Role of Acidic Sphingomyelinase in Fas/CD95-mediated Cell Death*

(Received for publication, July 8, 1999, and in revised form, December 23, 1999)

Tsu Lin‡‡, Laurent Genestier‡‡, Michael J. Pinkoski¶¶, Arturo Castro‡, Shelby Nicholas‡, Rona Mogil‡, Francois Paris‡, Zvi Fuks, Edward H. Schuchman**, Richard N. Kolesnick‡‡‡, and Douglas R. Green‡§

From the ‡Department of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121, **Department of Human Genetics, Mount Sinai School of Medicine, New York, New York 10029, †Department of Radiation Oncology and ‡‡Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Engagement of the Fas receptor has been reported to induce ceramide generation via activation of acidic sphingomyelinase (aSMase). However, the role of aSMase in Fas-mediated cell death is controversial. Using genetically engineered mice deficient in the aSMase gene (aSMase−/−), we found that thymocytes, concanavalin A-activated T cells, and lipopolysaccharide-activated B cells derived from both aSMase−/− and aSMase+/+ mice were equally sensitive to Fas-mediated cell death, triggered by either anti-Fas antibody or Fas ligand in vitro. Similarly, activation-induced apoptosis of T lymphocytes was unaffected by the status of aSMase, and aSMase−/− mice failed to show immunological symptoms seen in animals with defects in Fas function. In vivo, intravenous injection of 3 μg/25 g mouse body weight of anti-Fas Jo2 antibody into aSMase−/− mice failed to affect hepatocyte apoptosis or mortality, whereas massive hepatocyte apoptosis and animal death occurred in wild type littermates. Animals heterozygous for aSMase deficiency were also significantly protected. Susceptibility of aSMase−/− mice to anti-Fas antibody was demonstrated with higher antibody doses (≥4 μg/25 g mouse). These data indicate a role for aSMase in Fas-mediated cell death in some but not all tissues.

* This research was supported by Mentored Clinical Scientist Development Award DK02445 and Glaxo Digestive Disease Basic Research Award (to T. L.) and National Institutes of Health Grants CA69381 to (to D. R. G.), CA42385 (to R. N. K.), CA52462 (to Z. F.), and HD28607 (E. H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby protected. Susceptibility of aSMase−/− mice were also significantly protected. Animals heteryzogous for aSMase deficiency were also significantly protected. Susceptibility of aSMase−/− mice to anti-Fas antibody was demonstrated with higher antibody doses (≥4 μg/25 g mouse). These data indicate a role for aSMase in Fas-mediated cell death in some but not all tissues.

** This research was supported by Mentored Clinical Scientist Development Award DK02445 and Glaxo Digestive Disease Basic Research Award (to T. L.) and National Institutes of Health Grants CA69381 to (to D. R. G.), CA42385 (to R. N. K.), CA52462 (to Z. F.), and HD28607 (E. H. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby protected.

†† These authors contributed equally.

§§ To whom correspondence should be addressed: Division of Cellular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121. E-mail: Dgreen52406@aol.com.

This paper is available on line at http://www.jbc.org

Experimental Procedures

Mice—Mice deficient in the aSMase gene (19) were genotyped by polymerase chain reaction of tail DNA as described previously (19). Primers 5′-AGCCGTGTCCTCTTCCTTAC-3′ and 5′-CGAGACTGTT-

The abbreviations used are: aSMase, acidic sphingomyelinase; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; ConA, concanavalin A; LPS, lipopolysaccharide; sFasL, soluble Fas ligand; mAb, monoclonal antibody.
GACGAGCACATC-3' were used to amplify a 269-base pair product that is specific for exon 2 of the wild type aSMase gene. Primer 5'-AGCGGT-GTCCCTCTCCCTAC-3' and the neomycin gene-specific antisense primer 5'-GGTACCGCGTATGTCGGT-3' were used to amplify a 523-base pair product that was specific for the mutant gene containing part of the neo cassette and exon 2 of the aSMase gene. Polymerase chain reaction consisted of 50 μl reactions containing 1× QiaGen polymerase chain reaction buffer (QiaGen, Carlsbad, CA), 1× QiaGen Q solution, 1 μg of DNA, 20 pmol of each primer, 0.5 mM dNTP, and 5 units of Taq polymerase (Life Technologies, Inc.). Polymerase chain reaction amplification conditions consisted of 32 cycles, 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. All mice were raise under specific pathogen-free conditions in the animal care facility at the La Jolla Institute for Allergy and Immunology or the Sloan-Kettering Cancer Center.

