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COMMENTARY

Chemotherapy by Slowing Glucosphingolipid Synthesis

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ABSTRACT. The hypothesis offered here is that many different illnesses could be treated by slowing the synthesis of glucosphingolipids (GSLs) with a suitable inhibitor. In people with inadequate hydrolases for the GSLs (e.g. Gaucher's disease), the lipids accumulate to a pathological degree. It should be possible to eliminate the accumulation by slowing the synthesis of the GSLs to match the ability of the patient to degrade them. In people with cancer, the tumors secrete excessive amounts of GSLs, which block the ability of the immune system to attack the tumor cells. By blocking the synthesis of tumor GSLs, it should be possible to enable the patient to generate antibodies and activated T cells that can destroy the tumor. Tumors exhibiting multidrug resistance may do so by synthesizing GSLs even faster than usual. It should be possible to restore the sensitivity of the tumor to anti-cancer drugs by inhibiting their synthesis of GSLs. Metastasis of tumors also appears to require the formation of GSLs, so an inhibitor should help block tumor dissemination. Diabetics tend to have high levels of blood glucose, which acts to stimulate kidney growth via more rapid synthesis of GSLs. This pathological growth can be blocked by inhibiting the formation of kidney GSLs. Viruses, bacteria, and bacterial toxins have been found to bind to specific GSLs of human and animal cells. Presumably, this binding leads to the damaging process of infection. It should be possible to treat such infections by depleting the host's body of its GSLs. BIOCHEM PHARMACOL **57**;6:589–595, 1999. © 1999 Elsevier Science Inc.

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The GSLs† comprise a group of ~300 naturally occurring compounds derived from the lipoidal precursor (ceramide), glucose, and assorted sugars. GlcCer, made from ceramide and UDP-glucose, is the precursor of all the other GSLs. GlcCer, LacCer, and the gangliosides (sialoGSLs) are the ones most easily detected.

The pressures of evolution have, no doubt, acted to set the tissue levels of the GSLs close to their optimal ones, so that whatever it is that they do, they do optimally. However, studies of cells and animals treated with GSL synthase inhibitors, and organisms naturally deficient in GSLs, have shown a curious lack of essentiality. It is true that the growth rate of the deficient cells is low but—in a laboratory environment—the cells and animals seem to be quite healthy. This article offers evidence suggesting that many people would benefit greatly by slowing their rates of GSL synthesis.

GLUCOSPHINGOLIPIDOSES

Gaucher's disease is a genetic disorder in which patients are unable to hydrolyze GlcCer at an adequate rate. Thus, the lipid accumulates in organs with a particularly high GlcCer turnover rate. Fortunately, the discrepancy between rate of accretion and rate of hydrolysis is rather small in most patients, so the lipid accumulates slowly. A similar problem is seen in patients who accumulate the more complex GSLs, as in Tay-Sachs disease, Fabry's disease, and gangliosidosis. For all these patients, it would seem obvious that there is a need to slow GSL synthesis to match the hydrolytic capability of the patient. This can be done with any of several available synthase inhibitors.

During the initial period of synthase inhibition, one should expect that the accumulated GSL would be eliminated gradually by hydrolysis and that the cells storing the GSL would regain normal appearance and function. The dosage of inhibitor can then be reduced to the maintenance level, balancing the rate of synthesis to match the residual hydrolase activity in the affected individual.

This concept, proposed several times—initially in 1980, and in greater detail in 1996 [1]—has been supported recently by a study with a mouse strain exhibiting a form of Tay-Sachs disease [2]. Here the inhibitor used was *N*-butyldeoxynojirimycin, which blocks GlcCer synthase, as well as an α-glucosidase. The study showed that the mice

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† *Abbreviations:* GlcCer, glucosylceramide; GSL, glucosphingolipid; IL, interleukin; LacCer, galactosylglucosylceramide; MDR, multidrug resistance (resistant); and PDMP, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol.

could be normalized by slowing the rate of GlcCer synthesis. However, it was not shown that accumulated lipid could be destroyed by the inhibitor.

