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. Anecic 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 cells 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

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 of 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; Erikson 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; Mura et al., 2001; Ks et al., 2003), and the inhibition of cytokine-
Materials and methods

Bovine ovaries (n=25) were collected from slaughtered bovine for immunohistochemistry (IHC) of follicles. The ovaries were fixated in ice-cold Earl's balanced-salt solution (Sigma Chemical Company, St Louis, MO, USA) and transported to the laboratory, sliced into thin slices and fixed in in situ paraffin carbaryl 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 μm) was measured using an ocular micrometer, and the follicles were classified into the following categories: healthy, antral active, or basal atretic, as previously described (Ivins et al., 2003). 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 Velzen et al., 1999) or 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 (Ivins et al., 2003). Tissue sections (10 μm) were cut and mounted on poly-L-lysine coated slides, dried at 37°C, rehydrated in ethanol, and air-dried. Sections were then baked at 4°C for 60 min. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution for 60 min. Non-specific binding was blocked with a 5% fetal bovine serum solution in PBS. Slides were incubated with a 1:100 dilution of anti-cytochrome c oxidase antibody (CYP17, to detect steroidogenic, thecal cells) at a dilution of 1:1,000 (Cox et al., 1985). Secondary antibody (rabbit antimouse Affiniti streptavidin-alkaline phosphatase) was used to visualize the primary antibody. The slides were stained with fast red substrate (Sigma Chemical Company, St Louis, MO, USA) and counterstained with hematoxylin.

Flow cytometry of ovarian cells

Flow cytometric analysis of ovarian cells was used to quantify cytochrome c staining in CL throughout the luteal phase relative to the incidence of apoptosis. Detection of CL was achieved using a FITC-conjugated anti-cytochrome c antibody (clone CY-90, Sigma, whereas apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay with the Apoptag kit (Phoenix Flow Systems Inc., San Diego, CA, USA). An Alexafluor-647 labeled fluorescein PRB-1 antibody was used to identify cycling cells (days 10-12). Controls consisted of exclusion of the enzyme in the labeling reaction. Ovarian cells from CL of cows during the estrous cycle (days 1-5) and from cows undergoing a luteolytic stimulus at 80% estrus (days 10-12) were examined for ovulation by transvaginal ultrasonographic scanning. With Day 0 corresponding to the day of ovulation, three different cows were examined for ovulation on Days 5-6 (developing CL), Days 10-12 (mature CL) and Day 18 (regressing CL) of the estrous cycle were removed (n=3 cows/stage). A population rich CL was assessed in OCT compound using liquid nitrogen and isopentane, and then prepared as frozen tissue sections (thickness, 6.8 μm).

