Mammalian sphingomyelinas have been implicated in many important physiological and pathophysiological processes. Although several mammalian sphingomyelinas have been identified and studied, one of these, an acidic Zn\textsuperscript{2+}-stimulated sphingomyelinas (Zn-SMase) originally found in fetal bovine serum, has received little attention since its first and only report 7 years ago. We now show that Zn-SMase activity is secreted by human and murine macrophages, human skin fibroblasts, microglial cells, and several other cells in culture and is markedly up-regulated during differentiation of human monocytes to macrophages. Remarkably, peritoneal macrophages from mice in which the acid SMase gene had been disrupted by homologous recombination secreted no Zn-SMase activity, indicating that this enzyme and the intracellular lysosomal SMase, which is Zn-independent, arise from the same gene. Furthermore, skin fibroblasts from patients with types A and B Niemann-Pick disease, which are known to lack lysosomal SMase activity, also lack Zn-SMase activity in their conditioned media. Chinese hamster ovary cells stably transfected with a cDNA encoding lysosomal SMase massively over-express both cellular lysosomal SMase and secreted Zn-SMase activities. Thus, Zn-SMase arises independently of alternative splicing, suggesting a post-translational process. In summary, a wide variety of cell types secrete Zn-SMase activity, which arises from the same gene as lysosomal SMase. This secreted enzyme may play roles in physiological and pathophysiological processes involving extracellular sphingomyelin hydrolysis.

Mammalian SMases\textsuperscript{1} have been implicated in many important physiological and pathophysiological processes, including lysosomal digestion of sphingomyelin, which is important for normal neuronal and vascular function (1); ceramide-mediated signal transduction, leading to cytokine-induced apoptosis, cellular differentiation, and various immune and inflammatory responses (2, 3); lipoprotein aggregation within the vessel wall, which is a key event in atherogenesis (4–7); and intracellular cholesterol trafficking and metabolism (8–10).\textsuperscript{2} Several different forms of mammalian SMases have been identified, including: (a) a lysosomal SMase that is present in all tissues, acts optimally at low pH, and shows no dependence on divalent cations (11). This enzyme, which is defective in types A and B Niemann-Pick disease (11), has been purified and cloned (12), and mice in which the gene encoding lysosomal SMase has been inactivated have recently been generated (13, 14); (b) a neutral, membrane-associated, Mg\textsuperscript{2+}-stimulated SMase that is found predominantly in brain and kidney (15) and that is known to arise from a separate gene from lysosomal SMase (16); (c) a cytosolic SMase that like Mg\textsuperscript{2+}-stimulated SMase has a neutral pH optimum but no dependence of divalent cations (17); and (d) an acidic, Zn\textsuperscript{2+}-stimulated SMase (Zn-SMase) so far reported only in fetal bovine serum and to a lesser degree in newborn human serum (18).

Although the molecular identity and functions are definitively known only for lysosomal SMase (15), two of the other forms, namely, Mg\textsuperscript{2+}-stimulated SMase and cytosolic SMase, also have been studied. Both have been partially purified (17, 19, 20), and these two enzymes, in addition to lysosomal SMase, have been implicated in ceramide-mediated signal transduction (17, 21, 22). The cellular sources, genetic origin, and functions of Zn-SMase, however, have remained totally unknown since the first and only report on this enzyme by Spence et al. in 1989 (18). Herein, we demonstrate that Zn-SMase activity is secreted by many cultured mammalian cell types, including human macrophages, human fibroblasts, and murine microglial cells. In addition, we show that Zn-SMase activity is encoded for by the same gene as lysosomal SMase (13, 14, 23) and that it arises independently of alternative gene splicing. Potential processes that might give rise to Zn-SMase, as well as possible functions of this enzyme and the implications of its absence in types A and B Niemann-Pick disease, are discussed.

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\textsuperscript{2} The abbreviations used are: SMase, sphingomyelinas; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HI-FBS, heat-inactivated FBS; LDL, low density lipoprotein; PSG, penicillin, streptomycin, and glutamine; Zn-SMase, Zn\textsuperscript{2+}-stimulated SMase; BODIPY, 4,4-difluoro-4-bora-3a, 4a-diazaindacene.
EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media, granulocyte/macrophage-colony stimulating factor, and other tissue culture reagents were obtained from Life Technologies. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65 °C (HI-FBS). Human serum was purchased from Gemini Bioproducts (Calabasas, CA) and was also heat-inactivated.

