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Expression and distribution of cytokeratin 8/18 intermediate filaments in bovine antral follicles and corpus luteum: An intrinsic mechanism of resistance to apoptosis?

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Summary. Apoptosis is a mechanism of cell elimination during follicular atresia and luteal regression. Recent evidence suggests sensitivity to apoptosis in some cell types is partly dependent upon cytokeratin-containing intermediate filaments. Specifically, cytokeratin 8/18 (CK8/18) filaments are thought to impart resistance to apoptosis. Here, cytokeratin filament expression within bovine ovarian follicles and corpora lutea (CL) was characterized and the potential relationship between cell-specific CK8/18 expression and apoptosis explored. Immunoprecipitation and western blot analysis confirmed CK8 associates with CK18 to form CK8/18 heterodimeric filaments within bovine ovarian cells. Immunostaining revealed populations of CK18-positive (CK18+) cells in healthy growing follicles that increased in postovulatory follicles. Atretic follicles at all stages of atresia also contained some CK18+ cells. However, no CK18+ cells were detected in primordial or primary follicles. In CL, developing CL contained a higher proportion of CK18+ cells (~35%, range 30-70%) than mature CL (~16%) and regressing CL (~5%; $P<0.05$, n = 3-5 CL/stage), suggesting CK8/18 filament expression diminishes over time, as luteal cells become more susceptible to apoptosis. Dual-fluorescence labeling for CK18 and a cell death marker (TUNEL labeling) confirmed this view, demonstrating less death of CK18+ than CK18- luteal cells throughout the estrous cycle ($P<0.05$). The results indicate differential expression of CK8/18 filaments occurs in cells of bovine ovarian follicles and CL throughout the estrous cycle. The prevalence and cell-specific pattern of cytokeratin expression in these structures is consistent with the concept these filaments might impart resistance to apoptosis in ovarian cells as is seen in other cell types.

Key words: Ovary, Apoptosis, Cytoskeleton

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Introduction

Cytoskeletal intermediate filament proteins known as the cytokeratins constitute a diverse class of elements that derive from a family of approximately 65 homologous proteins, forming six classes of molecules (Moll et al., 1982). The cytokeratins are obligate heterodimers composed of an acidic CK (Type I: numbered 9-20) paired with a basic CK (Type II: numbered 1-8). The cytokeratin 8/18 (CK8/18) filament is considered one the most abundant Type I: Type II filaments found in normal epithelia, cultured cell lines, and carcinomas. Functionally, CK8/18 filaments provide structural integrity to cells, but they also influence intracellular transport mechanisms and signaling (Singh et al., 1994; Eriksson et al., 2009). Recently, the expression of these filaments in certain types of epithelial cells has been implicated in the resistance of these cells to apoptosis. Mechanisms of protection include impairing cytokine receptor trafficking and cell surface expression (Gilbert et al., 2001; Marceau et al., 2001; Ku et al., 2003), and the inhibition of cytokine-

induced apoptotic intracellular signals (Caulin et al., 2000; Oshima, 2002; Ku et al., 2003; Gilbert et al., 2008). These observations support earlier suggestions that intermediate filaments regulate transport processes between the cell surface and nucleus, and influence nuclear events (Li et al., 1994; Singh et al., 1994).

In the ovary, the expression of intermediate filaments has been described for several species (Czernobilsky et al., 1985; Gall et al., 1992; Santini et al., 1993; Gallicano et al., 1994; Bukovsky et al., 1995; Nilsson et al., 1995; Ricken et al., 1995; van den Hurk et al., 1995; Pan and Auersperg, 1998; Loffler et al., 2000). Many of these reports describe the relative distribution of filaments such as vimentin, cytokeratin, and desmin within the ovary, attempting to elucidate the embryonic origins of certain ovarian cell types. For instance, vimentin immunostaining is generally found in cells of mesenchymal origin, whereas cytokeratin occurs in epithelial cells, and desmin resides in cells of myogenic origin (Steinert et al., 1984). In many of the above cited studies, vimentin and cytokeratins were detected within granulosal cells of follicles at various stages of growth and atresia, in luteal cells of the corpus luteum throughout the luteal phase, and in oocytes from both fetal and adult ovaries (Czernobilsky et al., 1985; Gall et al., 1992; Santini et al., 1993; Gallicano et al., 1994; van den Hurk et al., 1995; Nilsson et al., 1995). However, while these reports recognized a likely role of intermediate filaments in aspects of folliculogenesis, luteal function, and/or oocyte competence, the more recent discovery of CK8/18 filaments as a potential anti-apoptotic influence was not a consideration.

Apoptosis, particularly cytokine-induced apoptosis, has been implicated in the selective elimination of granulosal cells and oocytes during atresia of ovarian follicles (Kim et al., 1998; Bridgham and Johnson, 2001; Hu et al., 2001), and luteal cells during regression of the corpus luteum (Kuranaga et al., 2000; Pru et al., 2002; Taniguchi et al., 2002; Komatsu et al., 2003; Peluffo et al., 2009). In both atretic follicles and regressing corpora lutea, the cells undergoing apoptosis are generally scattered throughout the parenchyma (Kim et al., 1998; D'Haeseleer et al., 2006; Peluffo et al., 2007), suggesting cell death is triggered by local, possibly intrinsic factors. Considering recent evidence that cytoskeletal filaments, specifically cytokeratin-containing 8/18 (CK8/18) intermediate filaments, provide mechanisms of resistance to apoptosis in certain types of epithelial cells (Caulin et al., 2000; Gilbert et al., 2001, 2004, 2008), it is conceivable that these filaments could similarly influence the intrinsic susceptibility of ovarian cells to apoptosis during ovarian function.

The objective of the current study was to determine CK8/18 filament expression within bovine ovarian follicles throughout folliculogenesis and ovulation, and within the CL throughout the luteal phase. In addition, we initially explored the potential relationship between cell-specific CK8/18 filament expression and relative susceptibility of ovarian cells to apoptosis.

Materials and methods

Bovine ovaries (n=25) were collected from slaughterhouse specimens for immunohistochemistry (IHC) of follicles. The ovaries were immediately placed in ice-cold Earle's balanced-salt solution (Sigma Chemical Company, St Louis, MO, USA) and transported to the laboratory, sliced into thirds and frozen in ornithine carbamyl transferase compound (OCT; Miles Laboratories, Inc., Elkhart, IN).

