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Increases in Ceramide Levels in Normal Human Mesangial Cells Subjected to Different Cellular Stresses Result from Changes in Distinct Enzyme Activities and Can Influence Cellular Responses to Other Stimuli[1]

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Sphingolipids, ceramide in particular, have come to be regarded as having roles in cellular signaling, most recently being associated with stress and the cellular responses to stress. In the present study we first examined the mechanisms involved in the changes in cellular ceramide levels in normal human mesangial cells (NHMC) in the growth, quiescent, and senescent phases as well as those resulting from environmental stimuli. We found that in NHMC total ceramide levels increase in response to cellular stresses as a result of a combination of enzyme activities. Furthermore, different stresses cause different alterations in various enzyme activities, with age and growth influencing acidic enzymes, but cell density affecting neutral, resulting in final ceramide level increases which most likely are associated with distinct pools of ceramide. Secondly, we examined the influence of changes in ceramide levels on apoptosis induced by sphingosine and its methylated derivative *N,N*-dimethylsphingosine. We found that increases in cellular ceramide levels prohibited the apoptosis and caused a quiescent state in the cells. The data presented here provide additional insight into the roles of ceramide and related enzymes in cellular responses to stress and suggest a possible relevance to *in vivo* disease states.

Key words: apoptosis, ceramide, *N,N*-dimethylsphingosine, mesangial cell, stress.

Primary cells growing *in vitro* can be classified into four major phases: growth, quiescence, senescence, or apoptosis, each of which has its own set of inherent stresses. While in culture, additional environmental factors can act on the cells, at times enabling them to pass between phases or to signal other cell changes. These factors, which include the introduction of exogenous compounds or intrinsic influences such as cell culturing time, age (passage number), or density, act on cells to trigger internal signaling pathways and so are used as models for studying cellular processes.

Mesangial cells (MC) are an unusual type of contractile smooth muscle cell. In the normal glomerulus the regulation of mesangial cells and the surrounding matrix is tightly controlled. However, in many types of glomerulonephritis excessive MC proliferation is a major component of the disease process (*1*) and is associated with increased signaling from growth factors and cytokines, especially PDGF (*2, 3*). The resolution of the excessive proliferation and, thus, the recovery process is thought to involve apoptosis (*4, 5*). We previously showed that cultured normal rat mesangial cells were not susceptible to apoptosis by the sphingolipid sphingosine (Sph) or its methylated derivative dimethylsphingosine (DMS), but their transformed counterparts (*6*) were.

Sphingolipids, complex lipids consisting of a long chain amino base with an amide-linked fatty acyl group and a polar head group, have become recognized for their roles in cellular signaling (*7*). Ceramide in particular has been studied to determine its roles in various cellular processes (*8*), although its roles remain unclear. Ceramide has most recently been associated with stress and the cellular responses to stress (*9*). In the present study we examine mechanisms involved in the changes in cellular ceramide levels in normal human mesangial cells (NHMC) in the growth, quiescent, and senescent phases as well as those resulting from environmental stimuli. We further study the roles of these changes in intracellular signaling and the effect they have on the susceptibility of the cells to sphingosine-induced apoptosis.

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Abbreviations: MC, mesangial cells; NHMC normal human mesangial cells; Sph, sphingosine; DMS, *N,N*-dimethylsphingosine; SM, sphingomyelin; SMase, sphingomyelinase; MAPP, (1*S*,2*R*)-D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin; TLC, thin-layer chromatography; MsBM, mesangial cell basal media.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal human mesangial cells in the third passage were purchased from Clonetics and grown in mesangial cell basal media (MsBM) (Clonetics, San Diego, CA) supplemented with sodium pyruvate, sodium glutamate, penicillin-streptomycin, and 5% heat inactivated (30 min, 55°C) fetal bovine serum (FBS) (Hyclone, Logan, Utah). Cells were rendered quiescent by incubating the cultures for 48 h with MsBM supplemented as above with 0.25% bovine serum albumin in the place of FBS.

Sph, C2-ceramide, and C6-ceramide were purchased from Sigma Chemical (St. Louis, MO). DMS was prepared as previously described (*10*). (1*S*,2*R*)-D-*erythro*-2-(*N*-Myristoylamino)-1-phenyl-1-propanol (MAPP) was prepared according to published reports (*11*). Lipids were diluted in ethanol/water, 50:50, and added directly (final ethanol concentration was <0.01%). Growth factors were diluted into fresh culture medium and added to cells. PDGF-$\beta\beta$ and TNF-α were purchased from Boehringer Mannheim (Indianapolis, IN), TGF-β was from Sigma Chemical, and IL-1 was from Calbiochem (La Jolla, CA).

