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Ceramide: An Intracellular Mediator of Apopotosis and Growth Supression [and Discussion]

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Ceramide: an intracellular mediator of apopotosis and growth supression

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SUMMARY

Ceramide is an endogenous lipid molecule generated by hydrolysis of membrane sphingomyelin, in response to cellular stimulation by hormones and cytokines. Ceramide appears to have a role in mediating biological responses in a wide variety of cell types. These responses are generally considered anti-proliferative, but endpoints are varied and include differentiation, growth inhibition, senescence and apoptotic cell death. Mechanisms for ceramide action involve regulation of protein phosphorylation via stimulation of a serine/threonine protein phosphatase, a proline-directed kinase and possibly other direct and/or indirect targets.

1. INTRODUCTION

Sphingolipids are a diverse and ubiquitous class of eukaryotic lipids, once thought to be inert structural components of cell membranes. A potential role for sphingolipids as signalling molecules was identified with the demonstration that sphingosine, an endogenous sphingolipid metabolite, reversibly inhibits the pivotal enzyme, protein kinase C (PKC) (Hannun 1986). This concept was further supported by the subsequent elucidation of a sphingomyelin cycle, in which intracellular ceramide is generated in response to cytokine stimulation of HL-60 leukemia cells (Okazaki 1989).

Over the last decade, direct investigation of sphingolipid-mediated biology has implicated a role for ceramide in such key cellular processes as differentiation (Bielawska 1992; Dobrowsky 1994), cell-cycle arrest (Jayadev 1995), and programmed cell death (Obeid 1993). Although distinct, these processes may be considered variations on an antimitogenic theme, as all result in the reining of cell proliferation. The sphingomyelin cycle may, therefore, provide a signalling function neatly opposite to the phospholipid-mediated pathway which leads to growth activation. This chapter will review the evidence for ceramide as a signalling molecule, our current understanding of ceramide-mediated biology, and future directions in the study of these important lipid molecules. Emphasis will be placed on recent insight generated into the heretofore poorly understood mechanisms of cell growth suppression.

2. STRUCTURE AND LOCALIZATION

Sphingolipids are composed of an aliphatic amino base, which in mammalian cells is predominantly D-erythro-(2S,3R)-sphingosine, with a fatty acyl group in amide-linkage, and a hydrophilic head-group at the 1-position. This may be a hydroxyl group (ceramide), phosphorylcholine (sphingomyelin), or a carbohydrate, which may be neutral or acidic. When the fatty acyl group is removed, the resulting molecule is a lysosphingolipid. The extraordinary diversity of mammalian sphingolipids afforded by differences in head group, sphingoid base, fatty acid and stereoisoform is in sharp contrast to the simplicity of yeast sphingolipids, which are restricted to one major and two minor species. The relative invariance of yeast sphingolipids may present an advantage in the study of sphingolipid function, as will be discussed.

Sphingolipids have been shown to be located primarily in the plasma membrane in several organisms, including mammals, budding yeast and plasmodium. The hydrophilic head group is oriented outward toward the extracellular space, and the hydrophobic backbone lies in the outer leaflet of the plasma membrane. However, recent studies have provided evidence for the existence of distinct pools of sphingolipids within other cellular compartments. For example, the biosynthesis of sphingolipids appears to begin in the endoplasmic reticulum where ceramide is synthesized, and moves to the Golgi, where glycoprotein assembly occurs in specific Golgi subcompartments before vesicular transport to the plasma membrane. Sphingolipid turnover may also involve endocytic membrane flow from plasma membrane to lysosomal compartments, defining other potential pools of sphingolipids within the cell. More recently, a distinct pool of sphingomyelin localized at the inner leaflet of the plasma membrane has been identified and may be responsible for the generation of ceramide second messengers in response to cytokine stimulation (Linardic 1994).

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3. THE SPHINGOMYELIN CYCLE

To begin to address the hypothesis that sphingolipids function as lipid second messengers, investigations utilizing the HL60 human promyelocytic cell line were performed. This powerful cell line model is multipotential, retaining the ability to differentiate along discrete pathways, which gives rise to mature granulocytes, monocytes, eosinophils or macrophages, depending upon the specific differentiating agent to which it is exposed. Exposure of HL60 cells to the hormone 1-alpha, 25-dihydroxyvitamin D3, a known inducer of monocytic differentiation, elicited a rapid and reversible loss of membrane sphingomyelin, with peak effects at 2 h after stimulation (Okazaki 1989), long before morphological and biochemical evidence of differentiation had occurred.