Classification of follicular health

One 10-μm-thick section from each ovarian slice was cut and stained with hematoxylin and eosin. Sections were viewed on an Olympus BX50 microscope (Olympus, Australia Pty. Ltd., Mount Waverly, Australia) and follicles identified. The cross-sectional diameter from the follicular basal lamina of follicles (1-5 mm) was measured using an occular micrometer, and the follicles were classified into one of three categories: healthy, antral atretic, or basal atretic, as previously described (Irving-Rodgers et al., 2001). The morphology of the membrana granulosa was used to ascertain follicular health. Cell death was identified as intensely stained round or crescent-shaped pyknotic nuclei (Van Wezel et al., 1999) or as apoptotic nuclei.

Immunohistochemistry of follicles

Bovine ovaries embedded in OCT compound were used for IHC using an indirect immunofluorescence method. Methods for IHC have been reported previously (Irving-Rodgers et al., 2002). Tissue sections (10 μm) were cut from each of the frozen follicles using a CM1800 Leica cryostat (Adeal Pty. Ltd. Altona North, Vic, Australia) and serial sections were collected on SuperFrost Plus glass slides (HD Scientific Supplies, Pty Ltd, Sunshine, Vic, Australia) and stored at -20°C until use. Unfixed sections were dried under vacuum for 5 min before fixation in 10% neutral buffered formalin for 5 min at room temperature. After rinsing in 3x5 min changes of hypertonic phosphate-buffered saline (10 mM sodium/potassium phosphate with 274 mM NaCl, 5 mM KCl; pH 7.2; hPBS), sections were treated with blocking solution [10% (v/v) normal donkey serum (Sigma Chemical Company, St Louis, MO, USA)] in antibody diluent containing 550 mM sodium chloride and 10 mM sodium phosphate (pH 7.1) for 20 min at room temperature. Incubation in primary antibody was carried out overnight at room temperature. Primary antibodies included a mouse monoclonal anti-human cytokeratin-18 peptide (clone CY-90, Sigma) at a dilution of 1:1000; a mouse monoclonal antibody (CytoDEATH) to the caspase cleavage product of cytokeratin 18 (clone M30, Roche Applied Science, Mannheim, Germany) at a dilution of 1:50; a rabbit anti-human caspase 3 (ab4051, Abcam, Cambridge, UK) at a dilution of 1:100; a rabbit anti-murine EHS laminin

(L9393, Sigma) at a dilution of 1:100; and a rabbit anti-human cytochrome P450, steroid 17-alpha-hydroxylase antibody (CYP17; to detect steroidogenic, thecal cells) at a dilution of 1:1000 (Conley et al., 1995). Secondary antibodies were biotin-SP-conjugated AffiniPure Donkey anti-mouse (CK18 and CytoDEATH) or anti-rabbit secondary antibody (caspase 3, Laminin and CYP17) followed by Cy3-conjugated streptavidin or FITC-conjugated streptavidin (1:100) for single and dual immunodetection experiments. Secondary antibodies and fluorophores were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Tissue sections were counterstained with the nuclear stain 4',6-diamindino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR) at a concentration of 3 μM and cover-slipped using mounting medium for fluorescence observation (Dako Corporation, Carpinteria, CA). Tissue sections were observed and photographed with an Olympus BX50 microscope using an epifluorescence attachment and Spot RT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Tissue sections of ovarian surface epithelium were utilized as positive controls for CK18 immunostaining (van den Hurk et al., 1995, Perez-Martinez et al., 2001). Non-specific staining was assessed by substitution of the primary antibody with either normal mouse or normal rabbit sera (Sigma) at a dilution of 1:100, and was undetectable in all instances.

Classification of corpora lutea

Bovine corpora lutea (CL) were staged by ultrasonography and obtained by transvaginal lutectomy as described previously (Townson et al., 2002). This procedure has been approved by the University of New Hampshire Institutional Animal Care and Use Committee (IACUC # 021102). Briefly, cows were examined for ovulation by transrectal ultrasonographic scanning. With Day 0 corresponding to the day of ovulation, CL from cows at Days 5-6 (developing CL), Days 10-12 (mature CL) and Day 18 (regressing CL) of the estrous cycle were removed (n=3-5 cows/stage). A portion of each CL was processed in OCT compound using liquid nitrogen and isopentane, and then prepared as frozen tissue sections (thickness, 6-8 μm).

Immunohistochemistry of corpora lutea

Methods for IHC staining of frozen tissues sections of bovine CL have been described and validated previously in the laboratory (Townson et al., 2002). Briefly, frozen tissue sections were air dried, fixed in acetone for 10 min at 4°C, and transferred to 0.3% H_2O_2 in methanol at 4°C to quench endogenous peroxidase activity. Sections were rinsed 3x5 min in PBS-1% BSA before blocking with 10% normal horse serum (Sigma) in PBS-1% BSA for 30 min at room temperature. The sections were then rinsed 3x5 min in PBS-1% BSA prior to incubating with mouse anti-human cytokeratin-18 antibody (clone CY-90, Sigma) at 5 μg/ml in 10% normal horse serum, 2% normal bovine serum, and PBS-1% BSA overnight at 4°C. The next day the slides were rinsed 3x5 min in PBS-0.1% BSA, and incubated at 37°C for 30 min with biotinylated anti-mouse IgG (Vector/Novacastra Laboratories, Burlingame, CA, USA) applied at a 1:200 dilution in 10% normal horse serum and 2% normal bovine serum in PBS-0.1% BSA. Amplification of the antigen-antibody complex was achieved using avidin-biotin-peroxidase (ABC kit; Vector/Novacastra Laboratories) for 30 min at 37°C. Detection occurred by precipitating 3-amino-9-ethylcarbazole (AEC, Vector/Novacastra Laboratories) for 10 min at room temperature. The tissue sections were then rinsed, counter stained with hematoxylin, rinsed, and mounted with coverslips using aqueous mounting medium (Vector/Novacastra Laboratories). All incubations were done in a humidified chamber. Similar to above, nonspecific staining was assessed by omission of the CK18 antibody and by substitution of the CK18 antibody with an identical concentration of a non-specific, IgG1 isotype control (clone MOPC-21, Sigma), and was undetectable in all instances.