Enzyme Assays—SMase activities were measured by the method of Quintern and Sandhoff (*12*) modified as follows. For acidic SMase assays cells, grown and treated as indicated, were harvested by scraping, washed in Hanks Salt Solution with Mg^{2+}/Ca^{2+}, and centrifuged. The pellet was resuspended in lysis buffer (250 mM Na acetate, 1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 0.1 mM Na_3VO_4, 20 μM leupeptin, and 20 μM pepstatin, pH 5.0), sonicated briefly, incubated on ice for 20 min and microfuged 12,000 rpm for 5 min. Protein determination was by BCA method (Pierce, Rockford, IL). Activity was determined in a micellar assay with 0.02 μCi *N*-methyl-^{14}C sphingomyelin (Amersham, Buckinghamshire, England) and 40 nmol unlabeled sphingomyelin, dried in a glass tube, followed by the addition of 50 μl reaction buffer (250 mM Na acetate, 1 mM EDTA, pH 5.0) and sonication. Protein extract, 10 μg in 50 μl lysis buffer, was added, and the mixture incubated for 90 min at 37°C. After adding 800 μl chloroform/methanol (C/M) 2:1, the released ^{14}C-phosphocholine in the aqueous phase was measured by counting on a scintillation counter (Beckman Instruments, Palo Alto, CA) or identified by TLC using a solvent system of chloroform/methanol/acetic acid/water (100:60:20:5).

Neutral SMase was similarly measured using a neutral lysis buffer (20 mM HEPES, 10 mM $MgCl_2$, 2 mM EDTA, 10 mM β-glycerophosphate, 750 μM ATP, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, 0.2% Triton X-100, pH 7.4) and centrifuging the extract at 1,500 rpm for 5 min. Protein extract, 15 μg in 50 μl lysis buffer, was incubated with 0.02 μCi *N*-methyl-^{14}C sphingomyelin and 7.5 nmol in 50 μl neutral reaction buffer (20 mM HEPES, 10 mM $MgCl_2$, 250 μM ATP, pH 7.4) for 2 h at 37°C.

Ceramidase activities were measured according to methods previously published (*13*) with modifications. Cells were harvested as above, and pellets were resuspended in sucrose buffer (0.25 M sucrose, 1 mM EDTA). Micelles were prepared with 0.2 μCi 3-^{3}H-*N*-hexanoyl-sphingosine (C6-ceramide, New England Nuclear, Boston, MA), 25 nmol cold C6-ceramide, 100 μl of 0.25% Triton X100 in C/M 2:1 (0.1% for acidic) and 100 μl 0.4% sodium cholate in C/M (0.1% for acidic) which were dried down and then resuspended in 30 μl hot water and sonicated. After cooling, reaction buffer (0.125 M acetate, pH 4.5, or 0.125 M HEPES pH 8.5, or 0.125 M Tris pH 7.4 supplemented with 5 mM $MgCl_2$ and 1 mM PMSF) and cell extract were added, and the mixture incubated for 1.5 h at 37°C. The reaction was stopped by the addition of 800 μl C/M 2:1 and 100 μl water. After separation, the lower phase was washed with C/M/W 3:48:47 and dried down. TLC was performed in a solvent system chloroform/methanol/7 N NH_4OH/water 80:20:0.5:0.5 and visualized by autoradiography using XOMAT-AR X-ray film (Eastman Kodak, Rochester, NY). The band corresponding to ^{3}H-Sph was scraped and quantified by scintillation counting.

In the same conditions for the ceramidase assay, ^{3}H-C6-ceramide was replaced with 0.2 μCi ^{3}H-Sph and the reaction run and visualized by two different solvent systems. The amount of sphingosine converted to ceramide was <1/10 of the starting material in each of the three buffer systems (data not shown). Therefore, we concluded that under the experimental conditions no significant ^{3}H-Sph from the ^{3}H-C6-ceramide would be lost by conversion to ceramide. Additionally, it has been shown that C6-ceramide is a better substrate for the ceramidases than native ceramides (*13*).

Lipid Extraction and Measurements of Ceramide—For ceramide measurements, cells were scraped and washed as above. Cell pellets were microfuged to remove excess liquid. Lipids were extracted by three additions of methanol/chloroform 2:1, transferred to borosilicate tubes (13×100 mm) with Teflon lined caps and dried down under N_2. One-tenth of each sample was taken for total phospholipid analysis (*14*). For standards ceramide type III (Sigma) was added to tubes and dried down. Measurement of ceramide levels was accomplished using a modified version of the diacylglycerol kinase assay of Preiss *et al.* (*15*). Briefly, 20 μl of micelle buffer (7.5% *n*-octyl β-D-glucopyranoside, 5 mM cardiolipin, and 1 mM DTPA) was added to the test tube and sonicated, then 20 μl of reaction buffer (250 mM Tris HCl, 500 mM NaCl, 10 mM EGTA, and 25 mM Mg^{2+}) was added, and the mixture vortexed and sonicated. Cold ATP (10 mM) with (^{32}P)-γ-ATP 10^5 cpm per sample (New England Nuclear) was diluted in enzyme buffer (20 mM Tris HCl, 10 mM DTT, 1.5 M NaCl, 250 mM sucrose, and 15% glycerol) and 20 μl added to the tube and incubated at room temperature for 40 min. Phosphorylated ceramide was extracted by adding 1 ml C/M/W 100:100:1, 170 μl buffered saline solution (BSS; 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM MgCl, 5.6 mM glucose, 10 mM HEPES, pH 7.2) and 30 μl of 100 mM EDTA. The aqueous layer was removed, and the organic layer was washed 3 times with BSS. The organic layer was then dried under N_2 and resuspended in C/M 2:1. The final mixture was separated by TLC in a solvent system of chloroform/acetone/methanol/acetic acid/water at 10:4:2:2:1. Autoradiography was carried out using X-ray film (Kodak), and bands corresponding to ceramide-1-phosphate scraped and counted on a scintillation counter (Beckman).

