Human Vascular Endothelial Cells Are a Rich and Regulatable Source of Secretory Sphingomyelinase

IMPLICATIONS FOR EARLY ATHEROGENESIS AND CERAMIDE-MEDIATED CELL SIGNALING*

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We recently reported that macrophages and fibroblasts secrete a Zn²⁺-dependent sphingomyelinase (S-SMase), which, like lysosomal SMase, is a product of the acid SMase gene. S-SMase may cause subendothelial retention and aggregation of lipoproteins during atherogenesis, and the acid SMase gene has been implicated in ceramide-mediated cell signaling, especially involving apoptosis of endothelial cells. Because of the central importance of the endothelium in each of these processes, we now sought to examine the secretion and regulation of S-SMase by vascular endothelial cells. Herein we show that cultured human coronary artery endothelial cells secrete massive amounts of S-SMase (up to 20-fold more than macrophages). Moreover, whereas S-SMase secreted by macrophages and fibroblasts is almost totally dependent on the addition of exogenous Zn²⁺, endothelium-derived S-SMase was partially active even in the absence of added Zn²⁺. Secretion of S-SMase by endothelial cells occurred both apically and basolaterally, suggesting an endothelial contribution to both serum and arterial wall SMase. When endothelial cells were incubated with inflammatory cytokines, such as interleukin-1β and interferon-γ, S-SMase secretion by endothelial cells was increased 2–3-fold above the already high level of basal secretion, whereas lysosomal SMase activity was decreased. The mechanism of interleukin-1β-stimulated secretion appears to be through increased routing of a SMase precursor protein through the secretory pathway. In summary, endothelial cells are a rich and regulatable source of enzymatically active S-SMase, suggesting physiologic and pathophysiologic roles for this enzyme.

The enzyme sphingomyelinase (SMase)¹ (sphingomyelin phosphodiesterase; EC 3.1.4.12) catalyzes the hydrolysis of sphingomyelin to ceramide and choline phosphate (1). SMase reactions have been implicated in specific atherogenic processes (2–5) and in cell signaling events (6–8). For example, partial hydrolysis of lipoprotein sphingomyelin by bacterial SMase, via the generation of lipoprotein ceramide, leads to lipoprotein aggregation (2) and retention onto arterially derived matrix (3). Aggregation and retention of lipoproteins in the arterial wall are prominent events during atherogenesis (9–11), and these lipoprotein aggregates are among the most potent inducers of macrophage cholesteryl ester accumulation (“foam cell” formation) (2, 3, 12–14). Most importantly, recent findings indicate that subendothelial LDL, including aggregated LDL isolated from human lesions, is hydrolyzed by an arterial wall SMase (4). Regarding cell signaling, treatment of certain cell types with bacterial SMase results in cellular differentiation, cellular senescence, or apoptosis, which are thought to be triggered by ceramide-mediated signal transduction pathways (6–8). Moreover, cell-derived SMase activity and then cellular ceramide content rise when these cell types are treated with specific inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1β, and interferon-γ (6–8).

Experimental evidence suggests that products of the acid SMase (ASM) gene may have a prominent role in both atherogenesis and ceramide-mediated apoptosis. Hydrolysis of lipoprotein SM retained on subendothelial matrix would clearly be expected to be an extracellular event. In this context, our laboratory has reported that the ASM gene in macrophages and fibroblasts gives rise not only to lysosomal SMase (L-SMase) but also, via differential trafficking of the ASM precursor protein,² to a secretory SMase (S-SMase) (15). Importantly, S-SMase secreted by these cells, which is activated by physiologic levels of Zn²⁺ (15), can hydrolyze and cause the aggregation of atherogenic lipoproteins, even at neutral pH (16). Several cell culture studies have also implicated a role for acid SMase activity in cytokine-induced, ceramide-mediated apoptosis (17–19), and mice in which the ASM gene has been disabled by homologous recombination show defective radi-

¹ The abbreviations used are: SMase, sphingomyelinase; ASM, acid sphingomyelinase; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HI-FBS, heat-inactivated FBS; HUVEC, human umbilical vein endothelial cell; IL, interleukin; LDL, low density lipoprotein; L-SMase, lysosomal sphingomyelinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SM, sphingomyelin; S-SMase, secretory sphingomyelinas; o-MEM, α minimum essential medium.