Flow cytometry of luteal cells

Flow cytometric analysis of luteal cells was used to quantify cytokeratin 18 staining in CL throughout the luteal phase relative to the incidence of apoptosis. Detection of CK18 was achieved using a FITC-conjugated anti-cytokeratin 18 monoclonal antibody (clone CY-90, Sigma), whereas apoptosis was assessed by terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL), using an APO-BrdU labeling kit (Phoenix Flow Systems Inc., San Diego, CA, USA). An Alexafluor-647 labeled fluorescein PRB-1 antibody was used to detect and quantify the dead or dying cells. Controls consisted of exclusion of the enzyme in the labeling reaction. Luteal cells from CL of cows during the developing (Days 5-6), mature (Days 10-12), and regressing (Day 18) stages of the estrous cycle were fixed according to the manufacturer's protocol (Phoenix Flow Systems Inc,) and then later immunostained for CK8/18 expression and/or TUNEL. The cells were analyzed with a 4-color, dual laser FACSCalibur (BD Biosciences, Palo Alto, CA, USA), quantifying 10,000 cells for CK8/18 staining (FITC) and/or TUNEL (Alexafluor 647). Results were collected using Cellquest software (BD Biosciences) and analyzed with WinMIDI software (Joseph Trotter, Scripps University).

Immunoprecipitation and western blot detection of CK8/18 heterodimers in the bovine CL

To verify cytokeratin intermediate filaments in the bovine ovary consist of CK8 and CK18 heterodimers, a small portion of CL was snap-frozen in liquid nitrogen and then prepared for immunoprecipitation and western

blot analysis. Briefly, the snap-frozen tissue was homogenized in 1.5 ml immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 1% triton X-100; with 10 μl Protease inhibitor, 10 μl 0.1 M DTT, and 10 μl PMSF per ml of buffer just before use) using a mortar and pestle. The lysate was transferred to a microfuge tube, vortexed briefly, and then incubated on a rotary shaker for 30 min at 4°C. Subsequently the lysate was centrifuged and the supernatant (sample) transferred to a new microfuge tube. The supernatant was then pre-cleared by mixing 2 mg of sample in 1 ml volume with 10 μl normal rabbit serum and 50 μl protein-G Agarose beads (beads were washed with lysis buffer 5 times prior to use), with gentle shaking at 4°C for 1 hr. This step was followed by centrifugation at 12,000xg for 30 sec, and transfer of the supernatant to a new microfuge tube. Ten microliters (10 μl) of the mouse monoclonal anti-human cytokeratin-18 antibody (clone CY-90, Sigma) was added to the sample followed by incubation overnight at 4ºC with shaking. Then, 50 μl of pre-washed homogenous Protein G was added to the sample followed by incubation at 4ºC for an additional hour. The sample was centrifuged at 12,000xg for 30 sec, the supernatant was carefully removed, and the remaining pellet (i.e., containing the precipitated agarose-antibody-antigen complexes) was resuspended and washed with 0.5 ml lysis buffer for 5 min at 4°C with shaking. This wash step procedure was repeated two additional times. Following the last wash the agarose pellet was resuspended in 30 μl of 4x non-reducing sample buffer, 12 μl of reducing buffer, and 18 μl ddH_2O. The proteins were eluted from the beads by heating the suspension to 95°C for 10 min. For a negative control, 2 μl (2 μg) of non-specific mouse IgG was used in place of the monoclonal anti-CK18 antibody. The eluted proteins were resolved by SDS-PAGE and then probed by western blot analysis using a mouse anti-human CK8 antibody (clone C51, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistics

Quantification of CK18 and TUNEL immunostaining by flow cytometric analysis was repeated using separate CL dissociations for each stage of the luteal phase (n=3 CL per stage). Data were analyzed using a one-way analysis of variance (ANOVA) in the general linear model of Systat (Point Richmond, CA). Pair-wise comparisons were conducted using Fisher's LSD test, and the results are expressed as a percentage, with differences established at $P<0.05$.

Results

Immunohistochemical analysis of CK18 in bovine follicles

A total of 122 follicles, through all stages of follicular development (i.e., primordial, primary, secondary, tertiary, and ovulatory), were examined for CK18 expression. The antral follicles were further classified as healthy (70 follicles) or atretic (44 follicles) based upon morphological criteria. Primordial and primary follicles (Fig. 1A, asterisks) did not show any specific immunoreaction for CK18 (Fig. 1B,C); however, CK18 was quite evident as filamentous staining in cells throughout the granulosal layers of small growing follicles (Fig. 1C). In larger antral follicles (Fig. 1D), this staining was confined to granulosal cells adjacent to the follicular basal lamina (Fig. 1E,F). However, not all antral follicles contained CK18-positive (CK18+) cells. In addition, CK18 staining was seen in a minority of cells within the theca interna of healthy antral follicles, and was expressed in both CYP17-positive and CYP17-negative cells (Fig. 1F). Ovarian surface epithelium exhibited strong, positive immunostaining for CK18 (Fig. 1G), whereas in negative control tissue sections staining was absent (Fig. H,I).

Similar to observations of growing follicles, CK18+ cells in ovulatory follicles (Fig. 2A) were distributed throughout the granulosal layers, with relative expression of CK18 filaments increased immediately postovulation (Fig. 2B). As before, CK18 staining was filamentous and detected in both CYP17-positive and CYP17-negative cells within ovulatory follicles (Fig. 2C,D,E).

Atretic follicles (Fig. 3A) also contained some CK18+ cells with intense cytoplasmic staining. The pattern of distribution of CK18+ cells was consistent with the relative phase of follicular atresia. In early antral, atretic follicles, CK18+ cells were present in the most antral layers of the follicle (Fig. 3B,C). In advanced atretic follicles, CK18+ cells were distributed throughout the follicle (Fig. 3D), particularly in basal atretic follicles, in which the granulosal layer had separated from the basal lamina (Fig. 3E). Atretic follicles with CK18+ cells also contained a subpopulation of cells stained with the CytoDEATH antibody for the caspase cleavage product of cytokeratin 18 (compare Fig. 3F,G). Moreover, an atretic follicle (Fig. 3H) with CytoDEATH-positive cells (Fig. 3I) also stained positively with an antibody to caspase 3 (Fig. 3J).