Initial studies revealed that, concomitant with sphingomyelin loss, choline phosphate and ceramide were generated in response to exposure to monocytic differentiating agents. Further studies demonstrated that a neutral, magnesium-independent cytosolic sphingomyelinase was activated in response to the action of vitamin D3 in HL60 cells, accounting for the formation of ceramide.

Ceramide, which is structurally the simplest of sphingolipids, seemed an attractive candidate to carry out the second messenger function in a signalling pathway initiated by vitamin D3 stimulation. To verify this possibility, a number of short-chain, cell permeable ceramides were synthesized and their effect on HL60 cell growth and differentiation evaluated. It was found that HL60 cells treated with low micromolar concentrations of several cell-permeable ceramides underwent growth inhibition and monocytic differentiation, analogous to that seen with hormone exposure. This effect was both dose-dependent and stereospecific, the natural D-erythro stereoisomer being the active form. In addition, a closely related species, D-erythro-dihydroceramide, was inert in the HL60 assays (Bielawska 1992; Bielawska 1993).

This exciting finding strongly supported the notion that vitamin D3 and other monocytic differentiating agents exerted their effects on HL60 cells via the generation of ceramide. It became possible to entertain the idea of a sphingomyelin cycle (figure 1), in which extracellular ligands bind to their specific receptors and stimulate the activation of a neutral sphingomyelinase, resulting in hydrolysis of membrane sphingomyelin and generation of a pulse of intracellular ceramide. Ceramide would presumably interact briefly with a downstream target before reincorporating into sphingomyelin.

The elucidation of the sphingomyelin cycle provided the first clear evidence that sphingolipid metabolites generated in response to hormonal or cytokine stimulation of cells could function as signalling molecules. Since its discovery, the sphingomyelin cycle and ceramide have been implicated in mediating the biological effects of various receptor-ligand interactions (vitamin D3, IL-1β, interferon γ (IFNγ), tumour necrosis factorα (TNFα), and nerve growth factor (NGF)) in a number of cell types, including lymphocytes, myelocytes, fibroblasts and glioma cells (reviewed by Hannun 1994). These findings have begun to define ceramide's key role in the regulation of mammalian cell growth and differentiation events. In addition, Strum and colleagues have recently demonstrated the generation of ceramide in HL60 cells treated with 1-β-D-arabinofuranosylcytosine (ara-C), a nucleoside analogue chemotherapeutic agent, thus implicating ceramide as a possible mediator of responses to both physiologic and nonphysiologic stimuli (Strum 1994). As a result of the establishment of the sphingomyelin cycle's importance, the mechanisms of sphingomyelinase activation and the downstream effects of ceramide generation have been the focus of ongoing investigations.

4. ACTIVATION OF SPHINGOMYELINASE

Despite the generalization of the sphingomyelin cycle to a wide variety of cell lines and nontransformed cells, the HL60 system has remained a fruitful one. Recent studies have shown that TNFα stimulation of HL60 cells results in the very early generation of arachidonic acid, prior to the onset of sphingomyelin hydrolysis (Jayadev 1994). The addition of micromolar concentrations of exogenous arachidonic acid (AA) can mimick the effects of TNFα on sphingomyelin hydrolysis, ceramide generation and the transient inhibition of proliferation. Exposure of intact cells to the phospholipase A2 (PLA2) activator, melletin, induced responses similar to those observed with addition of exogenous AA. Interestingly, an arachidonate-responsive neutral, magnesium-independent sphingomyelinase activity was identified in *in vitro* assays, although melletin was completely inert in the same assay. Taken together, these results suggest a

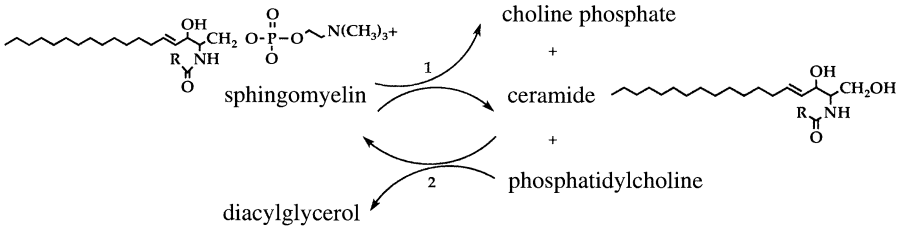

Figure 1. The sphingomyelin cycle: Sphingomyelin is cleaved by sphingomyelinase to yield ceramide and choline phosphate (1); sphingomyelin is regenerated from ceramide via the transfer of choline phosphate from phosphatidylcholine (2). R is a fatty acyl chain of variable length.

direct activation of the sphingomyelin (SM) cycle sphingomyelinase by AA.