Age- and gender-matched littersmates (wild type, aSMase knock-out and heterozygote) 9 to 12 weeks of age were injected via the retro-orbital sinus with anti-mouse Fas antibody Jo-2 (3–5 μg/25 g mouse body weight) in a total volume of 100 μl in 0.9% saline. Mice were checked hourly for mortality. Kaplan-Meyer actuarial analysis was performed using the computer-assisted system for patient data entry and retrieval (CASPER) statistical package. For pathologic analysis, animals manifesting an agonal breathing pattern or survivors 60 h post-injection were sacrificed by CO2 asphyxiation.

Cell Isolation and Flow Cytometry Analysis—Spleen cells and thymocytes were isolated by gently meshing the respective organs between two frosted slides followed by filtering through cotton gauze. Two-color FACS analysis was performed with a Becton Dickinson FACScan flow cytometer. The data were analyzed with the Cell Quest program.

All antibodies were obtained from Pharmingen (San Diego, CA). Antibodies and reagents used were as follow: FITC-conjugated and unconjugated anti-Fas (Jo-2), FITC-conjugated anti-CD4 and phosphatidylethanolamine-conjugated anti-CD8a, FITC-conjugated anti-B220 and phosphatidylethanolamine-conjugated anti-CD3.

Concanavalin A (ConA)- and Lipopolysaccharide (LPS)-activated Spleen Cell Blasts—ConA-activated T cell blasts were produced by stimulating Ficoll (Amersham Pharmacia Biotech)-purified mononuclear cells from naïve spleen for two days with 5 μg/ml ConA (Sigma). LPS-activated B cells blasts were produced by culturing Ficoll-purified mononuclear cells from whole spleen stimulated with 15 μg/ml lipopolysaccharide (Sigma).

Apoptosis Assays—Cell death was assessed by flow cytometry by double staining with propidium iodide and FITC-conjugated annexin V (Pharmingen). Unless otherwise stated, cell death was reported as percentage propidium iodide uptake. Similar results were obtained when % annexin V staining was used to assess cell death, except that it resulted in higher sensitivity and background. Soluble Fas ligand (sFasL) was a kind gift from J. Tschopp (University of Lausanne, Switzerland). Cell death was induced with flag-tagged sFasL by cross-linking it with an anti-Flag mAb (M2, 1 μg/ml (Sigma) as described previously (20).

Measurement of Ceramide Levels—Ceramide levels were measured as described previously (15). In brief, ceramide was quantified by the diacylglycerol kinase assay as 32P incorporated upon phosphorylation of ceramide to ceramide 1-phosphate by glycerol kinase from Escherichia coli (Biomol, Plymouth Meeting, PA). LPS blasts were starved for 2 h in RPMI containing 2% bovine serum albumin and then treated with different monoclonal antibodies for the indicated times. Ceramide 1-phosphate was resolved by TLC using CHCl3/CH2OH/CH3COOH (6: 15.5, v/v) as solvent. Authentic ceramide 1-phosphate was identified by autoradiography at 0.025, and the ceramide concentration was determined by comparison to concomitantly run standard comprised of known amounts of natural ceramide type 3 (Sigma) and normalized to 3H triglyceride introduced during lipid extraction.

RESULTS

aSMase−/− Mice Express Normal Levels of Fas—Before assessing the susceptibility of aSMase−/− cells to undergo Fas-mediated apoptosis, we examined surface expression of Fas. We found that thymocytes from aSMase−/− mice expressed normal levels of Fas compared with wild type aSMase+/+ littermate controls (Fig. 1a). Fig. 1b demonstrates that naive spleen cells from both mice expressed little or no levels of Fas, and upon ConA activation, Fas expression increased equally in both. Similar results were obtained with LPS-activated spleen cells (data not shown). Therefore, Fas expression is not affected by the absence of aSMase.

aSMase−/− Lymphocytes Are Not Protected from Fas-mediated Cell Death—To directly assess the contribution of aSMase in Fas-mediated cell death, we analyzed the susceptibility of lymphocytes from aSMase−/− mice to undergo apoptosis from Fas−/− mice to die from Fas-mediated cell death with anti-Fas mAb (Jo-2). As shown in Fig. 2a, thymocytes from aSMase−/− littermates from both aSMase−/− and aSMase+/+ mice were equally sensitive to cell death induced by anti-Fas antibody. Similarly, ConA- and LPS-activated spleen cells from both aSMase−/− and aSMase+/+ mice were equally sensitive to Fas-mediated cell death (Figs. 2, b and c).

