Differential expression of acidic cytokeratins 18 and 19 during sexual differentiation of the rat gonad

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This work is dedicated to the memory of Professor Alfred Jost, deceased on February 3, 1991
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Summary
The expression of cytokeratins (CKs) 8, 18 and 19 was analyzed in male and female rat gonads from the undifferentiated stage (12.5 days of gestation) until two weeks after birth by indirect immunofluorescence, using specific monoclonal antibodies anti-CK 8 (LE41), anti-CK 19 (LP2K) and anti-CK 18 (LE65 and RGE53). In the undifferentiated blastema, the somatic cells were stained for CK 8 and CK 19, whereas no detectable immunoreactivity for CK 18 was obtained. The same staining CK pattern was observed in ovaries, in the somatic cells of ovigerous cords and in primary follicles. The staining was progressively decreasing in growing follicles after one week after birth. At the onset of testicular differentiation, when the first Sertoli cells differentiate in the gonad of 13.5-day old male fetuses, positive staining for CK 18 became evident, in addition to CK 8 and CK 19 expression. In the following days, CK 8, CK 18 and CK 19 were detected in Sertoli cells in the differentiating seminiferous cords, but progressively the reactivity for CK 19 decreased and was no longer observed after 18.5-19.5 days of gestation. In all cases, CKs were found to be coexpressed with vimentin, and germ cells were negative for both vimentin and CKs. The results reported here show first, that CKs are expressed before sexual differentiation in gonadal blastema in which no epithelial organization is observed, and second, that there is a CK 18/CK 19 shift in expression during morphogenesis of the testis which is not observed in the differentiating ovary. Future studies will have to determine whether these differences in CK expression are due to epitope-masking phenomena or to the regulation of CK synthesis.

Key words: cytokeratins, testis, ovary, fetus.

Introduction
Cytokeratins (CKs), the largest class of intermediate filament (IF) proteins, represent a multigene family comprising at least 20 different polypeptides in human, grouped into type I (acidic, CK 9 to CK 20) and type II (basic or neutral, CK 1 to CK 8) subfamilies according to immunological and biochemical criteria (Franke et al., 1981; Moll et al., 1982, 1990). The expression of at least one CK of each type is necessary for the formation of the heterotypic tetrameric structure which is the basic unit of CK IFs (Franke et al., 1981; Moll et al., 1982, 1990). The expression of at least one CK of each type is necessary for the formation of the heterotypic tetrameric structure which is the basic unit of CK IFs (Franke et al., 1981; Moll et al., 1982, 1990). Depending on the epithelia, different combinations of CK polypeptides have been observed, such that CK patterns appear to be specific to given epithelial tissues (Franke et al., 1981; Moll et al., 1982, 1990). During embryogenesis, CKs are the first IF proteins detected (Jackson et al., 1980). In the mouse, they have been described before implantation, as early as the 4- to 8-cell stage (reviewed by Lehtonen et al., 1988). Later, they are present in ectoderm and endoderm. Vimentin is the subsequent IF protein expressed in mesodermal cells differentiating on day 8 in the mouse (Franke et al., 1982). In addition to the general acceptance of tissue specificity in the expression of IF proteins (Osborn and Weber, 1982), it has been hypothesized that CKs could be used as a marker for tissues derived from ectoderm or endoderm and vimentin as a marker of mesodermal origin (reviewed by Viebahn et al., 1988). Observations of atypical IF expression in early embryos, such as CKs in mesenchyme and vimentin in epithelia (Lane et al., 1983; Lehtonen et al., 1983; Erickson et al., 1987; Viebahn et al., 1988; Page, 1989), have cast doubt upon this hypothesis and suggest that the expression of CKs and vimentin may not be correlated to either germ layer derivation or epithelio-mesenchymal organization (Erickson et al., 1987; Viebahn et al., 1988; Page, 1989), but rather to a functional and differentiative state of the cell (Viebahn et al., 1988). An example of differential expression of IF proteins according to the stage of development is furnished by mammalian Sertoli cells. In adult testis, Sertoli cells contain IF of vimentin type and neither CKs nor...
desmosomes (Franke et al., 1979). They were, thus, identified as epithelial cells deriving from mesenchymal cells (Franke et al., 1979). During fetal and early neonatal life, Sertoli cells coexpress vimentin and CKs 8 and 18 (Paranko et al., 1986; Stosiek et al., 1990a). The expression of CKs has been interpreted as indicating an epithelial origin of Sertoli cells (Paranko et al., 1986). The disappearance of CKs occurring two weeks after birth in the rat, when important developmental modifications take place, has suggested the existence of a relationship between testicular maturation and IF expression (Paranko et al., 1986). Reexpression of CKs has been observed in human senile and atrophic testes (Stosiek et al., 1990a) and in rat adult Sertoli cells when they are cultured in vitro (Guillou et al., 1990).

