Fas and Fas ligand protein and mRNA in normal and atretic mouse ovarian follicles

S. J. Dharma, R. L. Kelkar and T. D. Nandedkar*

National Institute for Research in Reproductive Health, JM Street, Parel, Mumbai-400012, India

Apoptosis is the underlying mechanism of follicular atresia in the mammalian ovary. However, the apoptotic pathways governing this ovarian process are not completely elucidated. In the present study, expression of Fas and Fas ligand, the proximal members of the death receptor pathway, was evaluated in mouse ovarian follicles using immunofluorescence and *in situ* hybridization. Normal or atretic follicles were obtained from immature female

Swiss mice after administration of 10 iu equine chorionic gonadotrophin for 48 or 72 h, respectively. Expression of both Fas and Fas ligand mRNA and protein was observed in granulosa cells of normal and atretic follicles. Although the oocytes of normal follicles failed to show any staining, those of atretic follicles stained intensely for Fas, indicating that the presence of Fas in the oocyte determines the fate of the follicle.

Introduction

Coordination between oocyte and granulosa cells is an essential prerequisite to normal follicular development. Interactions between granulosa cells through gap junctions and between granulosa cells and oocytes through the microvilli in the zona pellucida have been reported (Canipari, 2000). Studies in rat (Peluso and Pappalardo, 1994; Peluso *et al.*, 1996) and bovine (Luciano *et al.*, 2000) granulosa cells have demonstrated that cell–cell contact plays a vital role in inhibiting granulosa cell apoptosis and regulating proliferation. Hence, a role for gap and tight junctions between granulosa cells and oocytes in preventing granulosa cell apoptosis has been proposed. Moreover, the molecular reprogramming that leads to oocyte maturation is under the regulatory control of signals from the cumulus cells, in response to gonadotrophin stimulation. Therefore, Moor *et al.* (1998) suggested that the key to oocyte maturation and subsequent embryo viability resides in the follicular compartment rather than the oocyte.

However, studies in mice *in vitro* have demonstrated that the oocyte promotes proliferation of granulosa cells from preantral and antral follicles (Vanderhyden *et al.*, 1992) and also regulates steroidogenesis through its secretory factors (Vanderhyden and Macdonald, 1998). Hence, the control of ovarian follicular development and function has been attributed to the oocyte (Eppig, 2001; Eppig *et al.*, 2002). Therefore, it appears that oocyte–granulosa cell interaction, finely tuned by factors expressed in both these follicular components, plays

a vital role in propelling the follicle either towards ovulation or degeneration (that is, atresia).

Morphological and biochemical studies have shown that the demise of both somatic and germ cells in the ovary is mediated by apoptosis (Hughes and Gorospe, 1991; Morita and Tilly, 1999). Apoptotic changes have been observed in granulosa cells of atretic follicles (Hseuh *et al.*, 1994) and expression of members of the *Bcl-2* gene family (Tilly *et al.*, 1995; Morita *et al.*, 1999; Nandedkar and Dharma, 2001) and the tumour necrosis factor α receptor (TNF α -R) family have been reported in various species during atresia (Guo *et al.*, 1997; Kim *et al.*, 1998, 1999; Porter *et al.*, 2000) and luteolysis (Roughton *et al.*, 1999). Studies *in vitro* have demonstrated that apoptosis induced by serum withdrawal in bovine granulosa cells is mediated by the Fas–Fas ligand pathway (Hu *et al.*, 2001). It remains unclear whether the oocyte plays a role in inducing–signalling apoptotic changes in the follicle and whether it expresses any of the known apoptotic factors.

Fas is a cell membrane integral glycoprotein similar to TNF α -R, whereas Fas ligand (Fas L) is a type II membrane protein homologous to TNF α . Apoptosis is induced in Fas-bearing cells when Fas L binds to Fas, leading to the activation of the Fas-associated death domain (FADD). The death domain, in turn, forms a docking site for the autocatalytic activation of procaspase 8 (Chinnaiyan *et al.*, 1995; Nagata and Goldstein, 1995; Ashkenazi and Dixit, 1999). Once activated, caspase 8 can stimulate other downstream proteases such as caspase 3. These proteolytic effectors of cell death cleave key cellular substrates, thereby disorganizing the cellular assembly and executing apoptosis (Thornberry *et al.*, 1997; Thornberry and Lazebnik, 1998).