Because engagement of Fas can potentially lead to the activation of multiple pathways leading to cell death, we considered the possibility that aSMase mediates Fas-mediated cell death more rapidly than the other pathways. Hence, it is plausible that the lack of differences in Fig. 2, a–c, was due to an examination of cell viability 16 h after anti-Fas stimulation, by which time an alternative pathway may have resulted in cell death independently of aSMase. To address this issue, we examined cell death at earlier time points (4, 6, and 12 h). As shown in Fig. 2d, cell death in thymocytes from aSMase+/+ and aSMase−/− mice occurred at the same rate following treatment with anti-Fas antibody. Similar results were obtained with ConA- and LPS-activated T cells (data not shown).
sFasL, when cross-linked, readily induces cell death in Fas-sensitive cells (20). We examined the possibility that aSMase is involved in cell death induced by cross-linked sFasL. Fig. 3a demonstrates that aSMase+/+ and aSMase−/− thymocytes were equally susceptible to cell death induced by cross-linked sFasL. Similarly, aSMase+/+ and aSMase−/− ConA- and LPS-activated spleen cells were also examined for their susceptibility to cell death mediated by cross-linked sFasL, and again, no differences were observed (Fig. 3, b–c).

Fig. 3d summarizes our results from several independent experiments comparing cells from aSMase+/+ and aSMase−/− mice for their susceptibility to ligation of Fas with anti-Fas antibody or sFasL. In the figure we compare the extent of cell death of thymocytes cultured with various doses of anti-Fas for 16 h. Data shown are representative of at least two separate and independent experiments. Vertical bars represent S.D. of each assay, done in triplicate.

To assess the involvement of aSMase in Fas-mediated hepatic injury, we injected 8-week-old aSMase+/+ and aSMase−/− mice intravenously with various doses of anti-Fas and monitored survival after injection. Fig. 4 demonstrates that at 3 μg/25 g mouse of anti-Fas intravenous there was a striking difference observed between the aSMase−/− and aSMase+/+ mice. Animals lacking aSMase were completely resistant to the effects of the antibody (all mice surviving at 24 h continued to survive). Fig. 5 shows the appearance of the livers from an aSMase−/− and an aSMase+/+ mouse following intravenous injection with 3 μg/25 g mouse of anti-Fas antibody. Extensive damage in the +/+ liver was apparent at both the macroscopic (insets) and microscopic levels, with extensive apoptosis of the hepatocytes seen as chromatin condensation. In contrast, the liver of the representative anti-Fas-treated aSMase−/− mouse appeared completely normal.

Furthermore, at this dose of antibody there was also a significant resistance observed in aSMase−/− heterozygotes (7/11 survivors) compared with +/+ mice (2/11 survivors, p < 0.03 versus +/+). Homozygous−/− animals were completely resistant to intravenous injection of 3 μg/25 g mouse anti-Fas in this experiment (11/11 survivors) (the data from homozygous animals are part of the survival study in Fig. 4).

However, at doses exceeding 4 μg/25 g mouse, aSMase+/+ and aSMase−/− mice were equally susceptible to the lethal effect of anti-Fas. Gross and microscopic examination of the liver revealed that all aSMase+/+ and aSMase−/− mice, which died from anti-Fas injection, showed evidence of significant hepatic injury (Fig. 5 and data not shown). Similarly, we found no difference between the aSMase−/− and aSMase+/+ mice in their susceptibility to >5 μg anti-Fas injected intraperitoneally (data not shown). These studies indicate the existence of aSMase-dependent and -independent apoptotic pathways in Fas-mediated cell death and show that the aSMase-dependent pathway...
can be bypassed by supralethal doses of anti-Fas antibody.