² S. L. Schissel, E. H. Schuchman, K. J. Williams, and I. Tabas, submitted for publication.
tion- and endotoxin-induced apoptosis in vivo (20, 21). The products of the ASM gene that mediate these cell signaling events have not been identified, but S-SMase might be an excellent candidate, since the outer leaflet of the plasma membrane is rich in SM (22).

The endothelium is central to these processes. Our previous work on S-SMase focused on macrophages (15), which enter the arterial wall in response to the initial retention of lipoproteins in the subendothelial matrix (5, 23) and therefore may contribute to lipoprotein aggregation after lesion initiation. Lipoprotein aggregation also occurs, however, in prelesional susceptible segments of the subendothelium (10), and thus the endothelium would be a likely candidate for a source of S-SMase in the prelesional arterial wall. Likewise, recent in vivo studies have shown that the endothelium is the key tissue in cytokine-induced, ASM-mediated apoptosis (20, 21). Moreover, cytokines are important regulators of endothelial function (24, 25), atherosclerosis, and apoptosis (26, 27), which raises the possibility that cytokines influence endothelial secretion of S-SMase.

In this report, we demonstrate that cultured endothelial cells, including human coronary artery endothelial cells, are an even more abundant source of S-SMase than are macrophages. Furthermore, we show that the secretion of S-SMase by endothelial cells is strongly regulated by cytokines known to be present in atherosclerotic and inflammatory lesions. The mechanism of this regulation is primarily through an alteration in the cellular trafficking of the ASM precursor protein. Thus, the vascular endothelium is likely to be a major, regulatable source of S-SMase that may contribute to atherogenesis and inflammation.

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Millicell-CM 0.4-μm culture plate inserts were from Millipore Corp. Tissue culture media and other tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65 °C (HI-FBS). Human recombinant cytokines were obtained as follows: interferon-β and interferon-γ from Biogen (Cambridge, MA), interleukin-1β from R & D Systems Inc. (Minneapolis, MN), [9,10-3H]palmitic acid (56 Ci/mmol) was purchased from DuPont NEN, and all organic solvents were from Fisher.

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the method of Lowry et al. (40), and subjected to SDS-PAGE and immunoblotting.

The conditioned medium was concentrated, dialyzed against buffer A, and subjected to the same two rounds of DEAE-Sephaloc chromato-
graphy as above. The combined flow-through fractions from the sec-
ond DEAE-Sephaloc step were then concentrated and dialyzed against
10 mM Tris-HCl, 500 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM CaCl2,
0.1% Nonidet P-40, and 0.02% NaN3, pH 7.2 (buffer B). This solution
(<1 ml) was added to a 1-ml concanavalin A-Sepharose column and
incubated with the column exactly as described for the DEAE-Sephaloc
step. The column was then washed with two volumes of buffer B
containing 10 mM methylglucopyranoside, followed by two bed volumes
of buffer B containing 1 mM methylglucopyranoside. This last eluate
was concentrated, assayed for protein concentration by the method of Lowry
(40), and subjected to SDS-PAGE and immunoblotting.

Northern Blot of S-Mase mRNA—Total cellular RNA was isolated
from HUVECs using RNaZol B (Tel-Test, Inc., Friendswood, TX). Ap-
proximately 10 μg of RNA was separated on a 1% agarose gel and
blotted onto a nylon membrane. The membrane was hybridized in a
QuickHyb hybridization solution (Stratagene) with a 1.6-kilobase pair
fragment of human ASM cDNA (41) that was labeled with
EcoRI–XhoI

