Cell Death and Differentiation (2001) 8, 100–102

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Letter to the Editor

Niemann-Pick Disease *versus* acid sphingomyelinase deficiency

Dear Editor,

Types A and B Niemann-Pick Disease (NPD) result from the inherited deficiency of acid sphingomyelinase (ASMase)[1–4] activity which is required for hydrolysis of sphingomyelin to ceramide.[4,5] Type A NPD is a severe neurodegenerative disorder of infancy usually fatal by 3 years of age. Type B NPD patients have minimal or no neurologic involvement and often survive into adulthood.[4] Although it has been assumed that the differences between types reflects the level of residual ASMase activity, in other lipid storage disorders there is evidence that modifying factors, such as the GM2 activator protein of Tay Sachs disease, influence the disease course.[6,7]

Interest in experimental NPD has recently escalated following the establishment of cell lines and animal models defective in ASMase activity.[8–10] These reagents have also been used to elucidate the role of ceramide as a second messenger in the induction of apoptosis. Thus, a number of laboratories have shown that EBV-transformed B cells from patients with point mutations in *asmase* are defective in radiation-induced apoptosis,[10–12] while disparate results have been obtained with these cell lines regarding CD95-induced death.[13,14] Although this approach has proved insightful, these lines are unstable and manifest variability depending on the patient source, EBV subtype and the *asmase* point mutation.

The *asmase* knockout mouse, originally generated in our laboratory, has proven to be a valuable addition to our armamentarium. Endothelium in the lung and throughout the central nervous system of these mice are almost completely resistant to induction of apoptosis with doses as high as 100 Gy, while littermates display dose-dependent apoptosis beginning with as little as 1–5 Gy.[10,16] Similar protection of endothelium has now been observed in microvessels throughout the small intestine.[17] In contrast, thymic cells from these mice display no resistance to apoptosis.[10,15] ASMase deficient mice have also been reported to display defects in hepatocyte apoptosis, liver failure and animal lethality upon intravenous injection of Jo2 anti-CD95 antibodies.[15] In contrast to the defect in radiation-induced death, which cannot be overcome by increasing the radiation dose, the defect in hepatocyte apoptosis and lethality could be bypassed with higher amounts of antibody.[15] Importantly, animals heterozygous for *asmase*, which represents the clinically benign human carrier state, display half of the protection from CD95-mediated damage, indicating gene dosage not NPD determines the outcome.[15]

In addition to these defects in apoptosis induction, ASMase deficient mice manifest a marked defect in the ovarian developmental program.[18] A failure to normally delete oocytes in *asmase* mutant females during embryogenesis results in ovarian hyperplasia at birth.[19] Further, oocytes isolated from adult ASMase deficient females are markedly resistant to doxorubicin-induced apoptotic death.[20] A ceramide metabolite, sphingosine 1-phospate, which has been proposed to generally antagonize ceramide-induced apoptosis, similarly prevents drug-induced apoptosis of superovulated oocytes.[18] Moreover, radiation-induced oocyte loss in adult wild-type female mice, the event that drives premature ovarian failure and infertility in female cancer patients[21] is prevented by sphingosine 1-phosphate therapy *in vivo*.[20]

A recent article in *Cell Death and Differentiation* by Nix and Stoffel[22] reported marked biochemical alterations and membrane dysfunction in cells derived from their line of *asmase* knockout mice. Hepatocytes displayed a twofold increase in plasma membrane sphingomyelin with secondary increases in glycosphingolipids, changes indicative of the NPD phenotype. Further, murine embryonic fibroblasts (MEFs) derived from these mice displayed a dramatic reduction in caveolin content and a resistance to isolation of caveolae, plasma membrane sphingolipid rafts regarded as signaling microdomains. This was accompanied by reduced signaling through tyrosine kinases in T lymphocytes, lymphopenia, the absence of proliferation of T cells in response to anti-CD3, reduced expression of the anti-apoptotic adapter FLIP, and a paradoxical increase in apoptosis of anti-CD3 pre-treated splenocytes upon activation of CD95.