Ceramide generation in aSMase\textsuperscript{−/−} Lymphocytes after Anti-Fas Signaling—Although our results suggest that aSMase is not required for Fas-mediated cell death in B and T cells, we cannot rule out the possibility that ceramide is involved in this process. Other enzymes such as neutral sphingomyelinase and ceramide synthetase have been shown to be capable of producing ceramide. Therefore, it is possible that enzymes other than aSMase can potentially induce an increase in ceramide level after Fas engagement. To help clarify this issue, we examined ceramide levels from LPS-activated B cell blasts from aSMase\textsuperscript{−/−} and aSMase\textsuperscript{+/+} mice after Fas engagement. As shown in Fig. 6, aSMase\textsuperscript{−/−} LPS-activated B cells initially appear to produce slightly more ceramide than aSMase\textsuperscript{+/+} cells after engagement with anti-Fas. At later times, the cells from aSMase\textsuperscript{−/−} animals were capable of producing similarly high levels of ceramide to those of wild type animals. Thus, although this result is consistent with our observation that aSMase is
not necessary for Fas-mediated cell death in B and T cells, it does not rule out the possibility that ceramide plays a role in the process of Fas-mediated cell death in these cells.  

**aSMase** 

asMase Do Not Develop Lymphoproliferative Disease—As a result of a viral transposon insertion in the intron of the Fas gene, lpr mice express no functional Fas (28, 29). These mice consequently develop severe lymphoproliferative disease resulting in massive enlargement of lymphoid organs, largely as a result of massive accumulation of CD3^+^ B220^+^ T cells. Therefore, signaling through Fas suppresses the development of lymphoproliferative disease. Interestingly, recent evidence suggests that the Fas-mediated signal, which prevents this lymphoproliferative disease, may be distinct from the Fas-mediated signal that leads to cell death. T cells from transgenic mice that overexpress crmA, a potent caspase 8 inhibitor, have been shown to be resistant to Fas-induced cell death yet do not develop lymphoproliferative disease (30). Although we saw no evidence that aSMase plays a role in the Fas-mediated signal that leads to lymphocyte death, we investigated the possibility that aSMase may be involved in the Fas-mediated signal that prevents this lymphoproliferative disease. Fig. 7, however, demonstrates that the spleens of aSMase^−/−^ mice are of normal size and do not display an accumulation of large numbers of CD3^+^ B220^+^ T cells. Similar results were observed with lymphocytes from the lymph nodes of aSMase^−/−^ mice (data not shown).

**DISCUSSION**

Ligation of Fas engages a potent and rapid signaling mechanism that results in apoptosis in a variety of cell types (26, 31–34). This signaling generally involves the recruitment of an adapter protein, FADD/Mort-1, to the cytoplasmic domain of Fas (2, 4). FADD, in turn, recruits procaspase 8 molecules, which transprocess each other to the highly active mature form (35). Caspase-8 then processes and activates its substrates, including other caspases, leading to apoptosis (3).

Fas ligation also induces the production of ceramide, and since ceramide is known to be capable of triggering apoptosis, this pathway may contribute to Fas-mediated cell death (12–14). Several studies have implicated aSMase as the enzyme responsible for hydrolyzing sphingomyelin to produce ceramide following Fas ligation, and aSMase activity increases following ligation of this receptor (11, 36). How this occurs remains unclear, although some recent reports have shown that aSMase activation following Fas ligation is dependent on caspase activity, presumably through the action of caspase 8 (15, 37, 38). We have confirmed this using caspase inhibitors but were unable to detect caspase-mediated cleavage of...
Role of Acidic Sphingomyelinase in Fas-mediated Cell Death

aSMase, which might account for it. Nevertheless, if aSMase and the ceramide pathway function downstream of the Fas signals discussed above to contribute to apoptosis, this is of vital importance to our understanding of this death process. The experiments described herein were undertaken to determine this.

The simplest interpretation of our results is that aSMase is not required for Fas-mediated apoptosis of thymocytes, T cell blasts, or B cell blasts. Consistent with this notion, aSMase−/− animals do not manifest an lpr-like phenotype. In contrast, our results in vivo indicate a conditional requirement for aSMase in Fas-mediated death. At an optimal dose of anti-Fas antibody (3 μg/25 g mouse, intravenous), the effect of the aSMase null mutation was dramatic; these animals were significantly resistant to the highly lethal effects of this antibody (Fig. 4). This was mirrored in the appearance of the hepatocytes; apoptosis in the +/- animals treated with this dose of anti-Fas was not seen in the treated −/− animals. Furthermore, significant protection was afforded in the aSMase heterozygotes, indicating a sensitivity to gene dosage. The heterozygous animals, like the human carrier state, do not manifest Niemann-Pick disease, indicating that aSMase effects on apoptosis are independent of any potential deleterious effects of this disease process. Supralethal amounts of anti-Fas can override this effect, resulting in death of the −/− animals. However, if limiting Fasl is available for some physiological effects, it is unlikely that in such cases aSMase is essential to the maintenance of the Fas-mediated apoptotic signal. Our data suggest selective preference for such a role for aSMase depending not only on the strength of inductive signal but also on cell type.