While no suitable animal model exists for Gaucher's disease, it has been shown in normal mice that PDMP (a potent inhibitor of ceramide glucosylation) and chlorpromazine (a tranquilizer) rapidly lower the level of tissue GSLs. L-Cycloserine, an inhibitor of the first step in sphingosine synthesis, augmented the reduction. No toxic effects were seen.

From these findings, it appears likely that simple inhibitors could readily replace the currently used very expensive treatment with human β-glucosidase or a risky bone marrow transplantation.

GLUCOSYLCERAMIDE AS A GROWTH STIMULATOR

The organs that accumulate the most GlcCer in Gaucher patients (spleen, liver, and the marrow in the weight-bearing long bones) grow abnormally large. In the case of the spleen, the large size can result in excessive blood cell destruction (i.e. the organ is rather normal despite its enlarged state). In the case of bone marrow, the growth takes place inside a rigid cage of bone, so that the inner part of the bone dissolves, leaving a thinned, fragile shell. My basic proposal is that GlcCer or a closely related GSL is a growth stimulator. Here are some of the experiments supporting this hypothesis.

(a) Analysis of the blood of Gaucher patients revealed elevated levels of the growth factor M-CSF (2- to 8-fold) [3]. Another cell component associated with cell proliferation, protein kinase C, was found to increase in MDCK cells whose level of GlcCer had been increased by treatment of the cells with conduritol B epoxide [4]. This inactivator of the β-glucosidase that catabolizes GlcCer (and, therefore, causes GlcCer accumulation) produced an elevation of the kinase in both of its forms, membrane-bound and cytosolic. The increase in GlcCer also increased the ability of the cells to incorporate [^{3}H]thymidine. As one would expect from its inhibitory effect, PDMP produced opposite changes.

(b) A similar stimulation of growth and GlcCer and DNA formation was seen in mouse skin when bromoconduritol B epoxide was applied to the surface to produce GlcCer accumulation. Intracutaneous administration of GlcCer also stimulated epidermal DNA synthesis, while simultaneous treatment with conduritol B epoxide plus GlcCer resulted in an additive increase in DNA synthesis [5].

(c) Young mice rendered glucosidase-deficient with conduritol B epoxide were found after 7.3 days to have significantly enlarged brains (+13%) and liver (+9%) [6].

(d) Stimulation of cell growth by exogenous GlcCer was demonstrated with Ehrlich ascites carcinoma cells growing in mouse abdomens [7]. A suspension of GlcCer (a very insoluble lipid) was injected i.p. into the mice each day for 8 days. The volume and number of tumor cells were found to be 52% higher than in untreated mice. As expected, the volume of cells in mice treated with PDMP was markedly lower than in the controls (−66%). This showed that the supplemental (exogenous) GlcCer was insufficient to overcome the loss in GSLs resulting from the PDMP.

(e) Gangliosides from the plasma of mice carrying Ehrlich ascites cells were also found to stimulate tumor growth [8]. The gangliosides were taken up in PBS with the tumor cells, and the suspension was inoculated i.p. into normal mice. Control mice were injected with similar cells that were treated with the plasma gangliosides from healthy mice. After 2 days, the number of tumor cells found in the peritoneum was found to be double that seen in the control mice. Perhaps the stimulation of proliferation was due to hydrolysis of the gangliosides to form GlcCer or, conversely, perhaps the stimulation by GlcCer in other studies was due to faster conversion to gangliosides.

(f) When a suspension of GlcCer was injected i.p. into normal mice, the liver was found to grow rapidly. Within 23 hr there was a 15–30% increase in the weight of total liver and liver DNA, lipids, and protein [9]. In other words, the liver simply grew—it was not distended by hydration or by accumulation of a single substance. It is possible that the enlargement was due, partially or completely, to formation of a specific cell type, such as macrophages, which seem to have a propensity for accumulating GlcCer.

(g) Kidney growth in young male mice is much greater than in females. This parallels the differing changes with age in GlcCer synthase specific activity. Injection of testosterone into the young males and females induced a higher level of GlcCer synthase in the kidneys and stimulated kidney growth markedly [10]. Apparently as a result of a coordinating control system, GlcCer glucosidase decreased. This effect thus augmented the effect of the increase in GlcCer synthase. Conversely, the female hormone 17β-estradiol slowed kidney growth and lowered GlcCer synthase activity (only in female kidney). The hormone also raised the glucosidase activity.

