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REVIEW ARTICLE

Killing cancer cells by poly-drug elevation of ceramide levels

A hypothesis whose time has come?

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Many papers have shown that sphingolipids control the balance in cells between growth and proliferation, and cell death by apoptosis. Sphingosine-1-phosphate (Sph1*P*) and glucosylceramide (GlcCer) induce proliferation processes, and ceramide (Cer), a metabolic intermediate between the two, induces apoptosis. In cancers, the balance seems to have come undone and it should be possible to kill the cells by enhancing the processes that lead to ceramide accumulation. The two control systems are intertwined, modulated by a variety of agents affecting the activities of the enzymes in Cer-GlcCer-Sph1*P* interdependence. It is proposed that successful cancer chemotherapy requires the use of many agents to elevate ceramide levels adequately. This review updates current knowledge of sphingolipid metabolism and some of the evidence showing that ceramide plays a causal role in apoptosis induction, as well as a chemotherapeutic agent.

Keywords: apoptosis; cancer chemotherapy; ceramide concentration; ceramide metabolism; glucosylceramide synthesis inhibition; hormone effects on ceramide; multidrug resistance; sphingomyelin-ceramide pathway.

BACKGROUND

Information about sphingolipids has always occupied a small space in biochemistry textbooks and their involvement in clinical problems is best known through their roles in a few rare genetic errors (mainly Gaucher and Tay-Sachs). However, in the last 10 years or so, a flood of papers showed a remarkable degree of involvement in important health issues, such as cancer, viral and bacterial infections, brain function, the skin barrier, and atherosclerosis. Figure 1 shows a summary of sphingolipid metabolic processes.

The major synthetic enzymes are shown, omitting the steps by which the sphingols (mainly sphingosine) are formed. The two phosphate esters are mitogenic but only the sphingosine ester has received much study [1–3]. Glucosylceramide (GlcCer) is also mitogenic, possibly not as strongly as the esters but it occurs at a much higher concentration. It also acts as the precursor of lactosylceramide (GalGlcCer) and a large assortment of more highly glycosylated glucosphingolipids (GSLs), some of which are mitogenic while others inhibit proliferation or promote apoptosis. Among these active GSLs are GalGlcCer and the sialic acid derivatives, the gangliosides. The effects of sphingolipid hydrolases can be visualized by imagining that the arrows in the Figure are reversed. Not shown in this scheme, are the lyso sphingolipids, such as sphingosylphosphorylcholine, which seems to have mitogenic properties. The reader seeking more basic information about these reactants should consult sphingolipid review articles [4–7], which also discuss the many factors relating Cer to apoptosis.

Ceramide lies in the midst of this cluster of metabolites, being formed by the *de novo* route and by hydrolases that act on the more complex sphingolipids, mainly sphingomyelin and GlcCer. Its concentration is reduced by the actions of a kinase, hydrolases, a glucosyltransferase (GlcCer synthase), and sphingomyelin synthase. This multiplicity of anabolic and catabolic enzymes makes it difficult to identify mechanisms by which Cer and other agents seem to induce apoptosis, a reagent that should lead to an increased Cer level may also increase the formation of counter-apoptotic Cer metabolites.

Not only do the rules of mass action apply, modified by local K_m values, but also dynamic modulation of enzyme turnover. For example, an increased level of Cer acts to induce a higher level of GlcCer synthase activity [8–10], so that measuring the level of Cer in a cell without also measuring (at least) the levels of GlcCer, sphingomyelin, and Sph1*P* can lead to the belief that Cer does not produce apoptosis. Adding exogenous Cer to cell media may not induce apoptosis if the cell studied has a naturally high rate of glucosylation; in fact, this could lead to enhanced proliferation rather than apoptosis. These obvious relationships are made more complicated by reports that Cer can accelerate Cer synthesis. In one example of this phenomenon, an elevated level of Cer leads to increased sphingomyelinase activity, which leads to a cycle of gradually increasing Cer production [11]. The balance between the pro- and anti-apoptotic growth controllers

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Abbreviations: Cer, ceramide; GlcCer, glucosylceramide; GSH, glutathione; GSL, glucosphingolipid; MDR, multidrug resistance; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; P-gp, P-glycoprotein; PPPP, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; ROS, reactive oxygen species; Sph1*P*, sphingosine-1-phosphate.

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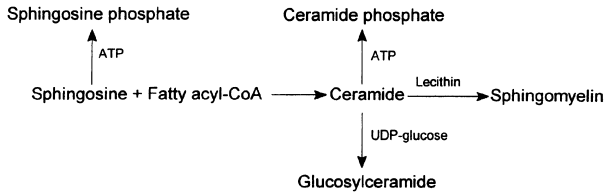

Fig. 1.The major synthetic pathways of the simple sphingolipids.

seems to be more critical than the absolute concentration of any single sphingolipid. The relative concentrations of Cer and Sph1*P* have been likened to a 'rheostat', determining whether a cell will tend to die or proliferate [1]. A 'see-saw' or 'two-pan balance' would be a more appropriate simile. This 'balance concept' can be stated differently. Apoptosis can be produced in two ways: by (a) elevating a cell's Cer concentration or (b) by lowering the concentration of the sphingolipids that produce proliferation.

The main hypothesis of this paper comes logically from the above concept: chemotherapy is a multifaceted problem requiring poly-drug action. An elevated ceramide level can simply slip out from under the therapist's touch due to the ability of cells to convert the new ceramide molecules to neutral (sphingomyelin) and/or anti-apoptotic ceramide derivatives (Sph1*P* and GlcCer). Thus the ideal therapeutic cocktail should contain inhibitors of: (a) ceramide glucosylation, (b) ceramide hydrolysis, (c) ceramide phosphorylation, and (d) sphingosine phosphorylation. In addition, the cocktail should include stimulators of: (a) *de novo* Cer synthesis, (b) sphingomyelin hydrolysis, and (c) GlcCer hydrolysis. Drugs capable of producing these actions are already known (Table 1) and most are commercially available, but only some have been approved for human use. Curiously, reviews and research reports on this subject have typically mentioned only one or two of these pathways, disregarding the fluid dynamics of Cer metabolism. Drugs able to stimulate the phosphohydrolase acting on Sph1*P* [12] or Cer1*P* might help, but none are presently known.

