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ELEVATION OF *DE NOVO* CERAMIDE SYNTHESIS IN TUMOR MASSES AND THE ROLE OF MICROSOMAL DIHYDROCERAMIDE SYNTHASE

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Ceramide is formed through sphingomyelin hydrolysis or *de novo* synthesis and may play a key role in cell growth, differentiation and apoptosis. To clarify which pathway tumor cells use to form ceramide and how its formation is regulated, we determined the levels of dihydroceramide and ceramide in mice inoculated with Sarcoma 180, B16 melanoma or Lewis lung carcinoma cells. The levels in these tumor masses were very high compared to those in other healthy tissues. The high levels were significantly reduced by a single administration of the dihydroceramide synthase inhibitor fumonisin B_1, but not by a sphingomyelinase inhibitor, sphingomyelin analog-1 (SMA-1), suggesting that the tumor cells have a very effective means of synthesizing dihydroceramide and ceramide. To investigate the characteristics of dihydroceramide synthase, we prepared microsomes from Sarcoma 180 tumor masses and healthy mouse liver cells, and compared their catalytic activities on dihydroceramide formation. A kinetic analysis using sphinganine and palmitoyl CoA as substrates revealed that the enzyme present in the tumor formed dihydroceramide 3 times more efficiently than that in healthy liver cells. Partial purification of dihydroceramide synthase from bovine liver microsomes revealed that the enzyme was present in healthy tissues as a 333 kDa form constructed of 47 kDa subunit proteins. However, gel filtration of the enzyme solubilized from the Sarcoma 180 tumor masses demonstrated that its molecular weight was 1,300 kDa. These results suggest that malignant transformation causes the cell to produce a form of dihydroceramide synthase with a larger than normal molecular mass; the increased molecular mass may account for the enzyme's increased catalytic efficiency.

Key words: *ceramide; dihydroceramide synthase; sphingomyelin; sphingomyelinase; tumor survival*

Ceramide has received much attention recently as an important mediator in regulating a cell's response to stress. Inducers of ceramide formation include tumor necrosis factor-α, interleukin 1β, Fas ligand, and 1,25-dihydroxy vitamin D_3.[1] Many of these agents produce ceramide through hydrolysis of sphingomyelin (SM) after the activation of sphingomyelinase (SMase). Studies using fungal metabolites, fumonisins, demonstrated, howeves, that ceramide formed through the *de novo* synthesis pathway is also implicated in apoptosis.[2,3] Fumonisin inhibits the activity of dihydroceramide synthase,[4,5] an enzyme in the *de novo* pathway that catalyzes *N*-acetylation of sphinganine to produce dihydroceramide. The conversion of dihydroceramide to ceramide is catalyzed by a desaturase present in microsomes.[6] Several chemotherapeutic agents, such as daunorubicin,[2] etoposide,[7] and hexadecylphosphocholine,[8] have been reported to induce *de novo* ceramide formation during apoptosis of tumor cells. These findings suggest that tumor cells may synthesize ceramide in amounts close to or under normal levels because the cells need to lengthen their lifespan by holding the apoptotic effects of ceramide to a minimum. Little is known, however, about the correlation between ceramide levels and tumor survival.

We have developed recently a LC/MS method to measure levels of C_{16}-dihydroceramide and -ceramide in cells.[9] The C_{16}-ceramide levels in cells have been reported to increase during apoptosis.[10] We inoculated mice with Sarcoma 180, B16 melanoma and Lewis lung carcinoma cells, respectively. We then used the LC/MS method to measure both C_{16}-dihydroceramide and -ceramide levels in the tumor masses and in samples of healthy tissues. We found that these tumor masses produced large amounts of both dihydroceramide and ceramide through the *de novo* synthesis pathway, but not through SM hydrolysis. This is in contrast to the normal ceramides accepted role. Further experiments revealed that the high levels of dihydroceramide were caused by a 3-fold increase in the catalytic activity of dihydroceramide synthase present in microsomes of the tumor cells. We then extracted dihydroceramide synthase from healthy bovine liver microsomes and ran the extract through a purification process. The partially purified enzyme was a 333 kDa form constructed of 47 kDa subunit proteins. Gel filtration of the enzyme(s) solubilized from microsomes of the mouse tumor masses and healthy liver cells showed that the enzyme produced in the tumor cells was a 1,300 kDa form. In the tumor masses, the increase in the enzyme's molecular mass might cause a corresponding increase in its catalytic efficiency. We also discuss why tumor cells accumulate *de novo* synthesized ceramide.

