Cumulus cells are required for the increased apoptotic potential in oocytes of aged mice

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Recent studies with female ICR mice have suggested that oocyte DNA fragmentation is one reason for poor oocyte quality and lower fertility associated with ageing. Since it was not determined if this increased ‘apoptotic’ potential in aged oocytes is due to changes within the oocyte itself or within the microenvironment of cumulus cells (CC) surrounding the germ cell, we sought to clarify if CC were required to affect the rate of apoptosis in oocytes maintained in vitro. Intact cumulus-oocyte complexes (COC) were retrieved by superovulation of virgin female ICR mice at 7 weeks (‘young’) or 34–35 weeks (‘aged’) of age. One-half of the COC in each group were incubated at 37°C in human tubal fluid medium under paraffin oil for 24 h. The other half of the COC in each group were denuded of CC and incubated under the same conditions (denuded oocytes; DO). Following incubation, COC were stripped of adherent CC by gentle pipetting. All DO were then fixed and checked by light microscopy for morphological changes characteristic of apoptosis. In young mice, the presence of CC had no significant effect on oocyte death rate (18 ± 9% and 14 ± 6% apoptotic oocytes in COC and DO, respectively; P > 0.05). However, in aged mice the percentage of CC-enclosed oocytes that underwent apoptosis was significantly greater as compared to the death rate in DO (48 ± 3% versus 19 ± 8% apoptotic oocytes, respectively; P < 0.05). This increased death potential was due to the presence of CC since the occurrence of apoptosis in DO of aged versus young mice was not significantly different (19 ± 8% versus 14 ± 6% apoptotic oocytes, respectively; P > 0.05). These results demonstrate that the age-dependent acceleration of apoptosis in oocytes maintained in vitro requires the CC.

Key words: ageing/apoptosis/cumulus cells/fertilization/gap junctions/oocytes

Introduction

The menopause marks the irreversible loss of reproductive potential in women. In contrast to spermatogenesis, which remains active until advanced age, female fertility depends upon a finite pool of oocyte-containing primordial follicles formed in the ovary during the perinatal period. This stockpile of germ cells is depleted throughout prepubertal and adult life to near exhaustion by the time of menopause (Richardson et al., 1987; Vom Saal and Finch, 1988; Tilly and Ratts, 1996; Martinmeau and Tilly, 1997). However, fertility is markedly compromised several years before the menopause, suggesting that factors aside from complete exhaustion of the follicle pool play a role in the loss of oocyte competency in ageing women (Sharma et al., 1988; Hughes et al., 1989; Steinkampf, 1991; Navol et al., 1991).

Recently, it was proposed that DNA fragmentation in oocytes associated with apoptotic death might be one of the reasons for poor oocyte quality and lower fertility in aged mice; however, in this study the role of cumulus cells (CC) was not taken into account (Fujino et al., 1996). Communication between the oocyte and its surrounding somatic cells is established very early in life when pre-granulosa cells encompass an oocyte to form a primordial follicle (Peters, 1979; Nicosia, 1983). Throughout follicular development, the granulosa cells communicate with each other and with the oocyte via gap junctions to ensure proper oocyte growth and maturation (Eppig, 1991). However, the influence of CC on oocyte apoptosis has not been studied, and this point is particularly relevant to the recent work of Fujino et al. (1996) with young and aged mice. Our objective was to clarify the work of Fujino et al. (1996) by determining if age-related change in the rate of apoptosis in mouse oocytes maintained in vitro requires the surrounding CC or, alternatively, is due to functional changes in the germ cell itself.

Materials and methods

Collection and culture of oocytes

Virgin female ICR mice at 7 weeks (‘young’) or 34–35 weeks (‘aged’) of age (Taconic Laboratories, Wilmington, NY, USA) were superovulated with 10 IU of equine chorionic gonadotrophin (eCG; Professional Compounding Centers of America, Houston, TX, USA) followed by 10 IU of human chorionic gonadotrophin (HCG; Serono Laboratories, Norwell, MA, USA) 48 h later. Cumulus–oocyte complexes (COC) were collected from the oviducts 16 h after HCG injection. One half of the COC in each age group (young, n = 63; aged, n = 90) were denuded of CC by a 1 min incubation in 80 IU/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA), followed by three washes with culture medium (denuded oocytes, DO). The remaining COC from each age group (young, n = 68; aged, n = 117) were left intact. Following isolation, COC and DO were cultured in 0.1 ml drops of culture medium (8–10 oocytes or COC per drop) under paraffin oil, and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO2/95% air. The culture medium used for all experiments was human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.5% bovine serum albumin.
Figure 1. Representative morphology of intact and apoptotic oocytes. Following in-vitro culture for 24 h, the numbers of intact (A) and apoptotic (B and C) oocytes in each experimental group were assessed by light microscopy. Note the occurrence of cellular condensation, budding and fragmentation in the apoptotic oocytes.

(Fraction V; Gibco-BRL, Grand Island, NY, USA). All studies involving animals described herein were approved by, and performed in strict accordance with, the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee and the NIH Guide for the Care and Use of Laboratory Animals.