32P by the random-priming procedure (Life Technologies, Inc.). The
membranes were incubated with 5% Carnation nonfat dry milk, and 0.1% bovine serum albumin) for
approximately 10 minutes at room temperature. The membranes were then incubated with mouse anti-
FLAG monoclonal antibody (1:1000) or rabbit anti-L-SMase antibody
(1:1000) in buffer D (buffer C containing 0.1% Tween 20, 3% nonfat dry milk, and 0.1% bovine serum albumin) for 1 h at room temperature.
After washing four times with buffer C containing 0.1% Tween 20, the
blots were incubated with horseradish peroxidase-conjugated sheep
anti-mouse IgG (1:20,000) or goat anti-rabbit IgG (1:20,000) for 1 h in
buffer D at room temperature. The membranes were subsequently
washed twice with 0.3% Tween 20 in buffer C and twice with 0.1%
Tween 20 in buffer C. Finally, the blots were soaked in the enhanced
chemiluminescence reagent (Pierce “Super Signal” kit) for 2 min and
exposed to x-ray film for 1 min.

Immunoprecipitation of S-SMase—20 μl of HUVEC conditioned me-
dium, 20 μl of affinity-purified anti-SMase antibody, and 160 μl of PBS
were incubated for 1 h at 4°C. 100 μl of Protein A-Sepharose (50 mg/ml
in 50 mM Tris buffer, pH 7.0) was then added to the incubation mixture,
and the slurry was mixed end-over-end for 18 h at 4°C. The suspension
was centrifuged for 1 min in a microcentrifuge. The supernatant was
harvested and assayed for S-Mase activity.

Statistics—Unless otherwise indicated, results are given as means ±
S.D. (n = 3); absent error bars in the figures signify S.D. values smaller
than the symbols.

RESULTS

Cultured Human Vascular Endothelial Cells Secrete Abundant
Amounts of S-SMase—Previous work from our laborato-
ries revealed that human and murine macrophages were a relatively
abundant source of S-SMase compared with other cell types, such as COS-7 and murine migroglial cells (15). As
shown in Fig. 1A, however, both human coronary and umbilical
vein endothelial cells (HUVECs) are a much more abundant
source of S-SMase; for example, HUVECs secrete almost 20-fold more S-SMase than human macrophages. Interestingly,
endothelium-derived S-SMase was partially active in the absence
of exogenously added Zn2+ (Fig. 1A), whereas S-SMase
secreted by macrophages and other cell types previously exam-
ined by us was almost entirely Zn2+-dependent (15). This
observation may be important in the regulation of endotheli-
um-derived S-SMase activity (see “Discussion”). The data in
Fig. 1B show that S-Mase activity in the cell homogenate, which
is not stimulated by exogenous Zn2+ and represents L-SMase
activity (15), is also very abundant in endothelial cells (recall
that a single enzyme accounts for SMase activity in the conditioned
medium of HUVECs).

We next addressed the polarity of secretion of S-SMase from
cultured endothelial cells. Cultures of endothelial cells were

3 95% of S-SMase activity from HUVECs was immunoprecipitated by
an affinity-purified anti-S-SMase antibody, and 97% of HUVEC S-
SMase activity was inactivated by chelation of Zn2+ with 10 mM EDTA
plus 10 mM 1,10-phenanthroline (see Footnote 2). These data indicate that
a single enzyme accounts for SMase activity in the conditioned
medium of HUVECs.
established on Millicell-CM inserts in 35-mm dishes so that S-SMase from the upper (apical) and lower (basolateral) chambers could be assayed separately. As a control for apically secreted enzyme that either “leaked” or was transcytosed into the lower chamber, we set up parallel dishes in which immunoprecipitation (15), wash with PBS, and then incubated with α-MEM/BSA again for 1 h. At the end of this 1-h incubation, medium above the cells (apical; cross-hatched bars) and medium under the insert (basolateral; solid bars) were assayed for SMase activity in the presence of 0.1 mM ZnCl₂. The results of two separate experiments are shown. Insert, as a control for leakiness of the cell monolayer or for transcytosis, FLAG-tagged S-SMase was added to the upper chamber of parallel dishes of those in experiments 1 and 2 and incubated with the cells as described above. At the end of the 1-h incubation, FLAG-tagged S-SMase in media above the cells (open arrow), which is most likely L-SMase and possibly some S-SMase in the secretory pathway. By comparison, medial staining was much weaker, although still specific. These data demonstrate that the high levels of intracellular SMase in cultured endothelial cells (Fig. 1B) reflect the situation in an actual vessel wall. In addition, we also noticed areas of dark staining on the luminal edge of some of the endothelial cells (closed arrow). This staining is specific (i.e., not in panel A) and does not appear lysosomal, and so it is possible that this signal represents S-SMase that has been secreted and perhaps retained on the cell surface.