*Correspondence
Email: cellbioirr@hotmail.com

In the present study, the expression of Fas and Fas L mRNA and protein has been studied in oocytes and granulosa cells of normal and atretic ovarian follicles. mRNA expression was studied by *in situ* hybridization and Fas and Fas L proteins were localized in ovarian follicles and denuded oocytes by immunofluorescence. Expression of Fas protein was compared in granulosa cells isolated from normal and atretic follicles by western blot analysis. Results indicate a possible role for the Fas–Fas L signalling system in oocyte–granulosa cell interaction leading to follicular atresia.

Materials and Methods

Animals

Swiss mice bred from the animal colony at the National Institute for Research in Reproductive Health were maintained at constant light (12 h light: 12 h dark), temperature (24°C) and humidity (60%), and were supplied with food and water *ad libitum*. The animal requirement for the present study was approved by the Institutional Animal Ethics Committee and experiments were performed in accordance with the guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals, India.

Model for follicular atresia

Twenty immature female mice aged 21–23 days and weighing 8–10 g were injected s.c. with 10 iu equine chorionic gonadotrophin (eCG) (PMSG; Sigma, St Louis, MO) and killed 48 h later by decapitation (normal group). Animals in a second group were killed 72 h after eCG administration (atretic group; $n=20$). Almost 90% of follicles in a normal group are antral follicles, and > 90% of the follicles in an atretic group are atretic (Nandedkar and Balchandran, 1982). For *in situ* hybridization, ovaries were excised, dehydrated through sequential grades of alcohol, cleared in xylene and embedded in paraffin wax. Sections (5 μm) were cut on a microtome and mounted on glass slides treated with amino-ethoxy-silane (Sigma). For indirect immunofluorescence, ovarian follicles were punctured with a 26 G needle to release the oocyte–cumulus complex (COC). These COCs were used instead of sections as the monoclonal antibodies for Fas and Fas L failed to label the proteins in ovarian sections. Denuded oocytes were obtained after treating the normal and atretic oocyte–cumulus mass with 1% (w/v) hyaluronidase (Sigma) for 5 min. The oocyte–cumulus mass or denuded oocytes were fixed in acetone at -20°C for 10 min, mounted on individual slides coated with poly-L-lysine (Sigma) and air dried.

In situ hybridization

In situ hybridization of Fas and Fas L mRNA was essentially the same as that described for c-myc mRNA by Nandedkar and Dharma (2001). The cDNA sequences for Fas and Fas L were taken from the published literature (Roughton *et al.*, 1999). These oligoprobes were tail-labelled with a digoxigenin oligonucleotide tailing kit (Roche Molecular Biochemicals, Mannheim). In brief, after deparaffinization and digestion with proteinase K ($10\text{ }\mu\text{g ml}^{-1}$ at 37°C for 30 min), sections were postfixed with 4% paraformaldehyde. After washing the slides in 0.1 mmol PBS l^{-1} , prehybridization was carried out at room temperature (25°C) for 1 h in prehybridization cocktail. This was followed by overnight hybridization at 42°C with digoxigenin-labelled cDNA anti-sense and sense oligoprobes dissolved in the prehybridization mixture at a concentration of $0.5\text{ pmol }\mu\text{l}^{-1}$. After stringent post-hybridization washings with various concentrations of SSC and incubation in Tris buffer (pH 7.4) for 10 min, the slides were blocked with 2% (v/v) normal sheep serum in Tris buffer containing 0.3% (v/v) Triton-X100 for 1 h at room temperature. Sections were incubated overnight in alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500 dilution; Roche). On the next day, slides were washed twice in Tris buffer for 5 min each. Colour reaction was carried out by incubating the slides in a mixture of nitro blue tetrazolium (Roche) and 5-bromo 4-chloro 3-indolyl phosphate (Roche) for 20 min at room temperature in the dark. Levamisole (0.2%, w/v) (Sigma) was added in the colour mixture to block endogenous phosphatases. After a wash in distilled water, slides were mounted in aquamount and viewed. The representative areas were photographed under a bright field microscope. For negative controls, the slides were subjected to an identical procedure using digoxigenin-labelled sense probes.