These experiments can be explained by a signalling pathway initiated by TNFα binding to its receptor, and inducing PLA2 activation. This interaction allows for the subsequent generation of AA, which directly activates a neutral, magnesium independent sphingomyelinase responsible for hydrolysis of a signalling pool of membrane sphingomyelin. Ceramide is thereby generated and presumably propagates the signal via interaction with its downstream target or targets.

Other mechanisms for the generation of a signalling pool of ceramide have also been proposed, such as the activation of the sphingomyelin cycle by diacylglycerol (DAG), as well as the generation or attenuation of cellular ceramide levels via pathways distinct from the sphingomyelin cycle (Schutze 1992). Although intriguing and perhaps indicative of the cell's repertoire of mechanisms for regulating sphingolipid metabolism, they remain to be explored and defined.

5. DIRECT AND INDIRECT TARGETS OF CERAMIDE

Consideration of ceramide as a novel lipid second messenger created the imperative to identify the direct molecular target(s) of ceramide. In keeping with the general schema of second messenger functions, evidence points toward the regulation of protein phosphorylation by one or more avenues as the mechanism of ceramide action.

Early studies investigating ceramide-mediated biology in rat T9 glioblastoma cells had shown these cells to be readily responsive to the action of cell-permeable ceramides. Low micromolar concentrations of C2-ceramide induced growth inhibition and rapid differentiation to an astrocyte phenotype, as measured by neuronal process formation. More detailed evaluation of the ceramide response revealed evidence for the specific dephosphorylation of several proteins after ceramide treatment. *In vitro* studies led to the identification of a CAPP activity (ceramide-activated serine/threonine protein phosphatase) in T9 cytosol. CAPP was stimulated three to fivefold by C2-ceramide, C6-ceramide and natural ceramide, at concentrations similar to those which elicited ceramide biological responses. However, no activation was seen in response to treatment with sphingosine, sphingomyelin or dihydroceramide (Hannun 1994).

The biochemical features of CAPP include cation-independence, inability to bind heparin sepharose, lack of inhibition by inhibitor 2, and an exquisite sensitivity to low nanomolar concentrations of okadaic acid. These features are characteristic of the protein phosphatase 2A (PP2A) subclass of protein phosphatases, a family of heterotrimeric enzymes with catalytic (C), structural (A), and variable regulatory (B) subunits. Clearly, not all PP2A activity is ceramide responsive, because CAPP activity could be resolved from a significant component of ceramide-unresponsive PP2A-like activity by anion exchange chromatography.

Investigations using highly purified preparations of various combinations of PP2A subunits revealed that neither the catalytic subunit alone nor the AC dimer were affected by direct exposure to ceramide. However, the complete heterotrimeric protein was activated by ceramide, and this activation was completely abrogated by previous treatment of the phosphatase with trypsin and heparin, both of which dissociate the B subunit from the complex. These studies imply that ceramide has a direct activating effect on heterotrimeric PP2A, and that this interaction requires the presence of the B subunit. CAPP activity has since been identified in several other cell types in which ceramide-mediated biology has been shown, including the budding yeast, *Saccharomyces cerevisiae*, and in HL60 cells. Crucial studies utilizing okadaic acid in HL60 cells showed that inhibition of CAPP activity by this agent abrogated the ability of ceramide to induce growth inhibition and other biological endpoints (see later). These studies indicate that ceramide exerts at least some of its biological effects, such as the inhibition of proliferation, via CAPP activation. The strong correlation between requirements in biological and biochemical assays would also support this notion.

In addition to activation of a phosphatase, ceramide may have other phosphorylation-regulating targets. Kolesnick and colleages observed phosphorylation of the EGF receptor in A-431 human epidermoid carcinoma cells exposed to either sphingosine, ceramide or TNFα, a known activator of the sphingomyelin cycle. These findings led to the discovery of a membrane ceramide-activated protein kinase presumed to be responsible for epidermal growth factor receptor (EGFR) phosphorylation on residue threonine 669 under these conditions. The specificity of this proline-directed kinase is, however, not clear, as sphingosine functions as well as C8-ceramide, and ceramide cannot activate the enzyme in crude preparations. The effect of ceramide may, thus, be an indirect one. In addition to the kinase activity identified in A431 cells, HL60 cell MAP kinase activity has been shown to increase in response to ceramide (Kolesnick 1994). The exact role of these kinases in mediating ceramide biology awaits further elucidation.

Recently, evidence from several laboratories has implicated ceramide in the regulation of one or more isoforms of PKC (Moscat 1994). Although preliminary, these results are intriguing and may uncover yet another powerful mechanism by which ceramide may, directly or indirectly, affect cell biology.