Differentiation of Sertoli cells in the fetal gonad is the first morphological evidence of testicular differentiation (Jost, 1972; Magre and Jost, 1980). By their progressive adhesion to each other, Sertoli cells determine the formation of epithelial structures, the seminiferous cords (Jost, 1972; Magre and Jost, 1980). In the present work, we have investigated the presence of CKs in the rat gonad before Sertoli cell differentiation and compared the CK patterns of expression in male and female gonads during fetal life and up until two weeks after birth. The immunohistochemical results reported here show that CKs are present in undifferentiated gonad and that acidic CKs 18 and 19 are differentially expressed according to the developmental stage of the testis and the sex of the gonad.

Materials and methods

Animals

Wistar CF rats (stock maintained by the CNRS, colony of R. Janvier, 53680 Le Genest, France) were used. Gonads were dissected from males and females from 12.5 days of gestation up to two weeks after birth. The age and sex of the fetuses were determined as described by Jost (1972).

Immunohistochemicals

Primary cytokeratin antibodies were murine monoclonal antibodies. The antibodies anti-cytokeratin 8, LE41 (Lane, 1982), anti-cytokeratin 18, LE65 (Lane, 1982) and anti-cytokeratin 19, LP2K (Stasiak et al. 1989) were either kindly provided by Dr E. B. Lane or purchased from Amersham (respectively RPN 1166, RPN 1160 and RPN 1165). Anti-cytokeratin 18, RGE53 (Ramaekers et al., 1983) was obtained from Immunochemicals (respectively RPN 1166, RPN 1160 and RPN 1165). Anti-cytokeratin 18, LE65 (Lane, 1982) and anti-cytokeratin 19, LP2K (Stasiak et al. 1989) were either kindly provided by Dr E. B. Lane or purchased from Amersham (respectively RPN 1166, RPN 1160 and RPN 1165). Anti-cytokeratin 18, RGE53 (Ramaekers et al., 1983) was obtained from Immunochemicals (respectively RPN 1166, RPN 1160 and RPN 1165). Anti-cytokeratin 18, LE65 (Lane, 1982) and anti-cytokeratin 19, LP2K (Stasiak et al. 1989) were either kindly provided by Dr E. B. Lane or purchased from Amersham (respectively RPN 1166, RPN 1160 and RPN 1165). Anti-cytokeratin 18, LE65 (Lane, 1982) and anti-cytokeratin 19, LP2K (Stasiak et al. 1989) were either kindly provided by Dr E. B. Lane or purchased from Amersham (respectively RPN 1166, RPN 1160 and RPN 1165).

Monoclonal antibody to vimentin (clone V9) (Osborn et al., 1984) was purchased from Boehringer Mannheim (No. 1017390).

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Antiserum to human plasma fibronecint and to mouse laminin raised in rabbits were obtained from Bethesda Research Laboratories (No. 6071 and No. 6265 respectively).

Secondary biotinylated antibodies were either anti-mouse (Amersham RPN 1001) or anti-rabbit (Amersham RPN 1004) immunoglobulins.

For immunohistochemistry, fluorescein-streptavidin was used (Amersham, RPN 1232). For immunoblotting, alkaline phosphatase-streptavidin was used (Amersham, RPN 1234).

Immunohistochemistry

Tissues were either immediately frozen at -20°C in embedding medium (Tissue-Tek II, OCT compound, Miles Laboratories) or fixed with paraformaldehyde, 4% in PBS, before freezing. Frozen sections of 5 μm thickness were cut with a cryostat Bright Instrument. The indirect immunofluorescence technique with amplification by the biotin-streptavidin system was used. Incubation with primary antibody (see dilutions in Table 1) for 1 hour was followed by several washes in PBS. Sections were then exposed sequentially to secondary biotinylated antibody (used at 1/200 dilution) for 30 minutes and, after repeated washings in PBS, to fluorescein isothiocyanate-conjugated streptavidin used at 1/100 dilution for 15 minutes. Specimens were mounted in PBS, 10% v/v, glycerol, 9% v/v, with 1% paraphenylenediamine (Johnson and Nogueira-Araujo, 1981) and examined under a Zeiss microscope equipped for epifluorescence.