Immunofluorescence

Oocyte–cumulus masses or denuded oocytes fixed on poly-L-lysine-coated slides were washed in PBS and the specimens were permeabilized by incubating in 0.01% (v/v) Triton-X100 (Sigma) in PBS for 5 min. This was followed by incubation in 3% (w/v) non-fat dried milk in PBS for 30 min to block nonspecific binding. Slides were then incubated with primary antibody ($5\text{ }\mu\text{g ml}^{-1}$ of hamster anti-mouse Fas or Fas L monoclonal antibody; Pharmingen, San Diego, CA) overnight at 4°C . Negative controls were incubated in 1% (v/v) SP₂O culture supernatants in PBS instead of primary antibody. After PBS washes, secondary antibody (R-Phycoerythrin conjugated mouse anti-hamster IgG; Pharmingen) was applied at a dilution of 1:1000 in the dark at room temperature for 1 h. Slides were washed in distilled water, air dried, mounted in glycerol and observed under blue

light (Carl Zeiss, Gottingen). Photographs were taken at $\times 400$ magnification using a 400ASA Kodak colour film.

Protein extraction and western blot analysis for Fas

Twenty immature (23-day-old) female Swiss mice were administered 10 iu eCG. Animals from one group ($n=10$) were killed 48 h later to provide normal ovaries and others ($n=10$) were killed 72 h later for atretic ovaries. Freshly excised ovaries were teased in ice-cold phosphate buffered saline (PBS, pH 7.4) to extract granulosa cells. The cell suspension was centrifuged at 1000 *g* for 5 min. The granulosa cell pellet was suspended in PBS (pH 7.4) containing 1% SDS (w/v) and 10% protease inhibitor cocktail (w/v). The cell lysate was then sonicated for 2 min on ice and centrifuged (13 000 *g*, 15 min). Protein content was determined with the Micro-BCA protein assay kit (Pierce Endogen, Rockford, IL). Equal amounts of protein (30 μ g) from normal and atretic ovaries were resolved on 10% SDS-PAGE and electrotransferred to nitrocellulose membrane (Bio-Rad Laboratories, Inc). The membrane was blocked with 5% non-fat dried milk (w/v) in PBS containing 0.05% (v/v) Tween-20. Primary antibody (Fas; Oncogene Research Products, Cambridge, MA) was applied overnight at 4°C. The membrane was washed three times in PBS (pH 7.4) with 0.05% Tween-20 (v/v) and incubated in horseradish peroxidase-conjugated rabbit antimouse secondary antibody (DAKO, Glostrup) at 1:1000 dilution for 1 h at room temperature. After three washes in 0.05% Tween-PBS, bands were visualized using 1% diaminobenzidine (Sigma)-0.3% H₂O₂ in PBS.

Results

In situ hybridization of Fas and Fas L in ovarian follicles

Both Fas (Fig. 1a,b) and Fas L (Fig. 1c,d) were expressed in the granulosa cells of normal (Fig. 1a,c) and atretic follicles (Fig. 1b,d) but expression of Fas was more intense than that of its ligand. However, Fas (Fig. 1b) but not Fas L (Fig. 1d) transcripts were observed in the oocyte of atretic follicles. Oocytes from normal antral follicles did not show any staining for the receptor (Fig. 1a) and its ligand (Fig. 1c). Controls hybridized with the sense strands of Fas (Fig. 1e) and Fas ligand (Fig. 1f) did not show any staining. In both normal and atretic follicles, theca as well as stromal cells failed to show expression of Fas and Fas L.