6. EXTENDING CERAMIDE BIOLOGY

The initiation of the sphingomyelin cycle, the transient generation of cellular ceramide metabolites, and stimulation of either kinase or phosphatase activities as a consequence of ceramide generation are presumed to be components of signalling pathways in a variety of cell types. The extracellular ligands and cellular receptors which initiate the sphingomyelin cycle, however, are likely to differ in each cell type. In the same manner, cells are likely to have harnessed the sphingomyelin cycle and directed its signalling po-

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tential toward different endpoints. In this respect, the sphingomyelin cycle is again reminiscent of the phosphatidylinositol cycle and PKC activation, which clearly directs cells to individualized biological endpoints which are tissue and cell-specific.

(*a*) *Differentiation in HL60 cells*

Initial studies in HL60 cells demonstrated the activation of the sphingomyelin cycle in response to mediators of monocytic differentiation. Exogenous ceramide reproduced growth inhibition and monocytic differentiation, supporting the role of ceramide in mediating these responses. It then became important to determine the downstream effectors of ceramide-induced differentiation.

HL60 cells exhibit multiple genomic mutations in both oncogenes and antioncogenes. One of the most notable aberrations is a 15- to 30-fold amplification of the c-myc protooncogene in comparison to normal cells. The high steady state mRNA levels which correlate with this amplification decrease dramatically when these cells are induced to terminal differentiation. Exposure of HL60 cells to either TNFα or micromolar concentrations of C2-ceramide resulted in a rapid (by 1–2 h) downregulation of c-myc mRNA levels, as determined by northern blot analysis (Kim 1991). TNFα responses were somewhat delayed in comparison with C2-ceramide, consistent with the time required for TNFα to elicit sphingomyelin hydrolysis and concomitant ceramide generation. Consistent with the inactivity of dihydroceramide on HL60 cell biology, no effect on c-myc mRNA levels was seen in response to this agent.

Nuclear run-on studies indicated that ceramide's effect on c-myc mRNA levels was due to a block to transcriptional elongation, without affecting transcription through the first exon, similar to the mechanism of TNFα. Of equal importance was the observation that exposure of HL60 cells to okadaic acid at concentrations which are specifically inhibitory to PP2A blocked the effect of ceramide and TNFα on c-myc downregulation. The effects of phorbol 12-myristate 13-acetate (PMA), which also downregulates c-myc expression, were resistant to inhibition by okadaic acid. Therefore, it seems likely that TNFα exerts its effect on HL60 cells via ceramide generation, which results in CAPP activation and subsequent downregulation of c-myc expression, in a manner distinct from the regulation afforded by PKC.

(*b*) *T9 glioma cells/NGF*

The effects of ceramide on cell differentiation have been extended to other cell systems. In T9 glioma cells, the addition of C2-ceramide caused a dose-dependent inhibition of proliferation and induction of differentiation.

The intriguing effects of ceramide on the growth and morphology of T9 cells prompted investigation of the possibility that the sphingomyelin cycle might be involved in mediating at least some of the effects of NGF on these cells. NGF is a polypeptide which is involved in the growth and differentiation of specific components of central and peripheral nervous systems. Its effects are presumed to be mediated through interactions with two receptor systems, distinguished by high and low binding affinity for the peptide. It was found that SM hydrolysis and ceramide generation did occur in response to treatment with NGF (Dobrowsky 1994). The effects were elicited at NGF concentrations of 1–4 nM, an IC50% which suggested involvement of p75NTR, the low affinity receptor. Expression of low amounts of p75NTR was seen in T9 cells, whereas no expression of p140trk, the high affinity receptor, was seen. To determine whether the low affinity receptor was capable of stimulating SM hydrolysis, NIH 3T3 fibroblasts, which do not express either high or low affinity NGF receptors, were transfected with a p75NTR-expressing vector. These cells responded to NGF treatment by activating SM hydrolysis and ceramide generation. Additionally, T9 cells made to express an EGFR-p75NTR chimeric protein consisting of the ligand-binding domain of the EGFR and the transmembrane and cytoplasmic domains of p75NTR, responded to EGF with SM hydrolysis and ceramide generation, unlike wild type T9 cells

(*c*) *Ceramide and apoptosis*

Early studies of ceramide-mediated biology were hampered by significant cytotoxicity. This cytotoxicity was, however, stereoisomer specific, implying that ceramide-induced cytotoxicity was likely to be the result of an interaction between ceramide and a biological receptor or target, rather than the result of a nonspecific detergent effect on membrane integrity. Additionally, D-erythro-dihydroceramide, a naturally occurring molecule identical to ceramide except for the absence of the C4–5 double bond in the long chain base, had absolutely no effect on cell growth or viability. Lastly, ceramide's cytotoxic effects were reminiscent of those produced by TNFα. These findings raised the possibility that ceramide-induced death might represent a regulated apoptotic cell death.