Flow Cytometry—For apoptosis studies, cells were incubated with lipids for 5.5 h and then harvested and stained as previously described (*6*). Briefly, culture supernatants

and washes were combined and pelleted by centrifugation. Cells were detached with Trypsin/EDTA, and the cells and washes added to the first pellet and centrifuged. Pellets were resuspended in buffer (100 mM Na Citrate with 0.1% Triton X100) containing 50 μg/ml propidium iodide then stored overnight at 4°C in the dark. Cells were analyzed using a Becton Dickinson FACScan (San Jose, CA).

RESULTS

Ceramide Levels Increase during Culture Time through Combined Regulation by Sphingomyelinases and Ceramidases—We first examined the effect of culture time and replacing the media with fresh media (feeding) on ceramide concentrations. As NHMC grew exponentially in culture (Fig. 1B), ceramide concentrations increased linearly with growth time (Fig. 1A) up to 400% ($p<0.001$). However, in the absence of feeding, growth slowed after day 3 (Fig. 1B), and ceramide increases slowed, and leveled off (fed to unfed $p<0.05$).

To study the possible mechanisms involved in the increase in ceramide, enzymes involved in ceramide formation and metabolism were examined. Both acidic SMase and neutral SMase activities were measured in regards to culture time and feeding. While in culture, acidic SMase increased linearly with time regardless of the addition of fresh media, until at 7 days it was 200% of the levels seen at 24 h (Fig. 1C, $p<0.01$). On the other hand, neutral SMase increased linearly with cell growth in the presence of serum, until it was 350% ($p<0.05$) of baseline values by day 8 (Fig. 1D). However, in the absence of fresh media, the cells stopped growing (Fig. 1B), and the neutral SMase activity plateaued (Fig. 1D).

Ceramidase activities were also examined to see if changes in activity might account for some of the ceramide increases. Acidic ceramidase decreased linearly with culture time, also regardless of feeding new media, until levels were undetectable (Fig. 1E, $p<0.05$), directly inverse to the acidic SMase activities. Neutral ceramidase, however, did not change significantly in response to growth or culture time (Fig. 1F). Cellular ceramide increases during culture time, then, result from a combination of increased acidic SMase and neutral SMase activities with some influence by decreased acidic ceramidases.

Cell Density and Culture Age Increase Ceramide Levels through Diverse Regulation by Sphingomyelinases and Ceramidases—In addition to cell growth and media, cell density and passage number can affect the health of a culture and the growth of cells in primary cultures. To study the effects of these phenomena on ceramide forma-

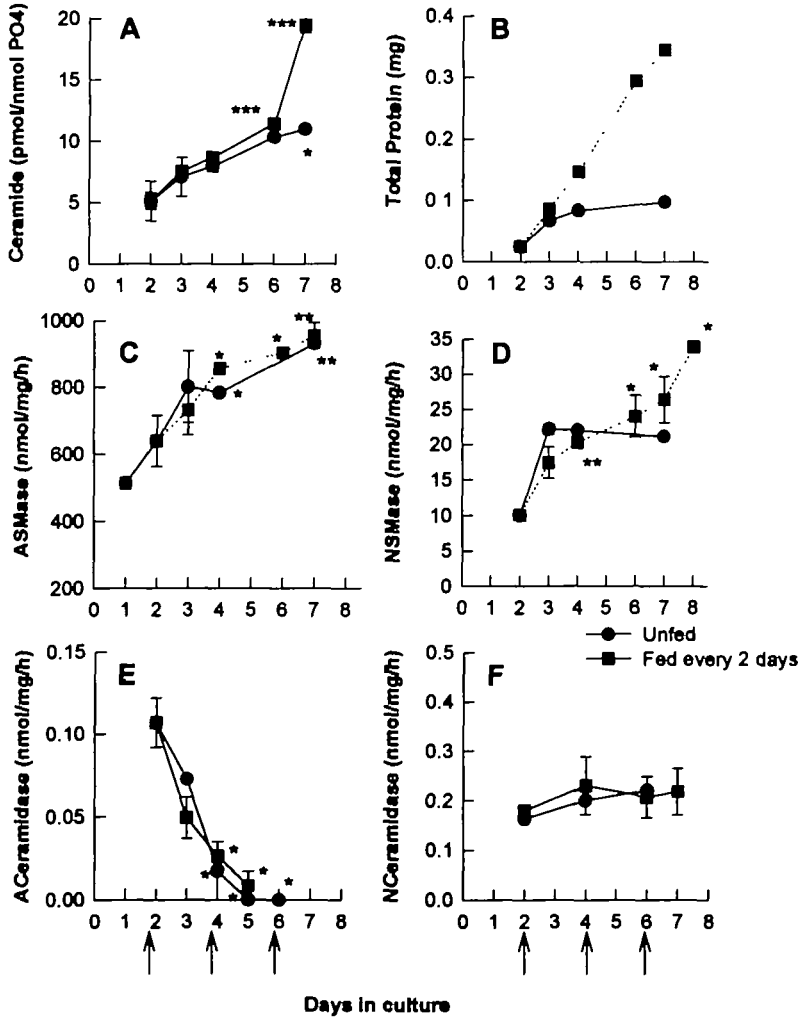

Fig. 1. **Effect of growth time and feeding on ceramide concentration and activities of related enzymes.** NHMCs (passages 6 to 7) were plated at 3,500 cells/cm² at day 0 and not fed (●) or fed every 2 days at arrows (■). Cells were harvested on the days indicated and assays conducted as stated in "EXPERIMENTAL PROCEDURES." (A) Ceramide concentrations. (B) Typical protein concentrations of harvested cells. (C) Acidic SMase activities. (D) Neutral SMase activities. (E) Acidic ceramidase activities. (F) Neutral ceramidase activities. A, C, D, E, F points are indicative of the mean of 2 or more separate experiments ± SE (some error bars are within the data point). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ with respect to first data point.