Fas-mediated apoptosis of T and B cells plays critical roles in the control of immune responses (39–43), and roles for Fas-mediated death in thymocyte maturation have been suggested (44, 45). Furthermore, Fas-mediated apoptosis of hepatocytes appears to be involved in liver damage as a result of viral hepatitis (46). Thus, the cells we examined for aSMase involvement in Fas-mediated apoptosis include those in which Fas is known to play a role in normal homeostasis or pathogenesis. Our studies do not rule out a role for ceramide in Fas-mediated apoptosis even in B and T cells, since ceramide production following Fas ligation still occurred (Fig. 6). It is possible that another enzyme such as neutral SMase can compensate for the absence of acidic SMase. Consistent with this possibility, Tonnetti et al. (36) recently demonstrated that engagement of Fas results in activation of both acidic and neutral SMase.

Our findings in lymphoid cells are consistent with those of Boesen-de Cock et al. (18), who examined Epstein-Barr virus-transformed B cells from Niemann-Pick patients with genetic defects in aSMase. They found that ligation of Fas induced both apoptosis and ceramide production in these cells and that, although transfer of wild-type aSMase gene into these cells restored aSMase activity, it had no effect on cell death induced by anti-Fas antibodies. Similarly, in another study, several inhibitors of aSMase activity failed to inhibit Fas-mediated apoptosis as well (47). These observations together with our studies using primary lymphoid cells from aSMase−/− mice support the idea that aSMase is not necessary for Fas-mediated apoptosis in lymphoid cells. This is in contrast to the studies of DeMaria et al. (17), who also employed Niemann-Pick lymphoblast lines and conclude that aSMase was required for Fas-mediated apoptosis and that restoration of wild type activity (by uptake of wild type protein) restored the response to Fas. This study suggests a role for aSMase in Fas-signaling in lymphoid cells but may be open to interpretation, since the virally transformed cell lines used may have lost susceptibility to Fas for reasons other than that of defects in the primary cells.

As noted above, our studies do not address the requirement for ceramide production in Fas-mediated cell death, but some recent observations suggest that it may not be required in lymphoid cells. In one study, a number of inhibitors of ceramide-induced apoptosis were identified, and these failed to affect Fas-mediated apoptosis, thus effectively separating the phenomena (48). In another study, a lymphoid line treated with a calcium ionophore showed a large increase in ceramide production but no apoptosis, although ligation of Fas induced both (38). Again, these results suggest that Fas-induced apoptosis and Fas-induced ceramide production can be separable events in lymphoid cells.

Signaling via aSMase and/or ceramide may be critically important for apoptosis in other systems. In radiation-induced apoptosis, a critical role for ceramide production was demonstrated (49, 50) as the lung endothelium from aSMase−/− mice was shown to be dramatically resistant to this form of cell death. Similar results were recently obtained for microvesSEL endothelium throughout the central nervous system (51). In contrast, thymocytes from aSMase−/− animals readily undergo radiation-induced cell death. Animals lacking p53 show the opposite pattern; thymocytes are resistant to radiation, whereas the lung cells remain fully susceptible (50).

REFERENCES

1. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O’Rouke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4961–4965

2 E. Skowronski and D. R. Green, unpublished observations.
Role of Acidic Sphingomyelinase in Fas/CD95-mediated Cell Death
Tese Lin, Laurent Genestier, Michael J. Pinkoski, Arturo Castro, Shelby Nicholas, Rona Mogil, Francois Paris, Zvi Fuks, Edward H. Schuchman, Richard N. Kolesnick and Douglas R. Green

*J. Biol. Chem.* 2000, 275:8657-8663.
doi: 10.1074/jbc.275.12.8657

Access the most updated version of this article at [http://www.jbc.org/content/275/12/8657](http://www.jbc.org/content/275/12/8657)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/275/12/8657.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 50 references, 19 of which can be accessed free at [http://www.jbc.org/content/275/12/8657.full.html#ref-list-1](http://www.jbc.org/content/275/12/8657.full.html#ref-list-1)