This was confirmed by the ability of ceramide to induce programmed cell death in U937 monoblastic leukemia cells (Obeid 1993). TNFα was found to stimulate sphingomyelin hydrolysis in a time frame and to a degree consistent with that observed in other cell lines in which the sphingomyelin cycle had been implicated. U937 cells exposed to micromolar concentrations of C2-ceramide underwent internucleosomal DNA fragmentation by 2 h, which was dose-dependent and inhibitable by the presence of $Zn2+$. The effect was specific for ceramide, in that dihydroceramide, oleic acid and DiC8 were unable to elicit the response. Interestingly, the fragmentation elicited by ceramide could be blocked by treatment of cells with PMA.

Subsequent to these studies, Quintans and colleagues demonstrated that ceramide induced apoptosis in the WEHI 231 murine B lymphoma cell line, and that IgM crosslinking elicited a fivefold increase in ceramide levels within 12–24 h (Quintans 1994). These findings implicate ceramide as the mediator (via programmed cell death) of clonal deletion in immature B cells.

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(*d*) *Ceramide, cell-cycle arrest and the retinoblastoma gene product*

Serum stimulation has long been a model for study of the proliferative response. Only recently, however, have investigators begun to utilize the converse model, serum deprivation, toward an understanding of anti-proliferative responses. Whereas it had been previously assumed that serum deprivation mediated its growth inhibitory effects via a passive response to the lack of stimulation by cytokines, it has become increasingly clear that serum deprivation, in fact, actively induces cell-cycle arrest and programmed cell death (PCD).

In addition to ceramide's role as an antiproliferative agent and mediator of PCD, it was also found to be an antiproliferative mediator of serum deprivation. In the Molt-4 leukemia cell line, it was found that by 12 h after serum withdrawal, the cells began to arrest in the G0/G1 phase of the cell cycle, as determined by fluorescence-activated cell sorting (FACS) analysis of cellular DNA content (Jayadev 1995). Arrest was maximal by 48 h, at which time over 90 % of cells were in G0/G1, compared to 61 % in control cultures. In addition to cell-cycle arrest, a small subset of the total cells underwent PCD.

Serum withdrawal was shown to elicit a profound degree of SM hydrolysis and ceramide generation in Molt-4 cells, significantly greater than previously observed after stimulation of HL60 cells with TNFα or T9 cells with NGF. This may be due to the activity of a particulate, magnesium-dependent sphingomyelinase, which appeared to be stimulated in response to serum deprivation. In addition, micromolar concentrations of ceramide resulted in G0/G1 arrest comparable to, but at an earlier time than, that observed with serum deprivation. The effect was dose dependent and specific, in that dihydroceramide was unable to elicit cell-cycle arrest.

Unlike that induced by serum deprivation, ceramide-induced cell-cycle arrest was associated with a significantly higher proportion of apoptotic cells, consistent with the effects seen in U937 cells. It had previously been observed in U937 cells that PMA was able to abrogate ceramide-mediated apoptosis. Therefore, the possibility that the lesser degree of apoptosis in serum deprived cells compared to ceramide treated cells might be due to simultaneous generation of DAG was investigated. Interestingly, it was found that, indeed, DAG levels increased three to fourfold over baseline in response to serum starvation. The simultaneous addition of exogenous DAG and C6-ceramide resulted in a profound cell-cycle arrest with a degree of apoptosis reminiscent of that seen with serum starvation. These results could be explained by a complex response to serum starvation involving the activation of two lipid signalling pathways.

Studies by Rani and colleagues provide additional evidence for ceramide-mediated cell-cycle arrest (Rani 1995). After exposure of NIH 3T3 cells overexpressing the IGF-1 (insulin-like growth factor-1) receptor to the glucosylceramide synthase inhibitor, PDMP (threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), they observed a time-dependent cell-cycle arrest at both G1/S and G2/M transitions. The effects of PDMP were attributed to the documented accumulation of cellular ceramide, and removal of PDMP from the media restored transit through the cell cycle. Cell proliferation in response to either serum or IGF-1 was completely abrogated in the presence of PDMP, while early signalling events were not affected.

The retinoblastoma gene product (Rb) is centrally involved in the regulation of mammalian cell cycle. The hypophosphorylated form of this nuclear protein is associated with maintenance of the G0/G1 phase of the cell cycle, and phosphorylation seems to be one event required for exit from G0/G1, prior to the onset of mitosis. To examine whether ceramide-induced cell-cycle arrest in the G0/G1 state might be mediated through an effect on Rb, the state of Rb phosphorylation in response to ceramide treatment of Molt-4 cells was determined (Dbaibo 1995). The addition of C6-ceramide, in concentrations similar to those achieved during serum deprivation, resulted in a time and dose-dependent dephosphorylation of Rb, as determined by western blot analysis.