tion, we plated cells from different passages at three densities (Fig. 2B) and measured the total ceramide concentrations after 48 h. In young cell cultures, ceramide levels increased up to 2-fold with increased density (Fig. 2A, $p<0.01$). Additionally, in cultures plated at the lowest density, ceramide levels increased as the passage number increased ($p<0.01$), and the levels in the older cultures reached the same concentrations as the cultures plated at high density.

While acidic SMase increased linearly with time, this increase appeared to be less dependent on cell density with only a 45% increase in activity in cultures seeded at very high density (Fig. 2C, $p<0.05$), although passage age did appear to have a role as older cultures also had increased activities even when plated at lower densities. Moreover, we earlier saw that neutral SMase increased with time only while cells were growing. This increase directly corresponded to cell density (Fig. 2D, $p<0.05$), especially in early passages, although in later passages there was some increase in activity (Fig. 2D).

As for the ceramidase activities, there was a 23% ($p<0.05$) decrease in acidic ceramidase activities in the more densely plated cultures (Fig. 2C). Again no significant change was seen in neutral ceramidase activity in regards to cell density (Fig. 2F).

As primary cells are grown *in vitro*, the cell growth slows and the number of senescent cells increases with the number of culture passages. In NHMC senescence becomes an experimental factor after passage 10. The ceramide concentrations were seen to increase significantly ($p<0.01$) in higher passages (Fig. 2A). We measured SMase activities in various passages. In high passage cultures acidic SMase activity increased dramatically ($p<0.05$) until it was more than twice the value of earlier passages (Fig. 3A). Neutral SMase, however, increased only in the very oldest cultures (Fig. 3C, $p<0.05$).

Another factor in the growth of primary cells is that of contact inhibition, which can cause quiescence and even senescence. As stated earlier and seen in Fig. 2, cells plated at higher density exhibit increased activity of acidic SMase and, even more so, neutral SMase resulting in higher ceramide levels. Similarly, cells grown to higher confluency show a dramatic increase in neutral SMase activities (Fig. 3D, $p<0.001$) in a linear fashion. However, acidic SMase (Fig. 3B) shows a less dramatic increase which is not significant until cells are >80% confluent, at which time

