A central role for ceramide in the age-related acceleration of apoptosis in the female germline

Gloria I. Perez,*1 Andrea Jurisicova,*† Tiina Matikainen,* Toshitake Moriyama,* Mee-Ran Kim,* Yasushi Takai,* James K. Pru,* Richard N. Kolesnick,‡ and Jonathan L. Tilly*

*Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts, USA; †Samuel Lunenfeld Research Institute, Mount Sinai Hospital, and Department of Obstetrics and Gynecology, University of Toronto, Toronto, Ontario, Canada; and ‡Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, New York, New York, USA

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SPECIFIC AIMS

1. To determine whether ceramide produced by cumulus cells and transported into oocytes could be responsible for driving the age-related increase in germ cell apoptosis.

2. To investigate whether oocytes collected from young and aged female mice respond differently to ceramide with respect to apoptosis induction.

PRINCIPAL FINDINGS

1. Ceramide is trafficked from cumulus cells into oocytes of aged mice before the onset of apoptosis

Using immunofluorescence based on a monoclonal antibody against ceramide, we detected abundant levels of the sphingolipid in most cumulus cells of freshly isolated cumulus oocyte complexes (COC) harvested from aged (34–35 wk postpartum) female mice, whereas comparatively lower levels were detected in the adjacent oocyte (Fig. 1A). Short-term (3 h) incubation of COC from aged mice resulted in complete loss of ceramide from the cumulus cells with a concomitant increase in the adjacent oocyte (Fig. 1B). By 24 h of incubation, ~50% of the oocytes within these COC had undergone apoptosis (Fig. 1G). In contrast, denuded oocytes incubated in parallel failed to show any increase in ceramide levels (Fig. 1C); and the incidence of apoptosis was <20% (Fig. 1G). Freshly isolated COC from young (7–8 wk) female mice possessed almost undetectable levels of ceramide in their cumulus cells, with higher levels in their oocytes (Fig. 1D), which remained constant during culture whether cumulus cells were present (Fig. 1E) or absent (Fig. 1F). The incidence of apoptosis in either cumulus-enclosed or denuded oocytes of young mice after a 24 h culture was low and comparable to that observed in denuded oocytes of aged females (Fig. 1G).

2. Interference with gap junction-dependent communication or disruption of lipid rafts prevents ceramide trafficking and apoptosis in COC of aged mice

Culture of COC from aged mice for 3 h in the presence of 10 μM glycyrrhetinic acid (GA), an agent that specifically uncouples gap junctions, prevented movement of ceramide from the cumulus cells into the oocyte. Concurrent with its ability to inhibit ceramide trafficking, GA completely suppressed the increased incidence of oocyte apoptosis in COC derived from aged females. Next, we disrupted biochemical reactions in membrane lipid rafts by incubating COC from aged mice with filipin (10–40 μg/mL) for 3 h. This efficiently blocked the increase in ceramide levels in oocytes and prevented, in a dose-dependent manner, apoptosis after a 24 h culture.

3. S1P prevents apoptosis in COC of aged mice but not ceramide trafficking

Treatment of COC from aged mice with 10 μM sphingosine-1-phosphate (S1P) abolished basal and the age-dependent increase in apoptosis observed in COC cultured for 24 h (Fig. 2), but an equimolar concentration of the inactive S1P analog, dihydro-S1P, was without effect (unpublished data). We observed that in COC from aged mice, treatment with S1P did not prevent the accumulation of ceramide in the oocyte after culture (unpublished data). This suggests that trafficking of ceramide from the cumulus cells was not affected by S1P, but rather that S1P specifically interfered with ceramide-promoted oocyte death.

† Correspondence: MSU, 4173 Biomedical Physical Sciences, East Lansing, MI 48824, USA. E-mail: perezg@msu.edu
4. Oocyte sensitivity to ceramide-induced apoptosis increases with advancing age

A second approach to confirming an age-dependent role for ceramide as a proapoptotic messenger in oocytes was undertaken by microinjecting natural long-chain (C16) ceramide (6 pl) or its vehicle into denuded oocytes from young and aged female mice. Microinjection of denuded oocytes from aged mice with an equimolar concentration of dihydro-C16-ceramide did not increase the incidence of oocyte apoptosis (unpublished data).