MATERIAL AND METHODS

Material

The following materials were obtained commercially: D-erythro-dihydrosphingosine (sphinganine), palmitoyl Co A, *N*-palmitoyl-D-sphingosine, *N*-palmitoyl-D-sphinganine, *N*-palmitoyl-D-SM and fumonisin B_1 from Sigma (St. Louis, MO); DMEM and FBS from GIBCO BRL (Grand Island, NY); leupeptin and antipain from the Peptide Institute (Osaka, Japan); DE-53 from Whatman International Ltd. (Maidstone, UK); and Sepharose CL-6B from Pharmacia Biotech AB (Uppsala, Sweden). SMA-1 was prepared in our laboratory.[11] It is an SMase inhibitor, in which the long alkenyl chain and the phosphodiester moiety of SM are replaced by a phenyl and an isosteric difluoromethylenephosphonic acid, respectively (Fig. 1).

Cell lines and treatments

Lewis lung carcinoma cells were supplied by the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). B16 melanoma and Sarcoma 180 cell lines were purchased from Dai Nippon Seiyaku (Osaka, Japan). C57BL/6 mouse-derived tumor cells were cultured in DMEM supplemented with 10%

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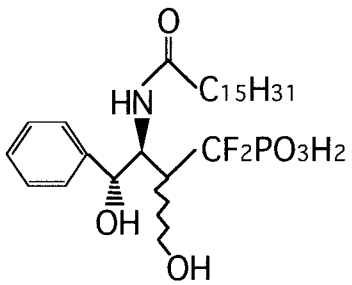

FIGURE 1 – Chemical structure of SMA-1.

FBS and antibiotics at 37°C in a humidified 5% CO_2 atmosphere. Confluent cultures of Sarcoma 180 cells in 175-cm^2 culture flasks were treated for 24 hr with fumonisin B_1 or SMA-1 (5 and 25 μM). The cells were harvested and centrifuged. The resulting pellets were subjected to LC/MS analysis for ceramide.

Animals and treatment

Male ICR mice (5 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in a light-controlled room (light on from 7:00–19:00) at a room temperature of 24 ± 1°C and a humidity of 60 ± 10% with food and water *ad lib*. Cells (5×10^5) from each cell line were inoculated into the right hind footpads of mice. After 2–3 weeks of growth, the animals were anesthetized and both the tumors and samples of healthy tissue were harvested for analysis. We injected fumonisin B_1 or SMA-1 (50 nmol each) directly into the Sarcoma 180 tumor masses of mice and, after a 2-hr treatment, determined the levels of C_{16}-ceramide. Animal treatment followed the animal care guidelines in Japanese Government Law No. 105 and Notification No. 6.

Determination of the amounts of C_{16}-dihydroceramide and -ceramide in tissues and cells