Immunohistochemical analysis of CK18 in bovine corpora lutea

In corpora lutea, CK18 immunostaining was evident throughout the parenchyma, detectable in cells morphologically similar to steroidogenic cells rather than endothelial cells or other cell types (Fig. 4). Similar to follicles, the relative abundance of CK18+ cells in the parenchyma varied by CL maturity. In developing CL, there was an abundance of CK18+ cells (Fig. 4A), consistent with the relative abundance of these cells immediately after ovulation (Fig. 2B). In contrast, mature and regressing CL had diminished numbers of

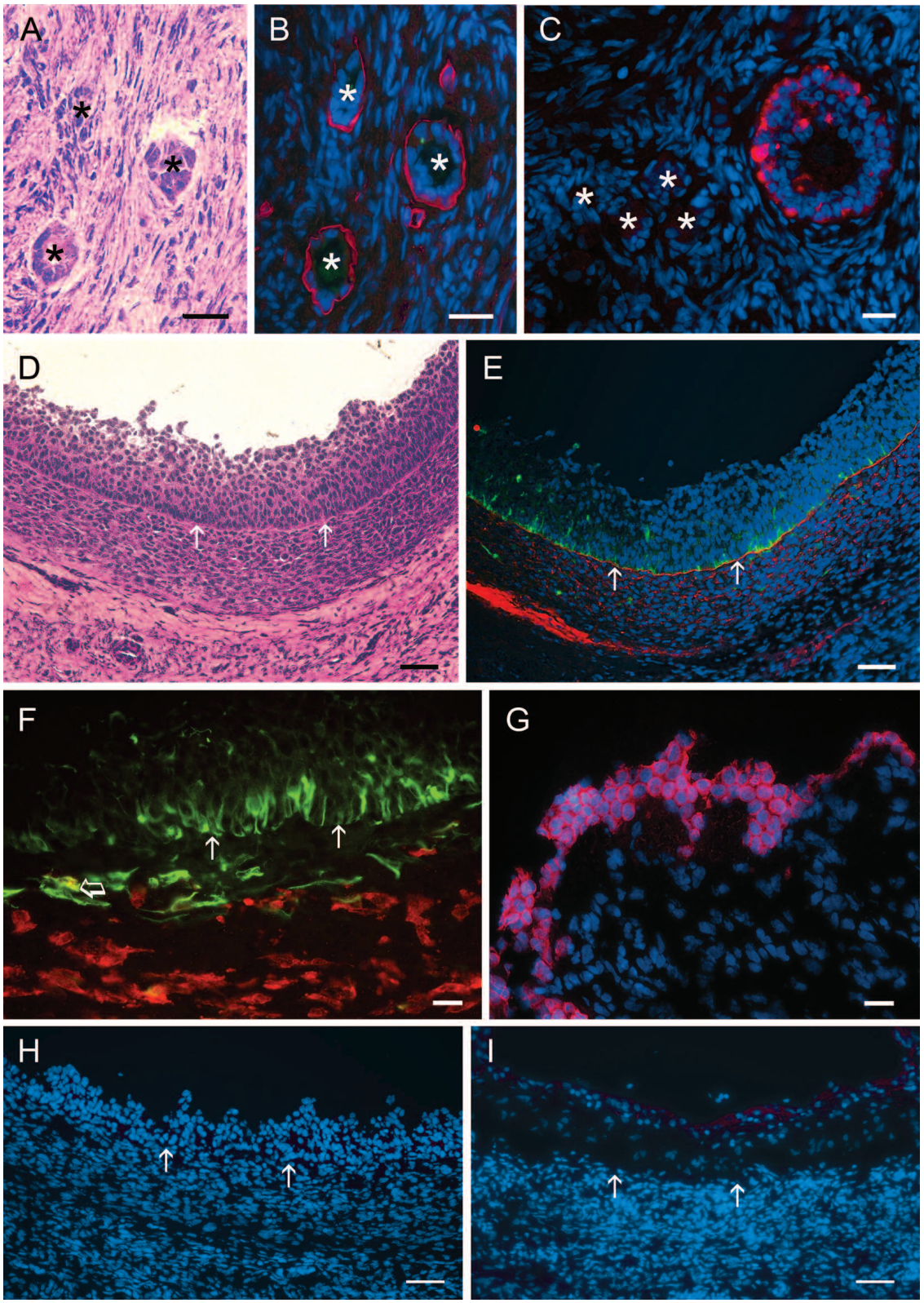

Fig. 1. Immunostaining of CK18 in healthy bovine primordial, primary and growing follicles **(A-C)**. Primordial and primary follicles (asterisks) are negative for CK18 **(B, C)**. **A.** Haematoxylin and eosin stained serial section to that shown in **B**. Laminin (red staining in B) is localised to the follicular basal lamina. **C.** CK18 is localized to the cytoplasm of granulosal cells in a growing follicle (red staining). **D, E**. Serial sections of an antral follicle stained with haematoxylin and eosin (**D**) and localization of CK18 (green) and laminin (red) **(E)**. **F.** CK18 (green) is localized to the basal granulosal cells and some thecal cells. Red staining is cytochrome P450c17 (CYP17). Open arrow, co-localization of CK18 and CYP17. **G.** Ovarian surface epithelial cells exhibit positive immunostaining for CK18 (red). **H, I.** Negative control sections of atretic follicles in which normal mouse serum **(H)** and normal rabbit serum (I) was substituted for the primary antibodies. Arrows indicate the position of the follicular basal lamina. B, C, E, H and I are counterstained with DAPI. Scale bars: A-C, F, G, 20 μm; D, E, H, I, 50 μm.

CK18+ cells (Figs. 4B,C). Non-specific staining for CK18 was undetectable in negative control sections of luteal tissue (Fig. 4D). Flow cytometric analysis of CK18+ luteal cells confirmed a higher proportion of CK18+ cells in developing CL (~35%, range 30-70%), which diminished with advancing age of the CL ($P<0.05$; Fig. 5).

Relative resistance of CK18+ luteal cells to apoptosis in vivo

Freshly dissociated luteal cells of developing and regressing bovine CL were compared using dual-fluorescence labeling for CK18 and apoptosis (TUNEL) and indicated no change in the relative proportion of cells that were both positive for these markers (i.e.,CK18+ and TUNEL+, ~2%, Fig. 6). Conversely, the proportion of cells that were CK18-negative (CK18-) and TUNEL+ increased with maturity of the CL (3% to 16% for Days 5-6 versus Day 18 CL, respectively, Fig. 6). Consistent with the results in which luteal cells were labeled only for CK18 staining (Fig. 5), numbers of CK18+ luteal cells in the dual-fluorescence labeling experiments decreased with advancing age of the CL (66% to 6% in Days 5-6 versus Day 18 CL, respectively, Fig. 6).

