Sphingolipids suppress preneoplastic rat hepatocytes in vitro and in vivo

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Sphingolipids can modulate cell growth, differentiation and apoptosis. In the present investigation, selective death of hepatocytes localized in enzyme-altered foci (EAF hepatocytes) was shown to be induced by sphingolipids. Sphingosine (20 μM) caused rapid cell death predominantly of EAF hepatocytes in vitro. During 4 h of such exposure, cytochrome c was released from the mitochondria into the cytoplasm and the number of cells demonstrating cleaved caspase-9 activity increased. The selective sensitivity of EAF cells to sphingolipid-induced death was attenuated by tumor necrosis factor-α. In previous studies we have demonstrated that EAF hepatocytes are resistant to Fas-mediated apoptosis, a resistance shown here to be reversed by low concentrations of sphingosine. Immunohistological staining revealed higher levels of glucosylated ceramide in EAF than in the surrounding tissue. Furthermore, an inhibitor of glucosylation enhanced the toxicity of ceramide towards EAF cells. TLC analysis suggested low levels of sphingosine in preneoplastic lesions. In in vivo experiments EAF-bearing rats were fed a diet supplemented with 0.1% sphingomyelin for 2 weeks. Sphingolipid feeding reduced the number of EAF and EAF area in the liver by 40–50% as compared with rats fed a control diet. These studies indicate that the turnover of sphingolipids in preneoplastic EAF hepatocytes is altered. This alteration may explain not only the increased sensitivity of EAF cells towards sphingolipid-induced cell death, but also the resistance of these hepatocytes to cell death involving sphingolipids as second messengers. Furthermore, sphingomyelin in the diet may prevent EAF development. It is suggested that the altered turnover of sphingolipids might be a target for chemoprevention of hepatocellular carcinoma.

Introduction

Resistance to apoptosis characterizes many different types of cancer cells. Such resistance can be acquired via various mechanisms and appears to be essential for the development of cancer. Pre-neoplastic lesions in rat liver, so-called enzyme-altered foci (EAF), are also resistant to apoptosis (1–3). The growth of these foci is induced by carcinogens and they appear to be resistant, e.g. to apoptosis mediated by Fas or triggered by genotoxicity (4). Furthermore, in many cases carcinogens increase the mass of EAF by inhibiting apoptosis (5) and withdrawal of the carcinogen leads to enhanced apoptosis in EAF and many EAF may thereby disappear (5,6). Although the underlying mechanisms are not fully understood, these observations suggest that regulation of apoptosis is altered in EAF.

Sphingolipids have been demonstrated to play important roles in signal transduction involved in various responses such as differentiation, proliferation and apoptosis (7). In a variety of cell types, ceramide is generated in response to stress caused, e.g. by exposure to irradiation, UV-light or chemotherapeutic agents and this substance may function as a second messenger in connection with cell death pathways (8,9). One of the several lines of evidence supporting this conclusion is that knockout mice lacking acid sphingomyelinase exhibit defects in liver cell apoptosis (10). In addition, the resistance of acid sphingomyelinase −/− hepatocytes to Fas-mediated apoptosis is reversed by ceramide (11).

Drugs, which elevate intracellular levels of sphingolipids and ceramide have been found to induce apoptosis in transformed cells in culture (7). Dysfunctional metabolism of ceramide and other sphingolipids in cancer cells may also give rise to multi-drug resistance (12). As well as inducing apoptosis in cancer cells in vitro, sphingolipids have been reported to inhibit carcinogenesis in vivo. For instance, dietary sphingolipids inhibit the growth of different stages of 1,2-dimethylhydrazine-induced colon tumors (13–15). Recently, dietary sphingolipids have also been shown to reduce the frequency of intestinal tumors in Min mice (16). Furthermore, ceramide analogs can prevent tumor growth and liver metastases in nude mice (17). Interestingly, in this same study the level of ceramide in human colon cancer was observed to be decreased.

In the present study our major question was whether preneoplastic cells are particularly sensitive to cell death induced by sphingolipids. In order to test the hypothesis, preneoplastic glutathione S-transferase (GST-P)-positive hepatocytes isolated from DEN-treated rats were exposed to ceramide, sphingosine or inhibitors of enzymes involved in sphingolipid metabolism and effects on cell death subsequently monitored in vitro. Furthermore, the effect of sphingolipid feeding on EAF development was studied in vivo.