The amounts of dihydroceramide and ceramide in tissues and cells were determined as described previously.[9] Briefly, tumor cell suspensions or homogenates of healthy or malignant tissues (250 μl each) were vigorously mixed with 4 ml of chloroform/methanol (2:1, v/v) for 15 min. After the addition of 1 ml water, the sample was vortexed and centrifuged. The lower layer was collected, and the chloroform was allowed to evaporate. The residue was dissolved in a solvent and subjected to LC/MS analysis. HPLC was carried out using a Gulliver 1500 series system (Jasco, Tokyo, Japan) equipped with a Develosil ODS HG-5 reversed-phase column (35 × 2.0 mm i.d., 5 μm, Nomura Chemical, Aichi, Japan). Mobile phases were as follows: A, 5 mM ammonium formate/methanol/tetrahydrofuran (5/2/3, v/v); and B, 5 mM ammonium formate/methanol/tetrahydrofuran (1/2/7, v/v) containing 0.01% formic acid. Elution was carried out at a flow rate of 0.2 ml/min with 70% mobile phase A-100% mobile phase B for 6.3 min in a linear gradient mode. LC/MS analyses were done with an electrospray ionization mass spectrometer (Finnigan-LCQ) and Navigator (Finnigan). Retention time (min) and m/z of inner standards were as follows. For C_8-ceramide, 7.20 and 426.1; for C_{16}-ceramide, 10.35 and 538.2. For C_{16}-dihydroceramide, they were 10.55 and 540.4.

Enzyme reactions

For the assay of dihydroceramide synthase, a mixture of sphinganine (20 nmol) in 25 μl of 20 mM Tris-HCl buffer (pH 7.4)/0.1% Tween 20/5% propylene glycol and palmitoyl CoA (40 nmol) in 25 μl of 20 mM Tris-HCl buffer (pH 7.4) was placed in a 12 × 100 mm screw-cap test tube. The enzyme reaction was started by adding 200 μl of enzyme source (100 μg protein). Incubation followed at 37°C for 30 min (conversion of dihydroceramide to ceramide by desaturase was not detected). For the assay of SMase, *N*-palmitoyl SM (20 nmol) in 50 μl of 20 mM Tris-HCl buffer (pH 7.4)/0.1% Tween 20 were mixed with 200 μl of enzyme source (100 μg protein) and incubated at 37°C for 60 min. The desaturase assay mixture contained *N*-palmitoyl sphinganine (20 nmol) in 50 μl of 20 mM Tris-HCl buffer (pH 7.4)/0.1% Tween 20 and 200 μl of enzyme source (100 μg protein). Incubation was carried out at 37°C for 30 min. All the enzyme reactions were terminated by adding 4 ml of chloroform/methanol (2:1, v/v). After termination, 1 ml of water was added, and the mixture was vortexed and centrifuged. The lower layer was collected, and the chloroform was allowed to evaporate. The residue was dissolved in a solvent and subjected to the LC/MS procedure to determine the amounts of ceramide and dihydroceramide.

Partial purification of dihydroceramide synthase

Partial purification of this enzyme from bovine liver microsomes was done using a modification of a method for purifying ceramide synthase from bovine liver mitochondria.[12] Briefly, the microsomal fraction (820 mg protein) was extracted at 4°C for 5 hr with a 2 vol mixture of 15 mM Tris-HCl buffer (pH 7.4)/0.25 M sucrose/0.15 M NaCl/0.8% sodium cholate/antipain/aprotinin/leupeptin (2 μg/ml each). After centrifugation at 105,000*g* for 45 min, the supernatant obtained was dialyzed at 4°C overnight against 25 mM Tris-HCl buffer (pH 8.0)/0.4% sodium cholate. The dialysate (30 mg protein/ml, 15 ml) was then applied to a DE-53 column (2.5 × 30 cm), pre-equilibrated with the dialysis buffer. The enzyme was eluted from the column with a linear gradient of NaCl in the Tris buffer from 0.15–1.5 M at a flow rate of 2 ml/min. The active fractions were collected and concentrated on a PM-10 membrane (Amicon). The concentrate (13 ml) was dialyzed at 4°C against 15 mM Tris-HCl buffer (pH 7.4)/0.15 M NaCl. The dialysate was next applied to a column of celite (1.5 × 7 cm), pre-equilibrated with the dialysis buffer. The pass-through fractions were collected and applied to a sphinganine-coated celite column (1.5 × 7 cm), which was prepared according to the method of Boulanger *et al*.[13] The enzyme was eluted with 15 mM Tris-HCl buffer (pH 7.4)/1.5 M KI at a flow rate of 0.3 ml/min. The active fractions eluted (10 ml, 5.7 mg protein) were dialyzed and concentrated on a PM-10 membrane. The concentrate was applied to a Sepharose CL-6B column (1.7 × 31 cm), pre-equilibrated with 15 mM Tris-HCl buffer (pH 7.4)/0.3 M NaCl, and the enzyme's molecular weight was determined at a flow rate of 0.1 ml/min.