While the complexity of the proposed cocktail seems absurdly high, it should be noted that empirical studies in cancer patients have shown the value of cocktails made from as many as five drugs. The reason poly-drug therapy has not been often used for cancer patients is that there has been a lack of a strong rationale for choosing specific drugs. Most of the anti-neoplastic drugs in current use have been discovered empirically or directed at specific inhibition of DNA metabolism rather than Cer elevation.

Even researchers studying the anti-neoplastic aspects of Cer metabolism have been slow to use poly-drug cocktails of Cer-enhancing drugs, probably because of the satisfying discovery that many cancer cells in culture have succumbed to apoptosis with only one drug, or a combination of two drugs. Few tests of this sort have been carried out in animals, which obviously pose a much more complex,

Table 1. Substances that elevate cellular ceramide concentration and produce apoptosis. SM, sphingomyeline; SMase, sphingomyelinase.

Drug or metabolite	Effect
Ceramide	Produces reactive oxygen, which destroys GSH, which stimulates SM hydrolysis. Induces faster synthesis of GlcCer. Produces nitric oxide [91].
PDMP, PPPP ('P-drugs')	Inhibit transferases that form GlcCer, GalGlcCer, globotriaosylCer, ganglioside GM3, and 1-*O*-acyl-*N*-acetylsphingosine.
N-Butyl deoxynojirimycin	Inhibits GlcCer synthase and α-glucosidase-1 (protein glucosidase).
N-Butyl deoxygalactonojirimycin	Inhibits GlcCer synthase.
Buthionine sulfoximine	Inhibits GSH synthesis, speeding SM hydrolysis and Cer synthesis.
N-Oleoyl ethanolamine	Inhibits acid ceramidase and GlcCer synthase, stimulates SMase [58,87].
threo-Sphinganine (Safingol)	Inhibits phosphorylation of sphingols.
N,N-Dimethyl sphingosine	Inhibits sphingosine kinase.
Phenylacetic acid	Lowers GSH, allowing faster SM hydrolysis [92].
Dexamethasone	Stimulates SMase [93] but also stimulates GlcCer synthesis and GlcCer hydrolase [94].
Ionizing radiation	Produces reactive oxygen, which destroys GSH and speeds SMase [95]. Also activates ceramide synthase [96].
PSC 833 (Valspodar)	Speeds Cer *de novo* synthesis [8] and SMase [56].
Dietary β-sitosterol	Stimulates Cer formation, slows growth, produces apoptosis [97].
Nitric oxide	Activates SMases, inhibits neutral and acid ceramidase [98].
D-*erythro*-2-Tetradecanoylamino-1-phenyl-1-propanol	Inhibits neutral/alkaline ceramidase [38].
Arachidonic acid	Directly stimulates SM hydrolysis by acid SMase.
Doxorubicin	Inhibits GlcCer synthase. Forms ROS, speeding SM hydrolysis.
All-*trans* retinoic acid and fenretinide	Slows GlcCer synthesis [99].
RU486 (mifepristone)	Inhibits ceramide glucosylation [74].
Tamoxifen	Inhibits GlcCer synthase [100].
Tumor necrosis factor	Promotes neutral SMase, ceramide synthase, and acid SMase [101].
Δ9-tetrahydrocannabinol (THC)	Speeds SMase [102].
Daunorubicin	Stimulates Cer *de novo* synthesis and/or SMase [103].
Staurosporine	Produces elevated Cer and apoptosis by unknown mechanism [104].
Arabinofuranosylcytosine (Ara-c)	Stimulates SMase [105].
Phorbol diester	Stimulates *de novo* synthesis of Cer [106].

adaptable test subject than a single cell type. Moreover tumors in humans and mice, once they have been given time to grow and evolve, are composed of many clonal cell types, not the (supposedly) single types used in culture research. The different clones surely differ in their susceptibility to a particular Cer-enhancing drug.

Cancer cells that are transferred from animal to animal are given little time to evolve, as researchers normally return to a frozen primary cell culture after several animal transfers, in order to maintain the uniqueness of the clone. Cells implanted into animals for drug testing are thus unrepresentative and may or may not respond to the one drug chosen for evaluation. However, cells that have developed the multidrug-resistance trait (MDR) are remarkably sensitive to apoptosis induction by Cer-enhancing drugs, especially by Cer glucosylation inhibitors. In the case of Ehrlich ascites carcinoma cells, which have been in experimenters' hands for many years, it is likely that more than one clone is present in many laboratories. This may explain the partial effectiveness of the inhibitor of Cer glucosyltransferase, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), which prevented cancer cell growth in 30–40% of the mice inoculated with the cells [13]. The surviving mice did not develop cancer when they were reinoculated with the cells and had apparently developed an immune response.

For reasons of economy, one might be tempted to use a limited poly-drug approach in animal trials, with just a few Cer-enhancing drugs. While this could yield 'promising results', it would kill only some of the clones present in the experimental tumor and eventually, in an actual patient, possibly allowing return of the tumor in the patient. Additionally, tumors in tissues that are androgen-dependent will respond better to a Cer-enhancement cocktail if the cocktail includes anti-androgens. Testosterone stimulates GlcCer synthesis and inhibits GlcCer glucosidase in murine kidney [14], so blocking its synthesis and receptors should prove a useful adjunct for poly-drug therapy of renal cancer. It is very probable that this approach would be useful also for prostate and lung cancer.

IS CERAMIDE SIMPLY AN IRRELEVANT BYSTANDER IN APOPTOSIS?

Despite literally hundreds of articles claiming to show an active role for Cer in apoptosis and many review articles supporting the claim, some researchers still question the meaning of these findings. From a clinical viewpoint, this question is itself somewhat irrelevant, because much evidence has appeared showing that cells can indeed be killed, by whatever mechanism, by exposing them to ceramide or to reagents which are known to increase ceramide concentration (Table 1). This does not mean that cells cannot be killed by other means, especially by metabolites such as caspase-3 that seem to be downstream from Cer. Conceivably, some cells are killed by one agent via two modes of action, due to Cer accumulation and some other action.