[9,10-3H]Palmitic acid (56 Ci/mmol) was purchased from DuPont from Amersham Corp. (Arlington, TX). [9,10-3H]palmitic acid (25 mCi, 450 nmol) was purchased from Dietsch-Bodi and Company (Boca Raton, FL). All other chemicals and reagents were purchased from Fisher Scientific Co. or Sigma.

Cell Culture—Human peripheral blood monocytes were isolated from normal subjects as described previously (25). Murine macrophage J774.A1 cells (ATCC TIB-67) were cultured in Ham's F-12 medium containing 10% HI-FBS. CHO-K1 cells (ATCC CRL-1650) were cultured in DMEM containing 0.2% bovine serum albumin for 12 h, washed twice in chloroform:methanol:acetic acid:water (50:25:8:4), and then suspended in anhydrous acetonitrile and centrifuged (molecular weight cut off, 30,000).

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 graphic symbols.

RESULTS

Initial studies were conducted with the J774.A1 murine macrophage cell line (27). As described in Experimental Procedures, confluent monolayers were cultured for 2 days in DMEM containing 0.2% bovine serum albumin prior to the collection of conditioned medium; under these conditions, the cells remained completely healthy as assessed by cell morphology, trypan blue exclusion, and cellular protein content. The medium was then collected and conditioned (dialyzed), and the cells were harvested and disrupted by brief sonication (cellular homogenate). Each of these two fractions was then assayed for SMase activity at pH 5.0 or 7.5, using [14C]sphingomyelin dispersed in 0.6% Triton X-100 micelles as substrate, in the presence of 0.1 mM sodium acetate, pH 5.0, or, where indicated, 0.1 mM Tris-HCl, pH 7.5, to bring the volume to 1.0 ml. The reaction was initiated by the addition of 40 µl of substrate (50 pmol [14C]sphingomyelin, 50 pmol [3H]sphingomyelin, or 2–20 nmol BM-DP-sphingomyelin) in 0.25 mM sucrose containing 3% Triton X-100 (final concentration of Triton X-100 in the 200-µl assay mix, 0.6%). When added, the final concentrations of EDTA, Mg2+, and Zn2+ were 5, 6, and 0.1 mM, respectively. The assay mixtures were incubated at 37 °C for no longer than 3 h and then extracted by the method of Bligh and Dyer (35). For the assays using [N-methyl-14C]sphingomyelin, the upper, aqueous phase, into which [14C]choline phosphate partitions, was counted by liquid scintillation. For the assays using [N-palmitoyl-9–10-3H]sphingomyelin and BODIPY-sphingomyelin, both of which are labeled in the hydrophobic ceramide portion of sphingomyelin (the lower, organic phase was harvested, evaporated under N2, and fractionated by TLC using chlorform:methanol (95:5). The ceramide spots were scraped and directly counted to quantify [14C]ceramide or extracted in chlorform:methanol:water (1:2:1) and assayed for fluorescence in a spectrophotofluorometer (excitation, 505 nm; emission, 530 nm) to quantify BODIPY-ceramide. Typically, our assay reactions contained approximately 20 µg of cellular homogenate protein and a volume of conditioned medium derived from a quantity of cells equivalent to approximately 50 µg of cellular protein.

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significant effects. The experiments were repeated at least three times, and representative data are shown. p values of <0.05 were considered statistically significant. Results were expressed as means ± S.D. (n = 4–5).

RESULTS

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were found when the reaction substrate was either $[^{14}C]$cho-
line-labeled sphingomyelin or fluorescent BODIPY-ceramide-
abeled sphingomyelin in 0.6% Triton X-100 micelles (data not shown). Further characterization of the secreted SMase activity revealed inhibition by AMP and by high concentrations of ZnCl$_2$ (6 mM), inactivity against $[^{3}H]$sphingomyelin in 0.1% Triton X-100 micelles, and relative heat instability compared with lysosomal SMase (data not shown). These properties exactly match those of an acidic, Zn-SMase activity of previously unknown cellular origin found by Spence et al. (18) in fetal bovine serum and to a lesser extent in newborn human serum. The Zn-SMase activity we observed in macrophage-conditioned medium, however, was clearly not a contaminant from FBS; the cells were grown for 48 h in FBS that had been heat-inactivated, which destroys the Zn-SMase activity in FBS (Ref. 18 and data not shown), and then the cells were washed extensively and incubated for 12 h in serum-free medium before collection of the conditioned medium. Furthermore, subsequent studies with Zn-SMase-negative cells, presented below, definitively exclude any noncellular source of this activity in our experiments.

