NOTE

Immunolocalization of Transforming Growth Factor- β 1 during Follicular Development and Atresia in the Mouse Ovary

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Abstract. In each estrous cycle not all follicles recruited grow to ovulate, as some degenerate through the process of atresia. Apoptosis is the mechanism underlying follicle atresia. TGF- β 1 has been implicated in the induction and promotion of apoptosis in many cell types and it is expressed in the ovary. In this study immunohistochemistry was used to localize TGF- β 1 in order to correlate the growth factor expression with morphological follicular atresia during diestrus in mice. Small and medium sized follicles had no staining for TGF- β 1 in the granulosa or theca cell layer. Weak to moderate TGF- β 1 expression was present in the theca cells of both large healthy and atretic antral follicles. Large healthy antral follicles showed weak granulosa staining but this was not observed in atretic follicles. Strong TGF- β 1 staining was present in the interstitial, corpus luteum and oocytes of all follicle stages. These results suggest that in the mouse TGF- β 1 promote follicular growth and differentiation rather than follicular atresia.

Key words: TGF- β 1, Follicular atresia, Follicular growth, Mouse ovary

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ALTHOUGH ovarian follicle atresia in mammals is a major event, the signals responsible for initiating this process have not been identified. However, there is evidence that apoptotic cell death is the molecular mechanism underlying this process in many species [1–5]. During any stage of follicle development granulosa and theca cell fate are determined by the balance of signals they receive from gonadotropins, steroids and intraovarian peptides [6].

Among the growth factors transforming growth factor- β 1 (TGF- β 1) belonging to the family of multi-functional cytokines is expressed in the ovary [7]. TGF- β 1 has been shown to induce apoptosis in a variety of tissues including uterine epithelial cells [8] and to enhance Fas-mediated apoptosis [9].

Granulosa cells are of epithelial origin and undergo apoptosis during follicular atresia [10]. Furthermore, granulosa cells express Fas receptor which has been implicated in follicular atresia in mice [11]. This information suggests that a relationship is likely to exist between atresia and TGF- β 1 expression.

In rodents, all follicles which reach the penultimate stage during diestrus undergo atresia since the ovulatory signal is absent. Therefore, during diestrus, follicular atresia is represented at almost all follicle stages. To determine whether there is a relationship between TGF- β 1 expression and follicular atresia, TGF- β 1 was localized by immunohistochemical technique in ovary sections from adult mice during diestrus.

Materials and methods

Antibodies

Rabbit raised polyclonal antibody specific to human TGF- β 1 was purchased from Biotechnology

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(Santa Cruz, CA, USA). The antibody does not crossreact with other TGF- β family members and was raised against a peptide corresponding to amino acids 328–352 mapping at the carboxy terminus of the precursor form of human TGF- β 1 (identical to corresponding mouse sequence) according to information provided with the antibody. The immunohistochemical staining kit with normal goat serum, goat anti-rabbit IgG and streptavidin was purchased from Nichirei (Tokyo, Japan).

Animals

Breeding ICR mice 7 weeks of age were maintained in an air conditioned facility on a 14L : 10D cycle, with food and water provided ad-libitum. Diestrus was determined by vaginal smears twice daily. After at least two 4-day consecutive estrous cycles three mice were sacrificed by cervical dislocation.

Histology

Ovaries were fixed in Bouin's solution for 12 h before being embedded in paraffin and were serially sectioned at 5 μ m. Every fifth section was immunostained for localization of TGF- β 1. The rest of the sections were stained with hematoxylin and eosin for determination of follicles undergoing atresia. Follicle classification was according to Pedersen and Peters [12]. The presence of at least 3 pyknotic nuclei or degenerating oocyte for stages 1–3 and 5 pyknotic nuclei for stages 4–7 in the largest cross section in which the oocyte was present was considered as undergoing atresia based on previous studies [13–14].