Co-precipitation of CK8 with CK18 in developing corpora lutea

Immunohistochemical staining of the bovine ovary revealed that the developing CL (i.e., Days 5-6 of the

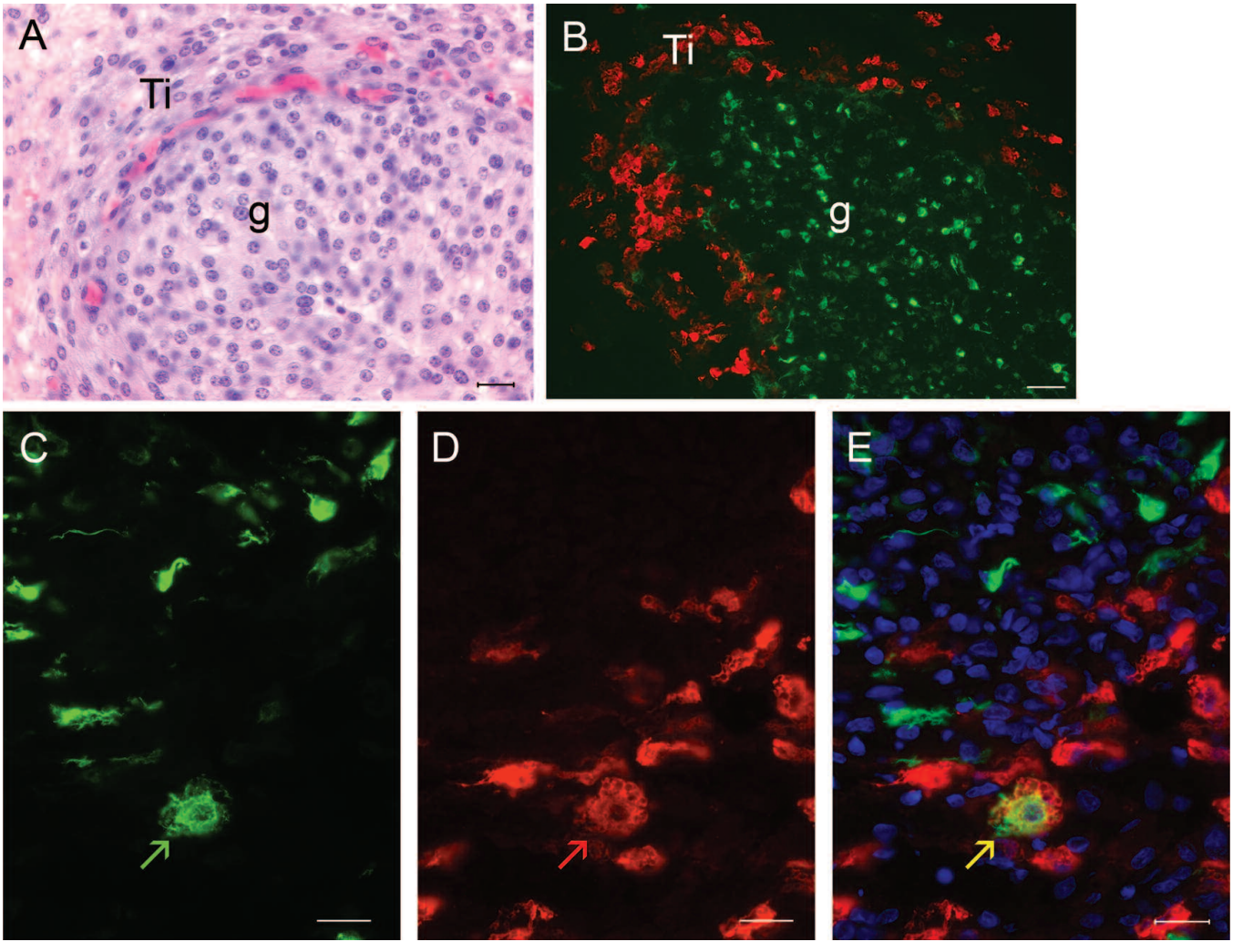

Fig. 2. Immunostaining of CK18 and cytochrome P450, steroid 17-alpha-hydroxylase (CYP17) in a postovulatory follicle **(A-E)**. **A.** Haematoxylin and eosin stained serial section to that shown in **B.** Theca interna (Ti) and granulosal (g) layers depicted. **B.** Numerous CK18+ (green) cells distributed throughout the granulosal layer (g), surrounded by predominantly CYP17 staining (red) in the theca interna layer (Ti). **C-E**. Higher magnification of theca-granulosal layer interface depicting immunostaining of CK18 **(C)**, CYP17 **(D)**, and co-localization of CK18 and CYP17 **(E)**, respectively (note colored arrows; cells in E also counterstained with DAPI). Scale bars: 10 μm.