Several other cell types were tested for the presence of secreted Zn-SMase activity, as shown in the first column of Table I. In terms of absolute activity, macrophages, fibroblasts, and CHO cells secreted the most Zn-SMase activity, but there was clearly detectable Zn-SMase activity in the conditioned medium of human monocytes, COS cells, and resting murine brain microglial cells. Note that differentiation of monocytes to macrophages in culture markedly increased secreted Zn-SMase activity. Differentiated macrophages also had the highest level of secreted Zn-SMase activity relative to cellular lysosomal SMase activity (third column in Table I).

Zn-SMase activity differs from lysosomal SMase activity in several important properties, including its requirement for Zn$^{2+}$, its heat instability, and its secretion from cells (see above). Nonetheless, the acidic pH optimum of Zn-SMase suggested a possible relationship to lysosomal SMase. For this reason, we tested conditioned media from peritoneal macrophages of mice in which one or both alleles of the acid SMase gene, which gives rise to lysosomal SMase (12, 23, 30), had been disrupted by homologous recombination (13). As expected, macrophage cellular homogenates from heterozygous acid SMase knockout mice contained approximately 60% the amount of lysosomal SMase activity as cellular homogenates from wild-type mice from the same colony, and macrophage homogenates from homozygous knockout mice had virtually no detectable lysosomal SMase activity (Fig. 2A). Remarkably, as shown in Fig. 2B, macrophages from heterozygous acid SMase knockout mice secreted approximately 40% of the amount of Zn-SMase activity as control macrophages, and the homozygous knockout macrophages secreted virtually no Zn-SMase activity. Experiments in which the wild-type conditioned medium was mixed with conditioned medium from the homozygous knockout macrophages indicated that the knockout cells did not secrete an inhibitor of Zn-SMase activity (data not shown). Thus, the Zn-SMase secreted by macrophages arises from the acid SMase gene.

Naturally occurring point mutations in the acid SMase gene that lead to inactive lysosomal SMase are observed in human patients with types A and B Niemann-Pick disease. To determine whether these mutations also lead to inactivation of the Zn-SMase, conditioned medium from cultured skin fibroblasts of a control individual, a patient with Niemann-Pick A disease (R496L mutation (28)), and a patient with Niemann-Pick B disease (S436R mutation (29)) were assayed for Zn-SMase. Lysosomal SMase activity in the control cellular homogenate was similar to that displayed in Table I, whereas the activity in homogenates from the two different Niemann-Pick patients was less than 1% of the control values (data not shown). As shown in Fig. 2C, this same pattern was found for secreted Zn-SMase: abundant activity in the conditioned medium of the control fibroblasts and barely detectable activity in the media of the two mutant cell lines. These data support our conclusion that lysosomal SMase and Zn-SMase arise from the same gene. Furthermore, the data indicate that the site of the two mutations, namely, exons 6 and 4, respectively (23, 28, 29), are important for both lysosomal SMase and Zn-SMase activities. Lastly, these data reveal for the first time that Niemann-Pick patients are lacking two distinct SMase activities.

Zn-SMase could arise either by mRNA modifications (alternative splicing, message editing, or cryptic polyadenylation) or by post-translational processes (protein modification, including proteolysis, or differential interaction with co-factors). To determine if alternative splicing was involved, we tested a CHO line that had been stably transfected with the predominant lysosomal SMase cDNA (i.e. type 1) (12, 30). Lysosomal SMase and Zn-SMase activities in cellular homogenates from transfected and control cells are shown in Fig. 3A. Consistent with previous results using COS cells transiently transfected with the type 1 cDNA (12), the stably transfected CHO cells had markedly increased cellular lysosomal SMase activity compared with control CHO cells. Somewhat surprisingly, the cellular homogenate from the transfected cells was also found to have substantially increased Zn-SMase activity compared with the control cellular homogenate. Notably, as shown in Fig. 3B, a massive amount of Zn-SMase activity was found in the conditioned medium from the transfected cells, as well as a much smaller amount of lysosomal SMase activity. Thus, Zn-SMase is made from a lysosomal SMase cDNA, indicating that the enzyme arises independently of alternative splicing. The other two processes for mRNA modification, namely, message editing and cryptic polyadenylation, require conserved nucleotide motifs (36, 37) that we did not find in the type I SMase cDNA sequence (12). Taken together, these results imply that Zn-SMase arises from post-translational processes.

