Estrous cycle dependent changes in expression and distribution of Fas, Fas ligand, Bcl-2, Bax, and pro- and active caspase-3 in the rat ovary

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Abstract

In the present investigation, the localization of proteins involved in ovarian apoptosis were studied throughout the estrous cycle in the presence of fluctuating hormone levels. Fas, Fas ligand, Bcl-2, Bax and caspase-3 mRNA expression and proteins were detected in all ovarian tissue extracts, though the amount of protein varied with the phase of the estrous cycle. Fas, Bax and caspase-3 protein levels were highest at diestrus and decreased thereafter towards metestrus. In contrast, Fas ligand and Bcl-2 protein levels were lowest at diestrus and increased toward metestrus. Immunohistochemistry revealed that the staining of the anti-apoptotic protein Bcl-2 was more pronounced in healthy preantral follicles than in atretic follicles. In contrast, the pro-apoptotic proteins Fas, Fas ligand, Bax and active caspase-3 were more predominantly present in atretic follicles. In the ovarian

surface epithelium (OSE), Fas, procaspase-3 and Bcl-2 immunostaining appeared independent of the phase of the estrous cycle. Fas ligand and Bax staining was detected particularly during proestrus in OSE cells surrounding the ovulatory follicles, while active caspase-3 was observed only in OSE cells at the postovulatory site during estrus. The proportion of luteal cells that stained positively for Fas, Bax and caspase-3 increased with the age of the corpus luteum, while Fas ligand and Bcl-2 immunostaining was strongest in newly formed corpora lutea and decreased thereafter. In conclusion, the components of the Fas signalling pathway were differentially expressed throughout the estrous cycle in a variety of ovarian cell types, which may correspond to hormone dependent survival mechanisms.

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Introduction

Programmed cell death or apoptosis is an essential component of normal reproductive function and development in the ovary. Most importantly, apoptosis attributes to the exhaustion of the oocyte reserve, either directly through germ cell death or indirectly through follicular atresia (Hsueh *et al.* 1994). In this regard, apoptosis has been proposed to be the major mechanism that determines female reproductive life span (Tilly 2001). Indeed, it has been shown that inhibition of apoptosis by selectively disrupting Bax function in female mice was accompanied by an increased primordial follicle pool size and a prolonged ovarian life span (Perez *et al.* 1999). Hence, a better understanding of programmed cell death in the ovary may help to develop novel therapies to treat females with ovarian disorders such as premature ovarian failure.

Throughout life, dormant primordial follicles are continuously recruited from the resting pool to start growing.

Until puberty, these follicles do not survive beyond the early antral stage. In postpubertal females, however, some early antral follicles may become selected during each estrous cycle and continue growth under the influence of elevated FSH levels (McGee & Hsueh 2000). Among this cohort, depending on the species, one or a few large antral follicles will reach the preovulatory stage and ovulate following the LH surge, whereas the remaining antral follicles that have been recruited during this growth wave will degenerate as a result of follicular atresia (Hsueh *et al.* 1994). Atresia, a process which involves apoptosis of granulosa cells, oocytes and eventually theca cells, can occur during any stage of follicular development, although early antral follicles are most vulnerable to undergo follicular degeneration (Hsueh *et al.* 1994, Markstrom *et al.* 2002). During each estrous cycle, other ovarian cell types in addition to follicular cells may also undergo apoptosis. Ovarian surface epithelial (OSE) cells covering the protruding preovulatory follicles degenerate from the

ovarian surface by apoptosis just prior to ovulation, to facilitate the release of the oocyte (Murdoch & McDonnell 2002). Moreover, corpora luteal cells arising from the ovulatory follicles undergo apoptosis during luteal regression, when ovulation is not associated with a fertile mating and implantation of the conceptus (Sakamaki *et al.* 1997, Kuranaga *et al.* 1999).

The role of proteins and genes involved in the regulation of apoptosis in the ovary is relatively well understood. However, the hormonal factors that trigger apoptosis in the various ovarian cells types during the estrous cycle are not yet clearly defined. The major players of apoptosis in the ovary are assumed to be the Fas system and the Bcl-2 family members (Guo *et al.* 1994, Kondo *et al.* 1996, Kugu *et al.* 1998, Kim *et al.* 1999, Roughton *et al.* 1999). It has been suggested that interaction between the Fas system and Bcl-2 family members may define the rate of apoptosis (Krammer 1999). Fas receptor (Fas/CD95) is a member of the tumor necrosis factor/nerve growth factor family, which is activated upon binding Fas ligand, leading to receptor aggregation and formation of a death-inducing signalling complex (DISC) (Krammer 1999). Once Fas has been activated, two distinct intracellular apoptotic pathways can be executed, depending on the level of released active caspase-8 (Fig. 1) (Scaffidi *et al.* 1998). In type I cells, high levels of caspase-8 at the DISC directly initiate cleavage of other downstream effector caspases, such as caspase-3, thereby initiating the execution phase of apoptosis. In type II cells, however, a small caspase-8 signal is generated, which in order to induce apoptosis, requires an amplification loop via the mitochondria involving cleavage of the pro-apoptotic Bcl-2 family member Bid (Scaffidi *et al.* 1998). It has been proposed that cleaved Bid stimulates dimeric binding of other pro-apoptotic Bcl-2 family members (e.g. Bax and Bak) to the mitochondria at the expense of anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-w, Bcl-x_L). This triggers cytochrome c release, which eventually results in downstream activation of effector caspases and cell death (Krammer 1999).