Expression of Fas and Fas L in the ovarian follicles by immunofluorescence

Weak or negligible expression for both proteins was observed in the cumulus cells but not oocytes of normal

follicles (Fig. 2a,c). In atretic follicles, the cumulus cells expressed both Fas (Fig. 2b) and Fas L (Fig. 2d) but expression of Fas appeared more intense than that of its ligand. However, the atretic oocyte expressed only death receptor Fas (Fig. 2b). Controls processed in the absence of primary antibody did not show any staining (Fig. 2e,f).

Expression of Fas in oocytes isolated from normal and atretic follicles by immunofluorescence

Localization of Fas protein in oocytes isolated from normal and atretic ovarian follicles after treatment with eCG is shown (Fig. 3). Although the oocyte from the normal follicle did not express the death receptor (Fig. 3a), the oocyte from the atretic follicle stained intensely for Fas protein (Fig. 3b) compared with the atretic oocyte processed as a negative control (Fig. 3c).

Western blot analysis for Fas

Western blot analysis was carried out with a view to obtain a semi-quantitative estimate of the amount of Fas protein in granulosa cells from normal and atretic follicles. Both normal and atretic granulosa cells expressed the apoptotic protein (observed as a band at 48 kDa; Fig. 4). However, expression of Fas was considerably higher in atretic granulosa cells than in the normal cells. Low molecular weight rainbow markers (Bio-Rad Laboratories, Inc.) were resolved simultaneously (Fig. 4, lane 1).

Discussion

The mouse model used in this study was eCG-induced follicular development and atresia. Normal follicles were observed 48 h after treatment with eCG, whereas in the absence of hCG, atretic follicles were observed 72 h after eCG (Nandedkar and Balchandran, 1982). This experimental murine model was based on: (i) the number of normal and atretic follicles; (ii) the number of ova shed in response to hCG (Nandedkar and Balchandran, 1982); (iii) the FSH-binding capacity of granulosa cells (Nandedkar *et al.*, 1985); and (iv) the uptake of FSH (Nandedkar and Balchandran, 1982). Furthermore, the follicular atresia in the present animal model demonstrated apoptotic changes in the granulosa cells as revealed by: (i) DNA fragmentation; (ii) DNA staining by acridine orange (iii) DNA-4,6-diamino-2-phenylindole (DAPI) flow cytometric analysis (Nandedkar *et al.*, 1996, 1998); and (iv) TdT-mediated dUTP nick-end labelling (TUNEL)-positive granulosa cells in the atretic follicles (Nandedkar and Dharma, 2001). A similar model for follicular atresia-apoptosis was also reported by Hughes and Gorospe (1991) demonstrating a DNA fragmentation ladder in the granulosa cells of atretic follicles. Thus, substantial evidence

Fig. 1. *In situ* hybridization analysis of Fas and Fas L mRNA in the normal and atretic mouse ovary. Bright field microscopic images of (a,c) normal and (b,d) atretic ovarian sections hybridized with (a,b) Fas anti-sense and (c,d) Fas L anti-sense oligoprobes. Expression of (a) Fas and (c) Fas L mRNA can be observed in granulosa cells but not oocytes of normal mouse ovarian follicles. Note the expression of (b) Fas and (d) not Fas ligand in atretic follicles. No specific hybridization was observed with the (e) sense Fas and (f) Fas ligand cDNA probes. Open and closed arrows indicate staining in the granulosa cells and oocyte, respectively. GC: granulosa cell; O: oocyte; TC: theca cells. Scale bar represents 16 μ m.

has been reported of differentiating normal and atretic follicles in the eCG-induced 48 h and 72 h mouse model established by the present authors and used in the present study.