**DISCUSSION**

The data in this report reveal that many different cell types secrete substantial amounts of a Zn$^{2+}$-stimulated SMase and...
**TABLE I**

Activities of Zn-SMase and lysosomal SMase in various cell types

| Cell type                  | Secreted Zn-SMase activity (A) | Cellular lysosomal SMase activity (B) | (A - B) × 100 |
|---------------------------|-------------------------------|--------------------------------------|---------------|
| J 774 macrophages         | 13.1 ± 0.02                   | 33.2 ± 0.2                           | 39.5          |
| Mouse peritoneal macrophages | 19.1 ± 0.5                   | 57.1 ± 3.9                           | 33.5          |
| Human monocyte-derived macrophages | 8.8 ± 0.07                 | 46.4 ± 0.4                           | 19.0          |
| Human skin fibroblasts    | 18.0 ± 0.2                    | 181 ± 5.6                            | 9.9           |
| CHO cells                 | 15.6 ± 0.3                    | 150 ± 9.1                            | 10.4          |
| Human monocytes           | 0.65 ± 0.04                   | 7.6 ± 0.4                            | 8.6           |
| COS-7 cells               | 2.31 ± 0.13                   | 26.6 ± 1.5                           | 8.7           |
| Murine brain microglial cells | 2.65 ± 0.06                 | 32.9 ± 3.4                           | 8.1           |

**FIG. 2.** Peritoneal macrophages from acid SMase knockout mice and fibroblasts from patients with types A and B Niemann-Pick disease have a deficiency in secreted Zn-SMase activity. Cellular homogenates (panel A) and serum-free conditioned media (CM) (panel B) were harvested from thioglycolate-elicited peritoneal macrophages (MPM) from wild-type mice with two normal copies of the acid SMase gene (+/+) or with one (-) or both (−/−) copies of the gene inactivated. Cellular homogenates (not shown) and conditioned media (CM, panel C) were also harvested from skin fibroblasts (Fb) from one control individual (Control), a Niemann-Pick A (NP-A) patient, and a Niemann-Pick type B (NP-B) patient. SMase activity was assayed using 250 nM [3H]sphingomyelin in Triton X-100 micelles in the presence of either 5 mM EDTA (hatched bars) or 0.1 mM ZnCl2 (solid bars) for 1 h at 37 °C at pH 5.0. ASM, acid SMase; NP-A, Niemann-Pick type A.

**FIG. 3.** CHO cells transfected with the type I acid SMase cDNA markedly overexpress both lysosomal SMase and Zn-SMase activities. Cellular homogenates (panel A) and conditioned media (panel B) were harvested from control CHO cells and from CHO cells transfected with the type I acid SMase cDNA (12). SMase activity was assayed using 250 nM [3H]sphingomyelin in Triton X-100 micelles in the presence of 5 mM EDTA (hatched bars) or 0.1 mM ZnCl2 (solid bars) for 10 min at 37 °C at pH 5.0.

that this enzyme arises from the acid SMase gene. These findings raise two important questions: (a) what is the nature of the post-transcriptional process that gives rise to Zn-SMase? and, most importantly, (b) what might be the biological function or functions of Zn-SMase? Regarding the first question, the fact that the same full-length cDNA gives rise to both the lysosomal and Zn2+-stimulated activities demonstrates that alternative RNA splicing is not involved, and the cDNA sequence does not contain motifs seen with RNA editing or alternative polyadenylation. Thus, we conclude that the processes must be post-translational. Regarding its secretion from cells, it will be important to determine whether Zn-SMase lacks mannose-phosphate residues, which target enzymes to lysosomes, whether it represents secreted mannose-phosphate-containing precursor enzyme that initially "escaped" lysosomal targeting and has not yet been re-internalized by cell-surface mannose-phosphate or other receptors, or whether it represents the discharge of mature, processed enzyme from lysosomes into the medium (see Refs. 38-40). In this regard, Sandhoff and colleagues (41) reported that human fibroblasts secrete 75-and 57-kDa proteins that were immunoprecipitated by antibodies made against fibroblast lysosomal SMase or human urine acid SMase. These proteins may represent secreted precursor and processed forms of acid SMase, respectively, and it is possible that one or both has Zn2+-stimulated SMase activity. In preliminary experiments with J 774 macrophages and acid SMase cDNA-transfected CHO cells, we have found that the anti-urine acid SMase antibody mentioned above was able to immunoprecipitate secreted Zn-SMase activity. Immunoblots with this antibody, however, revealed only one predominant band at 72 kDa in both conditioned media and cell homogenates. The 72-kDa band was much more intense in the conditioned medium of the transfected CHO cells than in conditioned medium of control CHO cells or of J 774 macrophages. Investigations are underway to determine how the secreted SMase acquires Zn2+ dependence despite a similar apparent molecular weight as the lysosomal form, which does not require added Zn2+ for enzymatic activity.

