Induction of Apoptotic Cell Death and Prevention of Tumor Growth by Ceramide Analogues in Metastatic Human Colon Cancer

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ABSTRACT

Dysfunction in the physiological pathways of programmed cell death may promote proliferation of malignant cells, and correction of such defects may selectively induce apoptosis in cancer cells. We measured the levels of ceramide, a candidate lipid mediator of apoptosis, in human metastatic colorectal cancer and tested it in vitro and in vivo effects of various ceramide analogues in inducing apoptosis in metastatic colon cancer. Human colon cancer showed over 50% decrease in the cellular content of ceramide when compared with normal colon mucosa. Application of ceramide analogues and ceramidase inhibitors induced rapid cell death through activation of various proapoptotic molecules, such as caspases and release of cytochrome c. Ceramidase inhibition increases the ceramide content of tumor cells, resulting in maximum activation of the apoptotic cascade. Normal liver cells were completely resistant to inhibitors of ceramidases. Treatment of nude mice with B13, the most potent ceramidase inhibitor, completely prevented tumor growth using two different aggressive human colon cancer cell lines metastatic to the liver. Therefore, B13 and related analogues of ceramide and inhibitors of ceramidases offer a promising therapeutic strategy with selective toxicity toward malignant but not normal cells. These studies also suggest that the ceramide content in cancer cells might be involved in the pathogenesis of tumor growth in vitro and in vivo.

INTRODUCTION

Metastatic colorectal cancer is common with ~100,000 new cases diagnosed each year in the United States (1, 2). Less than 20% of patients with metastatic colorectal cancer are candidates for curative surgery, and conventional chemotherapy is only marginally effective (3, 4). As a result, this condition has a dismal prognosis, warranting the development of new therapeutic strategies (5).

Induction of apoptosis in tumor cells, a form of physiological death in unwanted or dysfunctional cells, is an appealing therapeutic approach (6). Escape from apoptotic signals often accompanies tumor progression. For example, Naik et al. (7) showed in a mouse model that progression of islet cell tumors is associated with reduced susceptibility to apoptotic stimuli. Several groups have reported a higher incidence of tumor development in mice with genetic alterations of apoptotic mediators, such as Bcl-2 overexpression or Fas ligand deletion (8). Furthermore, the response to chemo- or radiation therapy in some cancers correlates with the induction of apoptosis within the tumors (9, 10). These findings suggest that dysregulation of the apoptotic pathway might be a pivotal point in the genesis of a variety of cancers. Although the mechanisms and mediators of apoptosis in malignant cells remain under investigation, restoration of the physiological death pathway holds promise as a novel treatment strategy for cancer (11–13).

Ceramide, a bioactive lipid arising from the hydrolysis of sphingomyelin or from de novo formation, has been proposed to play important roles in growth arrest, differentiation, and apoptosis in several cell culture models (14–17). For example, inhibition of ceramide formation with fumonisin B1 has been shown to prevent apoptosis in response to CPT11 and in response to angiotensin II (18, 19). Also, mice knocked out in acid sphingomyelinase show specific defects in endothelial and liver cell apoptosis (20, 21). Intracellular targets of ceramide are ceramide-activated phosphatases and ceramide-activated kinases, which in turn activate the apoptotic pathways, SAPK/JNK1 and the caspase cascade (17, 22).

Preliminary data have shown that malignant cells with low ceramide levels are resistant to apoptosis (23–25). Chmura et al. (23) reported that tumor cell lines with a defect in ceramide generation are resistant to radiation-induced apoptosis. In addition, patients with head and neck cancer resistant to radiotherapy had decreased ceramide levels in their tumor (25).

In this study, we evaluated the effect of ceramide on viability and growth of human colon cancer. We investigated two ceramide analogues (C2-ceramide and C6-ceramide), which have been shown to mimic the effect of natural ceramide, and two inhibitors of ceramidases (B13 and D-MAPP), critical enzymes involved in ceramide degradation. Ceramidase inhibition leads to elevation of intracellular natural ceramide (26). Therefore, we hypothesized that ceramide levels are low in human colon cancer, and that restoration of cellular ceramide activity might induce apoptosis and prevent cancer growth in vitro and in vivo.