Several studies have demonstrated the presence of Fas and Fas ligand during follicular atresia (Guo *et al.* 1994, Hakuno *et al.* 1996, Kondo *et al.* 1996, Kim *et al.* 1999, Cataldo *et al.* 2000, Dharma *et al.* 2003) and luteal regression (Sakamaki *et al.* 1997, Roughton *et al.* 1999). However, controversy exists about the distribution of these proteins during follicular development. The differences among these studies may in part be explained by the different experimental models used for the induction of apoptosis. In these models, apoptosis is often-induced artificially, for instance by gonadotrophin withdrawal or treatment with prostaglandin F_{2α}. This type of induced degeneration may differ in key aspects from normal spontaneous atresia or luteal regression. In fact, little is known regarding expression of either Fas or other components associated with Fas signalling under normal physiological conditions during the estrous cycle.

Figure 1 Schematic overview of Fas-induced apoptosis in type I and II cells. Fas is activated upon binding to Fas ligand, resulting in receptor trimerization and the recruitment of adapter proteins. When procaspase-8 becomes associated, it is autoproteolytically cleaved into active subunits that are released into the cytoplasm. In type I cells, active caspase-8 might then act directly to cleave other effector caspases, such as caspase-3, thereby initiating the execution phase of apoptosis. In type II cells, however, active caspase-8 cleaves Bid, a proapoptotic member of the Bcl-2 family. Cleaved Bid stimulates the binding of proapoptotic Bcl-2 proteins (Bax, Bak) to the mitochondria and inhibits association of anti-apoptotic Bcl-2 proteins (e.g. Bcl-2, Bcl-w, Bcl-x_L). This causes leakage of cytochrome c from the mitochondria into the cytosol, which in turn promotes formation of the apoptosome and triggers caspase-3. The activation of caspase-3 is considered to be the final executioner step in apoptosis and is responsible for cleavage of key substrates, such as DNA repair enzymes and cytoskeletal and nuclear scaffold proteins. Scheme adapted from Scaffidi and colleagues (Scaffidi *et al.* 1998).

Furthermore, the simultaneous assessment of relevant apoptotic proteins in the whole ovary throughout the estrous cycle has not been reported so far. We, therefore, investigated the localization and distribution of the Fas system and its related proteins, i.e. Fas, Fas ligand, Bcl-2, Bax, pro- and active caspase-3, during normal follicular development, atresia, ovulation and luteolysis in the rat throughout the estrous cycle. Furthermore, the corresponding mRNA expression and protein levels in whole rat ovaries were determined.

Materials and Methods

Reagents

Antibodies against Fas receptor (sc-715), Fas ligand (sc-956), procaspase-3 (sc-492), Bcl-2 (sc-1226), Bax (sc-526), and actin (sc-1616) were purchased from Santa

Cruz Biotechnology (SanverTech, Heerhugowaard, the Netherlands). The antibody against active caspase-3 (AF835) that detects only cleaved caspase-3 was obtained from R&D systems (ITK Diagnostics, Uithoorn, The Netherlands), while the antibody against caspase-3 used for Western blot analysis, which detects both the caspase-3 precursor of 32 kDa and the cleaved active caspase-3 fragment of 20 kDa, was obtained from Cell Signalling Technology (Westburg BV, Leusden, The Netherlands). Secondary biotinylated goat-anti-rabbit, rabbit-anti-goat or goat-anti-mouse IgG's were purchased from Vector Laboratories (Vectastain kit Elite; Vector Laboratories, Burlingame, CA, USA), while horseradish peroxidase-conjugated goat-anti-rabbit IgG was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). TRIzol reagent, acetylated BSA and 3,3'-diaminobenzidine tetrachloride were purchased from Gibco BRL (Invitrogen Life Technologies), Aurion (Wageningen, The Netherlands) and Sigma, respectively. The avidin-biotin complex (ABC)-peroxidase complex staining kit Elite was purchased from Vector Laboratories, the *in situ* cell death detection kit for TUNEL labelling was bought from Roche Diagnostics GmbH, while the Coomassie Plus protein assay reagent kit and supersignal chemiluminescent substrate kit (ECL) were bought from Pierce (Tattenhall, Cheshire, UK). The ImProm-II reverse transcription system kit, oligo dT primer, dNTPs and RQ1 Rnase-Free Dnase were obtained from Promega Corporation.

Animals

Female outbred Wistar rats (Harlan, Horst, The Netherlands) were housed in pairs and kept in a temperature- and light-controlled room (12 h light:12 h darkness) with free access to food and water. Estrous cycles were tracked by carrying out daily vaginal smears, and only the rats that showed two consecutive 4-day cycles were used for the experiments. A total of twenty three 16 to 19-week-old animals were sacrificed at four different stages of the estrous cycle, i.e. diestrus ($n=6$), proestrous ($n=6$), estrus ($n=6$) and metestrus ($n=5$). The ovaries were carefully excised to avoid damage to the OSE. After excision, the left ovaries of 8 of these animals (two animals for each stage of the estrous cycle) were snap frozen in liquid nitrogen and stored at -80°C until use for RT-PCR and immunoblot analysis ($n=8$). The right ovaries of these 8 animals were processed for immunohistochemical analysis together with the ovaries of the other 15 rats. These ovarian tissues were fixed for 24 h in 4% buffered formalin, embedded in paraffin and stored at room temperature until immunohistochemical processing. The experiments described in the present study have been approved by the ethical committee for animal welfare of Wageningen University (Wageningen, The Netherlands).