5. Chronic ceramide deficiency increases the sensitivity of oocytes of young mice to ceramide-induced apoptosis

A possible explanation for the difference in response between oocytes of young and aged female mice to a...
cytosolic spike in ceramide is that oocytes from aged mice develop a heightened response to exogenous ceramide because of a prolonged deficiency in endogenous ceramide. To test this, we examined oocytes of young ASMase-null mice, which are chronically deficient in ceramide generation. Microinjection of C16-ceramide into denuded oocytes of young ASMase mutant females induced apoptosis to an extent comparable to that seen in COC of aged wild-type females. Moreover, culture of the ASMase-deficient oocytes in the presence of the ceramide synthase inhibitor FB1 (2.2 μM for 6 h) further increased the apoptotic response to microinjected C16-ceramide. In contrast, FB1 had no effect on the basal rate of apoptosis in wild-type or ASMase null oocytes, nor on the apoptotic response of wild-type oocytes microinjected with C16-ceramide.

6. Oocytes of aged mice also show elevated Bax expression

We explored the possibility that Bax availability changes in oocytes with increasing maternal age. In freshly isolated denuded oocytes, we detected a significant increase in the levels of bax mRNA in oocytes of aged vs. those of young mice. These changes in bax mRNA accumulation were mirrored by similar age-related increases in Bax protein levels. Moreover, culture of COC from aged mice with a cell-permeable Bcl-2 homology domain-4 (BH4) peptide (15 μg/mL) derived from the Bax antagonist Bcl-x<sub>L</sub> suppressed the age-related increase in the incidence of oocyte apoptosis to levels approaching those observed in denuded oocytes of aged females.

7. Ceramide acts upstream of Bax to induce oocyte apoptosis

To further delineate the relationship between ceramide and Bax in inducing oocyte apoptosis, oocytes were collected from young wild-type and bax null females, then treated with FB1 and microinjected with a ceramide antibody (6 pl) to sensitize the oocytes to exogenous ceramide (see above). Microinjection of C16-ceramide into denuded oocytes of young wild-type females induced apoptosis (57±7%; n=49 oocytes). In contrast, in bax null oocytes treated in a parallel fashion, C16-ceramide microinjection had no effect on apoptosis (6±4%; n=52 oocytes; P<0.001 vs. wild-type).

CONCLUSIONS AND SIGNIFICANCE

The finding that ceramide was trafficked from one cell type (cumulus cells) into another (germ cells) as a signal for apoptosis is striking and, to our knowledge, has not been reported previously. The ability of filipin to prevent trafficking of ceramide from cumulus cells into oocytes and the ensuing suppression of apoptosis in oocytes after filipin treatment are interesting when considered with past studies showing that ASMase-generated ceramide is required for apoptosis in female germ cells during fetal development and in response to pathological insults. Thus, the present findings extend these earlier observations by identifying a key role for ceramide in signaling the accelerated incidence of apoptosis in oocytes of aged female mice.

Another intriguing observation was the age-related sensitivity of oocytes to a cytosolic spike in ceramide levels with consequential induction of apoptosis. Thus, rapidly elevating ceramide levels in oocytes of aged females, whether by intercellular trafficking or microinjection, were consistently linked to the onset of apoptosis. While oocytes of young mice were refractory to ceramide microinjection, their susceptibility was restored by experimentally reducing ceramide levels in young oocytes by ASMase deficiency and FB1 treatment. Thus, the basal cytosolic levels of ceramide present in oocytes affect whether a spike in ceramide can trigger apoptosis.

We observed that oocytes accumulate bax mRNA and Bax protein with age. These findings, together with reports from others that ceramide can synergize with Bax in mitochondrial permeability transition, raise the possibility that the increased incidence of apoptosis noted in oocytes of aged mice is related to a change in ceramide sensitivity and Bax bioavailability. Such a conclusion is supported by a number of observations. First, ceramide is clearly involved in triggering apoptosis in oocytes of aged mice, as shown by the ability of S1P to prevent apoptosis in this model as well as that of ceramide microinjection to trigger apoptosis in denuded oocytes. Second, the age-related increase in the incidence of oocyte apoptosis was suppressed by the presence of the BH4 domain of Bcl-x<sub>L</sub>, a known antagonist of Bax function. Last, ceramide microinjection failed to induce apoptosis in Bax-deficient oocytes, providing evidence of a functional link between ceramide and Bax in this model of cell death.

In summary, these experiments have begun to sort out the molecular and cellular components of enhanced apoptosis in aging female germ cells, uncovering a novel role for the intercellular trafficking of ceramide as a key step in the process. Significant differences in ceramide levels, ceramide sensitivity, and Bax availability were identified between oocytes of young and aged female mice; these collectively raise the apoptotic threshold in the female germline with advancing maternal age. Such findings not only further define the interrelationships between components of the apoptosis machinery in female germ cells, but may also help elucidate the molecular basis for the accelerated rate of oocyte depletion observed in women prior to menopause.