Serial frozen sections were immunostained for vimentin (dilution: 1/4) or for extracellular matrix components fibronectin (dilution: 1/400) and laminin (dilution: 1/1000).

Negative controls included omission of either primary or secondary antibodies and replacement with PBS buffer.

Electron microscopy

When necessary the structure of the gonad was analyzed by electron microscopy. Tissues were fixed by either 2% paraformaldehyde, 0.5% glutaraldehyde or 1% glutaraldehyde and post-fixed in 1% osmium tetroxide as described by Magre and Jost (1980). Thin sections (1 μm thickness) were cut with an LKB ultramicrotome and stained with methylene blue and safranine (Muny et al., 1970). Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philips EM 300 electron microscope under 80 kV.

Two-dimensional gel electrophoresis and immunoblotting

Testes were dissected free of tunica albuginea and ovaries free of mesogenital tissue from either 19.5- or 20.5-day old fetuses. Cytoskeletal fractions were extracted as described by Franke et al. (1981) and Achtstaetter et al. (1986) by three successive centrifugations in salt buffers for 10 minutes at 4000 g. The pellets were solubilized in equal volumes of lysis buffers I and II (Franke et al., 1981; Achtstaetter et al., 1986). The first dimension of the gel electrophoresis was performed in a non-equilibrium pH gradient, NEPHGE (O'Farrell et al., 1977), and the second one, using the discontinuous system of Laemmli (1970), in SDS-polyacrylamide (10% acrylamide) gel according to the modifications described by Gosselin et al. (1989).

Separated polypeptides were either stained with Coomassie blue or transferred onto nitrocellulose sheets, pore size 0.45 μm (No. BA 85, Schleicher and Schuell) according to the method of Towbin et al. (1979). After incubation for 1 hour at room temperature in the blocking solution, TBS (Tris-NaCl buffer) containing 5% skim-milk, the nitrocellulose sheets were first exposed to cytokeratin monoclonal antibody (diluted in TBS supplemented with 0.5% skim-milk and 0.2% Tween 20) overnight at 4°C and then to biotinylated secondary antibody and streptavidin-alkaline phosphatase. Alkaline phosphatase activity was detected with substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.38 mM) (Sigma) and nitro blue tetrazolium (NBT, 0.4 mM) (Sigma) in
alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂). The anti-CK 8 antibody (LE41) and the anti-CK 19 antibody (LP2K) were used at a dilution of 1/5, and the anti-CK 18 antibodies (LE65 and RGE53) were used undiluted.

Moll’s international reference (Moll et al., 1982) was used to characterize the cytokeratins on the two-dimensional electrophoretic gels.

Results

Immunohistochemistry
Positive reactions with antibody LE41 were obtained on sections from both unfixed tissues and tissues fixed by paraformaldehyde (Table 1). For the three other CK monospecific antibodies (RGE53, LE65 and LP2K),

Fig. 1. Male undifferentiated gonads from 13.5-day old fetuses. Optical micrograph of a semithin section (A) and electron micrograph (B). In the caudal part of the genital tract, the as yet undifferentiated gonad (Go) appears as a blastema at the surface of the mesonephros (A). Germ cells (g) are identified by their large and roundish nuclei. Somatic cells are irregular in shape and not organized in epithelial structures (B). No basement membrane is seen in the gonad (B). W, Wolffian duct; mt, mesonephric tubules. A, x180; B, x4400.
positive reactions were observed only on unfixed tissues (Table 1). The broad-range CK antibody lu-5 stained both fixed and unfixed tissue (Table 1) with, however, differences in the results observed from fixed and unfixed tissues (see below).