Statistical analysis

All values are expressed as the mean ± SE and the significant levels between groups were assessed by Student's *t*-test.

RESULTS

Levels of dihydroceramide and ceramide in tumor-bearing mice and the role of de novo *ceramide synthesis*

Ceramide may play a regulatory role in cell cycle arrest, differentiation and apoptosis not only in healthy tissues, but also in malignant tissues. To the best of our knowledge, however, no report has appeared on the anabolism of ceramide in tumors. Figure 2 shows the C_{16}-ceramide and -dihydroceramide levels we measured in both the tumor masses and in samples of healthy tissues such as brain, liver, kidney and muscle (the tumor cells did not metastasize to these healthy tissues). In Sarcoma 180, B16 melanoma, and Lewis lung carcinoma masses, both ceramide levels (Fig. 2*a*) and dihydroceramide levels (Fig. 2*b*) were much higher than their levels in healthy tissues. Dihydroceramide is formed on microsome surfaces by *N*-acylation of sphinganine catalyzed by dihydroceramide synthase. Ceramide is produced by a dihydroceramide desaturase.[6] Ceramide is also formed by hydrolysis of SM, however, after activation of 1 or more SMase.[14,15] To clarify which pathway(s) induced the increase in ceramide levels in the tumor masses, we tested the effects of the dihydroceramide synthase inhibitor, fumonisin B_1[4,5] and a SMase inhibitor, SMA-1, on the mice. As described previously, SMA-1 inhibits the activity of SMase in bovine tissues, but not of SMase in *Bacillus cereus*.[11] The compound also suppresses TNF-α-induced

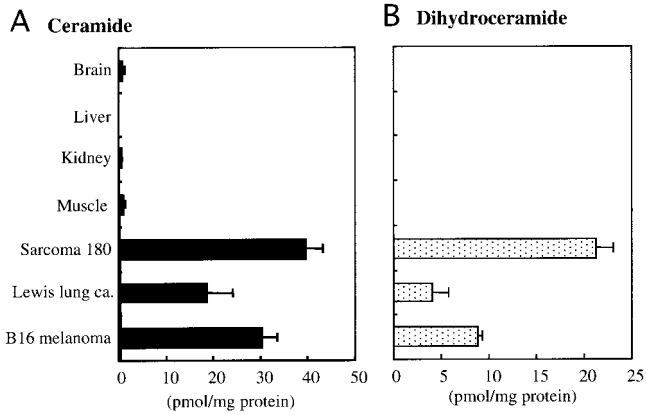

FIGURE 2 – Tissue levels of ceramide (*a*) and dihydroceramide (*b*) in tumor-bearing mice. Bars = the mean ± SE (n = 3).

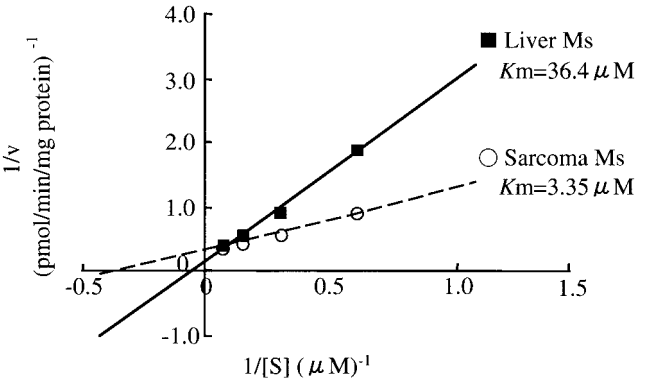

FIGURE 4 – Lineweaver-Burk plot analysis of C_{16}-dihydroceramide formation in the microsomes prepared from Sarcoma 180 tumor and normal liver tissues. Each point is the mean of duplicate experiments.