MATERIALS AND METHODS

Cell Lines. SW403 cells were purchased by American Type Culture Collection and grown in DMEM/F12 with 10% fetal bovine serum. Lovo cells were kindly provided by Dr. Mark Modrich (Department of Biochemistry, Duke University Medical Center) and grown in McCoy's 5A serum with 10% fetal bovine serum. Both cell lines were contained at 37°C in a humidified atmosphere containing 5% CO2.

Cell Isolation. Hepatocytes, Kupffer cells, and sinusoidal endothelial cells were isolated from male Wistar rats (250 g) as described elsewhere (27, 28). Briefly, hepatocytes were isolated by in situ perfusion of the liver with 0.013% collagenase (Boehringer Mannheim, Indianapolis, IN), followed by elutriation. For the nonparenchymal cell isolation, the rat liver was perfused with 0.013% collagenase and 0.25% Pronase (Boehringer Mannheim). The different cells were separated on a discontinuous density gradient. The fraction of Kupffer cells and endothelial cells was subjected to centrifugal elutriation (flow rates, 18 and 36 ml/min). The purity of the isolated cell cultures of hepatocytes, endothelial cells, and Kupffer cells was >90%, respectively, as assessed by phase-contrast microscopy, immunocytochemical analysis, and the ability to uptake fluorescently labeled actocyaninated human low-density lipoprotein.

The cells were cultured in Williams medium (Life Technologies, Inc., Gaithersburg, MD) and grown in McCoy's 5A serum with 10% fetal bovine serum.

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1 The abbreviations used are: SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling.
CERAMIDASE INHIBITION IN HUMAN COLON CANCER

ersburg, MD) containing 20% calf serum. The cells were maintained in a humidified 5% CO₂ incubator at 37°C, and the medium was changed 24 h after plating.

Ceramide Analogue. Short-chain ceramides (C2-ceramide and C6-ceramide) and selected inhibitors of ceramidase, D-MAPP and B13 (Fig. 1), were used in this study. Synthesis and physical characterization of the above compounds were described previously (16, 22). C2-ceramide and C6-ceramide were prepared by acylation reaction of (2S,3R)-sphingosine obtained in stereo- selective synthesis. D-MAPP and B13 were prepared by acylation of the respective amino alcohols with myristoyl chloride (26). B13 is a newly synthesized analogue of ceramide, and its molecular structure is similar to D-MAPP. Both compounds are potent blockers of ceramidase (26), a critical scavenging enzyme of ceramide, thereby elevating natural ceramide content in the cells.4

Cell Treatment. For the in vitro studies, the compounds of interest were dissolved in 100% ethanol. The final ethanol concentration in the culture medium was 0.1%. In pilot studies, we established normal cell growth and no decreased cell viability with 0.1% ethanol concentration in the culture medium.

Trypan Blue Staining. Cells were harvested by trypsinization and stained 1:2 with trypan blue. The percentage of staining cells was determined with a counting chamber at ×200.

[^3]HThymidine Assay. Tumor cells (50,000) were placed in a 96-well plate and grown in serum containing medium over night. Then, 1 μCi of[^3]Hthymidine was added to each well for 12 h. After the incubation time, the plates were washed, and the radioactivity of the remaining DNA was counted by a Wallace counter.

TUNEL Assay. The TUNEL assay was performed as described previously by Gao et al. (29). Briefly, after 4, 12, and 24 h of treatment with B13, the cells were incubated in freshly prepared 4% paraformaldehyde in PBS (pH 7.2) for 30 min. The cells were washed with PBS and finally placed in 100% ethanol. Then the cells were transferred on glass slides by cytopsin (800 rpm). The cells were treated with terminal deoxynucleotidyl transferase from calf thymus (Boehringer Mannheim) in the presence of fluorescein-dUTP and deoxynucleotide triphosphate, according to the supplier’s recommended protocol. This was followed by poststaining using horseradish peroxidase-conjugated anti-fluorescein antibody and development using diaminobenzidine/H₂O₂. Positive and negative controls were done using test sections pretreated with DNase I and staining without deoxynucleotide substrate, respectively.