It is well documented that the microenvironment in the follicle is essential in maintaining viability of granulosa cells and oocytes (Moor *et al.*, 1998) and that oocyte–granulosa cell interaction plays an important role in deciding the fate of the follicle (Canipari, 2000;

Eppig, 2001; Eppig *et al.*, 2002). During follicular atresia, apoptosis in the granulosa cells has been reported (Hseuh *et al.*, 1994). One of the major pathways in apoptosis is the Fas–Fas L pathway. Fas L binds to Fas membrane receptor to activate a further cascade of events leading to apoptosis. In the present study, Fas and Fas L proteins and mRNA were expressed in the granulosa cells of normal and atretic follicles. However, Fas protein and mRNA were expressed only on the oocyte of atretic follicles.

Fig. 2. Localization of Fas and Fas L protein in oocyte cumulus complexes of normal and atretic follicles by immunofluorescence. (b) Intense expression of Fas was observed in the oocyte and granulosa cells of atretic follicle. (d) Expression of Fas L was noted only in granulosa cells. Staining was negligible for Fas and Fas L in (a,c) normal follicles as well as (e,f) in negative controls incubated in SP₂O culture medium. Open and closed arrows indicate staining in oocyte and granulosa cells, respectively. Scale bar represents 16 μ m.

The specific expression of the *Fas* gene and protein on the atretic oocyte seems to play an important role. The Fas L on the granulosa cells possibly binds to Fas on the oocyte, leading to atresia of the follicle. As Fas L is expressed on

normal as well as atretic granulosa cells, the expression of Fas only on the atretic oocyte possibly determines the fate of the follicle, causing follicular atresia. Evidence has been reported of a Fas-mediated signalling pathway

Fig. 3. Expression of Fas protein in denuded oocytes from normal and atretic ovarian follicles by immunofluorescence. (a) Staining was weak in oocytes from normal follicles but (b) intense in atretic oocytes. (c) Immunoreactivity was not observed when SP₂O culture medium was substituted for primary antibodies. Scale bar represents 16 μ m.

Fig. 4. Western immunoblot analysis of Fas in normal (N) and atretic (A) mouse ovarian follicles. Fas was resolved as a 48 kDa band in the atretic sample. MW: molecular weight markers.

leading to apoptosis in mammalian ovarian granulosa cells (Porter *et al.*, 2000) and luteal cells (Roughton *et al.*, 1999).

The immunolocalization of Fas indicated by immunofluorescence in atretic oocytes could be the result of the presence of Fas in the cumulus cells. Oocytes were denuded and then immunofluorescence for Fas was

carried out to verify this hypothesis. The results revealed that the denuded oocytes from atretic but not normal follicles showed localization of Fas, confirming the findings of Porter *et al.* (2000) and further demonstrating the expression of Fas protein as well as the *Fas* gene in atretic oocytes, which possibly decide the fate of the follicle.

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References

- Ashkenazi A and Dixit VM (1999) Apoptosis control by death and decoy receptors *Current Opinion in Cell Biology* **11** 255–260
- Canipari R (2000) Oocyte–granulosa cell interactions *Human Reproduction Update* **6** 279–289
- Chinnaiyan AM, O'Rourke K, Tewari M and Dixit VM (1995) FADD, a novel death domain containing protein interacts with the death domain of Fas and initiates apoptosis *Cell* **81** 505–512
- Eppig JJ (2001) Oocyte control of ovarian follicular development in mammals *Reproduction* **122** 829–839
- Eppig JJ, Wigglesworth K and Pendola FL (2002) The mammalian oocyte orchestrates the rate of ovarian follicular development *Proceedings National Academy of Sciences USA* **99** 2890–2894
- Guo MW, Xu JP, Mori E, Sato E, Saito S and Mori T (1997) Expression of Fas ligand in murine ovary *American Journal of Reproductive Immunology* **37** 391–398