Immunohistochemistry of copepea lutea

Methods for IHC staining of frozen tissues sections of bovine CL have been described and validated previously in the laboratory (Towson et al., 2002). Briefly, frozen tissue sections were air dried, fixed in acetone for 10 min at 4°C, and transferred to 0.3% H2O2 in methanol at 4°C to quench endogenous peroxidase activity. Sections were rinsed 3x3 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 3x3 min in PBS-1% BSA prior to incubating with mouse anti-human cytokeratin-18 monoclonal antibody (clone 18-10, 10 μg/ml), normal horse serum, 2% normal horse serum, and PBS-1% BSA overnight at 4°C. The next day the slides were rinsed three times in PBS-1% BSA, and incubated at 37°C for 30 min with biotinylated anti-mouse IgG (VectorNovacarta Laboratories, Burlingame, CA, USA) and then rinsed three times in PBS-1% BSA, washed in normal horse serum and 2% normal bovine serum in PBS-0.1% BSA. Amplification of the antigen-antibody complex was achieved using avidin-biotin complex (ABC kit, VectorNovacarta Laboratories) for 30 min at 37°C. Detection occurred by precipitating 3-amino-9-ethylcarbazole (ABC, Vector/Novacarta 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/Novacarta Laboratories). All incubations were done in a humidified chamber. Similar to above, non-specific 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 cytochrome c staining in CL throughout the luteal phase relative to the incidence of apoptosis. Detection of CL was achieved using a FITC-conjugated anti-cytochrome c antibody (clone CY-90, Sigma, whereas apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay with the Apoptag kit (Phoenix Flow Systems Inc., San Diego, CA, USA). An Alexafluor-647 labeled fluorescein PRB-1 antibody was used to identify cycling cells (days 10-12). Controls consisted of exclusion of the enzyme in the labeling reaction. Ovarian cells from CL of cows during the estrous cycle (days 1-5) and from cows undergoing a luteolytic stimulus at 80% estrus (days 10-12) were examined for ovulation by transvaginal ultrasonographic scanning. With Day 0 corresponding to the day of ovulation, three different cows were examined for ovulation on Days 5-6 (developing CL), Days 10-12 (mature CL) and Day 18 (regressing CL) of the estrous cycle were removed (n=3 cows/stage). A population rich CL was assessed in OCT compound using liquid nitrogen and isopentane, and then prepared as frozen tissue sections (thickness, 6.8 μm).
Cytokeratin 8/18 filaments in the bovine ovary.

Blot analysis. Briefly, the snap-frozen tissue was homogenized in 1.5 ml ice-cold 2% sodium dodecyl sulfate buffer (0.1 M Tris-Cl, pH 8.0, 177 mM NaCl, 2% 10% mercaptoethanol, 10% glycerol, and 1% Triton X-100) with 10 µl proteinase 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 microtube, 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 microtube. 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 4°C with shaking. Then, 50 µl of pre-washed homogenous Protein G was added to the sample followed by incubation at 4°C for an additional hour. The sample was centrifuged at 12,000rpm for 30 sec, the supernatant was carefully removed, and the remaining pellet (i.e., containing the precipitated agaro-antibody-antigen complex) was resuspended and washed with 0.5 ml 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 of 0.1 M Tris-HCl. The proteins were eluted from the beads by heating the suspension to 95°C for 10 min. For a negative control, 0.1 µl (2 µg) of non-specific mouse IgG was used in place of the monoclonal anti-CXK18 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 immuno-staining by flow cytometry 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 corpora lutea

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/layer 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, 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. 1H).

Similar to observations of growing follicles, CK18-positive cells in ovulatory follicles (Fig. 2A) were distributed throughout the granulosal/layer, 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. 3I).

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 endoplasmic reticulum 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. Hematoxylin and eosin stained serial section is that shown in B. Lamina (red staining in B) is localized to the follicular basal lamina. G. CK18 is localized to the cytoplasm of granulosal cells in a growing follicle (red staining). D, E. Serial sections of an atretic follicle stained with hematoxylin and eosin (D) and localization of CK18 (green) and lamin (red) (E). F. CK18 (green) is localized to the basal granulosal cells and some thecal cells. Red staining is cytokeratin α34βc (green) (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 IgG and normal rabbit serum (I) was substituted for the primary antibodies. Arrows indicate the position of the follicular basal lamina. B, C, 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 3-6 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 1-3 to Day 18 CL, respectively, Fig. 6).

Co-precipitation of CK18 with CK18 in developing corpora lutea

Immunohistochemical staining of the bovine ovary revealed that the developing CL (i.e., Days 3-6 of the