Agarose Gel Analysis of DNA Fragmentation. The cells were harvested by trypsinization. The DNA was extracted using the Gibco DNA laddering kit (Life Technologies Inc., Gaithersburg, MD). The isolated DNA was quantified at 260 nm. Samples (5 μg/lane) were electrophoresed on a 2% agarose gel at 30V in TAE buffer (40 mM Tris-acetate, 10 mM EDTA, and 20 mM glacial acetic acid, pH 8.4) for 3 h. The DNA was finally visualized on an UV transilluminator.

Annexin V Assay. Surface expression of phosphatidyl serine was determined by Annexin V staining. Cells (10⁶) per milliliter were harvested and exposed to Annexin V and propidium iodide according to the recommendation of the manufacturer (R&D, Minneapolis MN). Annexin V and propidium iodide staining were determined by cell flow cytometry.

Ceramide Measurement. Lipids were extracted using the Bligh and Dyer procedure (30). This method involves the lysis of cells or tissue with organic solvent, followed by dilution with chloroform and water to obtain phase separation. Approximately 100% of the biological ceramides, diacylglycerols, and sphingosine are extracted into the organic phase under these conditions. The critical parameters of the lipid extraction are the ratios of chloroform: methanol:water 1:1:0.9 (v/v). Efficient lipid extraction into organic phase should be performed as follows. Cells (1–2 × 10⁶) are separated from medium, washed with cold 1× PBS, pelleted, lysed with 3 ml of chloroform:methanol (1:2, v/v), and vortexed vigorously. Extraction of neutral lipids into the chloroform phase is continued by adding 0.8 ml of water, 1 ml of chloroform, and finally 1 ml of water. After every step, strong vortexing is recommended. Finally, the organic lower phase (2 ml) is separated from the upper one (3.8 ml) by centrifugation and is used for analytical purposes. Lipid extraction from the tissues was performed after homogenization with buffer (0.25 M sucrose, 25 mM KCl, 50 mM Tris, and 0.5 mM EDTA, pH 7.4). The right amount of homogenate for diacylglycerol kinase assay was established through the protein measurement.

The level of the endogenous ceramide was determined by DK kinase according to the published method (31). Briefly, ceramide standards (50–1000 pmol) and lipid extracts from the experimental samples are sonicated for 5 min with 20 μl of 3.75% (w/v) octylglucoside-12.5 mM dioleylphosphatidylglycerol, made up in 1 mM DTPA, followed by the addition of 70 μl of the reaction mixture containing 120 μM HEPES buffer (pH 7.0), 100 mM LiCl, 25 mM MgCl₂, 2 mM EGTA, 2 mM DTT, and 5 μl of diacylglycerol kinase (7 μg/ml). After 10 min, the reaction is started by adding 10 μl of 10 mM ATP in 20 mM imidazole buffer (pH 6.6) and 1 mM DTPA. After vigorous mixing, the reaction mixture is left at room temperature for 0.5 h, and then 3 ml of chloroform: methanol 1:2 (v/v) are added, and the reaction mixture is mixed vigorously for 1 min. Bligh and Dyer extraction is continued by adding 700 μl of 1% perchloric acid, followed by 1 ml of chloroform and 1 ml of 1% perchloric acid. Phases are separated by centrifugation (3000 rpm for 5 min). 1.5 ml from the lower phase (total, ~2 ml) is transferred to the new vial, and the organic phase is dried down under nitrogen. Dry lipids are resuspended in 50 μl of chloroform-methanol (1:1, v/v). Twenty μl of this solution are applied to a TLC plate, and [32P]ceramide phosphate is resolved in a chloroform: acetone:methanol:acetic acid:water (10:4:3:2:1, v/v) solvent system. The TLC plate is then exposed to X-ray film. The area corresponding to [32P]ceramide-1-phosphates (Rf = 0.63–0.68) is scraped and counted quantitatively by liquid scintillation counting. Alternatively, visualization and quantitation can be performed on a Molecular Dynamics Phosphorimage. Quantitation of ceramide mass is based on the specific activity of [32P]ATP and on the external standard normalization. Final results are shown as [32P]:ceramide phosphate/total lipid phosphorous. Pi is determined for an equal amount of phospholipids present in the lower phase of the lipid extract. The results are then expressed as ceramide/Pi (pmol/pmol).