One implication of the observations of Nix and Stoffel is that the apoptotic abnormalities in *asmase*$^{-/-}$ cells and tissues[10–18,20,23] did not result merely from ASMase deficiency, but rather were impacted by disruption of membrane microdomains in response to altered sphingolipid metabolism. It should, however, be pointed out that the phenotype of our ASMase deficient mouse line is different from that of Nix and Stoffel. MEFs from our mice display only a minimal increase in sphingomyelin content (*asmase*$^{+/+}$ 1120 ± 17 pmol/10^6 cells *vs.* *asmase*$^{-/-}$ 1350 ± 20 pmol/10^6 cells), and normal morphology and doubling times [*asmase*$^{+/+}$ ($t_{1/2}$)=47.8 ± 0.2 h *vs* *asmase*$^{-/-}$ ($t_{1/2}$)=46.8 ± 1.2 h]. Further, the level of caveolin-1 is unchanged as measured by Western analysis (Figure 1A), and its distribution is unaltered as assessed by immunofluorescence (Figure 1B). Isolation of detergent-insoluble membrane domains and their caveolin content were also unaffected in our *asmase*$^{-/-}$ MEFs (Figure 1C). Additionally, p38, c-Jun N-terminal, and extracellular signal regulated kinase activation in response to tumor necrosis