Confirming the role of Rb in mediating ceramide's effect on cell cycle, subsequent studies performed in retinoblastoma cells devoid of Rb showed them to be resistant to ceramide-mediated growth-inhibition. In addition, cell lines which expressed either the large tumor antigen of SV40 or E1A of adenovirus, two viral proteins known to bind and inactivate Rb, were also unresponsive to the growth-inhibitory effects of ceramide. In contrast, cell lines which expressed the large tumor antigen in which the Rb-binding site is rendered dysfunctional exhibited an intact growth inhibitory response to ceramide.

(*e*) *Ceramide and cellular senescence*

Cellular senescence is the finite life span phenotype and it has been considered analogous to terminal cell differentiation. Senescent cells are unable to undergo DNA synthesis or proliferative responses to serum stimulation. Using the WI-38 human diploid fibroblast model, it was demonstrated that this is explained in part by the inability of senescent human diploid fibroblasts to respond to extracellular mitogenic stimuli by activating phospholipase D (Venable 1994). This apparently leads to lack of a sustained DAG signal and lack of activation of PKC. This may explain the inability of senescent cells to transcribe c-fos. In addition, ceramide levels were found to be significantly elevated in senescent cells. A role for ceramide in inducing senescence was determined by its ability to induce Rb dephosphorylation and cell-cycle arrest in HDFs (human diploid fibroblasts). Interestingly, ceramide inhibited PLD (phospholipase D) activity such that exogenously administered ceramide in young WI-38 HDFs inhibited DAG generation and PLD activation, thus mimicking the lack of PLD activation seen in cellular senescence.

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(*f*) *Ceramide and IL-1-mediated PGE2 production*

There is growing evidence to suggest that second messengers generated from one signal transduction pathway may interact with the components of other signalling pathways. In support of this concept are several observations by Ballou and colleagues (Ballou 1992). They demonstrated that both sphingosine and C2-ceramide markedly enhanced the production of PGE2 in response to stimulation of human dermal fibroblasts with IL-1. Furthermore, they found that sphingomyelinase also augmented PGE2 production in response to IL-1, implicating ceramide generation as a contributor to the increased prostaglandin synthesis. Sphingomyelin hydrolysis and ceramide generation did, indeed, occur in response to IL-1 treatment. Cyclooxygenase is considered to be the rate-limiting enzyme in the synthesis of PGE2. Investigating the mechanism of ceramide's effect on PGE2 synthesis, Ballou observed that ceramide or sphingosine treatment resulted in an eightfold increase in the expression of cyclooxygenase gene mRNA and, subsequently, in an increase in cyclooxygenase protein levels. PLA2 levels, however, remained unchanged. These changes in gene expression occurred within 2 h of treatment and were transient. Interestingly, ceramide or sphingosine alone were unable to elicit PGE2 production, implying an interaction which may be on one or more levels, involving the modulation of IL-1 signalling by ceramide and/or other products of the SM cycle.

(*g*) *Ceramide in* Saccharomyces cerevisiae

The study of sphingolipid biology has been hampered by the complexity of sphingolipid species present in mammalian cells. In sharp contrast to the sphingolipid diversity of higher eukaryotes, *Saccharomyces cerevisiae* contains only three major sphingolipids, inositol-phosphate-ceramide (IPC), mannosyl inositol-phosphate-ceramide (MIPC), and mannosyl diinositol-phosphate-ceramide [$M(IP)_2C$]. Yeast ceramides are composed primarily of the long-chain base phytosphingosine, and to a lesser degree, dihydrosphingosine (in contrast to sphingosine) in amide linkage with a hydroxy C_{26} fatty acid. The study of sphingolipid function in yeast has yielded some interesting results which may be applicable to the understanding of mammalian sphingolipid biology, especially at the most basic levels.