Male mouse kidneys contain a relatively high amount of LacCer, but those of females contain none. The level of the stage-specific embryonic antigens (a family of complex GSLs) was found to be ~4 times higher in male mouse kidney.

The above observations support the hypothesis of a strong relationship between kidney growth and GlcCer or GSL levels. They also point to the potential value of blocking GlcCer synthesis in people with kidney cancer. The prostate gland and prostate cancer may react similarly.

(h) It is significant that Gaucher patients display an abnormally high incidence of cancers of the B cells, including multiple myeloma. Perhaps a cancer that would ordinarily take several years to become manifest becomes visible more rapidly if an organ's level of GlcCer is elevated.

(i) People with diabetes tend to develop enlarged kidneys that ultimately fail and cause death. It had been shown previously that the level of UDP-glucose in rodent tissues is

elevated when the blood sugar level is elevated. From a kinetic study of GlcCer synthase in normal rat kidney, we know that the enzyme is not saturated with UDP-glucose. This means that an elevation in UDP-glucose should produce a higher rate of GlcCer synthesis (unless the ceramide level is rate-limiting). These relations were modeled in diabetic rats, which were allowed to develop enlarged kidneys [11]. Injection of PDMP blocked the enlargement, as did normalization of blood glucose by insulin. This study indicated that diabetics whose control of hyperglycemia is inadequate may make too much UDP-glucose and GlcCer, with consequent excessive kidney growth. The use of drugs of the PDMP family ("P-drugs") in diabetics may prove useful as a preventative.

The same phenomenon may be involved in the tendency of diabetics to develop atherosclerosis, since Chatterjee [12] reported that LacCer stimulates growth of the smooth muscle cells of blood vessels. It could be important to see if the rate of LacCer synthesis in these cells (especially human cells) is indeed accelerated by high blood glucose levels. If so, treatment with a P-drug might normalize the growth of the cells and control atherosclerosis.

As a corollary to these observations, one can hypothesize that diabetics will show increased morbidity for some kinds of cancer because of the proliferative effect of GSLs. Epidemiological relationships between diabetes and cancer incidence have certainly been observed. P-drugs may contravene the correlation, and diabetics with cancer may be expected to respond to the P-drugs even more favorably than other people. It would be desirable to study the GlcCer synthase in different human organs to see if the enzyme is not saturated by the local normal level of UDP-glucose.

(j) Gangliosides have been observed to stimulate axonal sprouting in neuronal cultures. A drug (the optical enantiomer of PDMP) that induces elevated synthesis of the simpler GSLs and elevated ganglioside levels was found to stimulate the outgrowth of axons from neurons [13]. Conversely, a P-drug prevented ganglioside synthesis and neurite formation [13].

PDMP readily penetrates the brain, and it is possible that its therapeutic use to slow the biosynthesis of GlcCer in extraneural tissues would lead to neurological difficulties. This problem, if it does occur, could probably be treated by synthesizing a P-drug that does not enter the brain readily. On the other hand, the permeability of PDMP through the blood–brain barrier enhances its value in treating glucosphingolipidoses and brain cancer.

Complicating this story are observations that the proliferation of certain kinds of cells is *inhibited* by GSLs, particularly certain gangliosides. This has been seen with human bone marrow mononuclear cells [14]. However, the concentration of added GSL may be an important factor in determining whether the effect is stimulatory or inhibitory [15]. Unfortunately, most of the studies examining the correlation between GSLs and cell proliferation failed to analyze the cell lipids to measure the GSL uptake or to see if a GSL metabolite (not the exogenous lipid) might be the probable cause of the effect. Nevertheless, it is clear from many studies that GSLs can have profound effects on cell proliferation.

(k) Pooled human immunoglobulins for intravenous use inhibit the proliferation of peripheral blood lymphocytes in allogeneic mixed lymphocyte reactions and of autonomously growing human and mouse cell lines. This effect was found to be mediated by binding of the antibodies to membrane GSLs [16]. Pooled IgG given to patients often yields significant clinical improvement, especially in autoimmune disease, cancer, and infection. This is in agreement with the hypothesis that GSLs are involved in these disorders and that a P-drug would produce the same beneficial effect as the IgG (without the batch-to-batch variation in pooled IgG).

