Changes in Telomerase Activity in Experimentally Induced Atretic Follicles of Immature Rats

YOSHIKI YAMAGATA, YASUHIKO NAKAMURA, KENJI UMAYAHARA, AYako HARADA, HISako TAKAYAMA, Norihiro SUGINO and HIROSHI KATO

Reproductive, Pediatric and Infectious Science, Yamaguchi University School of Medicine, Minamikogushi, 1-1-1, Ube 755-8505, Japan

Abstract. Follicular atresia is characterized by apoptosis of granulosa cells, and telomerase plays an important role in the apoptotic process. To study the relationship between follicular atresia and telomerase activity, we investigated changes in telomerase activity and localization in experimentally induced atretic follicles of immature rats. Immature female Sprague-Dawley rats received 15 IU equine CG (eCG) subcutaneous injection. Rats were killed under ether anesthesia at 2, 3, 4, or 5 days after eCG injection. Telomerase activity in granulosa cells was measured by telomeric repeat amplification protocol (TRAP) assay, and telomerase localization in the ovary was examined by in situ TRAP assay. Telomerase activity was detected at high levels in granulosa cells on day 2 after eCG injection regardless of follicle size, and levels were significantly decreased in large follicles, with atretic changes, on days 4 and 5. No such decrease was observed in granulosa cells of small follicles. In the next experiment, rats received subcutaneous injections of estradiol (1, 10, or 50 μg/rat) to prevent follicular atresia or sesame oil as a control from day 2 to day 4 after eCG injection and were killed under ether anesthesia on day 5 after eCG injection. The changes observed in the large follicles on days 4 and 5 in oil treated rats were not observed with estradiol treatment. These findings suggest that the telomerase in granulosa cells is likely to play an important role for healthy follicle life and that loss of its activity may be associated with follicular atresia.

Key words: Telomerase, Follicular atresia, Granulosa cell, Estrogen

IN the ovary, only a few follicles proceed through ovulation; most residual follicles undergo atresia, which is characterized by apoptosis of granulosa cells [1, 2]. Recent studies have revealed the role of telomerase in suppressing apoptosis and modulating cell death [3-8]. Eukaryotic telomerases comprise multiple tandem repeats of guanine-rich DNA sequences, (TTAGGG)n [9, 10]. Telomerases function in protection, positioning, and replication of chromosomes. These DNA repeats are synthesized by an RNA-protein complex enzyme called telomerase.

Telomerase activity decreases dramatically during growth arrest and cell differentiation [11]. Although germ cells are known to have telomerase activity [12-15], little is known about the localization of telomerase in the ovary, and there is no data regarding changes in telomerase production or activity during follicular atresia. Among atretic and anti-atretic factors [1, 2], estradiol is considered as a survival factor. Estradiol production decreases in atretic follicles, and estradiol treatment prevents apoptosis of granulosa cells [16]. Kyo et al. reported that estradiol increased telomerase activity in a breast cancer cell line [17]. The effect of estradiol on telomerase activity in the ovary is unclear, however.

The objective of this study was to investigate localization and change in telomerase activity during experimentally induced follicular atresia. We also examined whether estradiol modifies changes in the follicles.
Materials and Methods

Animals

Experimental protocols were approved by the Yamaguchi University School of Medicine Committee for Ethics on Animal Experimentation and were designed according to the Guidelines for Animal Experimentation at Yamaguchi University School of Medicine in compliance with Japanese Law No. 105 and Notification No. 6. Immature 21 day old female Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan) were housed in a controlled environment under a 14 h light/10 h dark cycle and with free access to standard rat chow and water.

Induction of atresia and isolation of granulosa cells

Follicular atresia was induced in the rats as described previously [18]. Briefly, the immature rats were injected subcutaneously with 15 IU equine CG (eCG) in 0.15 ml saline. This stimulates growth and development of ovarian follicles for 2 to 3 days, after which the follicles undergo atresia because of waning trophic support due to metabolism of gonadotropins. According to their report [18], large follicles with more than 400 μm diameter were considered as healthy preovulatory follicles on day 2 or 3 and as atretic follicles on day 5 after eCG injection. Rats were killed under ether anesthesia 2, 3, 4, or 5 days after eCG injection. Ovaries were removed quickly, the surrounding connective tissue was removed, and granulosa cells were isolated by enzymatic needle-puncture, as described previously [19]. The granulosa cells were stored at −80°C until use. Several intact ovaries were placed immediately in Tissue-Tek OCT embedding compound and frozen in liquid nitrogen. Frozen specimens were stored at −80°C until use.