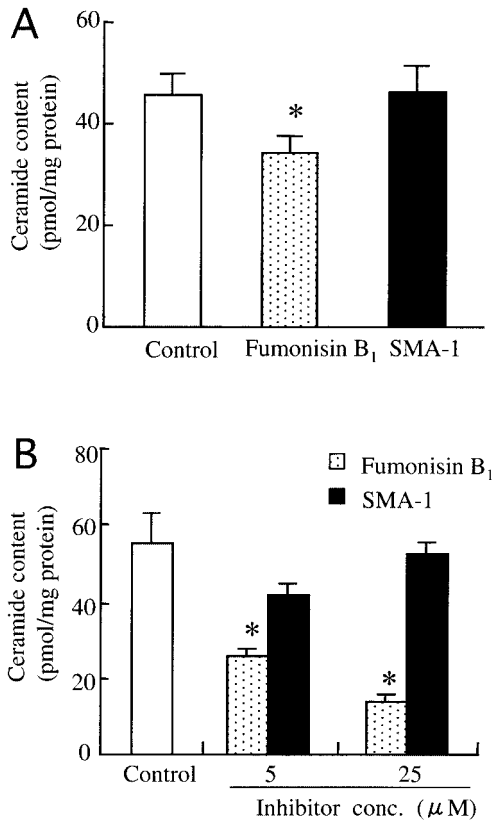

FIGURE 3 – (*a*) Effect of fumonisin B_1 or SMA-1 on the C_{16}-ceramide levels in Sarcoma 180 tumor masses in mice. We injected fumonisin B_1 or SMA-1 (50 nmol each) directly into the tumor masses. Bars = mean ± SE (n = 9). $p < 0.05$, compared to vehicle alone (control). (*b*) Effect of fumonisin B_1 or SMA-1 on the C_{16}-ceramide levels in cultured Sarcoma 180 cells. Bars = mean ± SE (n = 9). $p <$ 0.05, compared to vehicle alone (control).

apoptosis of PC-12 cells at a concentration of 0.1 μM by inhibiting the membrane SMase.[11] The inhibition of SMase resulted in a prevention of ceramide production in the cells. Therefore, the use of high concentrations (>1 μM) of SMA-1 in tumor cell culture experiments may completely inhibit the activity of SMase to produce ceramide via SM hydrolysis. Figure 3*a* shows that the administration of fumonisin B_1 significantly reduced ceramide levels. SMA-1, however, had no effect on ceramide levels. We then examined the inhibitor's effects on the C_{16}-ceramide levels in cultured Sarcoma 180 cells (Fig. 3*b*). Only fumonisin B_1 strongly reduced the ceramide levels. These results suggest that the high levels of ceramide and dihydroceramide in the tumor masses are produced by elevation of the *de novo* biosynthesis pathway, not by elevation of SM hydrolysis. The increase in both ceramide and dihydroceramide may be caused by an increase in both dihydroceramide synthase and desaturase activities, although Perry *et al.*[7] show that serine palmitoyltransferase is the initial and rate-limiting enzyme in the de novo pathway. To elucidate the enzymatic properties of dihydroceramide synthase, which are still poorly understood,[12,14] we prepared microsomal fractions from Sarcoma 180 tumor masses and healthy mouse livers and compared the catalytic efficiency of both fractions in dihydroceramide formation with sphinganine and palmitoyl CoA as the substrates. The K_m values of Sarcoma 180 and healthy liver microsomes for sphinganine were found to be 3.35 and 36.4 μM, respectively (Fig. 4). The V_{max}/K_m values of Sarcoma 180 and normal liver microsomes were calculated to be 1.07 and 0.37, respectively. These results suggest that the dihydroceramide synthase present in the microsomes of Sarcoma 180 cells catalyzes the conjugation of sphinganine and palmitoyl CoA 3 times more efficiently than it does in healthy liver. They also strongly suggest that the increase in *de novo* synthesized dihydroceramide observed in the tumor masses is caused, at least in part, by a malignancy-induced alteration of the enzymatic properties of dihydroceramide synthase.

