HISTOCHEMICAL STUDIES FOR COMPARISON OF REGULATORY MECHANISMS OF FOLLICULAR DEVELOPMENT IN MAMMALIAN OVARIES

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Introduction

In mammalian ovaries, only limited numbers (<1%) of follicles are selected for ovulation and the vast majority (>99%) of follicles undergo the degenerative process known as atresia. Recent studies have demonstrated that apoptosis of granulosa cells is the molecular mechanism underlying follicular atresia. Moreover, recent findings suggested that there are multiple pathways for apoptotic cell death. An understanding of the species-specific differences in the process of apoptosis in the follicular cells is essential for elucidation of the signal transmission process for selective atresia in the ovarian follicles. The present study was performed to histochemically examine the detailed differences in apoptotic appearance of porcine and bovine ovaries. As our preliminary histochemical study on porcine follicles indicated that the localization features were similar between proliferating granulosa cells and tumor necrosis factor a (TNFa), we investigated the detailed localization of TNFa in healthy to atretic granulosa cells of porcine ovarian follicles and the expression of TNF receptor-associated factor 2 (TRAF2) mRNA, which is a key effector of TNF pluripotent signals. Our results suggested that TNFa acts as a survival factor on granulosa cells of porcine follicles.

Materials and Methods

Porcine and bovine ovaries were fixed with 20% phosphate buffered formalin, pH 7.4, for *in situ* detection of DNA fragmentation by the TUNEL method and detection of proliferating cells and immunohistochemical localization of TNF© For *in situ* hybridization, porcine ovaries were frozen in

dry ice-isopentane and serial frozen sections were cut. The sections were fixed with 4% paraformaldehyde (PFA) in PBS, incubated with 0.2 N HCl, and digested with proteinase-K. They were post-fixed with 4% PFA in PBS and then hybridized with digoxigenin (DIG)-labeled oligo-DNA probes for TNFα or TRAF2 mRNA for 18 h at 37 °C. mRNAs were detected by alkaline phosphatase-conjugated sheep anti-DIG antibodies. The isolated granulosa cells prepared from follicles of pig ovaries were used for reverse transcription polymerase chain reaction (RT-PCR) Analysis. Total RNA was isolated and used to synthesize first strand cDNA using a T-Primed First-Strand Kit according to the manufacturer's protocol. The cDNA template was amplified in reaction buffer containing each specific primer by PCR. The PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and then images were digitally recorded using a FAS-III recorder.

Results and Discussions

In situ analysis of DNA fragmentation was performed on histological sections of porcine and bovine follicles using the terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method. As shown in Fig.1, compared with small follicles, higher percentages of apoptosis in granulosa and theca interna layers were seen in large follicles of porcine and bovine ovaries as compared to small follicles.

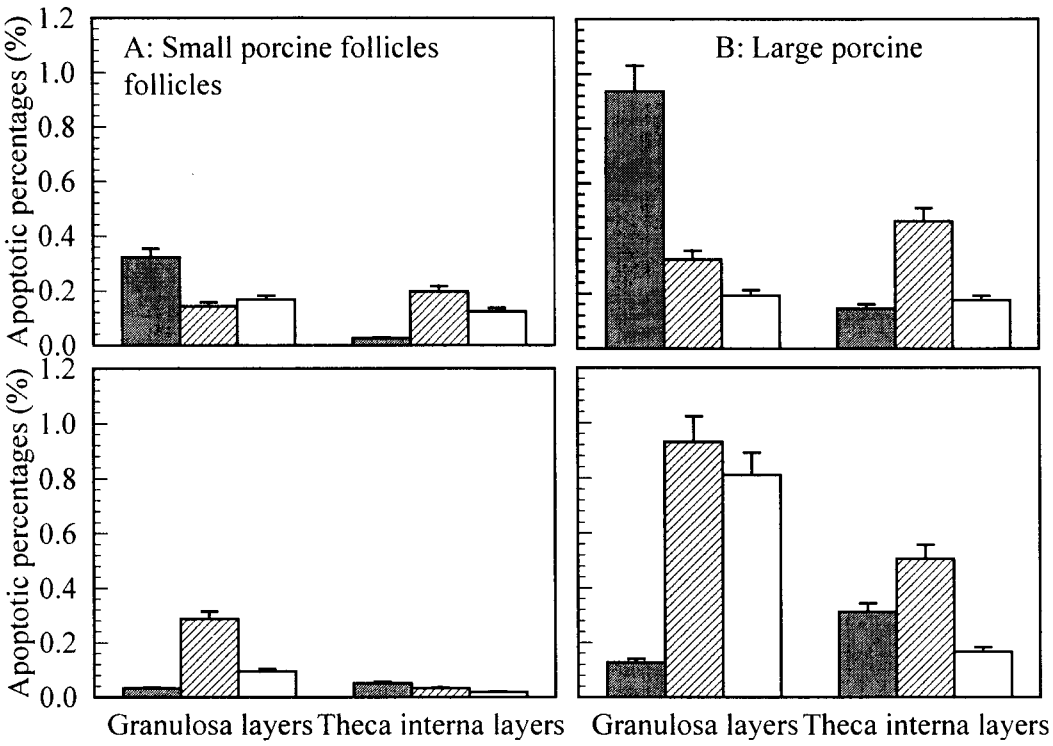
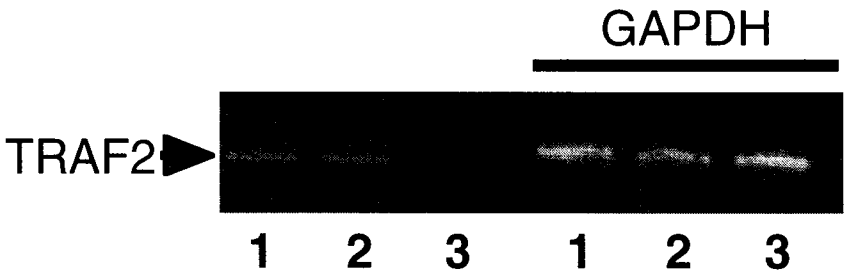