CANCER CHEMOTHERAPY AND GSL DEPLETION

The Peculiar Relationships between Tumors and Glucosphingolipids

This topic was reviewed in 1988, with many supporting references, and many of the hypotheses proposed here were offered then [17].

Novel GSLs, never before detected in normal tissue, have been isolated in small amounts from tumors. This automatically makes them potential actors in the lethal process of cancer formation and development. It is possible that significant properties of cancer cells stem from the novel GSLs. Unfortunately, none of the researchers who isolated the tumor GSLs appears to have studied their effects on tumor or normal cells. This is a major hole in the growing field of cancer GSL biochemistry.

Perhaps the novel GSLs occur in normal cells and perform vital functions, but their levels are so low that they are normally undetected. If chromosomal damage should occur in a single cell, causing the normal degradative enzyme (a specific hydrolase) to become defective, there would be gradual accumulation of the GSL, and this might lead to the lack of growth control that is typical of tumors. In effect, cancer would then be a *micro*sphingolipidosis in just a single cell type. GSL degradation usually or always involves special protein "cohydrolases," so a defective gene for such a protein would also cause accumulation of the lipid.

Hakomori [18] has attributed the appearance of the novel GSLs to "aberrant glycosylation"—the activation of a normally inactive gene that codes for a novel glycosyltransferase. Examples of such genes have indeed been found. Another form of aberrant glycosylation might exist in which the normally quiescent gene produces an activating protein that stimulates the synthesis of a slowly forming GSL. No such proteins are known, but it would be unusual to have a series of synthases that are not under control of this sort. Preliminary evidence for the existence of a natural stimulator of GlcCer synthesis was found in the kidneys of

polycystic mice [19]. These kidneys exhibit a high level of GlcCer, LacCer, and ganglioside GM3, and excessive proliferation.

Some of the GSLs initially discovered in tumors have subsequently been found to occur in much lower concentrations in normal cells. They can act as antigens, and attempts have been made to treat melanoma patients with monoclonal antibodies to ganglioside GD3, a GSL typically found in tumors. However, little benefit came from these attempts, possibly because of the limited amounts of antibody available.

It is significant that LacCer, originally thought to be a cancer-specific GSL, stimulates the proliferation of certain normal cell types [12]. Perhaps different GSLs are mitogens for different cell types, so there may be multiple cancer-promoting GSLs.

It is possible that certain tumors have a high content of a toxin-binding GSL, such as globoside. Such tumors might succumb to treatment with vero or Shiga toxin (in the case of globoside) or with botulinum or cholera toxin (for tumors rich in gangliosides). Conversely, these tumors might be very sensitive to the GSL-depleting action of a P-drug. Botulinum toxin has been used clinically in treating cases of excessive muscle contraction, suggesting an important role for gangliosides in muscle control. A P-drug might replace the toxin in such applications.

Glucosphingolipids as Blockers of Immunological Rejection

The chemical "foreignness" so typical of tumors has long provoked wonder at the inability of the body to mount an effective immunorejection reaction. Attempts have been made to develop "cancer vaccines" that might provoke a useful response from the tumor host, but these have generally been disappointing. Reports from several laboratories, especially those of Ladisch and of Ravindranath and Morton [20, 21], have indicated that tumors secrete ("shed") a water-soluble material that blocks the expected proliferative response by T cells. This material appears to be a mixture of gangliosides. Apparently tumors shed much of the gangliosides that they synthesize (see "e" above).

The blocking effect of gangliosides may devolve from their ability to block the binding of IL-2 to its receptors on T cells [22]. IL-2 normally acts to stimulate the proliferation of T cells, and gangliosides apparently bind competitively to the IL-2 receptors.

Shedding has been detected in some patients by determining the level in blood of tumor ganglioside. The concentration of the tumor-specific ganglioside was shown to be related to the body burden of tumor, responding to chemotherapy. It was accordingly proposed that specific ganglioside analysis could be used as a diagnostic tool and as a monitor of the effectiveness of therapeutic interventions. Ladisch and co-workers [14] found differing immunosuppressive effects with different gangliosides, which may explain why the normal mixture in plasma is not strongly immunosuppressive.