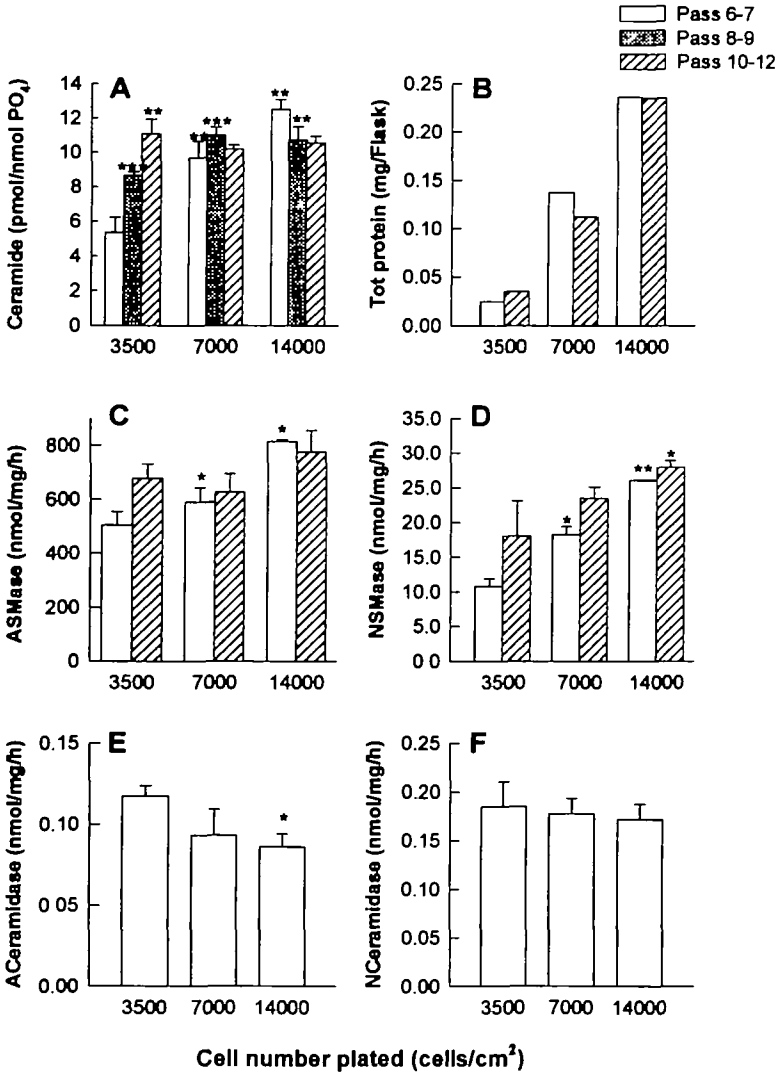

Fig. 2. **Ceramide concentrations increase and enzyme activities change with cell density and passage number.** NHMC at passages 6-7 (open bars), 8 to 9 (solid bars) or late passages >10 (hatched bars) were plated at the indicated cell number on day 0 and harvested at 40 h. Assays were conducted as stated in "EXPERIMENTAL PROCEDURES." (A) Ceramide concentrations. (B) Typical protein concentrations of harvested cells. (C) Acidic SMase activities. (D) Neutral SMase activities. (E) Acidic ceramidase activities. (F) Neutral ceramidase activities. A, C, D, E, F points are indicative of the mean of 2 or more separate experiments ± SD (some error bars are within the data point). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ with respect to first data point.

quiescence has begun. So, while acidic SMase activity seems dependent on both culture time and age, neutral SMase activity seems to be dependent mostly on cell density, and less on time in culture itself.

These studies indicate that during *in vitro* growth stresses placed on cells from a variety of conditions, including growth time, culture age, and cell density, cause total cellular ceramide levels to increase. However, different stresses affect different lipidases. Cell and culture age increase acidic SMase activities and decrease acidic ceramidase activity while cell density strongly affects neutral SMase.

Quiescence and Growth Stimulation Affect the Activities of Sphingomyelinases and Ceramidases—Quiescence can be imposed on primary NHMC by culturing them in the absence of serum. To measure the effect of quiescence and subsequent stimulation on SMase activities, cells at 70% confluence were incubated with MsBM containing 0.25% BSA for 48 h and then refed with MsBM/BSA or MsBM containing 5% FBS overnight, and the adherent cells tested for activity. As compared with activities found in cells growing in log phase at 70% confluency, acidic SMase activity increased by approximately 25% in quiescent cells. The addition of FBS to quiescent cells caused a 30% drop in activity from that of the unstimulated quiescent cells, returning to the levels of activity seen in growing cells (Fig. 4A). Neutral SMase activity exhibited a slight increase upon the removal of FBS, but the addition of FBS again returned the activity to the level seen in the continual presence of FBS (Fig. 4B).

To further study the effects of growth stimulation on the SMase activities, we examined various cytokines and growth factors. PDGF-$\beta\beta$, a known growth stimulant in NHMC (*16, 17*) caused responses similar to those of FBS stimulation in acidic SMase activities, giving a significant ($p<0.001$) drop of 28% in activity after 60 min stimulation with 10 ng/ml (Table I). This was shown to be highest at 60 min and dose dependent, with a maximum reached at 10 ng/ml (data not shown). Again, no change was apparent in the neutral SMase activity by PDGF-$\beta\beta$. Testing of ceramidase activities reflected the results seen in the culture time course in that PDGF-$\beta\beta$ increased acidic ceramidase by 10%, apparently working with the acidic SMase to raise the ceramide levels, but no significant effect was seen with either the neutral or basic ceramidase.

On the other hand, TGF-β, which is a growth inhibitor for NHMC and can act in a manner opposing PDGF-$\beta\beta$ (*17*), actually increased acidic SMase activity by 12% ($p<0.05$) and decreased neutral SMase activity by 15% ($p<0.001$) (Table I) which was seen to be both time and dose dependent (data not shown). Likewise, TGF-β decreased the acidic ceramidase by >20% ($p<0.001$), but increased the neutral ceramidase by 20% ($p<0.01$) again acting in a complementary fashion to the SMases.

TNF-α has been reported to have many varied effects on

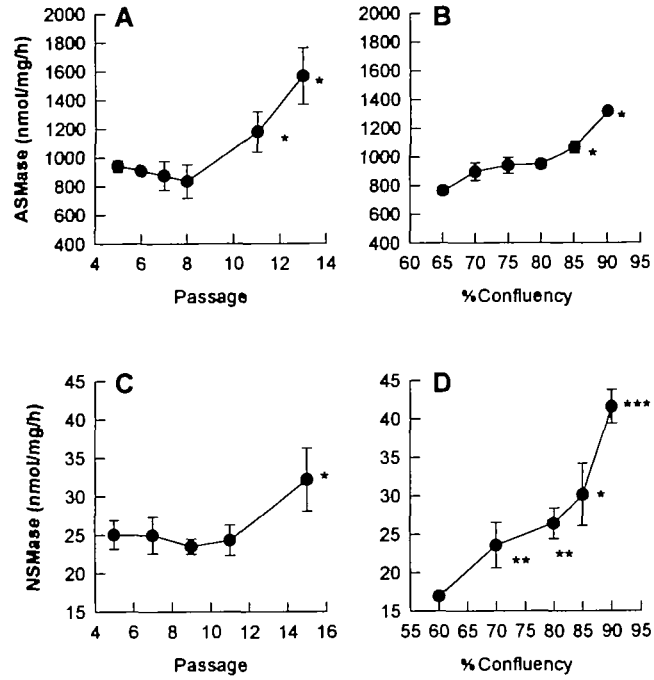

Fig. 3. **Sphingomyelinase activities in NHMC increase with cell age and culture confluency.** (A, C) Cells at various passages were cultured to medium confluency and Acidic SMase (A) or Neutral SMase (C) were measured as stated in "EXPERIMENTAL PROCEDURES." (B, D) Cells (passages <10) were split to 3,500 cells/cm² and cultured to the indicated confluency, then Acidic SMase (B) or Neutral SMase (D) activities were measured. Points are indicative of the mean of 2 or more separate experiments ± SE (some error bars are within the data point). $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ with respect to first data point.