Caspase 3-like Activity Assay. Caspase 3-like activity was determined by measuring the proteolytic of the specific substrate N-acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVd-CHO; Biomol, Plymouth Meeting, PA; Ref. 32). Caspase 3 is distinguished by its ability to cleave poly(ADP-ribose) polymerase during apoptosis. The cleavage site in poly(ADP-ribose) polymerase is COOH-terminal to ASP-216 (33). The upstream sequence, DEVD, is the basis for the substrate and inhibitors used in this assay. Liver tissue was quickly excised and sonicated in assay buffer [1 mM EDTA, 145 mM NaCl, 100 mM Tris, 0.1 mM DTT, 0.1% 3-(3-cholamidopropyl)dimethylamino)-1-propanesulfonate, and 10% glycerol]. The protein content was determined using the Bradford protein assay. The samples were diluted and incubated at room temperature with Ac-DEVD- AFC substrate in the presence or absence of the inhibitor AC-DEVD-CHO. AFC release was measured over 2 h in a fluorometer, using 400 nm excitation and measuring 505 nm emission. The AFC release was expressed as arbitrary fluorescence units per mg protein after subtracting the reading in the inhibited sample from the noninhibited sample.

In Vivo Model of Liver Metastases. The tumor cell lines (SW403 and Lovo) were twice washed in 1× PBS and finally dissolved in 0.2 ml of 1× PBS containing 2 million tumor cells. The abdominal cavity of the mice was opened by a median incision. The portal vein was exposed, and the tumor cell suspension (0.2 ml) was slowly injected into the portal vein. The abdominal cavity was closed by chromic gut 4.0, and the animals were allowed to awake after tumor cell injection. Two h after tumor cell injection, the mice either received 75 mg/Kg B13 dissolved in 10% ethanol, 30% Cremophor, and 60% saline or the solvent alone into the peritoneal cavity. The injection was repeated every 3 days up to a total number of five injections.

4 S. El Babaw, Y. Hammon, and A. Bielawska, unpublished observations.
RESULTS

Ceramide Levels Are Reduced in Human Colon Cancer. Because ceramide has been proposed to play a role in apoptosis (16, 22, 34, 35), we initially wondered whether ceramide levels may be deficient in human colon cancer. Ceramide levels were measured in fresh human specimens of primary and metastatic colon cancer obtained at the time of surgery. The specimens were immediately snap frozen at the time of resection. In each patient, ceramide levels in primary colon cancer were compared with normal colon mucosa. Ceramide levels were determined by the diacylglycerol kinase assay and TLC. Colon cancer specimens showed approximately half the levels of ceramide when compared with respective normal colon mucosa obtained in the same patient (Fig. 2).