**Fig. 2. Immunostaining of CK18 and cytokeratine 8/18 (CYP17) in a postovulatory follicle (A-E). A. Haematoxylin and eosin stained serial section to that shown in B, C. Haematoxylin and eosin stained serial section to that shown in B. D. C. Numerous CK18+ (green) cells distributed throughout the granulosa layer (g), immuno-stained CYP17 staining (red) in the theca interna layer (Ti), E. Higher magnification of theca-granulosa layer interface depicting immunostaining of CK18 (D), CYP17 (E), and co-localization of CK18 and CYP17 (F), respectively (note colored arrows; cells in G 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. C. CK18+ cells in atretic layers of early antral follicles (green in B, red in C). D. In advanced antral follicles, CK18+ cells (green in D) and E, F. Cytodeath (red staining) in the same follicle as in F, H. Haematoxylin and eosin stained serial section to those shown in I and J. G. Corresponding Cytodeath staining, J. Cytodeath staining (J, red cells) and Caspase 3 staining (J, red cells) in an atretic follicle. Arrows indicate the position of the follicular hilar lamina. B, C, G, I, J. Co-stained with DAPI. Scale bars: A, B, 50 µm; C-J, 20 µm.**
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Fig. 4. Immunostaining of CK18 in corpus luteum (A-D). A. Abundant CK18 cells (red staining) in a developing corpus luteum (PL) of cells depicted by arrows. B. Decreased CK18 expression in mature (PL) and regressing (Day 18) bovine CL, cells depicted by arrows and regressing corpus luteum (PL) cells depicted by arrows. D. Negative control (no primary antibody). Hematoxylin counterstained. Scale bars: 50 μm.

Fig. 5. Flow cytometric analysis of CK18 immunostaining on mixed luteal cells from developing (Days 5-12), mature (Days 10-12), and regressing (Day 18) bovine CL. The bar graph depicts the percentage of CK18+ cells among the three stages of the luteal phase (bars denoted by different letters are different, P<0.05, n=2-5 CL/age). The flow cytometric scatter plots depict representative results for the three stages of the luteal phase. The scatter plot is shown on the Y-axis; a right-shift along the X-axis indicates an increase in the percentage 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, a marker of cell death). Percentage of CK18- and TUNEL+ cells relative to controls is shown on the Y-axis, respectively. Note the higher percentage of CK18+TUNEL+ cells in Day 18 CL (16.2%) compared to Days 5-6 CL (3.1%).

Fig. 7. Representative western blot of CK8 in tissue lysate of a developing (Day 5-6) bovine CL following immunoprecipitation with anti-CK8 antibody. Lane one, detection of CK8 in total lysate prior to immunoprecipitation is expected. Lane two, detection of the CK8-40 complex formed following immunoprecipitation with a non-specific monoclonal IgG is shown, in lane three, the relative enrichment of CK8 following immunoprecipitation with the monoclonal, anti-CK8 antibody is shown. The molecular weight of CK8 is 52 kDa.

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 granulosa and luteal cells to apoptosis. Our results indicate expression of CK8/18 intermediate filaments within granulosa 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 granulosa cells during follicular atresia and luteal cells during luteal regression is similarly selective, affecting only discrete populations of cells (Sakamaki et al., 1997; Kim et al., 1998; Quirk et al., 2000; Taniguchi et al., 2002). Here we have found CK18 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 granulosa cells
undergo apoptosis during the initial stages of atresia. Not surprisingly, as atresia continues the number of granulosa cells that undergo apoptosis continues to increase, presumably extending to the most peripheral cells of the follicle. In contrast to cytochrome-induced apoptosis, however, the regulation of cell death in granulosa cells is evidently driven primarily at the level of ligand expression. Both healthy and atretic follicles, for instance, express cytokines such as Fas and TNF-a (Ghaffarifar et al., 2003). Indeed, recent changes in Fas receptor recognition cannot adequately account for the specificity of apoptosis of individual cells because Fas ligand and its receptor are present on healthy and atretic granulosa cells suggesting that a more complex regulation of apoptosis is taking place in the ovarian follicle (Porter et al., 2001). One possibility is that cytokine receptor expression on the surface of granulosa cells is intrinsically regulated, hence influencing the susceptibility of individual cells to Fas ligand and other cytokines. In the current study, CK18 expression in healthy growing follicles was confined to the granulosa one the basal lamina. These cells are also among the most resistant to apoptotic cell death. Our observations of CK expression in granulosa cells of growing follicles is consistent with previous studies (Czernobilsky et al., 1983, Santini et al., 1993) but at odds with one study in which CK immunoreactivity was not detected in pregranulosa or granulosa cells of primary and large follicles (van der Hurk et al., 1995). Such discrepancies might reflect differences in the extent of CK expression as they may be a consequence of differences in tissue fixation methods, specificity of the antibodies used, and/or immunohistochemical methods.