Exogenous estrogen treatment

Rats treated with 15 IU eCG were injected subcutaneously with different doses of estradiol (1, 10, or 50 μg) in 0.2 ml sesame oil or the same volume of sesame oil once a day from days 2 to 4 after eCG injection. On day 5 after eCG injection, rats were killed under ether anesthesia, and granulosa cells and ovaries were isolated and stored for TRAP or in situ TRAP assay.

Telomerase activity assay

Telomerase activity was measured by polymerase chain reaction-based TRAP assay with a Telomerase Detection Kit (Intergen, NY) as described previously [20]. Briefly, granulosa cells were removed from large follicles in PBS, pelleted by centrifugation for 5 min at 400 x g, and resuspended in 200 μl lysis buffer (0.5% CHAPS, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 10% glycerol, 10 mM Tris-HCl, pH 7.5). The lysate was incubated for 30 min at 4°C. The lysate was then centrifuged at 14,000 x g for 20 min at 4°C, and 160 μl of the supernatant was collected. The total protein concentration of the supernatant was determined with a BCA kit (Pierce, Rockford, IL). Cell extract (100 ng protein) was added to a reaction mixture containing 10 x TRAP buffer (15 mM MgCl₂, 630 mM KCl, 0.5% Tween-20, 10 mM EGTA, 0.1% bovine serum albumin, 200 mM Tris-HCl, pH 8.3), 50 x dNTP mixture (2.5 mM each of dATP, dTTP, dGTP, dCTP), TS primer (5'-AATCCGTGCAG CAGAGTT-3'), TRAP primer mixture (Intergen), and 5 units/μl Taq polymerase. For negative control, heat-treated sample was prepared by incubating the cell lysate at 85°C for 10 min to inactivate telomerase. For the first step, addition of the telomeric repeats by telomerase, the mixture was incubated at 30°C for 20 min. For the second step, amplification of the TS-telomerase product, the mixture was subjected to 35 cycles of amplification (94°C for 30 s, 72°C for 60 s). Each reaction product was amplified in the presence of a 36-base pair (bp) internal TRAP assay standard. Samples were loaded on a 12.5% non-denaturing polyacrylamide gel for separation by electrophoresis. Gels were stained with SYBr-Gold (Wako Pure Chemical Industries, Osaka, Japan), and photographic images were obtained. For quantification of the relative telomerase activity in each sample, the total density of characteristic was quantified using NIH image software. Values are expressed relative to control values.

In situ TRAP assay

In situ TRAP assay was performed as described previously but with slight modification [21]. Cryo-
sections (6 μm thickness) were cut at −20°C on a Reichert-Jung cryostat (Leica, Heidelberg, Germany), mounted on silane-coated slide glass, and air dried quickly. Twenty-five microliters solution containing 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 μM deoxynucleoside triphosphatase, 1 μg T4 gene protein, BSA (0.1 mg/ml), 2 units Taq polymerase, and 10 pmol FITC-labeled (5'-end labeling using Fluore Prime; Pharmacia Biotech, Uppsala, Sweden) TS forward primer (5'-AATCCGTCGAGCAGGTT-3') were placed within each frame, and slides were incubated for 30 min at 22°C in a dark box. After TS extension, 25 μM of the same solution but with 10 pmol of FITC-labeled (5'-end labeling) CX reverse primer (5'-CCCTACCCCTACCCCTACCTTA-3') was added. Samples were sealed with coverslips, heated to 90°C for 1.5 min to inactivate the telomerase, and then amplified in Takara PCR thermal cycler MP (Takara Biomedicals, Tokyo, Japan). The PCR conditions were 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s. The slides were washed in tap water and then sealed with a coverslip and Macalavain buffer/glycerin solution (1:1, v/v). Ovaries were examined under a fluorescence microscope equipped with a B-filter (Olympus, Tokyo, Japan).

**Statistical analysis**

Data were presented as the mean ± SEM and analyzed by one way-ANOVA and Duncan’s new multiple range test. Differences were considered significant at *p*<0.05.

**Results**

**Changes in telomerase activity in granulosa cells**

On day 5 after eCG injection, the number of granulosa cells with pyknotic nucleus was increased in the large follicles. As shown in Fig. 1, telomerase activity in granulosa cells was decreased significantly (*p*<0.01) in large atretic follicles on day 5 in comparison to that in healthy preovulatory follicles on days 2 and 3 after eCG injection. Regardless of follicle size, telomerase activity was stronger in granulosa cells than in theca or interstitial cells on day 2 after eCG injection (Fig. 2A, B). In large follicles with more than 400 μm diameter, the number of telomerase-positive granulosa cells decreased from day 2 to day 5 after eCG injection (Fig. 2C, E). In immature small follicles with less than 100 μm diameter, telomerase activity remained strong on days 4 and 5 after eCG injection (Fig. 2D).