Reverse transcription-PCR (RT-PCR)

The TRIzol method was used to extract total RNA from snap-frozen rat ovaries according to the manufacturer's instructions. RNA integrity was confirmed by running 2 μg RNA on a 1% agarose denaturing gel. The amount of RNA was estimated by spectrophotometry at 260 nm. Prior to RT-PCR, RNA samples were treated with RQ1 RNase-Free DNase, then purified by phenol-chloroform extraction followed by precipitation with ethanol. RT-PCR was performed in 20 μl reaction mixture containing 1 μg total DNase-treated RNA, 0.5 μg oligo dT primer, 0.5 mM dNTPs, 5 mM MgCl_2 , 4 μl ImProm-II $5\times$ reaction buffer and 1 μl ImProm-II reverse transcriptase at 42°C for 60 min. Then, reverse transcriptase was inactivated by heating at 75°C for 15 min. The synthesized complementary DNA was stored at -80°C until use. The PCR was carried out in 50 μl reaction mixture prepared on ice which contained 5 μl $10\times$ thermophilic reaction buffer, 1U Taq polymerase, 2 mM MgCl_2 , 0.2 mM dNTP, PCR buffer, 0.5 μM upstream primer, 0.5 μM downstream primer and 1 μl reverse transcription mixture (cDNA). The PCR primers used for amplification were synthesized by Isogen Bioscience on the basis of the rat DNA sequences described earlier for Fas (Kimura *et al.* 1994), Fas ligand (Suda *et al.* 1993), caspase-3 (Omezzine *et al.* 2003), GAPDH (Duvigneau *et al.* 2003), Bcl-2 and Bax (Sato *et al.* 1994, Tilly *et al.* 1995). The PCR conditions were 94°C for 5 min followed by 40 cycles of 94°C for 45 sec, 55°C for 60 s, and 72°C for 45 s, respectively and then 72°C for 5 min. After amplification, 10 μl of the PCR products were separated by electrophoresis on 1.5% agarose gels containing 0.002% ethidium bromide, visualized by u.v. light and photographed. The equal loading of the samples was demonstrated by RT-PCR analysis of the housekeeping gene GAPDH. Negative controls (reactions without reverse transcriptase and reactions without template) were performed to verify the absence of (DNA) template contamination. No products were observed in these control reactions.

Western blot analysis

Protein samples were prepared from snap-frozen rat ovaries using TRIzol reagent according to the manufacturer's instructions. Protein concentrations were determined by the Bradford assay (Bradford 1976) with the Coomassie Plus protein assay reagent kit and resolved in SDS-sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 1% β -mercaptoethanol and 0.003% Bromophenol Blue, pH 6.8) in a final concentration of 500 $\mu\text{g}/\text{ml}$. After boiling the samples for 10 min, the proteins (10 $\mu\text{g}/\text{lane}$) were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in TTBS (10 mM Tris pH 7.5,

100 mM NaCl, 0.1% Tween-20) for 1 h, and incubated overnight at 4 °C with a rabbit-anti-human polyclonal antibody against Fas and Fas ligand (diluted 1:500); caspase-3 (which reacts with both the 32 kDa pro-enzyme and the spliced 20 kDa active form of caspase-3, diluted 1:500), Bcl-2 and Bax (diluted 1:250), or with a goat anti-human polyclonal antibody against actin (diluted 1:1000) in blocking buffer. After washing three times with TTBS, the membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat-anti-rabbit IgG (Fas, Fas ligand, caspase-3, Bcl-2 and Bax) diluted 1:15000 or rabbit-anti goat IgG (actin) diluted 1:5000 in blocking buffer. The presence of antibody-protein complexes was detected by ECL. Relative quantification of ECL signals on films was performed using a two-dimensional scanning image densitometer (model GS-700; BIO-RAD Laboratories) followed by band analysis using Quantity One software version 4.2.1. Values are expressed as mean density ratio $\pm$ S.E.M. relative to that of the respective diestrus values from three independent experiments. Due to differences in exposure time of the separate blots and the fact that the intensity of the protein bands is, in part, dependent on the duration of the exposure time, no statistical analysis of the densitometric data was performed.

Immunohistochemistry

Immunohistochemistry was performed as described by Teerds & Dorrington (1995). Briefly, 5 μ m thick paraffin embedded ovarian sections were deparaffinized and treated with 1% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. The slides were subsequently washed in 0.01 M Tris-buffered saline (TBS pH 7.4), microwaved in buffered citrate for 10 min (only in case of procaspase-3), incubated with 0.1 M glycine in TBS for 30 min, and rinsed with TBS. Sections were blocked for 30 min with 10% normal goat or rabbit serum, and incubated at 4 °C overnight with rabbit-anti-human polyclonal antibodies against Fas, Fas ligand, Bcl-2, Bax (all diluted 1:200), active caspase-3 (concentration 10 μ g/ml), or with a goat-anti-human polyclonal antibody against procaspase-3 (diluted 1:100), respectively. A pilot experiment had revealed that these dilutions of the antibodies resulted in optimal staining. All antibodies were diluted in TBS containing 0.05% acetylated BSA. Slides were again washed in TBS and incubated for 60 min with the corresponding biotinylated goat-anti-rabbit or rabbit anti-goat IgGs respectively, diluted 1:200 in TBS containing 0.05% acetylated BSA. Sections were subsequently washed in TBS and incubated for at least 60 min with the components avidin (A) and biotin (B) of the ABC staining kit Elite. Both components (A and B) were diluted 1:1000 and the solution was prepared at least 15 min before use. Slides were washed in TBS, rinsed in 0.05 M Tris-HCl (pH 7.5) and finally bound antibody was visualized after the addition of a 0.6 mg/ml solution of

3,3'-diaminobenzidine tetrachloride (DAB) in Tris-HCl to which 0.03% H₂O₂ was added. The slides were subsequently counterstained with Mayer's haematoxylin. Control sections, in which the primary antibody was replaced by either normal rabbit or goat serum, were similarly processed. No staining was observed in these controls (Fig. 5 G). For each primary antibody, the intensity of immunostaining was determined by estimating the percentage of positively-labelled cells. The intensity of immunostaining was assigned as being absent (-), faint (-/+), moderate (+) and high (++) when respectively <1, 1-10, 10-50, 50-100 percent of the cells in the follicular or corpus luteum cross section stained positively. All OSE cells covering the surface of the ovary were counted.