The observation that discrete populations of granulosa cells within atretic follicles contain CK18 filaments or collagen I filaments suggests that these cells are undergoing cell death (as detected by the CytoDEATH antibody) suggests CK18 expression alone is not the sole determinant of sensitivity to apoptosis. The CytoDEATH staining observed in atretic follicles is particularly noteworthy because this antibody recognizes a portion of the CK18 (i.e., cleaved cytokeratin) described previously as associated with apoptotic cells (Chiu et al., 2001; Dandiale et al., 2004; Ueda et al., 2004; Ueda et al., 2005). However, our results suggest CK18-containing granulosa cells also eventually become vulnerable to apoptosis, especially during advanced stages of atresia, and the additional factors contribute to this process. Indeed, cell cycle progression (Quirk et al., 2006) and coloncinic-acid-sensitive, labile protein(s) (Porter et al., 2001) have been implicated as factors influencing granulosa cell susceptibility to apoptosis. In non-atretic cells, CK18 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., 1998), and by modulating the expression of cell death domain proteins (Gilbert et al., 2008). However, whether or not these or other mechanisms are related to CK filament expression within granulosa 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 CK18 filament expression diminishes over time, with the most prominent reduction in expression occurring during the late luteal phase. The decline of CK18+ luteal cells in the current investigation coincided with an increase in apoptosis, suggesting the loss of CK18 filament intermediates may predispose the cells to death. However, direct manipulation of CK18 filament expression in the New Hampshire Agricultural Experiment Station (the author wishes to thank A. Cherry, Jennifer J. Fortin, and Amy R. Liptak for their contributions to this manuscript) using the Conroy for the demonstration of CYP17 antibody, and Wendy Bonnar for immunohistochemistry of follicles.

References

Comparative study of testis structure, function, and thyroid hormone levels in control C57BL/6 mice and anti-mullerian hormone over expressing mice

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Summary. Anti-Mullerian hormone (AMH) is considered as a negative regulator of postnatal Leydig cell (LC) differentiation, because AMH over expressing mice (mi-hAMH mice) tests are deficient in LC. Therefore, in the present study Mt-hAMH mice was used as a model to examine the process of postnatal LC differentiation. Tissues structure-function studies were performed in age-matching Mt-hAMH and C57BL/6 (controls) mice to test whether components were quantified and circulating testosterone and thyroid hormone levels

Key words: AMH, Leydig Cells, Hypothyroidism, Testosterone

Introduction

Testosterone, primarily secreted by the Leydig cells in the testis, is important for general health and reproduction of the adult mammalian male. Therefore, postnatal differentiation of Leydig cells in the developing testis is an important process to the adult male to establish his adult Leydig cell population in the testis. Stem cells for the adult population of Leydig cells in the postnatal testis are shown to be the peritubular mesenchymal cells by many previous investigations (Roosen-Runge and Anderson, 1959; Lording and de Kreter, 1972; Mendis-Handagama et al., 1987) and studies from our laboratory have confirmed this fact for prepubertal (Ariyaratne et al., 2000a-c) as well as the adult rat (Ariyaratne et al., 2000d) using immunocytochemical labeling studies for 3β-hydroxy steroid dehydrogenase enzyme.

In the process of postnatal differentiation of Leydig cells, five cell stages have been identified (Mendis-Handagama and Ariyaratne, 2001). The mesenchymal cells surrounding the seminiferous tubules/peritubular mesenchymal cells are the stem cells; they are non-