Ceramide Analogues and Ceramidase Inhibitors Induce Cell Death in Cultured Human Colon Cancer. The ability of two ceramide analogues (C2 and C6) and two inhibitors of ceramidase (D-MAPP and B13) to induce cell death was determined in a human colon cancer cell line (SW403). SW403 cells were chosen because of their well-preserved epithelial phenotype, their similar production of tumor-specific antigens as in most human colon cancers, and their tumorigenic potency in nude mice. SW403 cells were exposed to different concentrations of each ceramide analogue and ceramidase inhibitor. Cell death was determined by trypan blue uptake, and DNA synthesis was significantly impaired by incorporation of [3H]thymidine. High doses of ceramides and ceramidase inhibitors were used because of the inhibition of ceramides by serum binding proteins. Exposure to 100 μM ceramide analogue or ceramidase inhibitor for 24 h resulted in significant increase in cell death compared with cells exposed to the solvent alone (Fig. 3A). The ceramidase inhibitor B13 (100 μM) was significantly more toxic than the other compounds with 90% cell death at 24 h. In a second approach, we investigated the effect of exposure to 100 μM B13, 85% of the colon cancer cells were TUNEL positive. Consistent with these findings was the appearance of DNA laddering by gel electrophoresis after 12 h of treatment (Fig. 5B). Translocation of phosphatidylserine to the outer surface of the cytoplasmic membrane, an early feature of apoptosis, was evaluated by the Annexin V binding assay. To exclude staining of phosphatidylserine on the inner surface of the cell membrane, which occurs

![Fig. 2. Ceramide content per total phosphate in patient tissues. Primary colon cancer of the large bowel contains significantly less ceramide than normal colon mucosa. Student’s t test: *, P < 0.05; n = 5 in each group. prim, primary. Bars, SD.](Image 56x595 to 284x741)

![Fig. 3. Cell viability after 0, 8, 16, and 24 h of treatment with 100 μM ceramide analogue (C2 and C6) or ceramidase inhibitor (D-MAPP and B13) compared with the control receiving solvent (0.1% ethanol) only (A). All four investigated lipids decreased the tumor cell viability with B13 being the significantly most potent compound. Twenty-four h exposure to 100 μM B13 resulted in 90% tumor cell death. In a second approach (B), the effect of four different doses (5, 10, 50, and 100 μM) of the most potent compound (B13) on tumor cell viability was evaluated. B13 induced tumor cell death in a time- and dose-dependent manner (n = 5). ANOVA: *, P < 0.05; Student’s t test: P < 0.05 when B13 was compared with any other group (n = 5).](Image 324x104 to 545x227)

![Fig. 4. [3H]Thymidine incorporation within 12 h exposure to ceramide analogues. 5 μM; □, 10 μM; ▯, 50 μM; □, 100 μM; compared with the control receiving solvent (0.1% ethanol) only. The two ceramide analogues C2 and C6 and the two ceramidase inhibitors D-MAPP and B13 reduced the amount of [3H]thymidine incorporation in a dose-dependent manner, with B13 being the most potent compound (Student’s t test: *, P < 0.05; n = 5). Bars, SD.](Image 1235)
when the cytoplasmic membrane is disrupted, we counterstained the cells with propidium iodide. Cells stained for Annexin V alone have early apoptotic membrane changes with intact cell membranes and are in an early phase of apoptosis. Cells stained for Annexin V and propidium iodide have membrane disintegration consistent with necrosis or a late stage of apoptosis in cell culture. Although Annexin V was minimally expressed (<12%) in untreated cells at all time points, B13-treated cells had significantly elevated Annexin V staining at 1 and 4 h of exposure (~40%; Fig. 6) with only minimal propidium iodide staining. After 4 h incubation, an increasing portion of cells was also positive for propidium iodide. Taken together, the data are consistent with B13 inducing apoptosis in these cells with rapid changes in the distribution of phosphatidylserine, followed by DNA fragmentation. Less specific features of cell death (propidium iodide staining) are typically noted at later stage of apoptotic death in cell culture.

We determined the effects of B13 on cellular content of ceramide and various mediators of the cellular machinery causing apoptosis. Ceramide content in SW403 cells was increased 2-fold at 4 and 12 h after exposure to B13 (Fig. 7). The release of mitochondrial cytochrome c, a potent activator of caspase 3, was elevated at 4 and 12 h of B13 treatment (Fig. 8). Finally, caspase 3 activity, the executor of the caspase cascade, demonstrated a 7-fold increase after 4 and 24 h exposure to B13 (Fig. 9A). Pretreatment of the cancer cells with a specific caspase 3 inhibitor (Ac-DEVD-CHO; Biomol) significantly
exposed to B13 alone. Exposure (4 and 12 h) to B13 results in a 7-fold increase of caspase 3 activation compared with the solvent (0.1% ethanol)-treated control (Co; Student’s t test: *P < 0.01; n = 5 in each group). In contrast, caspase 8 levels are comparable in control and B13-treated cells (Student’s t test; P = 0.4; n = 5 in each group). B. SW403 viability after 4 and 12 h of treatment with 100 μM B13 with and without simultaneous inhibition of caspase 3. SW403 cells treated with a specific caspase 3 inhibitor demonstrate significantly increased tumor cell survival at 4 and 12 h compared with SW403 cells exposed to B13 alone. Bars, SD.