**Secretion of S-SMase from Human Endothelial Cells Is Regulated by Cytokines**—Inflammatory cytokines are important constituents of atherosclerotic lesions and may contribute to various aspects of atherogenesis (26). A major target of these cytokines is the endothelium (24, 25). To determine if cytokines known to be present in atherosclerotic lesions affect endothelium-derived S-SMase, cultured human endothelial cells were exposed to IL-1β, interferon-γ, interferon-β, and IL-4. Each of the first three cytokines substantially increased the accumulation of S-SMase in the conditioned media of these cells, although IL-4 had no effect (Fig. 4A). In particular, IL-1β and interferon-γ increased S-SMase activity ~3-fold. Interestingly, the stimulatory cytokines led to a decrease in L-SMase activity (Fig. 4B). This pattern is distinct from that observed during monocyte-to-macrophage differentiation, in which both S- and L-SMase activities are increased (15).

**IL-1β Increases S-SMase Secretion by HUVECs via Alteration in the Trafficking of the ASM Precursor Protein**—We next sought to determine the mechanism of cytokine-mediated induction of S-SMase secretion by HUVECs. For these studies, we focused on IL-1β, and we first determined whether this cytokine increased SMase mRNA levels in HUVECs. As shown in Fig. 5, there was an ~40% increase in SMase mRNA in IL-1β-treated HUVECs, when normalized for glyceraldehyde-3-phosphate dehydrogenase mRNA and quantified by either densitometry or molecular imaging. Because differences less than 2-fold in Northern blot assays may not be significant and because the IL-1β-induced increase in S-SMase activity was greater than 3 times control, we conclude that most, if not all, of the induction by this cytokine was post-transcriptional. This conclusion is consistent with the finding that the cytokine-mediated increase in S-SMase is accompanied by a decrease in L-SMase (above).

Next, we asked whether the mechanism of IL-1β-induced S-SMase secretion could be the result of cellular lysis. For example, cellular release of certain molecules involved in inflammation may occur physiologically by this mechanism (45). To test this idea, the release of the cytosolic protein lactate dehydrogenase was assayed. Under conditions in which S-SMase was induced by IL-1β to 3.6 times control (Fig. 6A), lactate dehydrogenase release increased only 60% (Fig. 6B). Thus, at most, cellular lysis can account for only a small proportion (i.e., ~20%) of the IL-1β-induced increase in S-SMase.

We recently reported that S-SMase is secreted through a Golgi pathway that bypasses the lysosomal pathway of L-SMase. Thus, we considered three general mechanisms to explain how IL-1β increases the secretion of SMase: secretion of mannose-phosphorylated SMase during trafficking of the ASM precursor to lysosomes (e.g., due to saturation of the mannose 6-phosphate receptor; cf. Ref. 46), induction of secretion of intralysosomal SMase (cf. Ref. 47), or increased traffick-
ing of the ASM precursor protein through the normal Golgi secretory pathway. To begin, we assessed the mannose phosphorylation state of S-SMase from control and IL-1β-treated HUVECs by passing conditioned media from these cells over a mannose-phosphate receptor column (cf. Ref. 48). By Western blot analysis quantified by densitometry, only 7.9% of control S-SMase and 4.9% of S-SMase from the cytokine-treated cells bound to the column and could be eluted with mannose 6-phosphate. As a control for this experiment, we have previously shown that the massive secretion of SMase by ASM-transfected Chinese hamster ovary cells can be partly explained by secretion of mannose-phosphorylated SMase due to saturation of the mannose 6-phosphate receptor2; when conditioned medium from these cells was passed over the receptor column, 40.3% bound and could be eluted with mannose 6-phosphate. Based on this set of experiments, we conclude that IL-1β-mediated induction of SMase secretion by HUVECs is not due to increased secretion of mannose-phosphorylated SMase during trafficking of the ASM precursor to lysosomes.

