after stimulation with Fas or with ceramide analogues induced apoptosis (3, 34a), whereas Boucher et al. (1) show that ceramide elevation concomitant with activation of the TCR (and presumably elevation of DG) resulted in expression of differentiated functions and growth of the same cells.

A number of studies have directly evaluated the potential role for reciprocal regulation of apoptosis by ceramide and DG. Obeid et al. reported that phorbol ester pretreatment of U937 promonocytic leukemia cells blocked TNF- and ceramide-induced apoptosis (35). Similarly, Jarvis et al. reported that pretreatment of an HL-60 cell variant with either synthetic DG or exogenous phospholipase C blocked TNF- and ceramide-mediated apoptosis (31). Haimovitz-Friedman et al. (2) showed that phorbol ester or DG generated in response to basic fibroblast growth factor blocked ionizing radiation-induced apoptosis in primary cultures of bovine aortic endothelial cells, an event mediated by activation of neutral sphingomyelinase. Recently, Grant and co-workers studied the reciprocal regulation of apoptosis through DG and ceramide in detail, demonstrating that even non–tumor-promoting phorbol esters were capable of inhibiting ceramide-induced apoptosis and that down-regulation of PKC by prolonged exposure to phorbol ester abrogated DG inhibition (36). Hence, a substantial amount of evidence has been accumulated in a relatively brief time to argue that DG protects cells from the lethal effects of ceramide stimulation.

Since ceramide generation appears involved in both proliferation and apoptosis, events leading to apoptosis must somehow be suppressed in the presence of DG for proliferation and differentiated functions to prevail. However, more detailed information about the elements involved in the signal transduction pathway generated in response to a selective ceramide elevation will be required to ascertain which events mediate ceramide-induced apoptosis. Thereafter, it may be possible to delineate which of these elements proceed and which may need to be attenuated in the presence of DG to switch the downstream flow of events from apoptosis to differentiation or proliferation. The use of synthetic lipid analogues to generate isolated elevations in the cellular level of these second messengers should be invaluable for this purpose.

This commentary attempts to provide a framework to interpret data that appear contradictory by implicating ceramide in both cell proliferation and apoptosis. A similar conundrum surrounds the role of *myc* in these processes (37). The hypothesis that the effect of ceramide depends not only on the genetics of the system but also on the microenvironment, i.e., the context in which the signal is generated determines the outcome, is a testable one. In at least one system, preliminary data suggest that ceramide might serve, even within the same cell, as a signal for distinct and perhaps mutually exclusive events. If reciprocal regulation through DG and ceramide is a generic mechanism for regulation of apoptosis and proliferation, it may be possible to delineate the subset of intracellular signaling events that comprise an obligatory path committed to the apoptotic process. The studies of Boucher et al. (1) and Cifone et al. (3) clearly show that there is nothing inherent in the structure of ceramide that designates life or death.

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