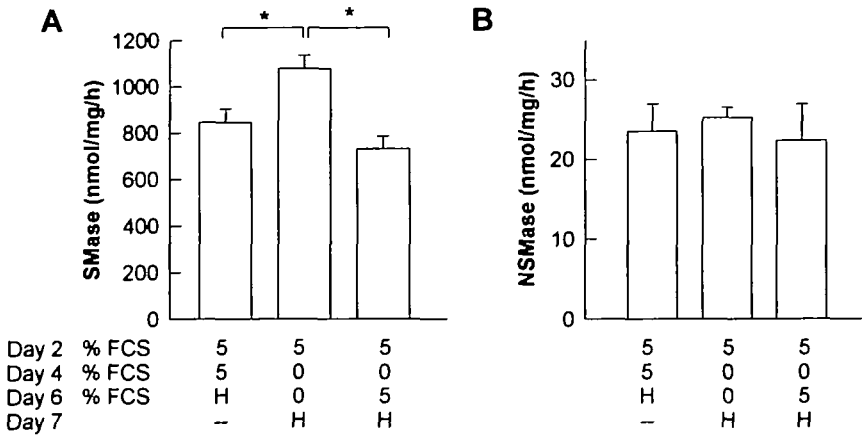

Fig. 4. **Comparison of growth, quiescence, and stimulation on sphingomyelinase activities.** NHMC (Passage 6-7) were plated at 3,500 cells/cm² and media replaced with fresh media with (5) or without (0) FBS and harvested (H) on the days indicated. SMase activities were measured as discussed in "EXPERIMENTAL PROCEDURES." (A) Acidic SMase activities. (B) Neutral SMase activities. Activities are expressed as a percentage of the control in log phase and presented as the mean of two or more separate experiments ± SD. $^*p<0.05$.

MC (*18*). We found that TNF-α had no effect on acidic SMase, but did increase neutral SMase by >20% (Table I, $p<0.05$). Likewise, TNF-α had no effect on the acidic ceramidase activity, but neutral ceramidase dropped by 15% ($p<0.05$), again complementing the rise in neutral SMase. IL-1, however, which is reported to be involved in signaling pathways not related to proliferation in MC (*19*) had no effect on either SMase under any conditions.

To summarize this data, the growth stimulus from PDGF-$\beta\beta$ or FBS acts to decrease the acidic SMase and increase the acidic ceramidase activities presumably to lower the ceramide content, but does not affect the neutral system. On the other hand, TGF-β acts to increase the acidic SMase and decrease the acidic ceramidase activity thereby increasing the ceramide content resulting from the acidic enzymes, yet it also acts to decrease the neutral SMase activity and increase the neutral ceramidase activity with the overall effect likely to be to lower the ceramide content arising from the neutral enzymes. Finally, TNF-α which increased neutral SMase also decreased the neutral ceramidase. The effects of various agents on related enzymes that are pH-dependent further indicates the likely importance of compartmentalization of ceramides in signaling (*8*).

Ceramide-Derived Quiescence and Growth Stimulation Affect the Susceptibility of NHMC to Sph/DMS Induced Apoptosis—We previously showed that unstimulated rat MC are not susceptible to apoptosis induced by Sph/DMS (*6*). Similarly, NHMC rendered quiescent did not respond to Sph/DMS by apoptosis. However, upon stimulation with either 10 ng/ml PDGF-$\beta\beta$ or 5% FBS (Fig. 5) the amount of apoptosis induced by sphingosine increased 2-fold ($p<0.05$) or over 3-fold ($p<0.005$) respectively, while DMS-induced apoptosis doubled for each ($p<0.05$).

Since ceramide levels were shown to be increased by SMases during quiescence, we examined the possibility that exogenously increasing cellular ceramide levels could in turn induce a state of quiescence in NHMC. Cellular ceramide levels were increased by the addition of ceramide analogues C2- or C6-ceramides or by the addition of the ceramidase inhibitor MAPP (10 μM) or bacterial SMase (0.5 U/ml) to cells growing in log phase. After 18 h the DNA distribution of the cells was tested by flow cytometry. All agents increased the percentage of cells in G_0/G_1 phase, indicating a quiescent state (Table II). Ceramide analogues increased the portion of cells in the quiescent state to 132-138% of the control ($p<0.01$) while SMase increased it to 124% ($p<0.01$). Furthermore, cultures which were rendered quiescent by the ceramide-increasing agents also lost the ability to undergo apoptosis induced by 20 μM Sph. Likewise, C2-ceramide inhibited this ability by 37% ($p<0.001$), C6-ceramide by 25% ($p<0.001$), SMase by 30% ($p<0.01$), and MAPP by 10%.