Is ceramide formed as the result of apoptosis?

The opinion of some 'anti-ceramidists', that the appearance of Cer in cells undergoing apoptosis reflects only a side-issue, has a basis in the nature of cell death. A dying cell loses its ATP and thus cannot carry out many synthetic reactions, yet the hydrolases, which in general do not need ATP, remain intact for a while. Three hydrolases could produce ceramide as cells die: GlcCer glucosidase, sphingomyelinase, and ceramide phosphate phosphatase. Ceramidase, which would be expected to degrade the accumulating ceramide, has the ability to synthesize ceramide from free fatty acids and sphingosine [15]. That reaction does not require ATP and could be active during cell death.

Studies have reported the rapid appearance during cell death of fatty acids (from ester-type phospholipids) and of ceramide (apparently from its major bound form, sphingomyelin). The first of the latter reports was the observation that decapitation of a mouse or rat and homogenization of the brain led to rapid ceramide synthesis by neutral, metal-catalyzed sphingomyelinase [16]. It is interesting that storage, instead of homogenization of the dissected brain led to much slower ceramide accumulation, possibly because sphingomyelin and sphingomyelinase are not close to each other in intact tissues. There is a certain possibility that cells undergoing apoptosis in culture (the system preferred by most researchers) eventually bring the hydrolases into contact with their sphingolipid substrates, especially during the usual 24-h incubation time that is needed to kill many cells. The weakness in the bystander death argument is that some studies have shown parallel development of apoptosis and the level of Cer.

The location argument

Another argument of 'anti-ceramidists' comes from consideration of the subcellular localization of sphingolipids and their enzymes. These substances seem to be localized in several kinds of membranous particles, yet a careful review of the literature tells us that all the factors producing elevated Cer levels were found, in specified cells, to slow cell growth or induce apoptosis or compete with anti-apoptogenic agents like platelet-derived growth factor [17], interleukins 10 and 13 [18], or epidermal growth factor [19]. In addition, the enzymatic reactions that convert Cer to nonapoptotic (sphingomyelin) or mitogenic products (GlcCer and Sph1*P*) have been observed to prevent apoptosis, as shown by the apoptotic effects of inhibitors of these enzymes. The 'location argument' assumes that Cer, once formed, may not be able to move freely between subcellular structures or gain access to the one site where apoptosis takes place. This belief is based in part on an unrealistic view of subcellular isolation techniques, which usually yield visibly heterogeneous membranes. Significant amounts of undefined membranous structures are usually discarded.

Intermembranous movement of lipids and other cellular components is a well established process that includes the sphingolipids [20,21]. Cer formed *de novo* in the endoplasmic reticulum moves to the cytosolic surface of specific Golgi membranes to be glucosylated. From there, the GlcCer is either transported to other membranes or internalized into the interior Golgi surface for further glycosylation. Some of the Cer has to flip over into the luminal Golgi surface and convert to sphingomyelin. Sphingomyelin synthase can also reversibly form Cer and

lecithin from sphingomyelin and diglyceride. Water-soluble sphingolipid transport proteins have been isolated and P-glycoprotein (the multidrug resistance protein) has also been shown capable of transporting sphingolipids. The saposins (activators of sphingolipid hydrolases in lysosomes) can also transport the lipids. Saposin C, which activates GlcCer glucosidase and forms Cer, is concentrated in light mitochondrial, mitochondrial, and microsomal fractions and (in a nonglycosylated form) in cell nuclei, suggesting that Cer can be formed in many locations [22]. These transporters may act as membrane 'scramblers' or 'flipases.'

Intramembrane movement of lipids can be shown by the bleaching-diffusion technique, a method that has not been applied to the Cer-apoptosis question. This kind of movement occurs with small (possibly homogeneous) lipid aggregates but the large highly dynamic aggregates (rafts or caveolae or glycolipid-enriched membrane microdomains) may be more fixed. Movement of intracellular lipids to the outer membrane and lipid-binding agents in the extracellular fluid is readily demonstrated. The Cer formed by sphingomyelinase at the cell surface is flipped over to the cytoplasmic face of the plasma membrane while phosphatidylserine flips to the external face [23]. This is a typical step in the apoptotic process. In addition, a transport protein, NPC1 (Niemann-Pick C protein 1), is involved in the transfer of cholesterol and sphingolipids from lysosomes/endosomes and the plasma membrane rafts that contain caveolin-1 [24]. These rafts also contain much GlcCer and the acidic GSLs (gangliosides), and much of the gangliosides that are shed into the extracellular fluid by tumors originate there [25].

No doubt the rates of release of Cer from the different sites of synthesis differ, which could explain why different studies have reported different appearance times, even multiple waves of appearance. While there is a need for more details of sphingolipid movement in cells undergoing apoptosis, the evidence suggesting that they are frozen in place has to be reevaluated.

The 'location argument' has drawn strength from the uncertainty as to the site where Cer produces apoptosis. While this uncertainty is not a reason to reject the large amount of evidence for apoptogenesis by Cer, it is an important question. Recent research reports suggest that Cer acts on mitochondria, which is a center of attraction for apoptosis researchers [26]. Mitochondria are visibly changed by added Cer or tumor necrosis factor-α. (The latter substance has often been observed to instigate Cer production.) Exogenous C_6-ceramide induced the formation of hydrogen peroxide in myeloid leukemia U937 cells [27]. On the basis of specific inhibitor effects, the authors concluded that reactive oxygen species (ROS), generated at the ubiquinone site of the mitochondrial respiratory chain, constitute an early major mediator in ceramide-induced apoptosis. The involvement of mitochondria was further indicated by the observation that Cer did not produce peroxide in cells deficient in mitochondrial respiration. Adding Cer to JB6 RT101 epidermal tumor cells has yielded ROS and, as may be predicted, destruction of GSH [28]. Hydrolysis of sphingomyelin by neutral, Mg^{2+}-dependent sphingomyelinase is under the control of a cell's oxido/reductase status; i.e. GSH inhibits sphingomyelinase [29,30]. This sequence explains the observation that ceramide added to U937 and HL60 myeloid leukemia cells and normal skin fibroblasts activated neutral sphingomyelinase, producing a further elevation of cellular ceramide [11]. Both short-chain and long-chain ceramides produced a large release of ROS and cytochrome *c* from mitochondria [31]. However several distinct differences between the two kinds of Cer were noted with respect to other electron flow changes.