Materials and methods

Donor animals and primary cell cultures

Female Sprague–Dawley rats were injected i.p. with diethylnitrosamine (DEN) (0.3 mmol/kg body wt; Sigma, St Louis, MO) dissolved in 0.15 M NaCl within 24 h after birth. At 3 weeks of age, these rats were weaned and injected thereafter with the same dose of DEN once each week (18). One week following the final of seven to 11 such injections, hepatocytes were isolated from these animals.

Hepatocytes were isolated employing collagenase perfusion and then seeded onto collagen-coated plates (2 × 10⁵ cells/35 mm plate; SARSTEDT, Sweden).
The cells were cultured in complete medium for 1.5 h and thereafter in serum-free RPMI 1640 medium (Life Technologies, Paisley, UK). To this latter medium various concentrations of the test substances were added, i.e. d-sphingosine, N-acetyl-d-sphingosine (C2-ceramide), N-stearyl-d-sphingosine (C18- ceramide), N-acetyl-D-methylsphingosine, glucoseno-deoxy-cholic acid (sodium salt; GCDC) or fumonisin B1 (dissolved in DMSO; Sigma) or tumor necrosis factor-α (TNFα, Biosource International, Camarillo, CA), d-Three-1-phenyl-2-decanoylamino-1-propanol (PDMP, Sigma) was added to the culture medium 4 h prior to the addition of the test substance. After completion of treatment, the cells were washed and fixed in 3.7% formaldehyde for 1.5 h.

Immunocytochemistry

After fixation, the cells were stained with rabbit polyclonal anti-GST-P antibodies (BIO23 Yp, Biotrin, Ireland) (4) or with rabbit polyclonal anti-cleaved caspase-9 antibodies (#9507, Cell Signaling Technology, Beverly, MA). In order to detect cleaved caspase-9, the plates were pre-treated with saponin (Sigma) (18) and, following incubation with the primary antibodies, stained using the labeled streptavidin–biotin procedure (LSAB 2 kit, DAKO, Denmark). Peroxidase-conjugated secondary anti-rabbit antibodies (P217, DAKO) were utilized to reveal GST-P. Peroxidase activity was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) as substrate.

Percentage of marker (GST-P or caspase-9)-positive cells was determined by examining at least 500 cells exhibiting characteristic hepatocyte morphology and located in several different randomly selected regions on each plate. All experiments were repeated at least three times employing different batches of cells. Statistical significance was evaluated utilizing the Mann–Whitney U-test. P value < 0.05 was considered statistically significant.

Glucosylceramide immunohistochemistry

The livers of rats receiving one dose of DEN within 24 h after birth and seven additional doses of DEN (0.3 mmol/kg body wt) were used for glucosylceramide immunohistochemistry. One week following the final injection, the liver was fixed in formaldehyde as described previously (19). Appropriate sections were stained overnight by incubation with rabbit anti-glucosylceramide antibodies (AIA) and, following incubation with the primary antibodies, stained using the labeled streptavidin–biotin procedure (LSAB 2 kit, DAKO, Denmark). Peroxidase-conjugated secondary anti-rabbit antibodies (P217, DAKO) were utilized to reveal GST-P. Peroxidase activity was visualized using DAB as substrate.

Western blotting

Cells (6 x 10^6/10 cm plate) were cultured in the same manner as for immunocytochemistry. For detection of cytochrome c, the cells were washed with phosphate-buffered saline and thereafter scraped off the plates and homogenized on ice in 50 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT and 1 mM PMSF. The resulting homogenate was centrifuged, the high-speed supernatant thus obtained subjected to SDS–polyacrylamide gel and thereafter transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Protein bands were identified and detected employing monoclonal mouse anti-cytochrome c antibodies (sc-2031, Santa Cruz Biotechnology), followed by secondary anti-rabbit peroxidase antibodies (P217, DAKO). Peroxidase activity was visualized using DAB as substrate.

DMS/SDS staining

Hepatocyte cultures from DEN-treated rats were exposed to C2-ceramide, C18-ceramide, sphingosine and an inhibitor of sphingosine kinase, DMS, or solvent (0.1% DMSO) control, for 24 h. Hepatocytes were then sonicated and subjected to 2D SDS–polyacrylamide gel electrophoresis. Protein spots were visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) as substrate. The gel was then stained with Coomassie Brilliant Blue R250 and destained with water.