The immunohistochemical staining procedures for each antibody were repeated at least 6 times on all 23 ovaries analyzed in this study.

In situ DNA labelling for apoptosis

The laboratory protocol for TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling) was used according to the manufacturer's instructions with some minor modifications adapted from Negoescu and colleagues. (Negoescu *et al.* 1996). Briefly, paraffin embedded ovarian sections were deparaffinized, microwaved in buffered citrate for 5 min, cooled rapidly and rinsed in PBS (pH 7.4). The slides were covered with the TUNEL reaction mixture (1:10 dilution of an enzyme solution containing calf thymus terminal deoxynucleotidyl transferase and label solution containing a nucleotide mixture of digoxigenin-11-2'-dUTP) for 60 min at 37 °C. Then, slides were blocked for 30 min with 5% non-fat dry milk and 10% normal horse serum in PBS. The slides were subsequently washed in PBS and incubated with a 1:4 dilution of peroxidase-labeled digoxigenin sheep Fab antibody for 30 min at 37 °C. After washing the sections in PBS, the DNA strand breaks were visualized with a DAB color reaction, and counterstained with Mayer's hematoxylin, as described above. Negative controls, by omitting terminal deoxynucleotidyl transferase TdT, were similarly processed. No staining was observed in these negative controls (data not shown).

Results

Expression of the Fas system in whole rat ovaries throughout the estrous cycle

To examine the presence of Fas receptor and signalling molecules throughout the estrous cycle, we determined their presence in whole rat ovarian tissue extracts at diestrus, proestrus, estrus and metestrus. Fas, Fas ligand, caspase-3, Bcl-2 and Bax mRNA and protein were detected by RT-PCR and Western blot analysis, respectively

Figure 2 Expression of Fas, Fas ligand, Bcl-2, Bax and caspase-3 mRNA and protein in whole rat ovaries throughout the estrous cycle. Ovaries were obtained from rats sacrificed at four different stages of the estrous cycle and Fas, Fas ligand, Bcl-2, Bax and caspase-3 mRNA and protein expression were determined by RT-PCR (A) and Western blotting (B), as described in Materials and Methods. (A) PCR products are visualized on agarose gels stained with ethidium bromide. Detection of GAPDH mRNA transcripts served as a positive control for amplification. Representative figures from three independent experiments are shown. (B) Western blot analysis of apoptotic and anti-apoptotic proteins. The panels above the graphs show a representative Western blot for each protein. The graphs represent a histogram of densitometric analysis of the immunoblots for each protein. The data were obtained from three independent experiments. Relative protein levels are expressed as mean density ratio $\pm$ S.E.M., relative to the diestrus value on the same blot, which were quantified by analysis of density intensity using Quantity One software. Actin protein immunoblot staining served as a positive (loading) control.

(Fig. 2). As shown in fig. 2A, mRNAs of all Fas signaling molecules studied were detected at all stages during the estrous cycle. The amplified PCR products were of the expected size, i.e. Fas (282 bp), Fas ligand (491 bp), caspase-3 (282 bp), Bcl-2 (349 bp), Bax (301 bp) and GAPDH (318 bp). The negative controls (cDNA synthesis performed without RT or without template)

revealed no expression for any of the investigated target sequences (data not shown). Western blot analysis revealed variations in the amounts of Fas (45 kDa), Fas ligand (40 kDa), caspase-3 (32 kDa), Bcl-2 (26 kDa) and Bax (22 kDa) protein during the estrous cycle (Fig. 2B). Fas, caspase-3 and Bax protein levels were maximal at diestrus and decreased towards metestrus. In contrast, Fas ligand

Table 1 Localization of various apoptotic proteins in the rat ovary

	Fas	Fas ligand	Bcl-2	Bax	Pro-caspase-3	Active caspase-3
Preantral follicles						
Healthy granulosa	–	–	+	–/+	++	–
Health theca	–/+	+	+	+	++	–
Atretic granulosa	–/+	–/+	–/+	–/+	++	+
Atretic theca	++	++	–/+	++	++	–/+
Antral follicles						
Healthy granulosa	–/+	–	+	–/+	++	–
Health theca	+	+	+	+	++	–
Atretic granulosa	+	–/+	–/+	–/+	++	+
Atretic theca	++	++	–/+	++	++	–/+
OSE						
Surface	+	–/+	+	–/+	++	–
At postovulatory site	+	+	+	+	+	+
Corpora lutea						
1st and 2nd generation	+	++	+	–/+	+	–/+
3rd and later generations	++	–/+	–/+	++	++	++

Immunohistochemical staining intensity: absent (–), faint (–/+), moderate (+), high (++)

and Bcl-2 protein levels were lowest at diestrus. Fas ligand protein levels increased towards metestrus, whereas Bcl-2 protein levels rose to a slightly lesser degree towards proestrus (Fig. 2B).