Next, we compared the zinc dependence of S-SMase and L-SMase activities from control and IL-1β-treated HUVECs. Zn²⁺ increased the enzymatic activity of S-SMase 1.77 ± 0.03- and 1.78 ± 0.17-fold from IL-1β-treated and untreated cells, respectively, whereas Zn²⁺ increased the enzymatic activity of L-SMase 1.13 ± 0.08- and 1.09 ± 0.04-fold from IL-1β-treated and untreated cells, respectively. Thus, S-SMase from IL-1β-treated HUVECs had a zinc dependence similar to that of S-SMase from untreated cells, not to that of L-SMase. These data support the idea that IL-1β-mediated induction of SMase secretion by HUVECs is not due to increased secretion of mannose-phosphorylated SMase during trafficking of the ASM precursor to lysosomes.

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To further test this conclusion, we took advantage of our previous observation that L-SMase has a lower apparent M₉ bound to the column and could be eluted with mannose 6-phosphate. As a control for this experiment, we have previously shown that the massive secretion of SMase by ASM-transfected Chinese hamster ovary cells can be partly explained by secretion of mannose-phosphorylated SMase due to saturation of the mannose 6-phosphate receptor²; when conditioned medium from these cells was passed over the receptor column, 40.3% bound and could be eluted with mannose 6-phosphate. Based on this set of experiments, we conclude that IL-1β-mediated induction of SMase secretion by HUVECs is not due to increased secretion of mannose-phosphorylated SMase during trafficking of the ASM precursor to lysosomes.

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than S-SMase on SDS-PAGE, presumably because of different post-translational modifications. Thus, we sought to determine if conditioned medium from IL-1β-treated HUVECs had increased amounts of higher Mr SMase, indicating increased flux through the normal secretory pathway or normal levels of the higher Mr form plus the appearance of the lower Mr form, indicating secretion or release of L-SMase from lysosomes (Fig. 7). It should be noted that the HUVEC enzyme, particularly HUVEC L-SMase, is not well recognized by our antibody, and so the immunoblot signal, particularly for the intracellular enzyme (L-SMase), is weak. Nonetheless, the data in Fig. 7 confirm the differences in Mr between L- and S-SMase in this cell type (compare lanes 1 and 3) and show that only the higher Mr S-SMase was detectably increased in the IL-1β-treated cells (compare lanes 3 and 4). By densitometry, this increase was approximately 2-fold. Even when the immunoblot was overexposed, the conditioned medium from the IL-1β-treated cells contained no evidence of a band at the Mr of L-SMase (data not shown). These data further support the conclusion that the increase in S-SMase from HUVECs induced by IL-1β is primarily due to an increased flux of the common SMase precursor through the Golgi-secretory pathway.

**DISCUSSION**

The data in this report demonstrate that endothelial cells are a rich source of active S-SMase, particularly in the presence of inflammatory cytokines. The potential physiologic relevance of these findings is related to the postulated role of the endothelium in extracellular SMase-induced lipoprotein aggregation and retention and to the demonstrated role of this tissue in cytokine-induced, acid SMase-mediated cell signaling (see Introduction and below). In addition, our results reveal several novel mechanisms for the regulation of S-SMase, particularly by alterations in intracellular protein trafficking.

We have previously shown that retained and aggregated lipoproteins in atherosclerotic lesions are hydrolyzed by an extracellular arterial wall SMase (4), and we found that S-SMase was the only SMase secreted by arterial wall cells (15). Mohan Das et al. (49) have reported that a magnesium-dependent SMase is externally oriented on neurons. We have not yet investigated whether this enzyme, which has not yet been cloned, is present on the surface of arterial wall cells. Nonetheless, we imagine that a secreted enzyme would have more access to lipoproteins retained on subendothelial matrix than a cell surface-bound enzyme. These points, together with the findings reported herein, lead us to propose the following model: basal levels of endothelium-derived S-SMase help initiate the atherosclerotic lesion by promoting lipoprotein retention and aggregation (5). Then, as T-cells and macrophages enter lesions (23, 26) and secrete cytokines and (at least in the case of macrophages) additional S-SMase, lipoprotein-SM hydrolysis and lipoprotein retention and aggregation would be amplified.