Fig. 9. A, caspase 3 and 8 activation after treatment with B13 or with the solvent alone (Co). Exposure (4 and 12 h) to B13 results in a 7-fold increase of caspase 3 activation compared with the solvent (0.1% ethanol)-treated control (Co; Student’s t test: *P < 0.01; n = 5 in each group). In contrast, caspase 8 levels are comparable in control and B13-treated cells (Student’s t test; P = 0.4; n = 5 in each group). B. SW403 viability after 4 and 12 h of treatment with 100 μM B13 with and without (■) simultaneous inhibition of caspase 3. SW403 cells treated with a specific caspase 3 inhibitor demonstrate significantly increased tumor cell survival at 4 and 12 h compared with SW403 cells exposed to B13 alone. Bars, SD.

Normal Liver Cells Are Resistant to B13 Treatment. The previous experiments demonstrated high activity of B13 in human colon cancer cells. The clinical use of this compound will be linked to its safety, particularly the lack of toxicity in normal cells. Liver metastases from colon cancer can be treated selectively by drugs injected directly in the blood supply of the liver, thus with a risk of toxicity mainly to hepatic cells. Therefore, we evaluated the toxicity of B13 in normal cells isolated from rat liver including hepatocytes, sinusoidal endothelial cells, Kupffer, and stellate cells. The cells were isolated by rat liver collagenase perfusion and subsequent gradient centrifugation. Cell viability was determined by trypan blue staining after 24 h exposure to 100 μM B13. None of these populations of cells demonstrated any decrease in cell viability (Fig. 10) at a dose associated with >90% cell death in the colon cancer cell line (see above). To investigate the mechanisms of resistance of normal hepatocytes against B13, we measured ceramide levels in hepatocyte cultures exposed to 4 and 12 h of 100 μM B13. No elevation of ceramide levels was detected in normal hepatocytes, despite maximum treatment with the ceramidase inhibitor B13. The baseline ceramide levels of cultured normal hepatocytes were twice as high as in the colon cancer cell line (data not shown). Similar results were obtained measuring ceramide levels in human specimens freshly obtained in the operating room. Liver metastasis from colon cancer had only half the ceramide levels compared with normal liver tissue (data not shown).

B13 Completely Prevents Liver Metastases from Human Colon Cancer in Vivo. The impressive effect of B13 in cultured human colon cancer cells combined with its innocuous effect on normal liver cells makes this compound attractive for the treatment of liver metastases from colon cancer. Experiments were designed to study the effects of B13 in an in vivo model of human colon cancer metastatic to the liver. For this purpose, we developed a nude mouse model (HST, thymic nude/nu) with selective implantation of human colon cancer cells into the liver. To avoid results that would be dependent on specific cell lines, two different human colon cancer cell lines were used (SW 403 and Lovo). Lovo was chosen as a second human colon cancer cell line beside SW 403 because it is derived from metastatic colon cancer and expresses an aggressive growth pattern typical for metastatic tumor growth. Briefly, two million cancer cells were injected into the portal vein of the nude mice (n = 7 mice in each treatment and control group). Macroscopic evidence of hepatic seeding occurred within 3 weeks. Each animal received five doses of B13 (75 mg/Kg) or the solvent alone, with the first dose injected into the peritoneal cavity a few hours after the tumor injection, and additional doses every 3 days afterward. Two and 5 weeks after tumor injection, the animals were sacrificed, and tumor growth was determined by macroscopic and microscopic evaluation (H&E staining). Each control animal developed large hepatic tumor masses. In the SW403 group, five of seven B13-treated mice remained completely tumor free. Similarly, in four of seven Lovo-treated mice, no malignant cell was detectable after B13 treatment (Fig. 11). The B13-treated mice, which developed tumor growth, had significantly lower tumor masses than the control-treated animals (1.2 g ± 0.2 g versus 2.8 g ± 0.8 g; Student’s t test, P = 0.03). We did not observe any signs of distress, such as a change in behavior or mutilation, in B13-treated animals. All B13-treated animals had a similar weight gain when compared with a nontreated control group. On careful macroscopic examination of the abdominal cavity, no signs of inflammation or serosal injury were detectable in mice treated with B13.