Purification and characterization of dihydroceramide synthase from bovine liver microsomes

Despite this enzyme's physiological and pathological roles in ceramide biosynthesis, we have found no report of its complete purification.[12,14] We therefore attempted to purify and characterize dihydroceramide synthase. We chose bovine liver microsomes as the enzyme source, because large numbers of microsomes are needed for enzyme purification. Table I summarizes the results of a typical purification procedure. The final enzyme preparation had a specific activity of 2,450 U/mg protein and a 3,182-fold purification. Figure 5 shows a DE-53 column chromatograph of this enzyme. In this purification step, dihydroceramide synthase activity was inseparable from the activity of desaturase. Dihydroceramide synthase was clearly separated from desaturase by gel filtration on Sepharose CL-6B, however, and its molecular weight was calculated to be 333 kDa (Fig. 6*a*). Analysis of the enzyme preparation by electrophoresis on 12% SDS-polyacrylamide gel revealed a major protein band at 47 kDa (Fig. 6*b*). These results suggest that, under physiological conditions, the microsomal dihydroceramide synthase is present as a macro-molecule constructed of subunit proteins. The molecular properties of the enzyme are very similar to those of other ceramide synthases purified

TABLE I – PURIFICATION OF DIHYDROCERAMIDE SYNTHASE FROM BOVINE LIVER MICROSOMES

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Microsomes	820	631	0.77	100	1.00
Sodium cholate extract	441	432	0.98	68.5	1.27
DE-53	5.69	740	130	117	169
Sphinganine-coated celite	0.06	138	2,300	21.9	2,987
Sepharose CL-6B	0.05	123	2,450	19.5	3,182

One unit is defined as the amount of enzyme which forms 1.0 pmol of dihydroceramide pre min at 37°C.

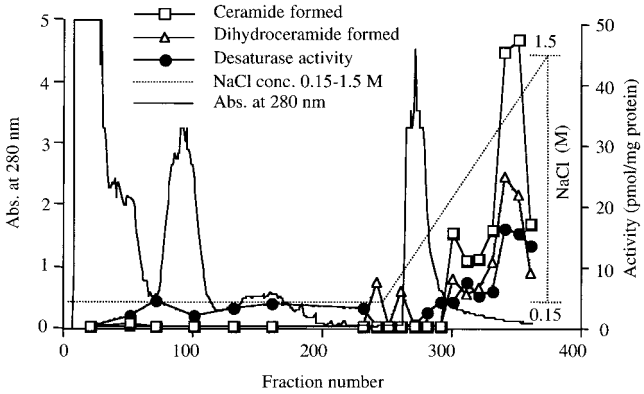

FIGURE 5 – DE-53 chromatography of dihydroceramide synthase preparation solubilized with 0.8% sodium cholate.

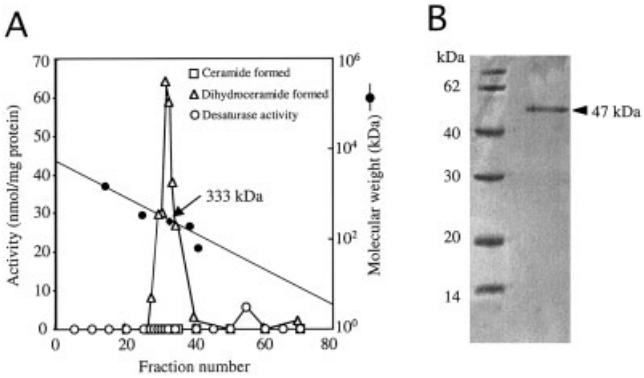

FIGURE 6 – Molecular weight and purity of the bovine liver microsomal dihydroceramide synthase. (*a*) Gel filtration chromatography on Sepharose CL-6B. Blue Dextran 2000 (2,000 kDa), ferritin (450 kDa), catalase (240 kDa), BSA (66 kDa) were used as molecular standards. (*b*) SDS-polyacrylamide gel electrophoresis of the final enzyme preparation in the presence of dithiothreitol. Phosphorylase b (97.4 kDa), BSA (66 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14 kDa) were used as the standards.

from bovine liver mitochondria[12] and rat liver microsomes.[16] Kinetic analyses using the purified enzyme demonstrated that the K_m value for sphinganine was 30.3 μM and that fumonisin B1 inhibited the enzyme in a non-competitive manner (data not shown).