We have also speculated that endothelium-derived S-SMase may play a role in ceramide-mediated apoptosis. This speculation is based upon several pieces of information. First, a product of the acid SMase gene plays an important role in endothelial apoptosis in vivo (20, 21). Specifically, the pulmonary endothelium of ASM knockout mice was the major tissue demonstrating a defect in ceramide generation and apoptosis in response to total body irradiation; radiation-induced apoptosis...
in thymocytes and splenocytes was much less diminished in the ASM knockout versus wild-type mice (20). Moreover, very recent studies in mice have shown that the endothelium is the target of tumor necrosis factor-α-mediated apoptosis after injection of lipopolysaccharide. This response is associated with an increase in endothelial ceramide content and is greatly diminished in ASM knockout mice, indicating involvement of a SMase arising from the ASM gene (21). Second, the data in this report show that endothelial cells are a rich and cytokine-regulatable source of S-SMase, a product of the ASM gene. Third, S-SMase may have more access to the most abundant pools of cellular SM (22). In contrast, since lysosomal membranes have little SM (50), signaling by L-SMase would have to occur during trafficking of the nascent enzyme to lysosomes or would require delivery of SM into lysosomes. Thus, we propose that apoptotic stimuli, such as radiation and cytokines, increase the amount of endothelium-derived S-SMase from a subthreshold basal level to a level capable of generating enough cellular ceramide to trigger or enhance cell-signaling events. It is important to note, however, that this hypothesis is based upon several controversial assumptions. For example, despite the compelling data obtained using ASM knockout mice (20, 21), there are some cell culture systems that have shown a role for the neutral, magnesium-dependent SMase in ceramide-mediated apoptosis (6). In addition, there are conflicting data regarding the role of cell surface versus intracellular pools of SM in ceramide-mediated signaling (cf. Ref. 51, and see “Discussion” therein). Nonetheless, the data presented in this report give impetus for further in vitro and in vivo studies on the role of endothelium-derived S-SMase in atherogenesis and ceramide-mediated apoptosis.

Regarding the regulation of S-SMase, the data in this report provide evidence to support at least three separate mechanisms for control of S-SMase. Two of these mechanisms, alterations in protein trafficking and accessibility to cellular zinc, are based upon the following model of how the ASM gene gives rise to both L- and S-SMase: when a common precursor protein derived from the ASM gene is mannose-phosphorylated and thus is targeted to lysosomes, it becomes L-SMase. During this targeting, the enzyme acquires cellular Zn\(^{2+}\) and so does not require exogenous Zn\(^{2+}\) for enzymatic activity. In contrast, when the common precursor is not mannose-phosphorylated, and thus is targeted to the Golgi-secretory pathway, it gives rise to S-SMase. In the secretory pathway, the enzyme does not acquire cellular Zn\(^{2+}\), so S-SMase secreted by macrophages requires exogenous Zn\(^{2+}\) for enzymatic activity.

Based on this model, we predicted that one level of control of S-SMase secretion might be the proportion of the common ASM precursor trafficked into the lysosomal versus secretory pathways. The current data indicate that this mechanism is indeed primarily responsible for the increase in S-SMase in response to IL-1α. How might a cytokine influence protein trafficking? The key regulatory step in the trafficking of the SMase precursor is mannose phosphorylation of the precursor by N-acetyl-glucosaminyl-1-phosphotransferase (52, 53), and we speculate that cytokines may affect (e.g. by protein phosphorylation) either the activity of this phosphotransferase or the suitability of the SMase precursor as its substrate. Further work will be needed to test this and other possible mechanisms.