It is interesting that other GSLs, GlcCer, GalGlcCer, and gangliosides GD3 and GM1, have been shown to produce an early burst of peroxide, followed by the mitochondrial permeability transition and cytochrome *c* release in isolated liver mitochondria [32,33]. Sph1*P* was also found to destroy GSH in PC12 cells [34], suggesting it is like the other simple sphingolipids in this respect. These effects may speed up sphingomyelin hydrolysis [30] and formation of additional Cer, as part of a homeostasis mechanism whereby excessive pro-apoptotic sphingolipids increase the level of Cer and thus steady the balance between proliferation and apoptosis.

Neutral/alkaline ceramidase has been found to be located primarily in mitochondria [35]; surely it acts on Cer that enters or is formed in the mitochondria. The enzyme probably normally exerts a protective anti-apoptotic effect in healthy cells. Other studies found ceramide synthase in mitochondria [36], as well as a high specific activity for acid ceramidase [37]. Blocking the neutral pH enzyme with an analog of ceramide, D-erythro-2-tetradecanoylamino-1-phenyl-1-propanol, produced Cer accumulation in cells and slowed growth [38].

Ceramide also gave rise to an elevation of a different form of ROS, nitric oxide, in aortic endothelial cells [39]. Exogenous C_2-Cer was taken up by the cell surface caveolae (a major site for cellular Cer) and this produced a rise in the endothelial isoform of NO synthase with subsequent formation of NO. The enzyme was translocated to an interior site in the cells. Both the H_2O_2 and NO produced by Cer act to destroy cellular GSH, which speeds the hydrolysis of sphingomyelin to form more Cer. This is another route for cyclic self-augmentation of Cer levels. Other studies also noted the ability of Cer to produce NO. Of special interest is the report [40] that short-chain ceramides not only up-regulated inducible NO synthase but also increased the level of tumor necrosis factor, a process in which src-related tyrosine kinases play an important role. This effect on tumor necrosis factor is the opposite of the ability of tumor necrosis factor to increase the production of Cer, making this one more Cer self-augmentation cycle. The various cycles of this sort probably explain why apoptosis typically takes many hours to develop: substantial increases in Cer concentration develop relatively slowly.

The bad-technique argument

While many methods for measuring the level of Cer have been used in apoptosis research, yielding the conclusion that Cer induces slowed proliferation and apoptosis, some doubt was cast on the enzymatic assay method that uses diglyceride kinase [41]. A new mass spectrometric assay method was applied to Jurkat T cells that had been treated with an anti-Fas IgM. While this method of elevating Cer concentration has been used successfully in other laboratories, the mass spectrometric method did not show Cer

accumulation, although apoptosis was seen. The enzymatic assay, however, did show an increase. This study, not yet confirmed in another laboratory, was a major source of skepticism in a discussion paper [42]. The topic of assay reliability was discussed further in several letters to *Trends in Biochemical Sciences* [43–46]. It is certainly true that researchers can easily become careless in their use of analytical methods, especially those involving sphingolipids, and editors should exert their powers to force authors to certify that they performed some simple validating tests. The doubt-provoking study [41] did not offer strong validation of the enzymatic assay, and it suffered by omission of an important step, the alkaline cleavage of contaminating diacyl glycerol that competes for the labeled ATP used in the assay. Further tests are needed to establish the validity of this source of doubt.

The Farber disease problem

People with genetically defective acid ceramidase (Farber disease or ceramidosis) accumulate much Cer in their lysosomes and one could ask why this Cer is not apoptogenic. The survival of such individuals seems to indicate that Cer accumulated in lysosomes cannot produce apoptosis. However examination of patients' cells has shown that the accumulated Cer induces formation of Mn^{2+} superoxide dismutase, which protects cells against peroxidative damage [47]. Moreover, examination of Farber colonocytes showed morphological signs of apoptosis [48]. Ganglioside GD3, a pro-apoptotic GSL that can accumulate when Cer levels are increased, was found to colocalize with active caspase-3 in Farber colon tissue. Perhaps part of the Cer escapes the lysosomes, generating anti-apoptotic and pro-apoptotic sphingolipids (the 'balance' situation described above). Neutral/alkaline ceramidase must metabolize much of the escaped Cer. Normally, Cer that is formed in lysosomes is hydrolyzed and the products are metabolized further.

A similar consideration applies to studies with Niemann-Pick patients, who have a low level of acid sphingomyelinase. In the type C disorder, cholesterol metabolism is strongly affected and major metabolic patterns are disturbed. Cholesterol binds to sphingomyelin, so changes in cholesterol levels can activate sphingomyelin conversion to Cer (shown by removing cholesterol from the cell surface with lipoprotein-deficient medium).

Other anti-Cer arguments

Other expressions of doubt have suggested that experiments with short-chain ceramides simply disrupted the physical structure of the plasma membrane and thus somehow produced apoptosis in an unnatural way. In the Hofmann/Dixit letter [45], a study of rabbit platelets treated with Cer and then thrombin was cited as evidence for membrane destabilization by Cer. This paper simply reported that C_2-Cer inhibited aggregation and arachidonic acid liberation, whereas C_6-Cer and C_8-Cer enhanced these responses [49]. This discovery is a far cry from even a hint of membrane disruption; apoptosis was not mentioned. The results could more realistically suggest differences in Cer metabolism that depend on Cer chain length. Another citation intended to summon up the destabilization hypothesis was a study of platelet aggregation in which the uptakes of C_2-sphingosine and C_2-sphinganine were compared [50]. Platelet lysis was obtained with the former Cer (not the saturated Cer) when added at ≈ 40 μM Cer, a level somewhat higher than those used in other studies. Because lysis was measured with platelets that had been prelabeled *in vitro* with [^{3}H]adenine instead of by measuring protein release or cell counts, one can wonder if the lysed cells had the same specific activity as the unlysed cells. In any event, platelets are very different from most cells, including cancer cells, because they are simply cell derivatives of limited complexity.