Table I. Selective induction of death of GST-P-positive hepatocytes by C2-ceramide, C18-ceramide, sphingosine and an inhibitor of sphingosine kinase, DMS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μM</th>
<th>% GST-P-positive cells remaining on the plates</th>
<th>Total number of cells remaining/ unit area (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2-ceramide</td>
<td>50</td>
<td>9.9 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>C2-ceramide</td>
<td>75</td>
<td>5.5 ± 2.8</td>
<td>60 ± 31</td>
</tr>
<tr>
<td>C18-ceramide</td>
<td>0.75</td>
<td>1.4 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35 ± 28</td>
</tr>
<tr>
<td>C18-ceramide</td>
<td>1.0</td>
<td>6.5 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69 ± 1.4</td>
</tr>
<tr>
<td>–</td>
<td>13 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sphingosine</td>
<td>10</td>
<td>5.4 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49 ± 0.42</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>20</td>
<td>0.93 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 ± 0.46</td>
</tr>
<tr>
<td>–</td>
<td>10</td>
<td>1.0 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49 ± 0.42</td>
</tr>
<tr>
<td>DMS</td>
<td>10</td>
<td>4.6 ± 3.9</td>
<td>56 ± 31</td>
</tr>
<tr>
<td>DMS</td>
<td>20</td>
<td>3.0 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 ± 27</td>
</tr>
</tbody>
</table>

Hepatocyte cultures from DEN-treated rats were exposed to C2-ceramide, sphingosine, DMS and C18-ceramide at the concentrations indicated for 24 h. The results are expressed as the percentage of GST-P-positive cells of total number of cells remaining on the plates or the total number of cells remaining on the plates. The data represent the means ± SD for the three independent experiments or the three different plates. *P < 0.05 compared with unexposed cells.
Exposure of cells to a long chain ceramide, C-18, also resulted in a selective loss of GST-P-positive cells (Table I). As expected (25) a lower concentration was needed to induce selection. When similar exposure to sphingosine, a metabolite of ceramide, was carried out for 24 h, the percentage of GST-P-positive cells decreased in a dose-dependent fashion (Table I). A 10 μM sample of sphingosine reduced the survival of GST-P-positive cells significantly, and this was associated with decreased number of cells on the plates.

The enzyme sphingosine kinase converts sphingosine to sphingosine-1-phosphate and inhibition of this enzyme by N,N-dimethylsphingosine (DMS) has been demonstrated to increase the level of ceramide in various types of cells (26). Treatment of hepatocyte cultures from DEN-treated rats with DMS was observed here to decrease total cell survival and to selectively decrease the survival of GST-P-positive cells, i.e. GST-P-negative cells were relatively resistant to this effect (Table I). A 20 μM aliquot of DMS reduced the relative number of GST-P-positive cells from ~10 to 3%.

In an effort to elucidate whether sphingosine and DMS exerts the effect on GST-P-positive cells by increasing the level of ceramide, an inhibitor of ceramidase, fumonisin B1 (9) was employed. However, fumonisin B1 (25 μM) treatment did not inhibit the death of GST-P-positive cells induced by a combination of sphingosine (10 μM) and DMS (10 μM), indicating a direct effect of sphingosine. In one typical experiment in fumonisins B1 treated cells the combination of sphingosine and DMS reduced the relative number of GST-P-positive cells from 7.0 ± 1.4 to 0.75 ± 0.70% (mean ± SD of three different plates).

In certain experiments, the percentage of GST-P-positive cells among all cells that detached from the plates was determined. It was found that the amount of GST-P-positive cells in the supernatant increased in proportion to the decrease in GST-P-% among cells remaining on the plates (data not shown). In other control experiments, sphingosine and ceramide were shown to induce cell death in hepatocyte cultures from untreated rats at concentrations similar to those required to cause death of hepatocytes isolated from DEN-treated rats (data not shown).

In general, the extent of cell death caused by exposure to sphingolipids was found to be dependent on cell density (data not shown). Because of a certain variability in the density of the primary cultures of hepatocytes, minor differences in responses to the same concentration were observed in different experiments. Variation within single experiments was small (compare with the effect of sphingosine in Table I). This suggests that the amount of e.g. sphingosine per cell, rather than the concentration in the medium, determined the responses.