A second level of regulation of S-SMase is via Zn\(^{2+}\)-induced activation (15). The zinc requirement of S-SMase is similar to that of matrix metalloproteinases (15, 43, 54, 55), and so under conditions in which these proteinases are active, such as in atherosclerotic lesions (56), one would expect S-SMase to be active as well. In addition, Zn\(^{2+}\) levels have been reported to be elevated in atherosclerotic (57) and inflammatory (58) lesions. Nonetheless, the accessibility of extracellular zinc, perhaps modulated by zinc-binding proteins such as metallothionein (59), may represent a regulatory mechanism. Our current results indicate that accessibility of cell-derived zinc is also relevant; S-SMase from endothelial cells was partially activated in the absence of exogenously added Zn\(^{2+}\) (Fig. 1). In contrast, S-SMase from macrophages, fibroblasts, and Chinese hamster ovary cells is almost entirely inactive in the absence of added Zn\(^{2+}\) (Fig. 1 and Ref. 15). Based upon our model (above), we propose that SMase in the secretory pathway of endothelial cells, unlike SMase in the secretory pathway of the other cells examined, has partial access to cellular pools of Zn\(^{2+}\). The findings that endothelial cells secrete abundant amounts of S-SMase and that this S-SMase is partially active in the absence of added Zn\(^{2+}\) suggest that endothelium-derived S-SMase has unique physiologic roles.

A third point of S-SMase regulation is extracellular pH. S-SMase, like L-SMase, has an acidic pH optimum when assayed in vitro using sphingomyelin in detergent micelles as substrate (15). Thus, S-SMase may be particularly active in environments in which the pH is relatively low, such as in advanced atherosclerotic lesions (60–63), in certain types of inflammatory processes (62, 64), and possibly after reuptake into acidic endosomes. Calahan (65) noted, however, that pH affects only the K_m, not the V_max of L-SMase. This finding suggests that access to SM is the issue, and we showed recently that S-SMase can extensively hydrolyze the SM of atherogenic lipoproteins (e.g., oxidized LDL) and lesional LDL at neutral pH (16). This point is of particular importance regarding our hypothesis that endothelium-derived S-SMase plays a role in early lesional events, where the arterial wall pH would be expected to be neutral. These observations may also be relevant to the hydrolysis of cellular sphingomyelin by endothelium-derived S-SMase in neutral pH environments.

In summary, endothelial cells, which we have postulated are important in subendothelial, extracellular lipoprotein SM hydrolysis and which others have shown are important in cytokine-induced, ASM-mediated cell signaling, are a rich and regulatable source of the ASM gene product, S-SMase. These findings have formed the basis of ongoing work directed at further testing the physiologic and pathophysiologic roles of endothelium-derived S-SMase.

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REFERENCES
1. Spence, M. W. (1993) Adv. Lipid Res. 26, 3–23
2. Xu, X. X., and Tabas, I. (1991) J. Biol. Chem. 266, 24849–24858
3. Tabas, I., Li, Y., Brocia, R. W., Wu, S. W., Swenson, T. L., and Williams, K. J. (1990) J. Biol. Chem. 265, 29419–29425
4. Schissel, S. L., Tweedie-Hardman, J., Rapp, J. H., Graham, G., Williams, K. J., and Tabas, I. (1996) J. Clin. Invest. 98, 1455–1464
5. Williams, K. J., and Tabas, I. (1985) Arterioscler. Thromb. Vasc. Biol. 15, 551–561
6. Hannun, Y. A. (1996) Science 274, 1855–1859
7. Spiegel, S., Foster, D. A., and Kolesnick, R. N. (1996) Curr. Opin. Cell Biol. 8, 159–167
8. Ballou, L. R., Launderkind, S. J., Rosolnice, E. F., and Raghow, R. (1996) Biochem. Biophys. Acta 1291, 273–287
9. Hof, H. F., and Morton, R. E. (1985) Ann. N. Y. Acad. Sci. 454, 183–194
10. Nievold, P. F. E. M., Fogelman, A. M., Mottino, G., and Frank, J. S. (1991) Arteriosclerosis. Thromb. Vasc. Biol. 11, 1795–1805
11. D’Sa, J. R., and Klop, K. F. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 4–11
12. Hof, H. F., O’Neill, J., Pepin, J. M., and Cole, T. B. (1990) Eur. Heart J. 11, 105–115
13. Khoo, J. C., Miller, E., McLoughlin, P., and Steinberg, D. (1988)
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