Lester and colleagues initially isolated mutant strains of budding yeast which were auxotrophic for sphingolipid long chain bases (Wells 1983). These strains could survive and grow normally only if supplied with exogenous dihydrosphingosine or phytosphingosine. The defect in these long chain base deficient (lcb) strains was ultimately shown to be a mutation in serine palmitoyl transferase, the first and rate limiting enzyme in the sphingolipid synthetic pathway (Buede 1991). The apparent dependence of lcb strains on supplemental sphingolipid precursors was the first indication in any species that sphingolipids are vital for survival. Suppressor strains were derived from these mutants which had acquired a second mutation allowing survival in the absence of long chain base supplementation (Dickson 1990). These sphingolipid compensation (slc) strains were found to synthesize unusual phospholipid molecules which contained the fatty acid component of yeast sphingolipids (Lester 1993). Slc strains survived in the absence of cellular sphingolipids, and it appeared that the phospholipid-sphingolipid hybrid molecules provided that sphingolipid function which was required for survival. However, slc strains retained certain interesting defects, including the inability to endure stressful conditions such as extremes of pH, temperature or osmotic stress. Growth under these conditions could be restored by supplementation of the media with phytosphingosine, indicating that sphingolipids are involved in protecting yeast from harsh conditions, possibly by mediating the appropriate stress response. This is supported by the observation that concentrations of specific sphingolipid species are markedly altered in response to temperature stress in yeast (A. Richards, unpublished observations). These findings are quite interesting, in light of the role that ceramide appears to play in mediating mammalian cell apoptosis and cell-cycle arrest, responses manifested under a variety of stressful conditions, such as serum deprivation and irradiation.

A different approach to the yeast model involved assessing the effect of exogenous cell-permeable ceramides on the growth of proliferating yeast cultures (Fishbein 1993). It was found that, similar to the effects observed in various mammalian cell lines, *S. cerevisiae* growth was markedly inhibited by micromolar concentrations of C2-ceramide. This effect was dose-dependent, stereospecific and could be elicited by phytoceramide, the naturally occurring yeast ceramide, but could not be elicited by exposure of cells to dihydroceramide. Interestingly, dihydroceramide in nanomolar concentrations was capable of partially preventing the effects of ceramide exposure. Because the synthesis of sphingolipids proceeds in the order of dihydroceramide formation, followed by phytoceramide (yeast) or ceramide (mammalian cell) formation, this observation raises the possibility that growth may be regulated at the level of the enzyme responsible for dihydroceramide conversion to its later product. In addition to the effects on growth, a ceramide-responsive phosphatase activity was identified in crude extracts of *S. cerevisiae*. The requirements for this phosphatase were identical to those required to mediate ceramide biology, supporting the role of the phosphatase in mediating these effects. These findings indicate that ceramide signalling is conserved in lower eukaryotes and underscores the fundamental nature of such pathways.

It remains to be seen what molecular mechanisms are involved in mediating ceramide biology and the stress response in simple organisms. It should be possible to manipulate the growth-inhibitory effects of sphingolipids in selection schemes designed to further dissect the components of sphingolipid signalling pathways in yeast. It is likely that such studies will yield further insights into what is becoming an increasingly complex and yet fundamental area of eukaryotic signalling.

cell type	inducer	biological response
HL-60	IFN, D3, TNF, ara-C	growth inhibition, monocytic differentiation
T9	NGF	growth inhibition, glial differentiation
lymphocytes molt-4	antigen, serum deprivation, steroids, irradiation	apoptosis, clonal deletion, cell cycle arrest
fibroblasts	IL-1	cox upregulation, PGE2 synthesis
U937	TNF	apoptosis

Figure 2. The sphingomyelin cycle: specific inducers and biological responses.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Lipids were previously considered to be static, structural components of cell membranes. It has become increasingly clear, however, that lipids and their metabolites play a key role in the signalling pathways which regulate cell growth, differentiation, and death (Hakomori 1993; Merrill 1993).

Whereas phospholipids have an established role, via the phosphatidylinositol cycle and other emerging pathways, in the transduction of mitogenic signals, it appears that sphingolipids contribute to the transduction of antimitogenic responses, via the sphingomyelin cycle. Cell-cycle arrest may be the initial event in this process, and the cell may then proceed either toward terminal differentiation, senescence or, alternatively, toward programmed cell death. This decision-point may be influenced by a number of factors, including the contribution of other signalling pathways. How these different signalling pathways interact under various circumstances, such as serum deprivation, remain to be elucidated.

The second messenger, ceramide, is generated in response to extracellular stimuli in a wide variety of cell types, from mammalian cells of hematopoietic and neural origin, to budding yeast (figure 2). These findings establish the fundamental nature of the SM cycle and indicate that, not only are sphingolipids ubiquitous, but their signalling functions also appear to be conserved throughout evolution. Clearly, ceramide may not be the only sphingolipid signalling molecule, and evidence is accumulating to support a role for sphingosine, phosphorylated forms of ceramide and sphingosine, sphingoglycolipids and lysosphingolipids in cell signalling events. This raises questions of how such sphingolipid metabolites interact with one another. The enzymatic steps involved in conversion of one species to another may be poised to control cellular events in their ability to regulate cellular levels of different sphingolipid species.