Immunolocalization of the Fas system during follicular development

To study the cellular distribution of Fas receptor and signalling molecules, immunohistochemistry was performed on ovarian sections. The localization and the intensity of the immunostaining for the various proteins of the Fas system in the ovary have been summarized in Table 1. Differences in immunostaining patterns were observed in healthy follicles at various stages of follicular growth (Fig. 3 and Fig. 4). In healthy follicles, immunostaining for Fas was detected in theca cells of secondary follicles onwards (Fig. 3A), whereas faint and inconsistent Fas staining appeared in granulosa cells at the early antral stage of follicular development (Table 1, staining not shown). Fas ligand immunoreactivity was present in theca cells, but absent in granulosa cells of healthy follicles throughout most of follicular development (Fig. 4A), though at proestrous some Fas ligand staining was observed in preovulatory follicles in the mural granulosa cells close to the basal membrane (Fig. 4A, insert). Bax immunostaining was also predominantly found in theca cells (Table 1, staining not shown). In granulosa cells, Bax immunoreactivity was faint and restricted to secondary and antral follicles (Table 1, staining not shown). Bcl-2 (Fig. 3C and 4B) and procaspase-3 (Fig. 3B) immunostaining was present in granulosa and theca cells of healthy follicles at all stages of development, though more fre-

quently present in dominant preovulatory follicles compared with smaller antral follicles. The number of cells staining positively for the spliced active form of caspase-3 as well as showing TUNEL activity was negligible in healthy follicles (Table 1, staining not shown). In atretic follicles, however, TUNEL labelling and immunoreactivity for active caspase-3 were often observed, predominantly in the apoptotic granulosa cells around the antral region but also in some scattered apoptotic theca cells (Fig. 4E and Fig. 4F). This increase in active caspase-3 staining and TUNEL labelling, occurring after the onset of atresia, was accompanied by increased staining intensity for Fas (Table 1, staining not shown) and Fas ligand (Fig. 3F) in granulosa cells. Fas (Table 1, staining not shown) and Fas ligand (Fig. 3F) staining was even more intense in theca cells of atretic follicles, which was accompanied by enhanced Bax staining (Fig. 3E). Moreover, the immunostaining for Bax (in theca cells only), Fas and Fas ligand (in particular in theca cells, but also in granulosa cells), and active caspase-3 and TUNEL labelling (mainly in granulosa cells) appeared to be more intense and widespread in severely atretic follicles (Table 1 and e.g. Fig. 3F and Fig. 4C,F) compared with moderately atretic follicles (Table 1 and Fig. 3E). In contrast, the intensity of the staining of the anti-apoptotic protein Bcl-2 declined through the progressive stages of atresia in both granulosa and theca cells (Fig. 3D). Immunostaining for procaspase-3 was uniform throughout follicular development (Table 1 and Fig. 3B) and its relatively high level in both granulosa and theca cells was not influenced by advancing atresia (Table 1 and Fig. 4D). There were no differences in the distribution or intensity of immunostaining patterns of above studied apoptotic proteins in

Figure 3 Immunohistochemical localization of various proteins involved in apoptosis in preantral follicles of cycling rats. Positive staining is shown as brown colouring of the cytoplasm of the cells. (A–C) Healthy preantral follicles; Fas (A) immunostaining was found in theca cells but not in granulosa cells, whereas procaspase-3 (B) and Bcl-2 (C) were observed in both granulosa and theca cells. D–F) In atretic preantral follicles; less Bcl-2 immunopositive cells were observed (D), while Bax (E) and Fas ligand (F) immunostaining was now also present in some granulosa cells of both moderately (E) and severely (F) atretic follicles. The immunohistochemical labelling experiments were repeated at least six times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), OSE (open arrowheads). Bar=20 μ m (A–C,E,F) or 40 μ m (D).

follicles that had become atretic at the various stages of follicular development (Table 1, Fig. 3D–F and Fig. 4C–E).

Immunolocalization of the Fas system during ovulation

In ovarian surface epithelial (OSE) cells, Fas (Fig. 3A and Fig. 5A), Bcl-2 (Fig. 5C) and procaspase-3 (Fig. 5E) immunostaining was generally observed throughout the entire estrous cycle (for summary of data see Table 1). In contrast, active caspase-3 immunoreactivity was observed only during estrus in some OSE cells at the postovulatory

site (Fig. 5F). Immunoreactivity for Fas ligand (Fig. 5B) and Bax (Fig. 5D) was heterogeneous, but in general, more frequently observed in OSE cells that lined the postovulatory site during estrus compared with OSE cells lining other areas of the ovary (Table 1, staining not shown).

Immunolocalization of the Fas system during luteolysis

The age of the corpora lutea was determined on basis of their morphology, as described by Greenwald & Rothchild (1968) and Erickson *et al.* (1993). Briefly,

Figure 4 Immunohistochemical localization of various proteins involved in apoptosis in antral follicles of cycling rats. Positive staining is shown as brown colouring of the cytoplasm of the cells. (A–B) Healthy antral follicles; Fas ligand (A) immunostaining was observed in theca cells only, though at proestrous some Fas ligand was observed in the mural granulosa cells of preovulatory follicles close to the basal membrane (insert), whereas Bcl-2 (B) was present in both granulosa and theca cells. (C–F) Atretic preantral follicles; Fas (C) and procaspase-3 (D) immunostaining are present in both granulosa and theca cells; staining for active caspase-3 (E) and TUNEL labelling (F) is often observed in granulosa with condensed nuclei as well as in some apoptotic theca cells. The immunohistochemical labelling experiments were repeated at least six times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), OSE (open arrowheads). Bar = 20 μ m (C–E) or 40 μ m (A,B,F).

corpora lutea derived from the most recent ovulation, consisted mainly of large luteal cells (large cells with a characteristic large round to oval nucleus) with only a minor number of fibroblasts (identified by the presence of an elongated nucleus) and apoptotic cells. These were classified as new corpora lutea of the first generation. The regressing corpora lutea of the previous estrous cycles were assigned as corpora lutea of the second, third and later generations. Corpora lutea of the second generation were larger than corpora lutea of the first generation and contained not only luteal cells but also patches with

significant numbers of fibroblasts, leukocytes (identified by a small compact nucleus and a relatively small amount of cytoplasm) and apoptotic cells. Corpora lutea of the third and later generations (two or more cycles old) were readily identified by their clearly smaller size compared with the first two generations, high numbers of fibroblasts as well as many apoptotic cells.