- Hseuh AJW, Billig H and Tsafiri A (1994) Ovarian follicle atresia: a hormonally controlled apoptotic process *Endocrine Reviews* **15** 707–724
- Hu CL, Cowan RG, Harman RM, Porter DA and Quirk SM (2001) Apoptosis of bovine granulosa cells after serum withdrawal is mediated by Fas antigen (CD95) and Fas ligand *Biology of Reproduction* **64** 518–526
- Hughes FMJ and Gorospe WC (1991) Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia *Endocrinology* **129** 2415–2422
- Kerr JF, Wyllie AH and Currie AR (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics *British Journal of Cancer* **26** 239–257
- Kim JM, Boone DI, Auyeung A and Tsang BK (1998) Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary *Biology of Reproduction* **58** 1170–1176
- Kim JM, Yoon YD and Tsang BK (1999) Involvement of the Fas/Fas ligand signalling system in p53 mediated granulosa cell apoptosis during follicular development and atresia *Endocrinology* **140** 2307–2317
- Luciano AM, Modina S, Gandolfi F, Lauria A and Armstrong DT (2000) Effect of cell-to-cell contact on *in vitro* deoxyribonucleic acid synthesis and apoptosis responses of bovine granulosa cells to insulin-like growth factor I and epidermal growth factor *Biology of Reproduction* **63** 1580–1585
- Moor RM, Dai Y, Lee C and Fulka J Jr (1998) Oocyte maturation and embryonic failure *Human Reproduction Update* **4** 223–236
- Morita Y and Tilly JL (1999) Oocyte apoptosis: like sand through an hour glass *Developmental Biology* **213** 1–17
- Morita Y, Perez GI, Maravei DV, Tilly KI and Tilly JL (1999) Targeted expression of Bcl-2 in mouse oocytes inhibits ovarian follicle atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis *in vitro*. *Molecular Endocrinology* **13** 841–850
- Nagata S and Goldstein P (1995) The Fas death factor *Science* **267** 1449–1456
- Nandedkar TD and Balachandran PK (1982) PMSG induced follicular atresia in mice *Indian Journal of Experimental Biology* **20** 353–354
- Nandedkar TD and Dharma SJ (2001) Expression of bcl-xs and c-myc in atretic follicles of mouse ovary *Reproductive Biomedicine* **3** 221–225
- Nandedkar TD, Parkar SG, Iyer KS, Mahale SD, Moodbidri SB, Mukhopadhyaya RR and Joshi DS (1996) Regulation of follicular maturation by human ovarian follicular fluid peptide *Journal of Reproduction and Fertility* **50** 95–104
- Nandedkar TD, Rajadhyaksha MS, Mukhopadhyay RR, Rao SGA and Joshi DS (1998) Apoptosis in granulosa cells induced by intrafollicular peptide *Journal of Biosciences* **23** 271–277
- Peluso JJ and Pappalardo A (1994) Progesterone and cell–cell adhesion interact to regulate granulosa cell apoptosis *Biochemistry and Cell Biology* **72** 547–551
- Peluso JJ, Pappalardo A and Trollice MP (1996) N-Cadherin-mediated cell contact inhibits granulosa cell apoptosis in a progesterone independent manner *Endocrinology* **137** 1196–1203
- Porter DA, Vickers SL, Cowan RG, Huber SC and Quirk SM (2000) Expression and function of Fas antigen vary in bovine granulosa cells during ovarian follicular development and atresia *Biology of Reproduction* **62** 62–66
- Roughton SA, Lareu RR, Bittles AH and Dharmarajan AM (1999) Fas and Fas ligand messenger ribonucleic acid and protein expression in the rat corpus luteum during apoptosis mediated luteolysis *Biology of Reproduction* **60** 797–804
- Thornberry NA and Lazebnik Y (1998) Caspases: enemies within *Science* **281** 1312–1316
- Thornberry NA, Rano TA, Peterson EP *et al.* (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis *Journal of Biological Chemistry* **272** 17907–17911
- Tilly JL, Tilly KI, Kenton ML and Johnson AL (1995) Expression of members of the Bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-X_{long} messenger RNA levels *Endocrinology* **136** 232–241
- Vanderhyden BC and Macdonald EA (1998) Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development *Biology of Reproduction* **59** 1296–1301
- Vanderhyden BC, Telfer EE and Eppig JJ (1992) Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles *in vitro*. *Biology of Reproduction* **46** 1196–1204

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