Immunohistochemistry

Immunostaining was performed as described by Flanders *et al.* [15] with minor modifications. Following deparaffinization and rinsing in phosphate buffered saline, tissue sections were incubated with 3% hydrogen peroxide in methanol for 10 min at room temperature to inactivate endogenous peroxidase activity. After this all steps were carried out at 4°C and slides were rinsed 3 times for 5 min each time in phosphate buffered saline between steps. Sections were incubated with normal goat serum for 10 minutes to prevent nonspecific staining followed

by primary antibody at a dilution rate of 1 : 10 for 24 h. This was then followed by secondary antibody and streptavidin peroxidase for 10 min and 5 min, respectively. Peroxidase was visualised with diaminobenzidine-tetrahydrochloride (DAB; Wako, Osaka, Japan) dissolved in 0.05 M tris-HCl buffer (pH 7.2) with 0.01% hydrogen peroxide. Finally, the sections were rinsed in distilled water, dehydrated and mounted. The slides that served as controls were not incubated with primary antibody but instead had normal serum. Slides were assessed and assigned staining intensities twice by the author and once by an independent observer.

Results

Follicle atresia was observed in follicles during all stages of development. In follicles in various stages of atresia, pyknotic nuclei were observed in the granulosa cell layer but not in the theca cell layer. The proportion of atretic follicles among the large follicles were 0.29 (stage 5b), 0.52 (stage 6) and 0.53 (stage 7).

Primordial (small) follicles in which the oocyte is surrounded by a single layer of cuboidal granulosa cells had weak or no TGF- β 1 staining in the oocyte (Fig. 1). In medium sized follicles, strong TGF- β 1 staining was observed in the oocyte but not in the granulosa and theca cell layers (Fig. 1). Large preantral follicles with more than three layers of granulosa cells showed strong oocyte staining and only a small proportion of these follicles had weak granulosa and moderate theca-staining. There was no TGF- β 1 staining in the granulosa cell layer of healthy or atretic follicles of stage 5b. Healthy stage 6 (early antral) and stage 7 follicles showed not only a strong oocyte staining but also weak to moderate granulosa cell layer staining for TGF- β 1 (Fig. 2). Interestingly, follicles in the process of atresia among stages 6 and 7 had strong oocyte staining but no staining in the granulosa cell layer (Fig. 3). Among stage 6 follicles, both healthy and those with typical signs of atresia had moderate TGF- β 1 thecal staining. However, the staining intensity in stage 7 follicles undergoing atresia was higher, moderate to strong compared to weak to moderate in healthy follicles (Table 1, Fig. 3). The interstitial layer surrounding the follicles showed moderate to strong TGF- β 1

Legend to figures 1 to 4 TGF- β 1 immunostaining in mice ovaries during diestrus. O, oocyte; GC, granulosa cells; TH, theca cells; IN, interstitial cells; A, antral follicles; PA, preantral follicles; DF, degenerating follicles.

Fig. 1. Moderate to strong staining in oocytes of preantral follicles (stages 4 and 5a) but no staining in the granulosa and theca layers. Weak staining in the interstitial. $\times 20$

Fig. 3. Degenerating follicle (stage 7) with weak thecal staining, pyknotic nuclei in the granulosa layer with no staining and corpus luteum with moderate staining. $\times 20$

Fig. 2. Healthy antral follicle (stage 7) with strong oocyte staining and weak granulosa staining but no staining in the theca layer. Granulosa of degenerating follicle has no staining but has weak theca staining shown by arrow. Interstitial compartment has moderate staining. $\times 20$

Fig. 4. No TGF- β 1 staining was observed when ovary sections were incubated with normal rabbit serum instead of anti-hTGF- β 1 antibody. $\times 20$

Table 1. TGF- β 1 staining in large follicles during diestrus in the mice ovary

Follicle stage	Follicle no. (%)	Granulosa cells		Theca cells		Oocytes	Interstitial cells
		Health follicles	Atretic follicles	Health follicles	Atretic follicles		
5b	69 (11.8)	—	—	—	—	++	++
6	60 (10.2)	$\pm$ to +	—	+	$\pm$ to +	++	++
7	38 (6.5)	$\pm$ to +	—	$\pm$ to +	+ to ++	++	++

Percentages were calculated based on total number of follicles from stage 3b to 7 inclusive.