Still a third citation described the production of apoptosis by added ceramides, including a natural mixture of long-chain ceramides [51]. The skeptics [45] were 'concerned' by the finding that the yields of apoptosis were different for the different chain lengths. It may be noted that long-chain Cer did enter the neurons despite the use of ethanol as solvent and the claim [42] that 'physiological' Cer does not enter living cells. Examination of the data in Fig. 1 shows that the differences were minor, and not statistically significant. While the question of purely physical effects of all substances of low polarity on membranes warrants serious concern, there is a marked lack of supporting evidence in the case of Cer. The ability of cells to convert short-chain ceramides to sphingosine and short-chain GlcCer and sphingo myelin [52] indicates that they behave like typical substrates rather than simple modifiers of the physical properties of the plasma membrane (i.e. they are not simply floating inertly in the plasma membrane; see also the data later in this paper on the different effects of the same Cer concentration in different cells).

Some experiments that found apoptosis preceding noticeable Cer accumulation may point to an independent mechanism of apoptosis induction but these constitute just a few pieces of evidence and their significance needs more study. As discussed above, apoptosis can be produced not only by Cer elevation but also by a reduction in the anti-apoptotic sphingolipids such as a stimulation of Sph1*P* hydrolysis.

Radford has listed objections to a primary role for Cer in radiation-induced cell damage, questioning how Cer can interfere with mitosis after the long delay typical of cell death after radiation (coming after the early cell death) [53]. Stimulation of Cer synthase in prostate cancer cells *in vitro* and *in vivo* with phorbol diester improved the response to radiation, raising the level of Cer and inducing early apoptosis [54]. It is possible that Cer levels can rise slowly by any of several cyclic self-amplification processes, based on the ability of Cer to generate H_2O_2 and nitric oxide and stimulate sphingomyelinase, described above in this paper. Surely the radiolytic damage to cells, which quickly yields ROS and GSH destruction, can be expected to yield Cer elevation and apoptosis [27,54]. Early destruction of cancer cells by radiation seems to owe much to this process, while delayed death of cancer cells may well owe much to a variety of radiolytic processes, including damage to DNA and agents of mitosis.

A systematic examination of the criteria needed to demonstrate apoptosis induction by ceramide follows below.

CRITERION (A): EXOGENOUS CERAMIDE SHOULD PRODUCE APOPTOSIS

Well over 100 studies have reported that adding ceramide to cells in culture almost always results in apoptosis and/or growth inhibition. A few studies have found that exogenous ceramide stimulates growth and, in some cases, this was traced to conversion to sphingosine or Sph1*P*.

Almost all studies with exogenous Cer have utilized a short-chain ceramide, C_2-, C_6-, or C_8-Cer made from sphingosine. Virtually all have yielded apoptotic effects or slowing of growth, except with MDR cells, which glucosylate (detoxify) the Cer very rapidly. The short-chain ceramides are used because they seem to dissolve in cell media and enter cells readily. However, it is likely that C_6-and C_8-ceramides precipitate as a fine suspension when their solution in organic solvent is added to the media. Researchers do not usually measure the percentage of added Cer that is actually absorbed by the cells.

These short-chain ceramides are typically active at inducing apoptosis at a concentration of 3–30 μM in the cell medium. We do not know how much of the absorbed Cer reaches 'the' site of apoptosis. This situation has seemed to some investigators to indicate a pharmacological effect rather than a 'natural' effect. To repeat the comment above: this does not rule out the therapeutic value of exogenous or endogenous Cer. The use of apoptotic agents other than ceramides has led to increases in Cer content of as much as 20-fold. This implies that Cer metabolism is or can be remarkably active. A study with long-chain ceramides, the much more typical ones containing C_{16} to C_{26} fatty acids, has shown much higher potency. Using a different solvent for these ceramides, dodecane/ethanol, Ji *et al*. [55] showed that apoptosis could be produced by as little as 25 nM ceramide. This is an impressive difference, supporting the conclusion that the common ceramides can be remarkably deadly to cells if they are in the right location. As dodecane is also insoluble in incubation media, it probably precipitates out in cell media together with the Cer in the form of very small droplets that fuse with the plasma membrane. Only a few other studies have been reported with this solvent. Unfortunately for the use of solvents that form Cer suspensions on dilution with media, the size of the particles and their ability to access cells may be very sensitive to variation in laboratory technique. Moreover the rate of uptake of the long-chain ceramides may be so slow in some cells that diversion to other products may dominate the apoptotic effect. There is a need to use true solubilizers (cyclodextrin derivatives, lipid transport proteins, or serum albumin) or suitable liposomal dispersions.

CRITERION (B): ENDOGENOUSLY FORMED CERAMIDE SHOULD ALSO INDUCE APOPTOSIS

Many techniques are available for apoptosis production by elevating Cer levels in cancer cells, but only the major ones are mentioned in Table 1. Most of the agents in the table produced apoptosis in susceptible cells, but in some publications, resistant cells were also reported or a second Cer-elevating agent was needed for apoptosis. This is the result of the balance problem: cells differ in the importance of any particular enzyme involved in Cer metabolism. These differences in susceptibility weaken the claims that Cer effects are due to nonspecific physical effects on plasma cell membranes.

The reader may be surprised to note that the table includes commercial drugs in current or proposed use for cancer chemotherapy, but this is to be expected if Cer accumulation is a practical therapeutic approach.

CRITERION (C): USE OF TWO CERAMIDE-ELEVATING DRUGS SHOULD PRODUCE APOPTOSIS MORE READILY THAN EITHER ONE ALONE

This kind of finding strengthens the proposal that cancer cells could be killed more readily by use of several Cer-elevating drugs. Here is a short list of studies in which two modalities proved more effective than one.