Immunoreactivity for Fas, Fas ligand, Bcl-2 and Bax, pro- and active caspase-3 protein was always present in all corpora lutea, independent of the stage of development. However, their immunostaining patterns changed during

Figure 5 Immunohistochemical localization of various proteins involved in apoptosis in ovarian surface epithelial cells (OSE) of cycling rats. Positive staining is shown as brown colouring of the cytoplasm of the cells. (A-E) On the ovarian surface; flat-to-columnar OSE cells show a clear positive immunostaining for Fas (A), Fas ligand (B), Bcl-2 (C) and procaspase-3 (E), while immunoreactivity for Bax (D) was mainly restricted to OSE cells at the postovulatory site during estrus. (F) At the time of estrus, at the postovulatory site, some OSE cells had now become immunopositive for active caspase-3 (see also insert) as well. (G) Control ovarian section incubated with normal rabbit serum instead of primary antibody. The immunohistochemical labelling experiments were repeated at least six times with similar results. Theca cells (TC; arrows), granulosa cells (GC; arrowheads), oocytes (Oo), OSE (open arrowheads), corpora lutea (CL). Bar=10 μ m (A-E and insert of F), 40 μ m (F) or 100 μ m (G).

luteal development. In the new corpora lutea of the first generation, many luteal cells stained positively for both Fas ligand (Fig. 6A) and Bcl-2 (Table 1, staining not shown) at metestrus, while in the corpora lutea of later generations, less luteal cells were positive for Fas ligand and Bcl-2 (Table 1, staining not shown). Additionally, we observed a change in the histological localization of Fas ligand during luteal regression. In corpora lutea of the first generation, Fas ligand was predominantly localized in the luteal cells, whereas in corpora lutea of later generations, Fas ligand was less frequently observed in luteal cells and more often in patches consisting largely of infiltrating leukocytes. The number of luteal cells that showed Bax (Fig. 6B), Fas (Fig. 6C-D), pro- and active caspase-3 (Fig. 6E) immunostaining increased with the age of the corpora lutea.

Discussion

This is the first study in which the expression and localization of various components of the Fas apoptotic machinery have been examined by RT-PCR, Western blot analysis and immunohistochemistry throughout the

estrous cycle in the rat ovary. Fas, Fas ligand, caspase-3, Bcl-2 and Bax mRNA expression and protein levels were detected in all ovarian tissue extracts, though the amount of protein varied with the phase of the estrous cycle. Fas, Bax and caspase-3 protein levels reached a maximum around diestrus and steadily decreased thereafter towards metestrus. In contrast, Fas ligand and Bcl-2 protein levels were lowest at diestrus and increased, in particular Fas ligand, towards metestrus. We speculate that the reduced levels of the apoptotic proteins Fas, Bax and caspase-3, and to a lesser extent the increased protein levels of Fas ligand and the anti-apoptotic protein Bcl-2 around the time of ovulation, may reflect a physiological survival mechanism to ensure ovulation of the dominant follicles and subsequent corpus luteum formation. Indeed, immunohistochemistry revealed that Bcl-2 was more frequently observed in dominant preovulatory healthy follicles. At estrus, following ovulation both Fas ligand and Bcl-2 were prominently present in the newly formed corpora lutea, while in older generations of corpora lutea, the immunostaining of these proteins diminished. In addition, the relatively prominent immunostaining at diestrus of the apoptotic proteins Fas, Bax and caspase-3 in corpora lutea of the third and later generations, may explain the

Figure 6 Immunohistochemical localization of various apoptotic proteins in corpora lutea of cycling rats. Positive staining is shown as brown colouring of the cytoplasm of the cells. (A) Early corpus luteum of the first generation (1stCL) at metestrus; clear immunostaining for Fas ligand is present in large luteal cells. (B–D) Corpora lutea of different ages; immunostaining for Bax (B) and Fas (C–D) was faint in corpora lutea of the first and second generation (2ndCL) while high in corpora lutea of the third generation (3rdCL). (E) Corpus luteum of the third generation; active caspase-3 immunopositive cells often had condensed and fragmented nuclei (arrowheads) that are indicative for apoptosis. Fibroblasts, characterized by their elongated nuclei, are indicated by asterisks. The immunohistochemical labelling experiments were repeated at least six times with similar results. Bar=20 μ m (A,C–E) or 40 μ m (B).

increased levels of these proteins at this stage of the estrous cycle, as observed by Western blot analysis.

In follicles, Fas ligand expression in granulosa cells was limited to some mural granulosa cells at proestrus, whereas this factor was predominantly localized in theca cells throughout most stages of follicular development. Theoretically, Fas ligand expressing theca cells can mediate apoptosis through binding to Fas receptor either on leukocytes thereby withstanding an attack by leukocytes (Kim *et al.* 1998), or to adjacent Fas-expressing theca cells. Although rat thecal/interstitial cells do undergo Fas-induced apoptosis *in vitro* (Foghi *et al.* 1998), we as well as some other groups (e.g. Palumbo & Yeh 1994) observed only a few apoptotic, TUNEL-positive, theca cells in atretic follicles, despite the abundant presence of Fas. Some of these apoptotic theca cells also stained positively for active caspase-3, which is in line with observations in humans (Matikainen *et al.* 2001) but in contrast to studies in mice (Fenwick & Hurst 2002). This suggests that theca cells *in vivo* are moderately resistant to Fas-induced apoptosis, though the mechanism by which these cells can resist apoptosis is at present not clear. Theca cells of both healthy and atretic follicles displayed intense positive

immunostaining for procaspase-3, implicating that the resistance to apoptosis is not a consequence of the absence of caspase-3. Apparently the activation of caspase-3 in theca cells is a tightly regulated process and seems differentially regulated from caspase-3 activation in for instance granulosa cells.