Figure 1A documents the time-course of these effects of sphingosine on GST-P-positive cells during 4 h of incubation. A significant decrease in GST-P-% was induced already after 2 h incubation, a reduction associated with decreased total cell survival. The percentage of cells remaining on the plates decreased from 61 ± 1.7% after 1 h exposure to 36 ± 4% after 4 h of exposure. These effects of sphingosine rapidly reached their maximal level, being only slightly greater after 24 h of incubation. In order to characterize the mechanism(s) involved in the cell death observed here, we examined the possible effects of sphingolipids on mediators of apoptosis signals. Release of mitochondrial cytochrome c was enhanced after 1–4 h of exposure to sphingosine (Figure 1B). Caspase-9 activity was monitored employing immunocytochemical staining with antibodies directed specifically towards cleaved caspase-9. A significant increase of activated caspase-9 expressing cells after 1 h of such exposure was monitored (Figure 1C). Primary cultures of hepatocytes have recently been shown to be resistant to ceramide-induced apoptosis (27). However, similar concentrations of ceramide did, indeed, induce cell death in our cultures. The reason for this apparent discrepancy is not known, but may involve the use of different conditions for cell culturing.

We have demonstrated previously that GST-P-positive cells from DEN-treated rats are resistant to Fas-mediated apoptosis (4), a process shown by others to involve sphingolipids (11). Consequently, the effect of sphingosine on the resistance of GST-P-positive cells towards Fas-mediated apoptosis was examined here by using glycochenodeoxycholic acid (GCDC), a cholic acid known to activate Fas in hepatocytes (28). As depicted in Figure 2A, the resistance of GST-P-positive cells...
against GCDC was reversed by sphingosine, 10 μM of this compound abolishing the effect of GCDC on the percent of GST-P-positive cells totally. In contrast, sphingosine had no effect on the total cell death caused by GCDC, as reflected by the number of cells remaining on the plates (Figure 2B). In this experiment 10 μM sphingosine alone had no effect on the percent of GST-P-positive cells (Figure 2A) or on the number of cells remaining on the plates (data not shown).

The cytokine TNF-α exerts a number of biological effects, including activation of several key enzymes involved in sphingolipid/ceramide metabolism (29,30). Thus, TNF-α has been demonstrated to activate sphingomyelinase and sphingosine kinase and thereby increase the level of sphingosine-1-phosphate in cells (30). Figure 3A documents the ability of TNF-α to protect against the decrease in the relative number of GST-P-positive cells induced by sphingosine. This protective effect apparently did not extend to GST-P-negative cells, as the total numbers of cells on the plates were not significantly affected by the presence of TNF-α (Figure 3B).

Sphingolipids have been shown to influence cell proliferation primarily by increasing the intracellular level of sphingosine-1-phosphate, which apparently serves as a second messenger in connection with this process (7). Therefore, the effect of sphingosine on the levels of p27, cyclin E and D2, which all vary during the cell cycle, were monitored. Exposure to this substance for 4 h was associated with a concentration-dependent decrease in p27 expression and an unchanged level of cyclin E (Figure 4), whereas the level of cyclin D2 was increased by 24 h of exposure. Higher concentrations of sphingosine were used in these studies due to the need for high cell density for western blot analysis. Together with the effects obtained with DMS (Table I) and TNFα (Figure 3) the data suggest that added sphingosine might modulate signal transduction, influencing both cell death and proliferation.

Glucosylation of ceramide confers resistance to apoptosis on many types of cancer cells (31). As shown in Figure 5, cell death induced by ceramide was potentiated by PDMP, an inhibitor of such glucosylation (32), which decreased the relative number of GST-P-positive cells from 2.5 ± 0.2 to 0.2 ± 0.2%. No significant effect of PDMP on total cell survival was observed (data not shown). These findings prompted us to...
examine the level of glucosylceramide (GlcCer) in EAF tissue. An immunohistological study indicated the presence of higher levels of GlcCer in many EAF compared with the surrounding tissue, which exhibited only weak staining (Figure 6A). It was seen that the staining in EAF tissue was concentrated to cytoplasmic vesicular structures.

Lipid extracts from nodules and surrounding tissue were separated by TLC. As can be seen, in Figure 6B nodular extracts gave smaller dots with the retention time corresponding to sphingosine than extracts from surrounding tissue. For sphingosine-1-phosphate smaller dots were obtained in two of these extracts. No marked difference of C18 ceramide was detected. The data suggest alterations in sphingosine content of EAF tissue. However, a more definitive analysis is needed to confirm this and to elucidate possible other changes in sphingolipid content.