(a) The KB-3-1 cell line succumbed to the apoptotic action of PSC 833 (valspodar), which was shown to act by greatly increasing the ceramide content of cells [8]. Inclusion of a common anti-neoplastic agent, vinblastine (which also causes increases in ceramide), resulted in even greater ceramide accumulation and loss of viability.

(b) With KG1a leukemia cells, which apparently make ceramide relatively slowly, treatment with tumor necrosis factor-α did not generate Cer or apoptosis [56]. However combination with PSC 833 increased the Cer concentration and neutral sphingomyelinase activity, and produced apoptosis. PSC 833 alone led to a threefold increase in inner plasma membrane sphingomyelin content and neutral sphingomyelinase activity.

(c) MCF-7-AdrR cells, which are resistant to the toxic action of doxorubicin and have a high level of GlcCer synthase activity (characteristics of MDR cells), were found to be insensitive to C_6-ceramide as well as to tamoxifen (an inhibitor of Cer glucosylation). However, a mixture of the two reduced cell viability to 42% and elicited apoptosis [57].

(d) *N*-Oleoyl ethanolamine, which blocks acid ceramidase and should cause accumulation of Cer, did not produce apoptosis in cultured sensory neurons from neonatal mice. (In general, normal cells seem to be somewhat less affected by Cer-elevating drugs than cancer cells.) Exogenous long-chain Cer, without the inhibitor, increased survival, even in the absence of nerve growth factor in the medium. However, the combination of the two produced apoptosis [51]. The anti-apoptotic action of Cer alone was apparently due to rapid hydrolysis to sphingosine, which may have been converted to Sph1*P*. A similar study with the ethanolamine amide, which reported that the inhibitor blocks ceramide glucosylation, found that the amide potentiated apoptosis induced by C_6-ceramide [58].

(e) C_6-Ceramide was found to produce apoptosis in CHP-100 neuroepithelioma cells, an effect that was markedly potentiated by PDMP, a potent inhibitor of ceramide glucosylation [59]. At the concentration of PDMP used here, there was no effect on cell viability when administered alone.

CRITERION (D): CERAMIDE SHOULD REACT WITH ENZYMES AND PROTEINS

If Cer is simply an ash from the fire of apoptosis, one would not expect it to show direct activity on enzymes, but it does in fact stimulate two enzymes that regulate apoptosis and cell growth, protein phosphatase-1 and protein phosphatase-2A [60]. The stimulation was accomplished with long-chain Cer and cell-free enzymes. Prolonged incubation of 3T3 cells with PDMP, which forces accumulation of ceramide, led to the production of three phosphorylated proteins [61].

Ceramide has also been shown to act directly on several enzymes, activating a protein kinase (CAPK), a guanine-nucleotide exchange factor (Vav), and protein kinase C zeta (ξ). At a concentration as low as 0.1 μM, C_8-Cer was found to rapidly stimulate the phosphorylation of epidermal growth factor receptor [62].

Ceramide derived from neutral sphingomyelinase activation is thought to be involved in modulating MAP kinases, phospholipase A_2 (arachidonic acid mobilization), and ceramide-activated protein phosphatase (CAPP), while ceramide generated through acid sphingomyelinase activation appears to be primarily involved in NF-κB activation. Cer resulting from the lysosomal acidic sphingomyelinase regulates phosphatidylinositol 3-kinase, which plays a role in the balance between apoptotic and anti-apoptotic lipids [18]. Other downstream targets for ceramide action include COX, interleukin-6 and interleukin-2 gene expression, retinoblastoma protein, c-Myc, c-Fos, c-Jun and other transcriptional regulators.

In one study, ceramide was found to bind specifically to two other protein kinase C isoenzymes, the alpha and delta forms, but not to the epsilon or zeta forms [63]. This was shown with a photoaffinity analogue of Cer. The alpha form increased in activity as the result of the combination. A similar situation exists regarding the formation of a complex between ceramide and the endosomal acidic aspartate protease, cathepsin D [64]. The binding resulted in formation of enzymatically active cathepsin D.

These effects suggest that ceramide is a metabolite with a wide repertoire of actions rather than an inert product of decay.

CRITERION (E): ACTIVE CERAMIDE SHOULD HAVE A SPECIFIC CHEMICAL STRUCTURE

Studies comparing ceramides or Cer-like substances have shown that apoptosis almost always requires ceramides that exhibit the D-erythro structure and the *trans* double bond typical of sphingosine (i.e. acyl sphinganine is usually found to be inactive). Researchers who add sphingolipids containing sphinganine should keep a factor in mind: while a desaturase can act on the saturated ceramide, the desaturation is not instantaneous. However the short-chain ceramides apparently cannot be desaturated [65]. It is true, as Hofmann and Dixit have proposed [45], that even slight differences in structure also affect the physical properties of the ceramides, but these seem like second-order phenomena.

The double bond in active ceramides is in the allylic position, which renders the hydroxyl at C3 quite chemically reactive and sensitive to oxidation. It is not surprising that such ceramides are involved in oxido/reduction reactions (see above). The quinone group in mitochondrial ubiquinone [27] may well oxidize unsaturated Cer or form a condensation product with it. A miniature version of the allylic region in sphingosine derivatives, (*S*)-3-hydroxy-4-pentenoic acid, stereospecifically undergoes oxidation in mitochondria, where the allylic ketone condenses with GSH to form a thio ether and potentiates cell death [66]. The hydroxy acid does not attack cytosolic GSH and one might guess the same is true for Cer. Thus it appears that only a small portion of cellular Cer is involved in apoptosis via mitochondrial damage.

Hofmann and Dixit, in their critique of the Cer apoptogenesis hypothesis [42], noted that Liu *et al*. [30] had found that oxidized GSH (GSSG) was also a sphingomyelinase inhibitor. From this, they concluded that the redox state of the cell is unlikely to affect sphingomyelinase action. However there are multiple redox levels within cells and the ROS produced by Cer converts GSH and GSSG to products containing S-linked oxygen and S-linked NO [39]. GSSG is of little relevance as it occurs at a much lower concentration than GSH [30]. ROS, particularly nitrogen-containing ROS, oxidize other cellular sulfur compounds, which explains the manifold effects of increases in Cer.