The majority of the TUNEL positive follicular cells as well as active caspase-3 staining were restricted to apoptotic granulosa cells in atretic follicles, which is consistent with earlier observations (Boone & Tsang 1998). Although the intensity of Fas staining in granulosa cells was increased in atretic follicles, Fas ligand and Bax staining remained faint in these granulosa cells. The physiological significance of this observation is not known and emphasizes our relatively limited understanding of the factors and mechanism that may cause apoptosis in granulosa cells in follicles at the onset of atresia. As will be discussed below, some recent studies suggest that growth factors and steroids may play a role in the initiation of granulosa cell apoptosis. On the other hand, as Fas ligand also exists in a soluble form that can move freely among cells, it is possible that a yet unknown factor may trigger a switch from membrane-bound Fas ligand to the soluble

form in theca cells, which in turn may diffuse to the granulosa cells. At the same time, Fas expression in granulosa cells of these follicles is enhanced and binding of soluble Fas ligand could then trigger apoptosis in these cells.

In the ovary, the loss of secretion of crucial hormonal survival factors is thought to stimulate atresia. For example, the gonadotrophins FSH and LH have been shown to inhibit apoptosis in cultured preovulatory follicles (Chun *et al.* 1994), possibly by suppressing Fas expression (Bridgham & Johnson 2001). Moreover, reduced FSH exposure leads to caspase-mediated apoptosis in granulosa cells at the early antral stage of follicular development, a process which was associated with increased levels of Fas and Fas ligand in the rat ovary (Kim *et al.* 1998). These observations are extended by our immunohistochemical data showing that Fas and Fas ligand were faintly present to absent in granulosa cells of healthy antral follicles during the follicular phase of the estrous cycle when gonadotrophins levels were high.

Beside gonadotrophins, growth factors and sex steroids are important local regulators of follicular survival. In cultured preovulatory follicles, the growth factors EGF, TGF- β , bFGF and IGF-I as well as the steroid estradiol, have been shown to inhibit apoptosis (Chun *et al.* 1994). The exact mechanism how these growth factors inhibit apoptosis has only been partially elucidated. EGF, TGF- β and bFGF may inhibit granulosa cell apoptosis through a tyrosine kinase dependent pathway (Tilly *et al.* 1992). A link with the Fas system was made in a more recent *in vitro* study in which Quirk and colleagues reported that these produced growth factors locally in the ovary that acted by inhibiting Fas ligand-induced apoptosis, an effect that was apparently not directly related to the level of Fas expression (Quirk *et al.* 2000a). In another study, the same group demonstrated that IGF-1 inhibits Fas ligand-induced apoptosis by activation of Akt kinase, which in turn can inactivate the caspases 8 and 9, and some proapoptotic members of the Bcl-2 family, all factors involved in Fas ligand-induced apoptosis (Hu *et al.* 2004). Estrogens have been proposed to enhance Fas ligand expression, a process that could be antagonized by the estrogen receptor inhibitor, tamoxifen (Sapi *et al.* 2002). In concert with these data, the present study showed that the amount of Fas ligand in ovarian tissue extracts was elevated shortly after estrogen dominance. There are indications that another steroid, progesterone, also plays a role in the regulation of granulosa cell apoptosis. The appearance of progesterone receptors on the surface of granulosa cells made these cells insensitive to Fas ligand-induced apoptosis, an effect that could be counteracted by treatment with the progesterone receptor antagonist RU 486 (Quirk *et al.* 2004). All these studies point to the important role of locally produced factors in the regulation of follicular atresia, in most cases through either direct or indirect interaction with the Fas system. On the other hand, a possible role of other mechanisms in the control of

follicular atresia, e.g. such as by influencing intracellular calcium levels (Peluso 2003), can at present not be excluded.

In our study, the Bcl-2 family proteins, Bcl-2 and Bax, were observed in both granulosa and theca cells throughout all stages of follicular development. In the primate and human ovary, Bcl-2 and Bax expression has been observed in granulosa cells as well, but not in theca cells (Vaskivuo *et al.* 2001, Depalo *et al.* 2003). This suggests that there may be differences among species concerning the presence and function of Bcl-2 family members in the ovary, and that one must be cautious in extrapolating the results obtained in the rat to other species.

The staining intensity of the anti-apoptotic protein Bcl-2 declined through the progressive stages of atresia in both granulosa and theca cells. In contrast, a higher degree of expression of pro-apoptotic proteins, i.e. Fas and Fas ligand (in particular in theca cells, but also in granulosa cells), Bax (only in theca cells) active caspase-3 and TUNEL labelling (mainly in granulosa cells) was observed in atretic follicles versus healthy follicles. The staining for these proteins appeared to be more intense and widespread with advancing atresia. These findings suggest that the expression of Fas-related proteins is increased as an integral part of the apoptotic process and is not due to follicular growth, since a similar apoptotic executioner program occurs among all stages of follicular atresia. These observations also suggest that the Fas death receptor in theca cells of atretic follicles may act along with the Bcl-2 family proteins to elicit an apoptotic response, characteristic for type II cells. On the other hand, theca cells do not undergo massive apoptosis like granulosa cells, suggesting that a regulatory mechanism determines theca cell susceptibility for Fas signalling activation and cell death.