Finally, as the molecular mechanisms of ceramide-mediated biology are elucidated, it may become possible to define the relationship between aberrations in sphingolipid signalling and disease states, such as tumor formation, immunodeficiency, tissue injury and storage diseases. It is anticipated that such an understanding will provide novel and specific avenues for the treatment and control of human diseases.

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Discussion

R. F. Irvine (*Department of Development and Signalling, The Babraham Institute, Cambridge, U.K.*). The lack of effect of dihydroceramide is interesting. Why is this not converted to ceramide, thus becoming active?

Y. A. Hannun. There is another layer of regulation of this entire pathway, which is topological. Dihydroceramide is made either in the late ER or Golgi, and the double bond is introduced in the late Golgi. The head group is then added in the Golgi and the molecules are exported to the plasma membrane. It seems likely that one function of this separation is to keep 'biosynthetic ceramide' away from the plasma membrane where 'signalling ceramide' is generated.

R. H. Michell (*CCRIS, Medical School, University of Birmingham, U.K.*). How many sphingomyelinases are there in cells, where are they located and how are they regulated?

Y. A. Hannun. There is an acidic sphingomyelinase that is deficient in Niemann-Pick patients, and which has been cloned. Fibroblasts from such patients have been very valuable in our studies: most of the effects I discussed appear to be unrelated to this enzyme. We have partly purified a cytosolic Mg^{2+}-independent neutral sphingomyelinase which we believe attacks an internal pool of sphingomyelin: I tend not to believe the usual 'textbook' claim that most of the sphingomyelin is at the cell surface. There is also a membrane-bound neutral sphingomyelinase that requires Mg^{2+} and which I think is responsible for attack on the external pool at the cell surface.

Question. What is Professor Hannun's view of the data, e.g. from Bell or Spiegel, that purport to implicate ceramide in MAP kinase activation and mitogenesis, particularly in 3T3 cells?

Y. A. Hannun. Ceramide was never shown to be mitogenic, and we have been unable to observe any increase in cell numbers in ceramide-treated fibroblasts. The reported assays were of thymidine uptake, and the stimulations shown were rather small. When we did BrDU incorporation studies and then examined at the cells, the stimulated incorporation looked rather spotty, which suggested that we might be

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looking at a DNA repair response rather than mitogenesis. We have never been able to reproduce the observation that ceramide can activate MAP kinase In our hands, treating cells with sphingomyelinase or TNF tends to activate Jun kinase or SAP kinase.

Question. How good is the correlation between the sensitivity of cells to TNF-induced apoptosis and to ceramide-induced apoptosis?

Y. A. Hannun. All TNF-sensitive lines seem also to respond to ceramide, but there are lines which can respond to ceramide but not to TNF. These cells are proving very valuable, because they seem to have a defect in their regulation of sphingomyelinase.

M. Wakelam (*University of Birmingham, U.K.*). Because it is assumed that the signalling ceramide is often generated in the outer leaflet of the plasma membrane, how does Professor Hannun imagine that it influences intracellular events?

Y. A. Hannun. Direct studies on this question have not been done with ceramide, but they have been done elegantly with diacylglycerol. Diacylglycerols are non-polar lipid molecules that equilibrate very quickly between the two leaflets of artificial and biological membranes, and ceramides are physicochemically similar and probably behave similarly. There are no known ceramide-binding proteins in the cells, so natural ceramides generated in the plasma membrane probably have access to both surfaces of the membrane but do not move to other membranes.

Question. Can added survival factors antagonize the ceramide generation and induction of apoptosis produced by apoptotic factors?

Y. A. Hannun. We have not studied this extensively. In the serum removal model, there is an early time window during which re-addition of serum will reverse all of the effects, including ceramide accumulation. We have not tested other survival factors.

Question. Is there any transient accumulation of ceramide at any stage of the cell cycle, such as at G0/G1?

Y. A. Hannun. No.

M. Wakelam. When short-chain diacylglycerols are added to cells, they are quickly removed by the diacylglycerol kinase pathway. However, short-chain ceramides appear to have have much longer-lived effects. Is the implication that the enzymes that metabolize, and thus inactivate, ceramides are much less active than those that handle diacylglycerols?

Y. A. Hannun. Yes, that is probably a fair conclusion. Di-C_2-ceramide is very stable, the C_6 compound is somewhat less metabolically stable, and the longer chain ceramides are metabolized more quickly (and are taken up by cells very slowly).

E. Rozengurt (*Imperial Cancer Research Fund, London, U.K.*). What are Professor Hannun's views of reports that ceramide activates PKC-ζ?

Y. A. Hannun. PKC-ζ remains 'a kinase in search of a lipid activator', with which we have been working for several years. We have never managed to achieve any specific activation by ceramide, over and above the effects of phosphatidylserine.

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