A recent paper reported that a P-drug prevented cultured tumor cells from shedding gangliosides into the medium, from which they were taken up by the target fibroblasts [23]. It would seem important to test the inhibitor in tumor-bearing animals and look for the actual formation of antitumor antibodies and T cells. The same P-drug has been tested *in vitro* with over 80 human tumor cell lines and found to inhibit the growth of every cell line almost equally well. This bodes well for the possibility of blocking all tumors with P-drugs. Unpublished work from two laboratories (Jin-ichi Inokuchi and James Shayman) has yielded new P-drugs that are much more effective against GlcCer synthase.* The growth-inhibiting P-drugs cause accumulation of ceramide, the lipoidal precursor of GlcCer. Ceramide is presently receiving much study as a participant in the apoptotic process, possibly acting via stimulation of a protein phosphatase-2A [24].

A preliminary test of the pro-immunoresponse effect of a P-drug was reported 11 years prior to the above experiment [23]. Mice were inoculated i.p. with Ehrlich ascites carcinoma cells, followed 1 day later by 10 daily injections of PDMP [7]. Untreated (saline-injected) controls died ~24 days after tumor inoculation, while 50–70% of the treated mice were still alive after 60 days. About 30% of the treated mice were alive and healthy-looking several months later, apparently permanently cured.

Nine of the cured mice were challenged with a new tumor cell inoculum, and eight were found to be immune to the new tumor cells. It appears that the GSL-depleted tumor cells were able to act as effective antigens in ~11 days. Unfortunately, the actual presence of antibodies or activated T cells was not determined.

It is important that the P-drug, unlike most antineoplastics, did not damage the immune system or affect blood cell concentrations. Surprisingly, virtually all published tests with other antineoplastics have not included a test for the generation of an immune response. Cancer researchers are beginning to recognize the importance of generating an immunological response, which ensures that the (apparently) cured patient will destroy tumor cells that survived surgical excision, radiation, or chemotherapy. The recent popularity of drug tests in immunologically disadvantaged (nude) mice is an unfortunate rejection of this concept. While testing human cancer cell lines in nude mice gives a hint as to the eventual usefulness of a new drug in humans, the test omits a vital criterion.

A similar drawback exists for the new NIH approach to improved quantitation of growth inhibition. In this elegant procedure, cancer cells are inserted into hollow porous fibers, which are implanted into an animal. Test drugs can penetrate the fibers but cell-mediated immunity cannot be generated.

On the basis of the above observations, I think that a

*Cited with permission.

very useful cancer "vaccine" would consist of cancer cells that are removed from the body of the patient and cultured a few days in the presence of a P-drug to deplete them of their GSLs. At the same time, the patient should be treated systemically with the same P-drug to deplete the remaining tumor cells and thereby block their interference with the immune response. The depleted cells, with an adjuvant, should then be reinjected. This inoculum, with its severely reduced ability to shed gangliosides, should act as a good antigen. As Ravindranath, Morton, and co-workers have pointed out, whole cells are more strongly antigenic than a single component [25].

In mice, PDMP was found to be attacked rapidly by cytochrome P450; thus, it left the body rapidly [26]. When inhibitors of P450 were used, much higher tissue levels of PDMP were achieved, and the biological half-life was greatly prolonged. One of the inhibitors used was cimetidine, a popular over-the-counter drug. Cimetidine has shown some promise as an enhancer of immune responses, possibly by inhibiting suppressor cells. Thus, it may have two beneficial effects—prolonging the action of P-drugs and helping the patient develop antibodies to the tumor. I suggest that it be co-administered with a P-drug in future animal experiments.

Glucosphingolipids as Mediators of Multidrug Resistance

The major cause of death from cancer is the appearance in *previously* treated patients of a mutated tumor, one that is resistant to the drugs that originally appeared to stop tumor growth and even some new drugs. Perhaps the most common mechanism for the appearance of MDR is the synthesis in tumors of abnormally high amounts of MDR proteins, such as MDR1.