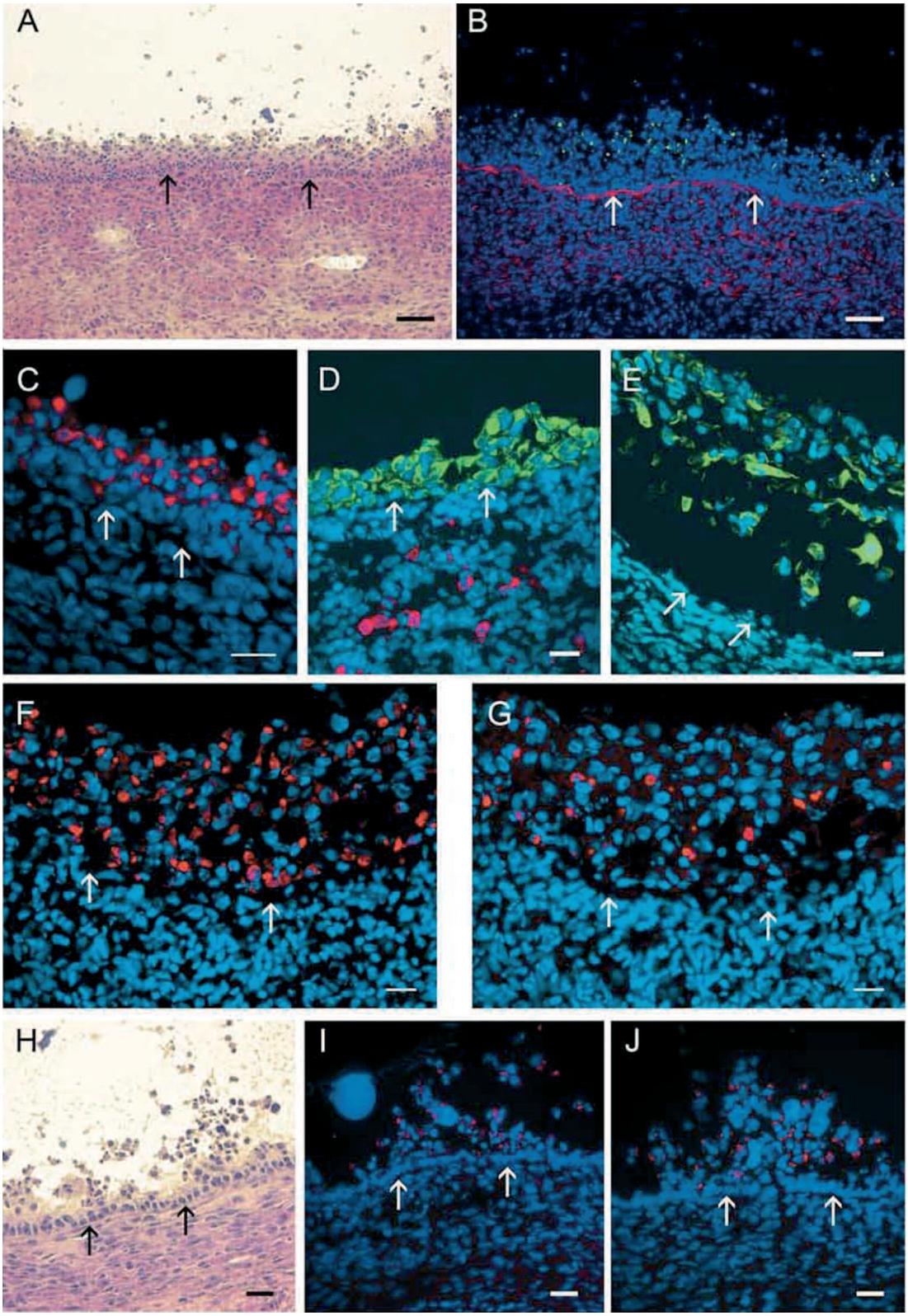

Fig. 3. Immunostaining of CK18, CYP17, CytoDEATH, and Caspase 3 in atretic antral follicles **(A-J)**. **A.** Haematoxylin and eosin stained serial section to that shown in **B**. **B, C.** CK18+ cells in antral layers of early atretic follicles (green in **B**; red in **C**). **D-F**. In advanced atretic follicles, CK18+ cells (green in **D** and **E**; red in **F**) are evident throughout the membrane granulosa. CYP17-positive cells (red staining in **B** and **D**) are also evident. **G.** Sparce CytoDEATH-positive cells (red staining) in the same follicle as in **F, H**. Haematoxylin and eosin stained serial section to those shown in **I** and **J**. **I, J.** Corresponding CytoDEATH staining (**I**, red cells) and Caspase 3 staining (**J**, red cells) in an atretic follicle. Arrows indicate the position of the follicular basal lamina. B, C-G, I, J. Counterstained with DAPI. Scale bars: A, B, 50 μm; C-J, 20 μm.

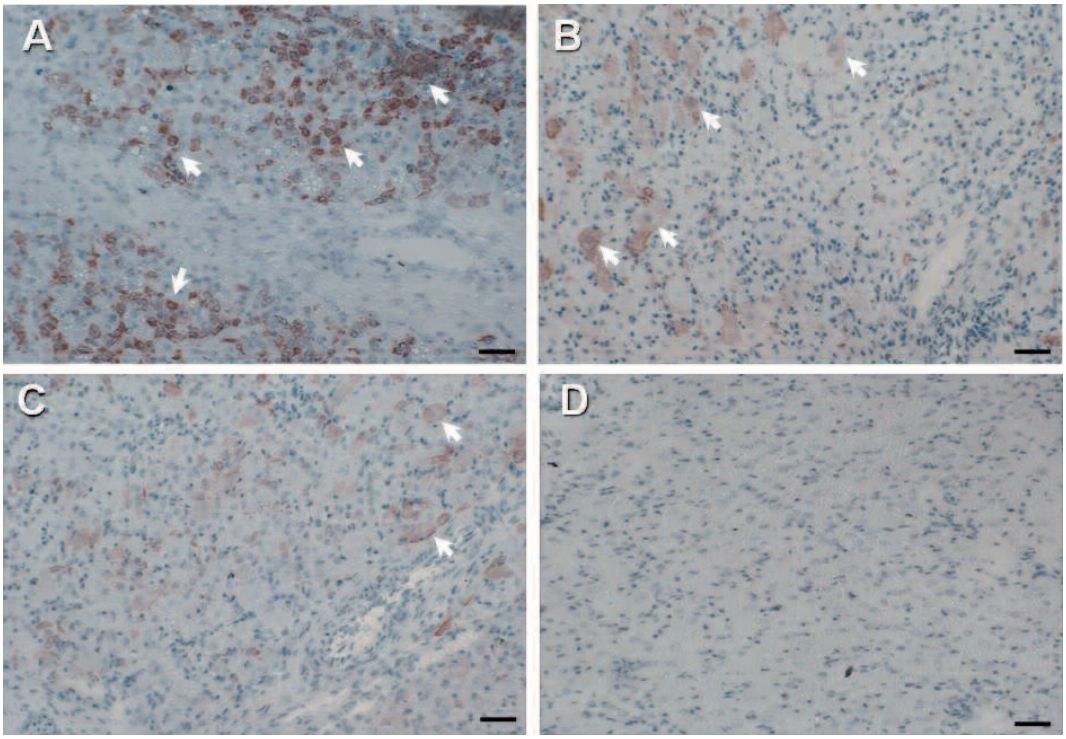

Fig. 4. Immunostaining of CK18 in corpora lutea (**A-D**). **A.** Abundant CK18+ cells (red staining) in a developing corpus luteum (clusters of cells depicted by arrows). **B, C.** Diminished CK18 expression in mature (**B**; individual cells depicted by arrows) and regressing corpus luteum (**C**, individual cells depicted by arrows). **D.** Negative control tissue section of corpus luteum. A-D. Hematoxylin counterstained. Scale bars: 50 μm