DISCUSSION

Apoptosis, a physiological and pathophysiological mechanism of cell death, can be initiated by extracellular and intracellular mechanisms that trigger a complex machinery of proapoptotic proteases and mitochondrial changes, leading to the activation of specific endonucleases and DNA fragmentation (36–38). Knowledge about mediators involved in this process is just emerging (11). In the present study, we found that ceramide is significantly decreased in human colon cancer when compared with normal colon mucosa. Ceramide prevented cell death at 4 and 12 h after exposure to B13 (Fig. 9B). In contrast, caspase 8 activity, the most upstream cell membrane-related mediator of the caspase cascade, was unaltered by exposure to B13.

Fig. 10. Twenty-four-h, B13-treated (100 μmol, ■) versus solvent-treated (●) normal rat hepatocytes (Hep), normal rat Kupffer cells (KC), and normal rat sinusoidal endothelial cells (SEC) in isolated cell cultures. Normal liver cells do not demonstrate any reduced viability compared with the control, despite B13 treatment.
analogues or the administration of ceramidase inhibitors, which restore cellular ceramide content, activate the apoptotic pathway in cancer but not normal cells. When testing the most potent ceramidase inhibitor (B13) in two in vivo models of human colon cancer metastatic to nude mice liver, we found complete inhibition of tumor growth without detectable toxicity.

Ceramide is an emerging lipid mediator, with proapoptotic activities in cancer cells (15, 16, 22). Ceramide accumulation in cells can be induced by a variety of extracellular stimuli, such as TNF-α, Fas ligand, IFN-α, radiation, and chemotherapy (16, 22, 39–41). Ceramide activates proapoptotic mechanisms in three major ways: (a) ceramide acts indirectly to activate caspases, a well-characterized group of cysteine proteases, which leads to activation of caspase 3 (22), the executor of apoptosis causing activation of endonucleases and DNA fragmentation; (b) ceramide activates the stress-activated protein kinases (SAPK/JNK; Ref. 34) pathway. SAPK/JNK activation may result in phosphorylation of c-Jun, which in turn induces apoptosis by a caspase 3-dependent mechanism (34, 42); and (c) ceramide up-regulates TNF receptor 1 and Fas receptors on the cell membrane and, therefore, may enhance the effects of antitumor T-lymphocytes and other Fas ligand- and TNF-dependent processes (9).

Recent data suggest a role for ceramide in the pathogenesis of cancer and failure of conventional therapy (21, 23–25). For example, resistance to radiation therapy has been associated with a significant decrease in cellular ceramide production (21, 23–25), whereas others have reported that a defect of ceramide production is associated with multidrug resistance (43, 44). Another evidence implicating ceramide in the development of cancer is the observation that mice subjected to a diet rich in sphingomyelin, which is cleaved into ceramide and sphingosine inside the gastrointestinal lumen, are resistant to 1,2-dimethylhydralazine-induced colon cancer (21). Our finding that human colon cancers, primary and metastatic to the liver, contain only half of the ceramide content of normal tissue obtained in the same patient is consistent with a role of this lipid mediator in the pathogenesis of cancer.