Change in the molecular weight of dihydroceramide synthase in tumor masses

We solubilized dihydroceramide synthases (100 mg protein each) from the microsomes of Sarcoma 180 tumor masses and

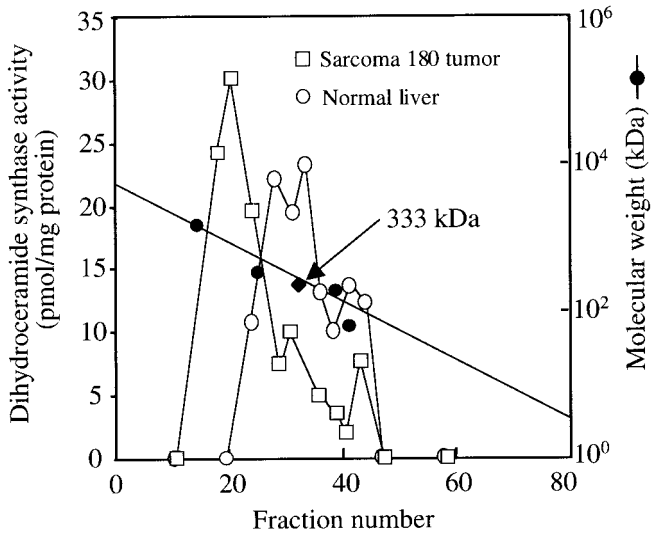

FIGURE 7 – Gel filtration on Sephadex CL-6B of dihydroceramide synthase solubilized from microsomes of Sarcoma 180 tumor or normal liver of mice. Blue Dextran 2000 (2,000 kDa), ferritin (450 kDa), catalase (240 kDa), BSA (66 kDa) were used as molecular standards. The data are results from 1 of 2 experiments that produced very similar results.

healthy liver cells of mice, and determined their molecular weight by gel filtration on a Sephadex CL-6B column (1.7 × 31 cm), equilibrated with 15 mM Tris-HCl buffer (pH 7.4)/0.15 M NaCl/0.2% sodium cholate. As shown in Figure 7, the activities of the Sarcoma 180 tumor enzyme were eluted near the column bed volume. Its molecular mass was estimated to be 1,300 kDa. The elution profile of mouse liver enzyme was the same, however, as that of the purified bovine liver enzyme (333 kDa). This suggests that dihydroceramide synthase is present in the tumor mass in a larger molecular weight form, but not simply in an aggregated form, because the gel filtration was carried out in the presence of sodium cholate.

DISCUSSION

Ceramide action and the regulation of its formation have recently attracted great attention, because of its possible roles in cellular differentiation, proliferation, and apoptosis.[17] Ceramide is formed by SMase-mediated hydrolysis of the plasma membrane SM or by the *de novo* synthesis pathway in cells. Better understanding of the ceramide formation system and its signaling might afford relevant insights into the pathogenesis of cancers, cardiovascular and neurodegenerative diseases and provide novel strategies for their therapy.[18] Little is known, however, about the regulatory mechanism of ceramide production and its link with specific signaling systems in tumors.

We have demonstrated that the tumor masses formed in mice have high concentrations of both dihydroceramide and ceramide.