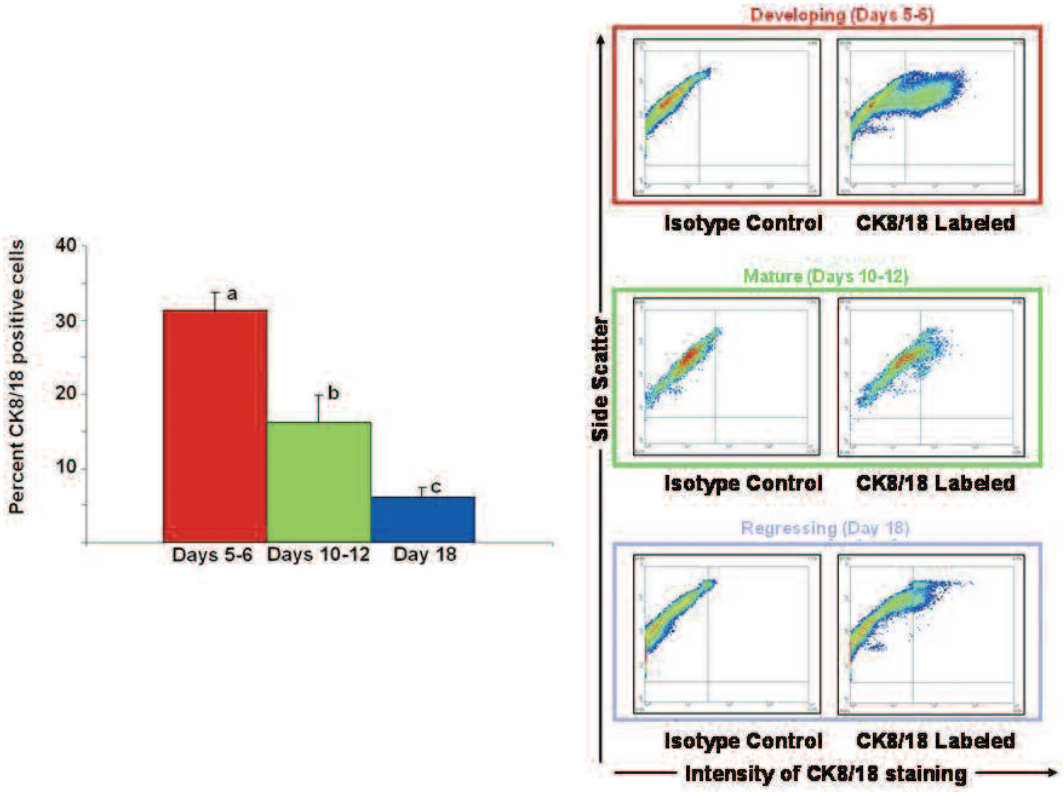

Fig. 5. Flow cytometric analysis of CK18 immunostaining in mixed luteal cells from developing (Days 5-6), mature (Days 10-12), and regressing (Day 18) bovine CL. The bar graph depicts the average percentage of CK18+ cells among the three stages of the luteal phase (bars denoted by different letters are different; $P<0.05$, n=3-5 CL/stage). The flow cytometric scatter plots depict representative results for the three stages of the luteal phase. Side scatter is shown on the Y-axis, a right-shift along the x-axis indicates an increase in the proportion of cells that are CK18+.

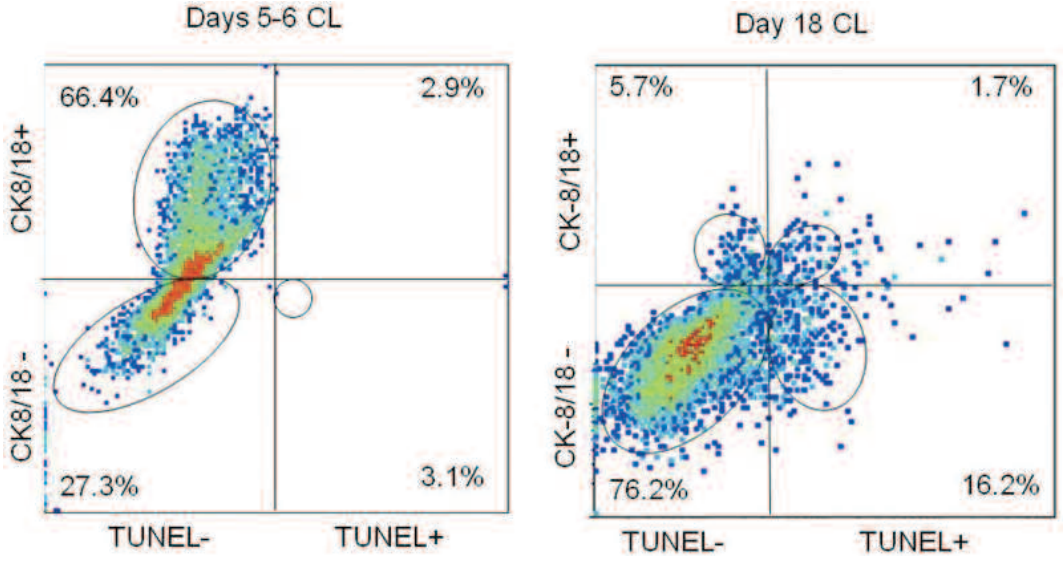

Fig. 6. Flow cytometric analysis of luteal cells of developing (Days 5-6) and regressing (Day 18) bovine CL dual-labeled for CK18 and TUNEL (a marker of cell death). Percentage of CK18+ and TUNEL+ cells relative to controls is shown on the y- and x-axis, respectively (n=3 CL/stage). Note the higher percentage of CK18-/TUNEL+ cells in Day 18 CL (~16%) compared to Days 5-6 CL (~3%).

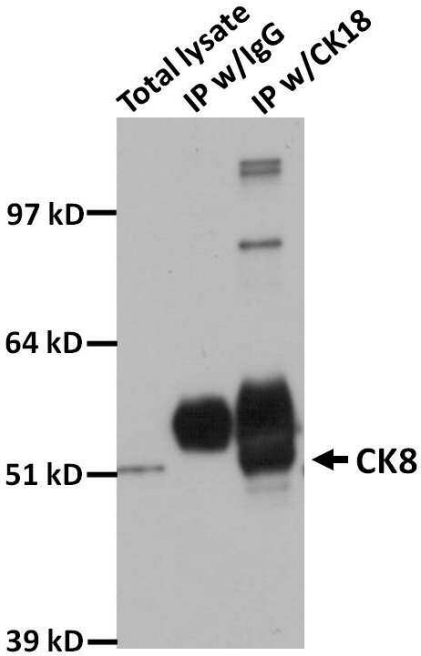

Fig. 7. Representative western blot of CK8 in tissue lysate of a developing (Days 5-6) bovine CL following immunoprecipitation with anti-CK18 antibody. In lane one, detection of CK8 in total lysate prior to immunoprecipitation is depicted. In lane two, detection of the protein complex formed following immunoprecipitation with a non-specific, monoclonal IgG is shown. In lane three, the relative enhancement of CK8 following immunoprecipitation with the monoclonal, anti-CK18 antibody is shown. Relative molecular weight of CK8 is 52 kD.

luteal phase) was the most abundant tissue-specific source of CK18 expression in this study. Hence, we utilized developing CL for immunoprecipitation and western blot analysis to verify the CK-containing intermediate filaments within these cells consisted of heterodimers of CK8 protein associated with CK18 (Fig. 7). In total luteal lysate, CK8 was readily detectable at a relative molecular weight of 52 kDa using the anti-CK8 antibody (Lane 1, Fig. 7). Immunoprecipitation of the luteal lysate with anti-CK18 antibody enhanced the abundance of CK8 (Lane 3, Fig. 7), verifying the dimerization of CK8 with CK18 in the formation of the intermediate filaments. The anti-CK18 antibody used for immunoprecipitation in this instance was identical to that used for IHC and flow cytometric analysis throughout the current study.