An important recent report states that MDR cells, compared to the "wild type" (parental) tumor cells, contain abnormally high amounts of GlcCer and other simple GSLs [27]. A P-drug was found to lower the GSL content of the resistant cells and to restore the sensitivity of the cells to an antineoplastic drug [28]. Significantly, other drugs known to neutralize the MDR effect were found to lower cellular GSL levels, suggesting that they act like the P-drug. *In vitro*, one of these drugs was found to directly inhibit GlcCer synthase, in further support of the conclusions.

Increased expression of ganglioside GM2 was observed in two Adriamycin®-resistant cell lines, in contrast to the parental lines [29]. Increased mRNA for GM2 synthase was also found in the resistant cells. An antibody, active against GM2, displayed antitumor activity in human tumor cells both *in vitro* and *in vivo*. These observations resemble and support those from the Cabot group and suggest that GSL synthases in MDR tumors act to block chemotherapy and immunorejection by rapidly forming and shedding gangliosides.

It would be wise to conduct testing of *any* proposed antineoplastic drug with the MDR variants of tumor cell lines, as well as the wild types.

Insightful observations along this line have come from van Meer and colleagues [30]. They found that MDR1 acts to translocate GlcCer and other lipids from the inner surface of the plasma membrane to the outer surface. At the outer surface, the lipids can be caused to shed by contact with lipid-binding proteins in the medium (albumin, lipid-carrying proteins, and serum lipoproteins). It is possible that MDR1 action is promoted by a high level of GSL or that its concentration in the plasma membrane can be induced by GSLs.

Glucosphingolipids as Factors in Metastasis

Metastasis of cancer cells, a major problem in cancer treatment, apparently requires GSLs. 3LL murine Lewis lung carcinoma cells are known to exhibit greatly enhanced metastatic potential when they are supplemented with GSLs by preincubation with gangliosides. When 3LL cells were incubated with 5 μM PDMP for 6 days, the cells contained only ~20% of their normal GSL level, and the GlcCer level was only 4% of normal [31]. The depleted cells were then injected i.v. into mice, which were killed after 20 days. The control cells yielded 28 pulmonary tumor colonies per mouse, and all the mice exhibited colonies in other tissues. GSL-depleted cells yielded only 8.5 pulmonary colonies per mouse and no extrapulmonary colonies.

Depleted cells that were allowed to regain some of their GSL content for 24 hr (by addition of PDMP-free medium) recovered all their metastatic capability, showing that tumors do not need a full complement of GSLs for colonization-metastasis. Depleted cells were unable to bind to dishes coated with laminin, a matrix protein that may be required for metastasis (see also Ref. 24).

GLUCOSPHINGOLIPIDS IN INFECTIONS

Many papers have shown that viruses, pathogenic bacteria, and bacterial toxins bind to the GSLs of the host. It seems very likely that the binding is necessary to produce pathological changes, and it is plausible to think that depletion of the host's GSLs would have beneficial effects. While protection against infection via lifelong, chronic depletion may be impractical, depletion after symptoms have developed should slow further development of the infection and give the host time to develop immunity. An example of pre-infection protection by PDMP has been described for cultured human A498 kidney cells [32]. The GSL-depleted cells, brought into contact with pathogenic *Escherichia coli*, bound only ~1/7 of the number of bacteria bound by untreated cells.

A potentially important observation was that the binding of the bacteria to the cells resulted in elevated production of IL-6. Generally, a high level of IL-6 in the circulating blood is associated with a poor prognosis in cancer patients. Depleting the human cells of their GSLs

not only reduced binding by the *E. coli*, but it also reduced the production of IL-6. Thus, the depletion process may have two kinds of beneficial effects.

Although pathogens may also bind to non-GSL components of mammalian cell surfaces, the depletion approach has enormous potential.

REALITY CHECK

A proposer of a widely ranging set of hypotheses must be careful to restate the biochemist's "Miranda Warnings": (a) life is far more complicated than any review article can say, and (b) extrapolating from tissue culture experiments to intact animals, particularly humans, may be dangerous to one's contact with the real world. Nevertheless, GSL depletion may really be extremely valuable. A deeper reality check can be obtained from over 400 pages of recent data about the remarkable complexities of GSL effects [33].

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