**Effects of estrogen on telomerase activity**

Treatment with 10 or 50 μg estradiol significantly (*p*<0.01) increased telomerase activity in granulosa
cells (Fig. 3) and prevented atretic changes in granulosa cells of preovulatory large follicles on day 5 after eCG injection (Fig. 4C). The number of telomerase-positive granulosa cells in large follicles decreased on day 5 in oil injected rats (Fig. 4A, B) but did not decrease in 50 μg estradiol injected rats (Fig. 4C, D).

Discussion

We observed telomerase activity in granulosa cells of all follicles on day 2 after eCG injection and decreased telomerase activity in preovulatory large follicles from day 2 to day 4 after eCG injection in
Telomerase and Follicular Atresia

Fig. 3. Effect of estradiol on telomerase activity in granulosa cells on day 5 after eCG injection. The representative ladder bands are shown (A), and data are expressed as percentages of the control value (B). Values are shown as mean ± SEM of 5 rats.

*p<0.01 vs control.

conjunction with atretic changes. Hughes and Gorospe [18] reported that granulosa cells showed apoptosis on day 4 after eCG injection in rats. Taken together, these findings suggest that there is a close relation between follicular atresia and loss of telomerase activity.

Telomere length is considered an internal clock for the cell [11, 12], and expression of telomerase is directly related to apoptosis [3–8]. Telomerase activity in granulosa cells is likely to be essential for healthy follicle growth [22], and loss of telomerase may induce granulosa cells to undergo apoptosis, which is observed as follicular atresia. However, it is unclear whether the loss of telomerase activity induces atresia or the atretic change induces the loss of it. Although the precise role of telomerase in reproductive organ is not fully understood yet, telomerase-deficient mice showed reduced size of their gonads with increased apoptosis [23]. Telomerase-deficient female mice showed low ovarian weight, low ovulated-oocyte number, and poor embryo development; whereas their ovaries showed a full spectrum of follicular development [23]. Telomerase activity declines in large follicles compared with small follicles in bovine, and this large follicle pool contains atretic ones [22]. In our preliminary study, telomerase activities were detected in granulosa cells of small follicles before eCG injection and also in small follicles of neonatal rats (unpublished data).

Five days after injection of eCG, granulosa cells in large atretic follicles showed loss of telomerase activity, but those in small antral follicles showed strong telomerase activity and no atretic changes. Corpora lutea formed from preovulatory follicles after ovulation also have a telomerase activity and lose it during regression in pseudopregnant rats (unpublished data). The present study, together with the previous findings, suggests that telomerase may be not essential but is likely important for follicle-life in any growing stage, and that those follicles may become atretic when they lose telomerase activity. eCG may influence not only the size and number of preovulatory follicles in rodents but also the characteristic decrease in telomerase expression.

Regarding estrogen as an anti-atretic factor, Dhanasekaran and Moudgal [24] reported that lack of estrogen is the principal trigger of atresia of premature follicles, and Hughes and Gorospe [18] reported serum estrogen levels falling precipitously to basal levels on day 4 after eCG injection. There was no corpus luteum or atretic follicles on day 5 in rats treated with eCG and estradiol. Moreover, estradiol alone cannot induce preovulatory follicular growth in immature rats [25, 26]. Therefore, the present results indicated that estradiol supplementation rescued preovulatory follicles from atresia and maintained telomerase activity in their granulosa cells. In recent studies, Kyo et al. [17] showed that estrogen activated telomerase via direct and indirect effects on human telomeric reverse transcriptase promoter.
(hTERT) in estrogen receptor positive MCF-7 cells, and Misiti et al. [27] showed that estrogen induced hTERT expression in telomerase-negative primary ovary epithelial cells. Estradiol is likely to enhance telomerase activity in granulosa cells too, but, to the best of our knowledge, the effect of estradiol on telomerase activity in granulosa cells has yet to be examined.

On day 5 after eCG injection, serum estradiol concentration was higher in the estradiol treatment group (320 pg/ml) than in the control group (50 pg/ml). Serum testosterone concentration were not different between the two groups (unpublished data), although testosterone is a well known atretic factor. Thus, injected estrogen itself appears to play an important role in the atretic process likely through its influence on telomerase activity.

In summary, the present study provides the first evidence of a decrease in telomerase activity in granulosa cells in large follicles during experimentally induced atretic changes. This decrease may be caused by decreases in estradiol production by these follicles.

References

3. Kondo Y, Kondo S, Tanaka Y, Haqqi T, Barna BP,