Detection of apoptotic oocytes
At the end of the incubation period, COC were stripped of adherent CC by gentle pipeting (~20 times). All DO were then fixed and checked by light microscopy for morphological changes characteristic of apoptosis (Kerr et al., 1972). These criteria included the occurrence of cytoplasmic condensation (as indicated by retraction of the oolemma from the zona pellucida), membrane budding, and fragmentation of the oocyte into ‘apoptotic’ bodies of unequal sizes (Figure 1) (see also: Takase et al., 1995; Fujino et al., 1996; Perez et al., 1997; Tilly et al., 1997).

Data analysis
The percentage of oocytes that underwent apoptosis out of the total number of oocytes cultured per drop in each experiment was determined at the end of the 24 h incubation period. All experiments were independently repeated four times using three mice per age group for each experiment (12 mice total per age group). The combined data from the replicate experiments (mean ± SEM) were subjected to a one-way analysis of variance followed by Sheffe’s F-test with significance assigned at P < 0.05.

Results
Ovulation rates were significantly lower in aged versus young mice (22 ± 6 versus 48 ± 7, respectively; P < 0.05). In young mice, the presence of CC had no significant effect on oocyte death rate in vitro (18 ± 9% versus 14 ± 6% apoptotic oocytes in the COC versus the DO group; P > 0.05) (Figure 2). However, in aged mice the percentage of CC-enclosed oocytes that underwent apoptosis in culture was significantly greater than the death rate in DO (48 ± 3% versus 19 ± 8% apoptotic oocytes, respectively; P < 0.05) (Figure 2). This increased death potential was due to the presence of CC since the occurrence of apoptosis in DO of aged versus young mice was not significantly different following the 24 h incubation period (19 ± 8% versus 14 ± 6% apoptotic oocytes, respectively; P > 0.05) (Figure 2).

Discussion
The recent report of increased apoptotic potential in superovulated oocytes of aged mice following culture in vitro, when compared with the lower rates of spontaneous apoptosis observed in oocytes of young mice, has served as the basis for the hypothesis that ‘one of the reasons for the decline in the fertility in older women, i.e. >40 years old, may be that their oocytes have undergone apoptotic changes’ (Fujino et al., 1996). However, the experimental design of this study indicated that oocytes were retrieved post-HCG and immediately subjected to culture in vitro, implying that intact COC and not isolated oocytes were investigated (Fujino et al., 1996). Consequently, it could not be ruled out that the age-related increase in oocyte apoptotic potential observed in vitro involved the adherent CC intimately associated with the oocyte. Clinically, this point is important to clarify since the immediate
denuding of oocytes retrieved from older patients undergoing assisted reproductive technologies may be warranted if CC are in fact detrimental to oocyte survival in vitro.

Using morphological criteria recently established for the detection of apoptosis in individual oocytes (cellular condensation, budding and fragmentation into apoptotic bodies) (Takase et al., 1995; Fujino et al., 1996; Perez et al., 1997; Tilly et al., 1997), our goal was to clarify the findings of Fujino et al. (1996) using young and aged ICR mice to study the apoptotic potential of denuded versus CC-enclosed oocytes maintained in vitro. In young (7 week) mice, the percentage of oocytes that underwent apoptosis in 24 h when enclosed within CC masses was 18%, a value nearly identical to the percentage apoptosis (19%) reported by Fujino et al. (1996) following a 24 h incubation of COC obtained from ICR mice at 7–8 weeks of age. In our study the percentage of apoptosis in oocytes of COC obtained from aged mice was significantly greater (48%), and again this observation was comparable to the data obtained by Fujino et al. (1996) using COC harvested from female ICR mice at 40–48 weeks of age (68% apoptosis) when one considers that our aged mice were slightly younger (34–35 weeks old). Importantly, however, the age effect was entirely dependent on the presence of CC since denuded oocytes from both age groups exhibited levels of apoptosis that were not different from each other nor from the data obtained using COC of young mice. Therefore, the conclusion reached by Fujino et al. (1996) concerning the potential role of oocyte apoptosis in the age-related decline in fertility requires modification to indicate that this effect, at least in mice, requires the surrounding CC.

At present, the identity of the CC-derived factor, or possibly an oocyte-derived factor requiring direct modulation by CC, responsible for the increased apoptotic potential in the oocyte with age is unknown. However, many recent studies have shown that apoptosis in both ovarian granulosa and germ cells of diverse species is precisely regulated by a cohort of intracellular signals and gene products conserved through evolution (reviewed by Tilly, 1996; Tilly et al., 1997). Defects in these pathways can lead to dramatic changes in follicle endowment (Ratts et al., 1995), granulosa cell apoptosis (Knudson et al., 1995) and oocyte survival (Perez et al., 1997; Tilly et al., 1997). Moreover, one such system, involving Fas and Fas-ligand, has been proposed as a key mediator of communication between CC and the oocyte in the rodent and human ovary, possibly serving to induce oocyte apoptosis during atresia (Guo et al., 1994; Hakuno et al., 1996; Kondo et al., 1996). Therefore, with these data in mind, our studies have now focused on evaluating age-related changes in these death regulatory pathways in the ovary to define the basis of increased apoptosis in oocytes enclosed within CC masses obtained from aged mice.

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