We hypothesized that tumors with low ceramide content might be particularly sensitive to the restoration of ceramide. We observed that various ceramide analogues and ceramidase inhibitors induce rapid death by apoptosis of human colon cancer in cell culture. Apoptosis in the cancer cells was mediated by a dramatic activation of the caspase cascade. Whereas caspase 8, the most upstream membrane-linked mediator, remained unaffected by exposure to B13, cytochrome c was...
CERAMIDASE INHIBITION IN HUMAN COLON CANCER

rapidly released, resulting in caspase 3 activation. Blocking Caspase 3 by a caspase inhibitor increased tumor cell viability. This indicates that the effects of the ceramidase inhibitor B13 are dependent on the caspase cascade. The exact place of ceramide in this pathway cannot be determined from these experiments, but the lack of caspase 8 activation suggests that ceramide is localized between caspase 8 and the release of cytochrome c. Other effects of ceramide, e.g., directly on caspase 3, cannot be excluded.

B13 has emerged from these studies as a particularly active analogue of ceramide that inhibits ceramide metabolism and leads to accumulation of cellular ceramide. B13 is significantly more active in this experimental model than either short-chain ceramides or D-MAPP, a previously described inhibitor of alkaline ceramidase. The reasons for this enhanced potency are not fully determined but may relate to the enhanced solubility of B13 compared with the other ceramide inhibitors or ceramide analogues.

An important finding related to the possible clinical use of B13 was the lack of toxicity to normal cells in vitro and to the animals treated with B13. Because our interest is in colon cancer metastatic to the liver, we focused on the effects of B13 on normal hepatic cells including parenchymal (hepatocyte) and nonparenchymal (sinusoidal endothelial and Kupffer cells) cells. Both in vitro and in vivo administration of high-dose B13 was not associated with detectable toxic effects. Increased ceramide levels might be less toxic to cells with an intact sphingosine/ceramide pathway compared with tumor cells with disrupted ceramide generation. Additionally, normal hepatocytes, Kupffer cells, and sinusoidal endothelial cells have a low mitotic rate in cell culture and in vivo. Although i.p. injection of B13 did not induce bone marrow suppression or intra-abdominal inflammation in our model, the systemic use of B13 might have adverse effects on cells with a high mitotic rate. The rodent model alone is insufficient to fully determine the potentially toxic effects of B13, and large animal experiments are necessary prior to clinical use.

In our in vivo model, we found that the development of tumor metastases can be prevented by the ceramidase inhibitor B13. Several possible mechanisms exist for the in vivo efficacy of B13. Apoptosis could be induced in tumor cells within the hepatic sinusoids similar to our in vitro model of tumor cell death. Furthermore, up-regulation of proapoptotic membrane receptors, such as Fas or TNF-α, could facilitate the induction of apoptosis by extracellular mediators. Finally, tumor cell adhesion in the hepatic sinusoids might be negatively affected by the exposure to the ceramidase inhibitor B13. Although our in vitro experiments demonstrated that B13 induces cell death by activation of the caspase cascade and inhibits tumor cell growth, additional mechanisms might also be active in vivo. Future studies are necessary to determine the effects of ceramidase blockade on established metastases.

Several aspects of ceramide as a therapeutic agent have to be clarified in the future. It is unclear at this point if ceramide inhibitors are effective only in preventing metastases or if established metastatic disease can be treated successfully. The effect of several chemotherapeutic agents are linked to the induction of apoptosis and associated with an increase of cellular ceramide (10). A defect in ceramide generation might be associated with resistance to chemotherapy. A combination of chemotherapy and regulation of the level of the endogenous ceramide might have synergistic effects on selected tumors.

These findings have profound implications for the treatment of cancer. Our results emphasize that defects in the apoptotic pathway exist in colon cancer. The use of mediators of apoptosis to restore the deficient pathway is therefore a new and attractive strategy to selectively attack the cancer cells. Increasing the ceramide levels by blocking ceramidase was highly effective in inducing apoptosis and preventing growth of colon cancer. B13 appears to be a promising compound for the adjuvant treatment of primary or metastatic colon cancer.

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