Moreover, cells which were made quiescent by the

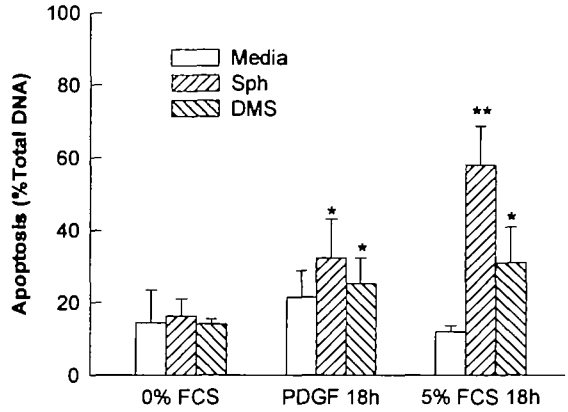

Fig. 5. **Growth factors enhance the susceptibility of NHMC to sphingosine-induced apoptosis.** Quiescent NHMC were pretreated for 18 h in the absence or presence of growth factors and then treated with 20 μM Sph (right-hatched bars) or DMS (left-hatched bars) or untreated (open bars) for 5.5 h and examined by flow cytometry for apoptosis. Activities are expressed as a percentage of total DNA and presented as the mean of two or more separate experiments±SD. $^*p<0.05$, $^{**}p<0.01$.

TABLE I. **Effect of 60 min treatment of growth factors on activities of ceramide forming enzymes in quiescent NHMC (Passage 6-7).**[a]

Growth factor	SMase activity		Ceramidase activity		
	Acidic	Neutral	Acidic	Neutral	Basic
Control	100.0±7.3	100.0±5.1	100.0±5.5	100.0±3.2	100.0±5.6
PDGF-$\beta\beta$ (10 ng/ml)	71.7±6.7***	96.2±3.3	108.5±5.0*	103.3±3.9	95.8±14.0
FBS (5%)	74.2±14.1*	96.8±5.5	96.8±11.5	103.3±11.9	103.7±5.3
TGF-β2 (10 ng/ml)	112.4±6.6*	84.8±5.9***	78.4±2.9***	119.8±2.5**	102.5±3.9
TNF-α (10 ng/ml)	99.2±4.8	122.3±10.4*	102.0±4.1	85.0±0.3*	107.4±7.6
IL-1 (10 ng/ml)	99.3±4.2	100.3±0.5	ND	ND	ND

[a]Values are presented as % untreated control±SD based on 2 or more separate experiments. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

TABLE II. **Effect of increased ceramide on quiescence and on sphingosine induced apoptosis[a] in growing and FBS stimulated NHMC.**

Pretreatment	Growing[b]		FBS stimulated[c]	
	G_0/G_1[‡]	$<G_0$	G_0/G_1	$<G_0$
Control	100.0±6.5	100.0±4.0	100.0±3.3	100.0±7.2
C2Cer (15 μM)	138.5±7.7**	63.4±3.3***	106.9±3.5*	74.5±4.0***
C6Cer (15 μM)	132.2±6.3**	75.7±3.0***	120.1±3.0*	67.5±8.3***
MAPP	114.8±8.2	90.7±10.6	107.7±9.0	84.2±8.5***
Bact SMase (0.5 U/ml)	124.4±3.7**	69.7±7.3**	111.5±2.1*	82.7±8.0*

[a]20 μM Sph for 5.5 h. [b]Cells in log growth phase. [c]Cells were rendered quiescent then stimulated to grow 5% FBS overnight. [‡]Values were determined by flow cytometry and are presented as % Control±SD. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

removal of serum were unable to return to growth phase after FBS stimulation in the presence of any of these agents ($p < 0.05$). And, again, the presence of ceramide-increasing agents inhibited Sph-induced apoptosis in serum stimulated cells by 15-33% ($p < 0.001$-0.01). These data indicate that quiescence involves, and can be induced by, increased cellular ceramide concentrations which then inhibit the ability of cells to undergo apoptosis in response to the addition of Sph.

DISCUSSION

The studies presented here indicate that during *in vitro* growth stresses placed on cells from a variety of conditions, including growth time, culture age, and cell density, cause cellular ceramide levels to increase. However, different stresses involve different enzymes resulting in increased total ceramide. In growing, young, healthy cells ceramide increases can be attributed to increases in both acidic and neutral SMases with some influence from acidic ceramidases. However, under stress ceramide levels increase for various reasons. Cell and culture age, as indicated by days cultured and passage number, cause an increase in acidic SMase activity, and some decrease in acidic ceramidase activity, while overcrowding of cells in culture activates primarily the neutral SMase. The end result of each being an increase in total ceramide levels, although it is important to remember that in NHMC the range of activity of acidic SMase is 10-fold higher than that of neutral SMase, and is therefore more influential on total cellular ceramide concentrations measured. These distinct mechanisms for increasing ceramide also support the theory that compartmentalization of the ceramide and distinct pools of sphingomyelin are important factors in cell signaling (*8*).

Recently, others have reported that ceramide levels are increased in other cell types, including leukemia and fibroblastic cells, in response to cell cycle arrest (*20*) or age (*21*). In fibroblasts, however, the increase in senescent cells was attributed to elevated neutral SMase activity with less increase in acidic SMase activities, although overall acidic SMase activities still appeared to be more than 10-fold higher than that of the neutral. This difference may be due to mathematical interpretation (the enzymatic specific activities when calculated give different results) or may simply reflect a difference in cell type as fibroblasts and MC are unrelated cell types and perform very different roles *in vivo*.