The most important issue related to secreted Zn-SMase is its biological function or functions. One possibility is extracellular sphingomyelin catabolism. For example, because microglia secrete Zn-SMase (Table I), perhaps in a regulated manner, this function may play a role in the catabolism of neuronal and glial sphingomyelin after nerve injury (42) and during demyelination (43, 44). Note that macrophages, an abundant source of secreted Zn-SMase (Table I), also appear to be involved in...
demelinating disorders in the brain (43). Another possible role of Zn-SMase is in "autocrine" or "paracrine" cell surface sphingomyelin hydrolysis, leading to various ceramide-mediated signaling pathways (2, 3) or to alterations in intracellular cholesterol trafficking (8–10, 45). Interestingly, some of the ceramide-mediated signaling pathways are thought to involve an acid SMase activity in early endosomes or some other plasma membrane-associated site (21, 22). A third possible function of Zn-SMase is related to the initial discovery of this enzyme in FBS and newborn human serum but not in adult serum (18). Although serum levels of Zn-SMase may not reflect levels of the enzyme in certain local tissue environments and despite the fact that both humans and induced mutant mice with acid SMase deficiency appear grossly normal at birth (1, 13, 14), this finding may implicate a role for this enzyme in developmental physiology (18). Finally, secreted Zn-SMase may play an important role in atherogenesis. In vitro, SMase can lead to LDL aggregation (4, 5), which is a prominent and important atherogenic event in prelesional areas and also in early macrophage-rich lesions (6, 7, 46, 47). In this regard, we have recently obtained evidence that LDL undergoes extracellular sphingomyelin hydrolysis in human and animal atherosclerotic lesions (48), and it is possible that secreted Zn-SMase contributes to this process. In another area related to atherogenesis, we have previously shown that βVLDL, an atherogenic lipoprotein rich in cholesterol and sphingomyelin, can engage in prolonged contact with the macrophage cell surface, during which the particle is partially degraded (49). Based on our current findings, it is possible that macrophage-secreted Zn-SMase participates in this process. Extracellular retained and aggregated LDL can also engage in prolonged contact with the macrophage cell surface and thus is another possible substrate for this enzyme. In support of these hypotheses, we recently found that under conditions in vitro designed to mimic the environment within atherosclerotic lesions (see Ref. 5), LDL-sphingomyelin is a substrate for Zn-SMase, even at pH 7.4.

An important issue is the physiological environment in which Zn-SMase is enzymatically active. Our finding of LDL hydrolysis at neutral pH, in addition to suggesting that Zn-SMase may be an atherogenic factor, shows that it is possible that physiological sources of sphingomyelin may be substantially hydrolyzed by Zn-SMase without requiring an acidic environment. Where acidic environments do exist, however, such as in inflammatory (50) or ischemic lesions of the arterial wall (51), in the vicinity of glycosaminoglycans (52), in the extracellular space of activated macrophages (53, 54), or in acidic endosomes following reinternalization by cell surface receptors (38–40), Zn-SMase may be particularly active. Regarding the requirement for Zn, extracellular concentrations of zinc are well within the range that is known to optimally stimulate Zn-SMase (18, 55), and zinc is also known to be present in the cerebrospinal fluid and in brain tissue (56). Interestingly, zinc levels have been reported to be elevated in human atherosclerotic lesions compared with normal arterial tissue (57) and in the central nervous system under certain pathological conditions (58, 59). Furthermore, other secreted proteins that require zinc, such as metalloproteinases, can be fully active in the extracellular environment (see above and Ref. 60).

Finally, an important question raised by our finding that Zn-SMase arises from the acid SMase gene is how much of the pathology seen in humans (1) and mice (13, 14) with acid SMase gene mutations is related to the deficiency of secreted Zn-SMase activity? Multiple and diverse abnormalities exist in these conditions, and the findings in this report now raise the possibility that at least some of these problems are due to the absence of secreted Zn-SMase. This issue, as well as others, will best be addressed using models, such as induced mutant mice, that selectively overexpress or underexpress Zn-SMase.

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