Discussion

Apoptosis contributes to follicular atresia and luteal regression in ovarian function, and in doing so impacts fertility. Yet the cellular mechanisms which influence apoptosis during these ovarian processes remain largely unresolved. In the current investigation we have discovered a potential intrinsic mechanism to account for the cell-specific sensitivity of ovarian granulosal and luteal cells to apoptosis. Our results indicate expression of CK8/18 intermediate filaments within granulosal cells and luteal cells is discrete and appears inversely related to the relative incidence of apoptosis in these cells, especially during luteal regression. In previous studies, others have observed that apoptosis of granulosal cells during follicular atresia and luteal cells during luteal regression is similarly selective, affecting only discrete population of cells (Sakamaki et al., 1997; Kim et al., 1998; Quirk et al., 2000; Taniguchi et al., 2002). Here we have found CK filament expression is similarly cell-specific, and we have provided initial evidence that CK18-positive cells are not concomitantly apoptotic.

In follicles, discrete populations of granulosal cells

undergo apoptosis during the initial stages of atresia. Not surprisingly, as atresia continues the number of granulosal cells that undergo apoptosis increases, generally extending to the most basal layers of the follicle. In the context of cytokine-induced apoptosis, however, the regulation of cell death in granulosal cells evidently does not occur at the level of ligand expression. Both healthy and atretic follicles, for instance, express cytokines such as Fas ligand (Dharma et al., 2003). Similarly, overt changes in Fas receptor expression cannot adequately account for the specificity of apoptosis of individual cells because Fas mRNA and protein do not differ between granulosal cells susceptible or resistant to Fas ligand-induced apoptosis (Porter et al., 2001). One possibility is that cytokine receptor expression on the surface of granulosal cells is intrinsically regulated, hence influencing the responsiveness of individual cells to Fas ligand and/or other cytokines. In the current study, CK18 expression in healthy growing follicles was confined to the granulosal cells aligning the basal lamina. These cells are also among the most resistant to apoptotic cell death. Our observations of CK filament expression in granulosal cells of growing follicles is consistent with previous studies (Czernobilsky et al., 1985, Santini et al., 1993), but at odds with one study in which CK immunoreactivity was not detected in pregranulosal or granulosal cells of primary and larger follicles (van den Hurk et al., 1995). Such discrepancies might reflect functional differences, but more likely are a consequence of differences in tissue fixation methods, specificity of the antibodies used, and/or immunohistochemical methods.

The observation that discrete populations of granulosal cells within atretic follicles contain CK8/18 filaments or contain the cleaved form of these filaments as they undergo cell death (as detected by the CytoDEATH antibody) suggests CK18 expression alone is not the sole determinant of susceptibility to apoptosis. The CytoDEATH staining observed in atretic follicles is particularly noteworthy because this antibody recognizes a phosphorylated form of cytokeratin (i.e., cleaved cytokeratin), described previously as associated with apoptotic cells (Chiu et al., 2001; Dinsdale et al., 2004; Ueno et al., 2005). By inference, our results suggest CK18-containing granulosa cells also eventually become vulnerable to apoptosis, especially during advanced stages of atresia, and that additional factors contribute to this process. Indeed, cell cycle progression (Quirk et al., 2006) and cycloheximide-sensitive, labile protein(s) (Porter et al., 2001) have been implicated as factors influencing granulosal cell susceptibility to apoptosis. In non-ovarian cell types, CK8/18 filaments provide protection to cells by impairing cytokine receptor expression and mobilization (Gilbert et al., 2001), by promoting anti-apoptotic intracellular signaling (Gilbert et al., 2004), and by hindering death domain proteins (Gilbert et al., 2008). However, whether or not these or other mechanisms are related to CK filament expression within granulosal cells is uncertain and merits further investigation.

In cells of the corpus luteum, the results of the current study and a previous study (Ricken et al., 1995) show CK8/18 filament expression diminishes over time, with the most noticeable loss of expression occurring during the late luteal phase. The decline of CK18+ luteal cells in the current investigation coincided with an increase in TUNEL+ cells, indirectly suggesting the loss of CK8/18 intermediate filaments may predispose the cells to death. However, direct manipulation of CK8/18 filament expression within the cells will be required to assess their relative impact on susceptibility to apoptosis. The observation of relatively high proportions of CK18+ cells in developing CL is also potentially important as others have shown that granulosal cells transitioning into luteal cells after ovulation are exquisitely resistant to cytokine-induced apoptosis (Porter et al., 2001). Acknowledging that here the number of CK18+ cells in developing CL varied considerably from cow to cow (range 30-70%), it is conceivable that the relative maturity of the CL and the location of CK18+ cells within the parenchyma (e.g., proximal or distal to the major vasculature) might have some bearing on the overall resistance of the tissue to apoptosis, or more specifically, its relative resistance to prostaglandin F2-alpha-induced regression (Inskeep, 1973). Of note in the current study is the observation that the highest proportion of TUNEL-positive cells occurred only in cells that lacked CK18 staining, regardless of stage of the CL. In addition, immunohistochemical evidence indicated that the type of luteal cells expressing CK18 morphologically resembled small and large steroidogenic cells rather than endothelial cells, fibroblasts, or other cell types. Lastly, immunoprecipitation and western blot evidence demonstrated that the CK filaments in luteal tissue consist primarily of CK8/18 heterodimers. These observations support those of previous studies (Nilsson et al., 1995; Ricken et al., 1995) acknowledging, however, CK18+ endothelial cells exist within the bovine CL, albeit in extremely small numbers (i.e., <5% of the total number of endothelial cells) (Fenyves et al., 1993).

In conclusion, the temporal expression of CK8/18 intermediate filaments within discrete populations of cells of bovine ovarian follicles and CL supports the concept that these cytoskeletal elements might confer resistance to apoptosis. In healthy growing follicles, CK18 staining is observed principally in granulosal cells aligning the basal lamina, which are among the most resistant to apoptosis throughout folliculogenesis. In CL, numbers of CK18+ luteal cells decline with advancing age of the tissue. The highest proportion of luteal cells lacking CK18 is associated with apoptosis at the time of luteal regression. The prevalence and pattern of CK18 staining in follicles and the CL strengthens the possibility these intermediate filaments impart resistance to apoptosis in ovarian cells as is seen in other cell types. However, further investigation is needed to conclusively

determine the role CK8/18 filaments within ovarian steroidogenic cells and whether they have a mechanistic influence on apoptosis during folliculogenesis, ovulation, and luteal function.

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