Earlier studies have shown that sphingomyelin feeding reduced the number of 1,2-dimethylhydrazine-induced colon tumors (13–15). The effect of sphingomyelin in diet (0.1%; the same concentration as used in ref. 14) on DEN-induced EAF is shown in Figure 7. Sphingomyelin reduced the number of EAF by 40%. Thus, rats fed the control diet exhibited 74 ± 7.4 EAF/cm² liver tissue and rats fed the sphingomyelin diet exhibited 44.6 ± 6.4 EAF/cm² (Figure 7A). EAF area fraction (EAF area/liver section area) was reduced by 50% (Figure 7B).

To study the effect on the smallest EAF the effect of sphingomyelin on the number of single- and double GST-P-positive hepatocytes (33) was monitored. Also, this parameter was reduced by sphingomyelin diet, from 2.3 ± 0.4 to 1.3 ± 0.3 positive cells/mm², a reduction by 43% (Figure 7C). No effect on liver morphology or growth of the animals was detected (data not shown).

**Discussion**

In the present study we have demonstrated that sphingolipids selectively induce cell death in preneoplastic EAF hepatocytes in vitro and that sphingomyelin in diet can reduce the number of EAF in vivo. Ceramide, sphingosine and an inhibitor of sphingosine kinase all activate a cell death pathway in such GST-P-positive cells in vitro. We also provide evidence suggesting that sphingolipids play a role in the resistance of these hepatocytes to apoptosis. An altered regulation of sphingolipid metabolism may explain both the increased sensitivity of EAF cells to death induced by sphingolipids and the resistance to cell death pathways in which sphingolipids act as second messengers.

It is well established that addition of sphingolipids to cells in culture or elevation of endogenous ceramide levels by, e.g. inhibition of sphingosine kinase gives rise to apoptosis in many types of cells (9). Accumulation of ceramide and sphingosine appears also to be involved in hepatocyte apoptosis in vivo (34). In this study the death induced selectively by various sphingolipids in GST-P-positive cells in vitro occurred rapidly. During 4 h of exposure, cytochrome c was released into the cytoplasm and the number of cells staining positively for activated caspase-9 increased. These observations indicate that apoptosis was induced in at least a fraction of affected cells, but it is not clear whether the death of all cells can be explained by apoptosis. For instance, sphingosine has been reported to induce both apoptosis and necrosis in a dose-dependent fashion, with partial overlap of the dose–response curves for these two effects (35).
It is well established that sphingolipids inhibit colon carcinogenesis in different models (13–17). The finding that dietary sphingomyelin reduces EAF in liver suggests that sphingolipids might have chemopreventive properties in this organ. EAF are the earliest preneoplastic lesions in rodent liver and the 40–50% reduction of EAF area fraction indicates that these lesions were suppressed. It remains to be elucidated whether the findings presented here are typical for all types of EAF.

Sphingolipids are prominent among components of food (39). Dietary sphingomyelin is slowly hydrolyzed in the intestine and it has been demonstrated in rat experiments that 2–5% of sphingomyelin administered by gavage is recovered in the liver within 2 h (40,41). An interesting question is whether, e.g. sphingosine, apparently taken up more efficiently than sphingomyelin (13), is more effective than sphingomyelin as chemopreventive agent.

Although the mechanism for the reduction of EAF remains to be elucidated, a low level of sphingosine in EAF tissue would provide a good explanation for the effects demonstrated here. The intracellular concentration of sphingolipids can be reduced by, e.g. enhanced glucosylation (42). Our immunohistological data are compatible with the finding that the constitutive rate of glucosylation of ceramide in EAF hepatocytes is higher than in surrounding non-EAF tissue. This finding was further supported from the pronounced potentiation of the effect of ceramide treatment in vitro observed here upon exposure to PDMP, a potent inhibitor of ceramide glucosylation (32). The mechanism underlying the enhanced glucosylation is of interest to elucidate in greater detail, especially in light of the elevated levels of glucosylceramide detected in many types of cancer cells (42).

Altogether, the present investigation indicates that the turnover of sphingolipids in preneoplastic EAF hepatocytes from DEN-treated rats is altered. This alteration may play a role in the adaptation of EAF to stress, conferring resistance to apoptosis induced by various extracellular stimuli. However, this altered turnover might at the same time give rise to increased sensitivity to sphingolipid-induced cell death, creating a possibility for eliminating preneoplastic cells. Additional studies, including in vivo studies with cancer endpoints, may provide a new strategy for chemoprevention of hepatocellular carcinoma.

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References


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