Under the topic of structural specificity, we should appreciate that several studies have found distinct differences in behavior between short-chain and long-chain ceramides, and between purely aliphatic ceramides and ceramides containing a fluorescent moiety. While fluorescence is a useful property, interpretation of the results of such sphingolipid experiments should be made cautiously.

CRITERION (F): THE FORMATION OF CERAMIDE OUGHT TO BE ACCOMPANIED BY LOSS OF A CERAMIDE PRECURSOR

In some studies showing accumulation of Cer as the result of stimulating sphingomyelinase, researchers have also reported loss of sphingomyelin, making it likely that the Cer was indeed formed by one or more SMases. In fact, the suggestion was offered that the loss of sphingomyelin was the cause of apoptosis. In most studies with an inhibitor of Cer glucosylation, GlcCer was found to disappear and Cer was found to increase, with production of apoptosis. In this case, the apoptosis was the result of two changes: the disappearance of a growth stimulator (GlcCer) and the appearance of the pro-apoptotic factor, Cer. In the production of apoptosis by stimulation of *de novo* sphingol biosynthesis, one ought to be able to demonstrate a decrease in the levels of serine and palmitic acid. In the stimulation of sphingol acylation, one ought to detect a lower level of sphingols and long-chain acyl-CoA.

Such 'coordinate assays' act to validate the experimenter's analytical method for determining Cer changes.

THE HYPERSENSITIVITY OF MULTIDRUG RESISTANT CELLS TO BLOCKADE OF CERAMIDE GLUCOSYLATION

This hypersensitivity was first reported in 1994 in a study of PDMP effects [67]. Two strains of CHO cells were

compared to an MDR strain of CHO cells, which makes much multidrug-resistance protein, P-gp. Incubation in 5 μM DL-PDMP for 5 days completely blocked the growth of the MDR cells while producing little or no effect on two other cell types. The latter required 30 or 50 μM PDMP to block growth completely, a typical effect seen in a wide variety of cancer cells.

A more detailed demonstration of the importance of the glucosylation pathway was shown in the laboratory of M. Cabot. This group found that MDR cells have a far higher activity of GlcCer synthase and content of GlcCer than the 'wild-type' parental cells [68,69]. Two additional GSLs, probably GalGlcCer and globotriaosylCer, were also greatly elevated. Incorporation of [^{3}H]palmitate into the GlcCer was ≈eight times faster in the MDR cells, suggesting that ceramide and sphingol synthesis was also much faster. Patients exhibiting high GlcCer levels in their tumors failed conventional chemotherapy [69], showing the relevance of this MDR aspect. These are very important points that distinguish normal tissue from MDR tumors and make the latter sensitive to control by Cer elevation therapy.

An additional test of the connection was made by transfecting GlcCer synthase into wild-type (parental) breast cancer cells [70]. Selected clones, activated with doxycycline, exhibited up to an 11-fold increase in the activity of GlcCer synthase and high resistance to apoptotic damage by doxorubicin (a common test for MDR). The transfected cells also became resistant to the toxic action of exogenous ceramide, unlike the parental cells. Evidently, as the authors concluded, this was due to rapid conversion of the added ceramide to GlcCer in the MDR cells. The parental cells, which did not glucosylate their Cer rapidly, were readily killed by exogenous Cer. A similar comparison with P388 leukemia cells and an MDR variant strain showed that C_6-Cer was somewhat more toxic to the parental strain [71]. Note that this difference in response to Cer by the two kinds of cancer cells is unlikely to support the belief that Cer uptake experiments give artefactual results. It was possible to block the resistance to doxorubicin damage by transfecting the MDR cells with an antisense polynucleotide active against ceramide glucosylation [72]. In other words, the MDR effect was induced simply by specifically raising the ability of the cells to accumulate GlcCer and removed by simply slowing GlcCer synthesis.

The same sensitivity to Cer accumulation was seen with the glucosyltransferase inhibitor, 1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP), and a group of KB carcinoma cell lines [73]. Here, the inhibitor produced marked apoptosis and decreased viability in the MDR cell lines, as well as loss of poly(ADP-ribose) polymerase (PARP). The PARP cleavage was quite visible even within 2 h. Direct addition of ceramide to MDR and parental cells showed the same kind of difference [74].

The important conclusion to draw from these studies is that the most aggressive type of cancer cell, the MDR cell, is very sensitive to apoptosis from its rapidly forming endogenous ceramide, provided that its diversion to mitogenic sphingolipids is blocked. Including a stimulator of Cer synthesis, like PSC 833, should augment the treatment. These are encouraging findings for patients who no longer respond to the usual treatments.

The high concentration of P-gp in MDR tumors may simply be an inductive response to the rapid production of GSLs which apparently have to be brought to the cell surface and expelled into the extracellular fluid by P-gp [20]. Thus P-gp may be responsible for GSL shedding by tumors [75] and its activity in expelling absorbed anticancer drugs is simply an accidental byproduct of the phenomenon. The dependence of P-gp on GSL levels was shown in neuroblastoma cells treated with DL-PDMP, which gradually leads to loss of cellular GSLs [76]. The GSL-depleted cells demonstrated slower ejection of labeled taxol and vincristine, which the authors attributed to a possible combination between the PDMP and P-gp (and/or multidrug resistance protein). However it is possible that the lowered concentration of GSLs led to a lower level of the transport proteins. Other evidence in this study, incidentally, suggested that taxol itself also inhibits GlcCer synthesis, which may account for its anti-neoplastic activity. Similar evidence for the ability of GSLs to promote drug resistance was obtained with colchicine-adapted colon carcinoma cells, which accumulated a different drug-resisting protein, MRP1, plus Cer, GlcCer, and GalCer [77].

THE PROLIFERATIVE EFFECTS OF GLUCOSYLCERAMIDE ACCUMULATION

A major theme in this paper is the importance of the balance between pro- and anti-apoptotic sphingolipids in which GlcCer plays a major role. While considerable evidence for this role has appeared, it is little known and warrants elaboration here. The first indication that GlcCer promotes cell proliferation and growth came from the observation that Gaucher disease patients gradually develop a greatly enlarged spleen and liver. These patients produce a defective, mutated form of GlcCer glucosidase, which is not active enough to prevent accumulation of GlcCer. Treating the patients with a modified form of β-glucosidase stops the accumulation, slowly eliminates stored GlcCer, and tends to normalize the enlarged organs.