Fig. 1. Changes in apoptotic percentages in granulosa and theca interna layers during follicular atresia in porcine (A and B) and bovine (C and D) ovaries. Dark columns, inner region; striped columns, middle region; and open columns, outer regions of granulosa and theca interna layer.

Marked increases in apoptotic cell number were seen in the theca interna layer of large follicles compared with those in small follicles. In summary, apoptotic cells were predominantly seen in the inner region of the porcine granulosa layer and in the middle region of the bovine granulosa layer. In the theca interna layer, apoptotic cells mainly appeared in the middle and outer regions of porcine follicles and in the inner and middle regions of bovine follicles. Moreover, the frequencies of apoptotic cells in granulosa and theca interna layers of bovine follicles were significantly lower than those of porcine follicles. These species-specific differences indicated that local mechanisms of regulation of granulosa cell apoptosis in atretic follicles and the apoptosis-inducing factor(s) or survival factor(s) for the granulosa cells may be different among mammalian species.

The TNF superfamily including Fas antigen or Fas/APO-1/CD95 protein consists of more than 20 structurally related type I transmembrane proteins eliciting a wide spectrum of cellular

responses including transcriptional gene activation and induction of apoptosis [1,2]. However, apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis remain to be determined. The functions of the TNF receptor superfamily are mostly mediated by the family of TNF receptor-associated factors (TRAF-1-6) [3]. The downstream effectors of TRAF signaling are transcription factors belonging to the nuclear factor κB (NF-κB) and AP-1 family [4-6], which have been shown to render cells protection from apoptosis via the transcription of antiapoptotic genes [7].



We showed that TRAF2 mRNA was expressed in healthy and early atretic, but not in atretic granulosa cells (Fig. 2). Interestingly, TRAF2 mRNA was detected in the granulosa cells close to the follicular BM of porcine follicles, and in this area we detected abundant TNFα and its mRNA, as well as many proliferating granulosa cells (Fig. 3).

Fig. 2. Detection of TRAF2 mRNA expression in healthy (lane 1), early atretic (lane 2) and atretic (lane 3) granulosa cells by RT-PCR.

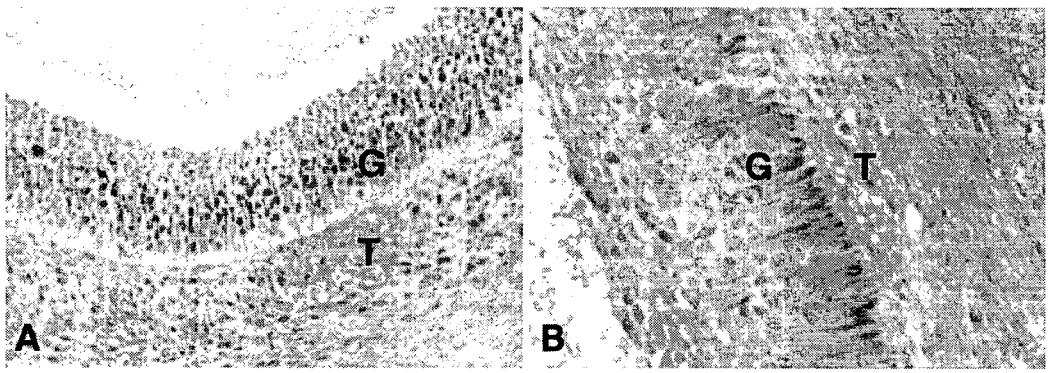


Fig. 3. Representative sections of porcine follicles. In healthy follicles, strong proliferating cell nuclear antigen (PCNA) and TNFα immunoreactivities were seen in the granulosa layer close to the follicular BM (A and B, respectively) and theca externa, and weak immunoreactivity was seen in the theca interna. In early and progressed atretic follicles, such immunoreactivity decreased. G,

granulosa layer; T, theca interna layer. X 200.

On the other hand NF-κB mRNA was expressed constitutively in the granulosa cells. We supposed that in the healthy granulosa cells some survival factor(s) transmitted the signals to NF-κB through TRAF2, but that apoptotic factors transmitted the death signals to NF-κB by another pathway(s) in atretic granulosa cells. Thus, it is likely that TNFα plays a role as a granulosa cell survival factor in the porcine ovary.

Acknowledgments

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