Slides were assessed and assigned staining intensities: —, negative; $\pm$, weak; +, moderate; ++, strong staining. Only staining patterns in large follicles are shown in the table.

staining regardless of stage of follicle development. Moderate to strong staining was observed predominantly in the large cells of the corpora lutea.

Discussion

The present study documents an immunohistochemical evaluation of TGF- β 1 distribution in healthy follicles and follicles undergoing atresia. TGF- β 1 has been shown to induce apoptosis in many types of tissues [8, 9]. Recently, it was shown that TGF- β 1 in combination with TGF- α can induce apoptosis in rat theca-interstitial cells in vitro [16]. In this study, however, TGF- β 1 expression was not observed in the granulosa cell layer of follicles in the process of atresia and was detected in healthy antral follicles. There was no significant incidence of pyknosis in the theca layer of follicles with typical signs of atresia in the inner part. No correlation between incidence of granulosa cell pyknosis and TGF- β 1 expression in theca cells was observed. The findings suggest that, in mice, TGF- β 1 may be implicated in ovarian follicle growth and cell differentiation rather than follicular atresia. Accordingly, it is speculated that in the mouse, as a result of atresia, granulosa cells do not acquire TGF- β 1 protein necessary for further maturation as observed in healthy follicles at the same stage of development.

Appearance of TGF- β 1 expression in granulosa cells at time of antrum formation may be correlated with its ability to modulate gonadotropin receptor expression and enhance steroidogenic activity [17–19]. The expression of TGF- β 1 in granulosa cells bordering the basement membrane of large healthy follicles indicate that these are the follicles which will eventually luteinize. This is consistent with the expression of the luteal marker enzyme 3 β -hydroxysteroid dehydrogenase which was co-localized in the same cells that expressed TGF- β 1 and T β R-II in the marmoset ovary [20]. The increased expression in theca of follicles undergoing atresia may be associated with its capacity to enhance progesterone production and differentiation while inhibiting LH stimula-

tion of theca cell androgen production [21, 22]. Stage 7 atretic follicles had a higher theca staining which may signify luteinization of theca cells in view of TGF- β 1's ability to promote theca cell differentiation [22].

Theca-interstitial production of androgens is important for ovarian estrogen production. However, when produced in excess quantities, it can lead to follicular atresia. Strong interstitial TGF- β 1 expression observed in this study may be necessary to keep androgen production in check [22].

TGF- β 1 was localized in oocytes of all follicle stages and in the theca and granulosa cells of large antral follicles, confirming previous findings [23].

In small and medium sized follicles, a number of possible physiological functions can be envisaged for TGF- β 1 staining in the oocyte and interstitial cells. In view of its inability in vitro to stimulate mouse oocyte maturation in meiotic arrest [24], TGF- β 1 may play a role in the maintenance of meiotic arrest in oocytes so that they can grow to mature size. Data in this study suggest that TGF- β 1 may act on granulosa cells in a paracrine manner to effect its growth promoting effects [25], or its inhibitory effect on EGF-induced follicular DNA synthesis [26] in preantral follicles.

In the present study, moderate to strong TGF- β 1 was observed in the theca but not in the granulosa cell layer of follicles showing signs of atresia in the inner part. This finding is in contrast with the data in the human ovary in which no TGF- β 1 expression was observed in atretic follicles [27]. The discrepancy between these findings may be attributed to species difference. In the marmoset ovary, however, strong TGF- β 1 expression was present in theca and granulosa cells of follicles with signs of atresia in the inner part. The expression disappeared when follicles became completely atretic [20]. In this study the stage of atresia was not determined therefore it is difficult to make comparisons.

In summary this study provides evidence that TGF- β 1 is implicated in ovarian follicular growth and cell maturation rather than follicular atresia in the mouse ovary.

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