An animal model of Gaucher disease was created by injecting a large amount of GlcCer, which became concentrated in the liver, producing a liver weight increase of ≈25% within 25 h [78]. The liver growth included increases in total protein, DNA, total lipids, thymidine kinase, and ornithine decarboxylase, all suggestive of a normal mitogenic reaction to the absorbed GlcCer. The extra liver disappeared fairly soon as the (normal) GlcCer glucosidase cleaved the GSL, showing considerable dependence on the lipid for the normal size of the organ.

Cultured cells also grow faster when exposed to inhibitors of GlcCer glucosidase (conduritol B epoxide and *N*-hexyl-glucosylsphingosine), which force GlcCer accumulation [79,80]. The same has been found in mice that were injected eight days with the epoxide: the brains were 13% heavier and the livers were 9% heavier [81]. The number of cells growing freely in the abdomen of mice inoculated with Ehrlich ascites carcinoma cells increased 50% after five daily injections of GlcCer [13]. As one would predict, inhibition of GlcCer synthesis blocks the growth of all cells tested, further supporting the role of

GSLs in proliferation. This use of glucosylation inhibitors looks promising for abnormally enlarged tissues [82].

Several kinds of experiments demonstrated the important role of GalGlcCer in promoting proliferation of the epithelial cells lining arteries [83]. Additional kinds of evidence for glucosphingolipids as stimulators of cell growth have been reviewed [84,85].

INTERPRETING EXPERIMENTS WITH ENZYME INHIBITORS

The literature on Cer apoptogenesis contains many examples of the use of inhibitors to explain the mechanism producing Cer accumulation and apoptosis. Unfortunately, most of these studies have made the simplistic assumption that the observed changes were due to simply slowing the enzymatic step. Little thought has been given to the concept that inhibiting an enzyme leads to depletion of the normal enzyme products' products due to normal catabolism of those products (e.g. PDMP causes not only depletion of GlcCer but also of the higher GSLs). In addition, there can be accumulation of the enzyme's substrates and diversion of these precursors to other bioactive products. A common example is the use of fumonisin B1, which blocks sphingol acylation and should therefore produce loss of Cer, stimulate growth, and prevent apoptosis. However, studies of this inhibitor, a significant public health problem because of its frequent contamination of cora, have shown that sphinganine and sphingosine accumulate. These compounds are active as producers of growth inhibition and stimulation, possibly undergoing faster phosphorylation to form sphingol phosphates. In the case of Swiss 3T3 fibroblasts, DNA synthesis was stimulated by the inhibitor [86]. Ketosphinganine, the intermediate in sphinganine biosynthesis, was found to undergo acylation with stearoyl CoA to form a keto Cer [37]. If fumonisin blocks this acylation too, it may well produce accumulation of the keto sphingol, a reactive sphingolipid which could be responsible for some of fumonisin's toxic effects.

Interpreting experiments using inhibitors is also fraught with the danger of inadequate knowledge of the reactions blocked by the inhibitor. For example, *N*-oleoyl ethanolamine is generally considered to inhibit only ceramidase, but inhibition of GlcCer synthesis has been reported [58]. In addition, exposing WEHI-231 cells to the amide activated neutral sphingomyelinase, thus elevating Cer production and ROS [87]. It also induced mitochondrial permeability, cytochrome c release, and apoptosis.

PDMP, which is usually described as an inhibitor of ceramide glucosylation, has also been found to inhibit galactosylation of GlcCer, sialylation of GalGlcCer, synthesis of globotriaosyl Cer, the enzymes that synthesize and hydrolyze 1-*O*-acyl C_2-ceramide, and the endoglycosidase that cleaves GSLs. However most of these actions simply block Cer consumption and cause loss of GSLs.

The concentration of inhibitor is an additional variable, since the different inhibitory effects depend on the different K_i values. At low concentrations of PDMP and a similar drug, PPPP, ceramide glucosylation was rather effective, but at higher concentrations Cer was found to accumulate, with consequent growth inhibition or apoptosis [88]. It is clear that ambiguity can result from failure to use more than one kind of inhibitor and analyze for more than a few sphingolipids.

WHAT IS TO BE DONE NEXT?

There is a clear need to analyze cells or tissues (normal and cancerous) for more than a few sphingolipids, and to use more than one kind of inhibitor, if one is to deduce a reasonably unambiguous interpretation of experiments on apoptosis or proliferation. The same applies to comparisons of different cell types, such as parental and MDR cells. Assays for different sphingolipid enzymes should be included. Science grows by making strong deductions, but experimenters must furnish strong data.

There is an urgent need to move to animal cancer experiments with poly-drug mixtures that can elevate Cer levels and lower the levels of anti-apoptotic sphingolipids. Once success is obtained with monoclonal tumor inoculations, tests should be run with more natural tumors consisting of complex, evolved clonal mixtures. MDR tumors, the most aggressive kind of tumors, should be examined for sensitivity to Cer poly-drug therapy. The use of immunologically impaired mice should be avoided as much as possible, since Cer poly-drug therapy can be expected to improve anti-tumor immunological responses [75,89]. Surviving animals should be tested for the formation of an immune response, by (at the minimum) reinoculating them with the same cancer cells.

Future studies of Cer uptake should more often use long-chain ceramides complexed with a truly water-soluble carrier, preferably a mixture of lipid-transport proteins. Future use of Cer glucosylation inhibition should utilize the newer, much more potent analogs of PDMP [90]. An intensive effort should be devoted to synthesizing and testing new drugs that might be more effective inhibitors or stimulators of Cer synthesis. Many 'lead' compounds are available (Table 1) as guides for the design. The compounds should be tested not only in specific sphingolipid enzyme assays, but also in intact cells that have been incubated with the test drug, as some drugs induce enzyme changes indirectly.

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