Little is known about the role of the Fas system in OSE cells. OSE cells have been suggested to undergo cycles of proliferation and degeneration that are associated with the ovulatory process. In ewes, it has been demonstrated that prior to ovulation, OSE cells surrounding the follicles destined to rupture, are highly exposed to both inflammatory agents and reactive oxidants, a condition which is accompanied by increased apoptosis and upregulation of p53 (Murdoch *et al.* 2001, Murdoch & Van Kirk 2002). In contrast, OSE cells located along the margins of ruptured follicles survive and express anti-apoptotic Bcl-2 protein, which probably functions to compensate for the insult following ovulation (Murdoch *et al.* 2001). A novel observation in the present study is that Bcl-2, Fas and procaspase-3 are moderately expressed in OSE cells throughout the estrous cycle at locations where apoptosis of these cells was negligible. In addition, Fas ligand and Bax appeared to be more frequently expressed in OSE cells that lined the follicles destined to ovulate, than in OSE cells covering the remaining part of the ovary. OSE cells expressing active caspase-3 were observed only at the postovulatory site. *In vitro* studies have shown that

cultured mouse OSE cells undergo apoptosis in response to Fas activation when pre-treated with IFN γ (Quirk *et al.* 1997). Hence, we would like to hypothesize that activation of the Fas death receptor pathway is blocked in OSE cells, until ovulation. The appearance of Fas ligand and Bax in OSE cells that surround the preovulatory follicles prior to ovulation, may in turn elicit the execution of apoptosis, indicating that Fas-induced signalling in apoptotic OSE cells is characteristic of type II cells (Krammer 1999). Based on our findings, we further hypothesize the following model for OSE cell survival associated with ovulation. Proteolytic factors, released prior to ovulation, may facilitate the rupture of the follicular stigma and induce apoptosis in OSE cells surrounding the apex of the follicle. The LH surge-initiated rise in progesterone may attribute to apoptosis of these damaged OSE cells (Murdoch *et al.* 2001, Murdoch & Van Kirk 2002). Indeed, progesterone has been shown to enhance p53 expression in sheep OSE cells (Murdoch & Van Kirk 2002) and to induce *in vivo* (Rodriguez *et al.* 1998) and *in vitro* OSE cell death via enhanced Fas/FasL signalling (Syed & Ho 2003). On the other hand, the high levels of estrogens, released with the follicular fluid at the time of ovulation, may play a role in the survival of undamaged OSE cells next to the rupture site (Murdoch & Van Kirk 2002), since *in vitro* studies have demonstrated that high concentrations of estrogens increase proliferation and suppress apoptosis in OSE cells (Murdoch & Van Kirk 2002, Ho 2003).

It has been well established that the Fas system may control structural involution of the corpora lutea (Roughton *et al.* 1999, Taniguchi *et al.* 2002). The incidence of luteal cell death appeared to be markedly delayed in mice lacking functional Fas, Fas ligand (Sakamaki *et al.* 1997), Bax (Perez *et al.* 1999) or caspase-3 (Carambula *et al.* 2002), whereas *in vitro* Fas ligand or agonistic-Fas antibodies-induced luteal cell death (Quirk *et al.* 2000b, Carambula *et al.* 2003). In the present study, the immunostaining intensity of various components of the Fas signalling pathway, i.e. Fas, Bax, pro- and active caspase-3, increased in luteal cells as the corpora lutea advanced in age, when the luteal cells lose their ability to secrete progesterone (Kuranaga *et al.* 2000b). *In vitro* studies have demonstrated that luteotrophic factors, like progesterone and human chorionic gonadotrophin, suppressed apoptosis in luteal cells by lowering Fas, Fas ligand, Bax and/or p53 expression (Matsubara *et al.* 2000, Sugino *et al.* 2000, Okuda *et al.* 2004). All these observations strongly suggest that in luteal cells of the rat the Fas death receptor acts in concert with a balance of pro- and anti-apoptotic Bcl-2 family members in luteal cells, which is in line with previous observations in various other species (Kondo *et al.* 1996, Sakamaki *et al.* 1997, Sugino *et al.* 2000). Therefore, these cells are presumably type II cells.

Increased levels of luteolytic factors, such as prolactin (Gaytan *et al.* 2001) and PGF $_{2\alpha}$ (Yadav *et al.* 2005) have

been shown to stimulate infiltration of Fas ligand expressing leukocytes into the regressing corpora lutea, which in turn could induce apoptosis in Fas-expressing luteal cells (Kuranaga *et al.* 2000a). Consistent with this assumption, we observed Fas ligand expression in infiltrating leukocytes in the regressing corpora lutea of the second and later generations. Additionally, we found a change in histological localization of Fas ligand during luteal regression, which has not been reported before. In newly formed corpora lutea, Fas ligand was predominantly localized in the luteal cells, while in the older corpora lutea of the third generation, expression was diminished in these cell types. We speculate that the appearance of Fas ligand in luteinized cells in newly formed corpora lutea may function to protect these cells against programmed cell death by inducing apoptosis of Fas expressing infiltrating leukocytes (Kuranaga *et al.* 2000a).

In conclusion, apoptosis in the reproductive tissues of both males and females is thought to occur after withdrawal of crucial hormonal support. Unlike the prostate and testis, however, apoptosis in the ovary and the uterus is linked to estrous cycle driven variations in hormonal secretion (Martimbeau & Tilly 1997). The reduced levels of the apoptotic proteins Fas, Bax and caspase-3, and the slightly higher levels of Fas ligand and the anti-apoptotic protein Bcl-2 in the rat ovary around the time of ovulation may reflect physiological survival mechanisms to ensure ovulation of the dominant follicles, corpus luteum formation and OSE cell repair. Hence, apoptosis of the various ovarian cell types seems to depend on hormonal support as well as on the presence of the Fas system and Bcl-2 family members.

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