The high levels of dihydroceramide and ceramide are caused by an increase in *de novo* synthesis of both compounds, not SMase-mediated SM hydrolysis. We reached this conclusion on the basis of 3 observations. First, a direct administration of fumonisin B_1 into the Sarcoma 180 tumor *in vivo* reduced the ceramide content, but the direct administration of SMA-1 had no such effect. Second, in experiments using Sarcoma 180 cells cultured *in vitro*, the inhibitors has a similar effect. Third, the kinetic analysis demonstrated that the dihydroceramide synthase present in the microsomes of Sarcoma 180 tumor cells formed dihydroceramide 3 times more efficiently than that it does in healthy liver. These results provide the first evidence that malignancy induces an increase in the catalytic efficiency of dihydroceramide synthase. The increase in not only dihydroceramide, but also ceramide levels suggests that the activity of dihydroceramide desaturase in the tumor also increases. Why tumors need such an accumulation of ceramide is an important question, because its major role in cells is thought to be the triggering of apoptosis. Ceramide, however, is a common biosynthetic precursor of complex sphingolipids such as SM and glycosphingolipids (GSLs). GSLs on the plasma membrane are involved in cell growth, differentiation and adhesion,[19] and SM also plays a critical role in cell to substratum adhesion.[20] Tumors might increase *de novo* biosynthesis of ceramide to produce the precursors of SM and GSLs. The temporary accumulation of ceramide, however, may trigger apoptosis in mouse tumors.[2,7,8] We found that the accumulated ceramide did not cause apoptosis. One possible explanation is that the net ceramide content we found in the tumor masses is not intracellular ceramide. In the epidermis of mammalian skin, ceramide is secreted into extracellular spaces to form keratinized cells and serves as a major component of the permeability barrier and a skin water reservoir.[21] The transformed mouse cells may secrete ceramide and dihydroceramide near the cell membranes. If the tumor cells translocated ceramide, however, the mechanism they used to do so is not known, although it is reported that P-glycoproteins function to transport phospholipids across cell membranes[22] and to reduce a small signaling pool of internalized SM.[23] Another possible explanation for the increase in ceramide levels is that, where the ceramide is formed may determine the lipid molecule's signaling function. The available evidence suggests that ceramide formed by SM hydrolysis triggers apoptotic death signaling in many cell types. The specific role of *de novo* synthesized ceramide in apoptosis, however, remains unknown.[9,24] Our findings suggest that the *de novo* synthesized ceramide in Sarcoma 180, B16 melanoma and Lewis lung carcinoma tumors may be linked to a survival signaling system related to cellular differentiation and proliferation.

The specific properties of dihydroceramide synthase are not well determined because its purification has not been achieved. We found that dihydroceramide synthase may exist in microsomes as a 333 kDa form constructed of subunit proteins. The molecular structure of this dihydroceramide synthase is similar to that of other ceramide synthases purified from bovine liver mitochondria. These synthases are a 240–260 kDa form constructed of subunit proteins.[12] The latter enzyme utilizes sphinganine as a substrate, and the K_m value is 144 μM.[12] It is important to note that dihydroceramide synthase is present in mitochondria, which are one of the targets for ceramide signaling.[17] The dihydroceramide synthase present in the mouse tumor microsomes has a larger than normal molecular mass; the increased mass appears to be the cause of the enzyme's increased catalytic efficiency.

In conclusion, tumors appear to require larger amounts of dihydroceramide and ceramide than normal tissues. The *de novo* synthesis pathway plays a key role; producing a high molecular weight microsomal dihydroceramide synthase that has increased catalytic efficiency. Neither SM hydrolysis nor GSL hydrolysis appears to contribute this ceramide production. Lower levels of microsomal SMase and the apoptotic protease caspase-3 in Sarcoma 180 tumor than those in normal liver (data not shown) suggest that *de novo* synthesized ceramide is important to tumor survival. A recent study by Riboni *et al.*[25] shows that ceramide levels are inversely associated with malignant progression in human glial tumors. They measured the ceramide levels by quantitative derivatization to ceramide-1-phosphate using diacylglycerol kinase and □ μγ-^{32}P □ ATP. Whether the low ceramide levels in high-grade tumors are associated with the decrease in *de novo* ceramide synthesis is unclear. This might be caused by the different cell type specificities. Further studies are needed to determine the roles of the different enzymes involved in ceramide accumulation and their regulation.

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