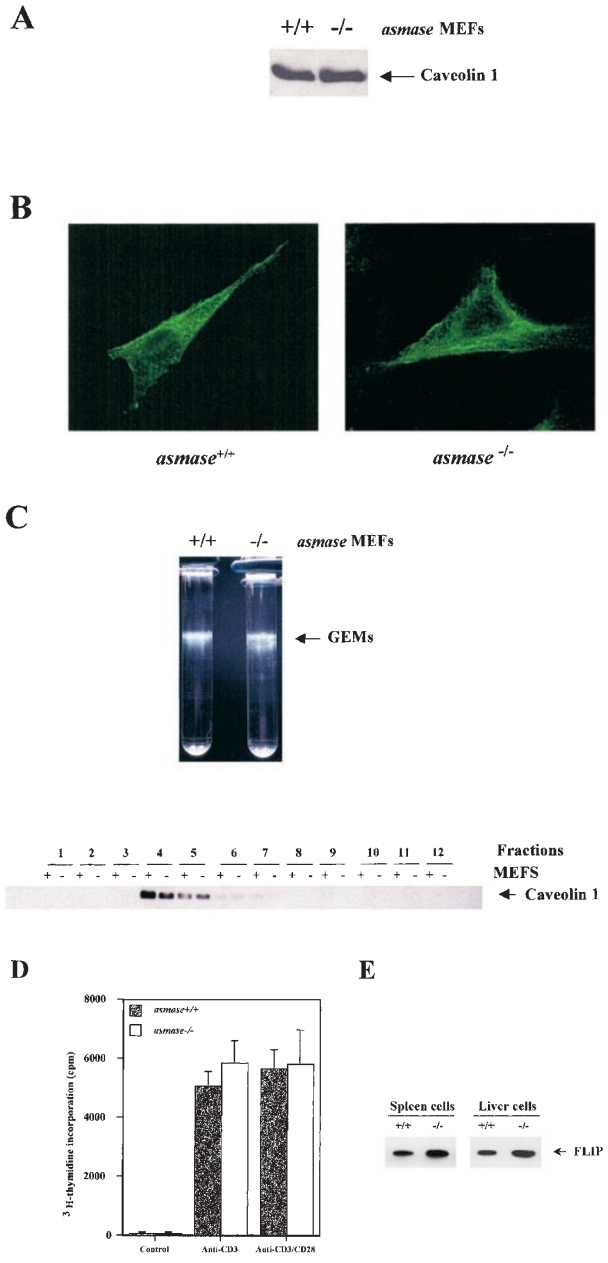

Figure 1 Comparison of *asmase*$^{+/+}$ and *asmase*$^{-/-}$ cells. *asmase*$^{+/+}$ and *asmase*$^{-/-}$ MEFs display similar caveolin-1 content by Western analysis (**A**) and distribution by immunofluorescence (**B**). In (**A**) caveolin-1 was detected in 30 μg NP-40 total cell lysate using rabbit anti-caveolin-1 N20 antibody (Cat.# sc-894, Santa Cruz Biotechnology Inc.). Similar results were obtained using a rabbit anti-caveolin antibody from Transduction Laboratories (Cat.#C13630, Lexington, KY). In (**B**) MEFs were fixed in methanol/acetone (1 : 1) as described by Nix and Stoffel,[22] and caveolin-1 detected by immunofluorescence using the Ab in (**A**) and a secondary FITC-labeled donkey anti-rabbit antibody. A goat anti-caveolin-2 N20 antibody (Cat.#sc-1858, Santa Cruz Biotechnology, Inc.) similarly revealed an identical distribution pattern in *asmase*$^{+/+}$ and *asmase*$^{-/-}$ MEFs. In (**C**), detergent-insoluble glycolipid-enriched membrane microdomains (GEMs) were isolated from *asmase*$^{+/+}$ (+) and *asmase*$^{-/-}$ (−) MEFs by discontinuous sucrose density ultracentrifugation (3×10^7 cells/gradient), fractions were collected and aliquots from each fraction were resolved by SDS–PAGE as described.[29] Caveolin-1 content was measured by Western blot analysis as in (**A**). In (**D**), spleen cells from *asmase*$^{+/+}$ and *asmase*$^{-/-}$ mice were stimulated for 18 h with 2 μg plate-bound anti-CD3 (clone 145-2C11, BD PharMingen) or anti-CD3 plus anti-CD28 (clone 37.51, BD PharMingen) and 10 μCi/ml [^{3}H]thymidine were added to 2×10^5 cells. After 18 h, the amount of radiolabeled thymidine incorporated into DNA was measured. Data (mean ± S.D.) are from three independent studies performed in triplicate. In (**E**), spleen or liver cells were isolated from *asmase*$^{+/+}$ and *asmase*$^{-/-}$ mice, purified by Ficoll and lysed in RIPA buffer. 2×10^6 cell equivalents were resolved by SDS–PAGE, transferred to nitrocellulose and blotted with an anti-FLIP$_L$ antibody (Cat.# sc-7109, Santa Cruz Biotechnology, Inc.)

factor, platelet derived growth factor and epidermal growth factor are identical to wild-type MEFs. The only defects we have found in these cells are specific reductions in apoptosis in response to environmental or pharmacologic stresses.[24] Similarly, we do not find substantive increases in sphingomyelin content of hepatocytes (*asmase*$^{+/+}$ 1150 ± 80 pmol/10^6 cells *vs* *asmase*$^{-/-}$ 1200 ± 85 pmol/10^6) or splenocytes (*asmase*$^{+/+}$ 950 ± 50 pmol/10^6 cells *vs* *asmase*$^{-/-}$ 980 ± 60 pmol/10^6) from 8-week-old animals. Nor do we detect lymphopenia, alterations in CD3- or CD3+CD28-induced proliferation of T cells (Figure 1D), alterations in tyrosine phosphorylation patterns of peripheral blood lymphocytes after PHA- or anti-CD4 GK.1 antibody or of splenocytes after anti-CD3 2C11 antibody, decreases in FLIP levels (Figure 1E) or paradoxical apoptosis of CD3 pre-treated splenocytes upon activation of CD95. Thus, the cells derived from our ASMase deficient mice do not display the panoply of defects observed by Nix and Stoffel.

Phenotypic differences also exist between the two *asmase* knockout mouse colonies, despite the fact that the technology used to generate these mice was similar.[8,9] Stoffel and co-workers reported that the life expectancy of their ASMase deficient mice was around 4 months,[9] with mice succumbing to advanced NPD. In contrast, we do not detect the earliest clinical manifestation of the disease, a resting tremor, until 12–16 weeks of age and our animals survive to 9.6 ± 0.4 (mean ± S.D.) months of age.[10,16] Although both knockouts are maintained in similar C57BL/6 × 129 backgrounds, the sub-strains of the 129 line are, in fact, different. Further, these ASMase deficient mouse lines were developed using non-isogenic stem cell clones. In this regard, extragenic strain specific enhancers and suppressors, such as polymorphisms or background mutations, and epigenic environmental factors, are well known to modify the expression of genetic phenotypes in mice. Whatever the reason for the differences, it is clear that the *asmase* knockout of Stoffel *et al.* develop NPD more rapidly than our knockout. In this regard, it should be emphasized that our and other studies demonstrating abnormalities in the apoptotic response to stress[10,15,16,18,23,24] were carried out in tissue samples derived from ASMase deficient mice younger than 12 weeks of age, before any biochemical, histological or clinical manifestations of NPD were apparent.

We agree with Nix and Stoffel that ASMase is likely to affect signaling within membrane microdomains. In fact, a developing literature shows that generation of the second messenger ceramide is occurring selectively within caveolae.[25–28] Further, investigators interested in using

asmase$^{-/-}$ models to evaluate defective apoptotic signaling, or to study disruption of membrane microdomains in experimental NPD, are urged to be cautious in selecting genetic reagents.

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