Additionally, that report found no influence from contact inhibition on SMase activity, whereas we clearly saw that neutral SMase activity increased linearly with cell density regardless of growth time. Again, these are different cell types and MC are thought to be more sensitive to surrounding cells due to their unique environment *in vivo* (*22*). It is quite plausible that neutral SMase has a signaling role in the cell-to-cell signaling termed contact inhibition which induces cell cycle arrest, usually reversible, since one pool of sphingomyelin resides in the cell membrane and a neutral SMase is thought to also be in the same location (*8*). However, this signaling is poorly understood, and any role neutral SMase or ceramide may have remains to be seen.

The later passage numbers of a primary cell culture reflect the cells becoming senescent. We saw substantial increases in acidic SMase, and additional increases in neutral SMase, activities in later passages. Interestingly, this data is similar to recent reports regarding elevated SMase activities in brain tissue from genetically engineered senescence-accelerated mouse (SAM) prone mice as compared to SAM resistant mice which also increased with the age of the mice (*23*). The activity was apparent over a pH range of 4-9, although it is known that neutral SMase activity is quite high in the brain (*24*).

Another fascinating report which supports our conclusion of a relation between acidic SMase and senescence is the identification of overexpressed genes coding for acidic SMase in fibroblasts from patients with Werner Syndrome (WS), a disorder characterized by premature aging (*25*). Elevated levels of acidic SMase mRNA were found in both WS and senescent normal human fibroblasts as compared to young fibroblasts.

Unlike senescence, quiescence is a reversible state of cell cycle arrest. Acidic SMase activity increased in quiescent cells but also decreased upon subsequent stimulation with growth factors. This data suggests that growth stimuli also act on the acidic system to decrease the resulting ceramide content, whereas the growth inhibitory signals, including aging or the addition of growth inhibitor factors such as TGF-β, act oppositely, to affect acidic lipidases to increase the ceramide concentrations. On the other hand, less evidence exists for the involvement of neutral enzymes in growth signaling, but argues for a role in other types of signaling including growth inhibition and cellular activation.

Data regarding growth factors and SMases which do not conform with ours have been reported by Kester's group (*26, 27*) based on studies in rat mesangial cells, which are known to differ significantly from NHMC in metabolic responses to many cytokines and factors including IL1 and TNFα (*22*). They found Sph to be associated with growth and with increased phosphorylation and activity of ERK, and also found that PDGF stimulated ceramidase activity. However, in these experiments cells were pretreated with the phosphatase inhibitor sodium vanadate. Vanadate is known to be insulin-mimetic and has been reported to stimulate a variety of signals in mesangial cells including stimulation of PLC or PKC, increased protein phosphorylation, and DNA synthesis (*28, 29*). Additionally, growth stimuli signaled by such factors as PDGF have been shown to be regulated by a vanadate sensitive phosphatase (*30*). Furthermore, we and others (*30, 31*) have shown that Sph inhibits ERK phosphorylation and activation, and we have provided evidence that this is accomplished *via* a kinase phosphatase (*30*). Any such phosphatase would likely be inhibited in their system.

NHMC in a quiescent state were not susceptible to apoptosis induced by Sph/DMS, but in the presence of FBS or PDGF the cells became susceptible. PDGF and FBS stimulate proliferation of NHMC in part by increasing MAP kinase activity (*33*). We have previously shown that Sph/DMS inhibit MAP kinase phosphorylation and presented evidence that this inhibition is involved in Sph/DMS induced apoptosis. The increase in susceptibility of the NHMC in the presence of these agents would support this conclusion.

There remains much discussion regarding the role of ceramide in apoptosis and cell death. Our study has indicated that total ceramide levels are increased from a variety of

factors and that large increases in cellular levels does not necessarily give rise to cell death. Our findings and others' (*20, 21, 34*) that increased ceramide levels can cause cell arrest strongly support the argument that not only is ceramide increase a result of stress, but that it likely has multiple roles in cellular responses to stress involving growth inhibition, growth arrest, or death (*9*). However, we have found an additional impact of increased ceramide and its ability to induce cell cycle arrest in that it is able to inhibit apoptosis by Sph, which we have shown to induce apoptosis upstream in the apoptosis cascade (*35*). This inhibition may be a secondary result of the cell cycle arrest or may reflect a direct involvement between ceramide and another cofactor. Some proposed factors include the retinoblastoma protein (*36*) or bcl-2 (*37*), a protein also reported to be involved in glomerulonephritis (*38*). Furthermore, *in vivo* these high ceramide levels may have negative implications for the resolution of excessive proliferation of MC through apoptosis and, ultimately, for the recovery process of glomerulonephritis.

In conclusion, we have found that in NHMC total ceramide levels increase in response to cellular stresses as a result of a combination of enzyme activities. Furthermore, different stresses cause different alterations in various enzyme activities, with age and growth influencing the acidic enzymes but cell density affecting the neutral, resulting in the final ceramide level increases most likely associated with distinct pools of ceramide. Increases in ceramide can also lead to a quiescent state which can prohibit the cells from engaging in apoptosis with potentially negative effects *in vivo*.

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