**Undifferentiated gonad**

As previously shown (Jost, 1972; Magre and Jost, 1980), at 12.5 days of gestation or at 13.5 days in the caudal part of the genital tract, male gonads were morphologically indistinguishable from female gonads. Gonadal primordia differentiated on the inner face of the mesonephros (Fig. 1A) and appeared as a blastema in which the somatic cells seemed to be identical (Fig. 1B). No epithelialization was observed (Fig. 1). No continuous basement membrane was visible either in electron microscopy (Magre and Jost, 1980) (Fig. 1B) or immunofluorescence studies (Agelopoulou and Magre, 1987) (Fig. 2A). Laminin (Fig. 2A) and fibronectin were distributed evenly between the cells (Agelopoulou and Magre, 1987). Immunohistochemical detection of CKs in gonadal blastema showed positive reactions with both anti-CK 8 antibody (Fig. 2B, F) and anti-CK 19 antibody (Fig. 2H) but not with anti-CK 18 antibodies (RGE53 or LE65) (Fig. 2G for LE65). The broad-range CK antibody stained gonadal blastema on sections of unfixed tissue (Fig. 2D), whereas it gave very few, if any, deposits on sections of fixed tissues (Fig. 2C). At this stage, the Wolffian duct was positive with anti-CK 8 (Fig. 2B, F), anti-CK 18 (Fig. 2G) and broad-range CK (Fig. 2C, D) antibodies but not with anti-CK 19 antibody (Fig. 2H). Mesonephric tubules were negative with all anti-CK antibodies tested (Fig. 2B-D, F-H). Observation of sections of fixed tissues showed that, among gonadal cells, only somatic cells were positive for CK whereas germ cells were negative (Fig. 2B). The staining of serial sections with an anti-vimentin antibody (V9) gave a positive reaction in all cells of the gonad, except for germ cells, as well as in mesenchymal and epithelial cells of the mesonephros (Fig. 2E).

**Differentiating testis**

At 13.5 days of gestation, at the onset of testicular differentiation, Sertoli cell aggregation determined the formation of differentiating seminiferous cords (Jost, 1972; Magre and Jost, 1980) which were negative for fibronectin (Fig. 3A) and laminin. A positive reaction with the two anti-CK 18 antibodies (Fig. 3C for LE65) appeared exclusively inside the differentiating seminiferous cords, whereas immunoreactivity for CK 8 (Fig. 3B) and CK 19 (Fig. 3D) was found, at this stage, inside the cords as well as in the as yet undifferentiated area of the gonad. On and after day 14.5 of gestation, the seminiferous cords became well-delineated (Fig. 4). They were outlined by a continuous basement membrane (Fig. 4) positive for fibronectin (Figs 4A, 7A) and laminin. Sertoli cells were polarized, displaying their nucleus and most of their cytoplasm at the periphery of the seminiferous cords (Fig. 4B). Germ cells tended to occupy the center of the cords (Fig. 4B). In early stages of testis differentiation, up to 16.5-17.5 days of gestation, all monospecific antibodies used, namely LE41 (Fig. 5A), LE65 (Fig. 5B), RGE53 (Fig. 6A) and LP2K (Fig. 5C), stained Sertoli cells. The broad-range CK antibody lu-5 gave a positive reaction in Sertoli cells on both unfixed and fixed tissue (Fig. 6B, C). As testicular differentiation progressed, CK 19 reactivity disappeared (Fig. 7C) and, from 18.5-19.5 days of gestation, Sertoli cells were stained only with broad-range CK antibody 8 (Fig. 7C) or 18 (Fig. 7D) antibodies but not with anti-CK 19 antibody (H). With all anti-CK antibodies used, mesonephric tubules (mt) are negative. ×180.

![Fig. 2. Male undifferentiated gonads. Immunofluorescence photographs of frozen sections in the caudal part of genital tracts from 13.5-day old fetuses. (A-C, E) 4% paraformaldehyde-fixed tissue; (D, F-H) unfixed tissue; (F-H) are serial sections. In the undifferentiated gonad (Go) no continuous basement membrane is observed, laminin is expressed evenly between gonadal cells (A). Laminin is absent from the mesenchymal tissue of the mesonephros and present under the surface epithelium (e) and in basement membrane around Wolffian duct (W) and mesonephric tubules (mt) (A). Anti-CK 8 stains positively somatic cells of the gonad (B, F). Germ cells (g) are negative (B). Note that CK 8 is visible in surface epithelium (e) in unfixed (F) but not in fixed tissues (B). The broad-range CK antibody (lu-5) gives a positive reaction in gonadal blastema on sections of unfixed tissue (D) but not on sections of fixed tissue (C). Somatic cells of the gonad and mesonephric cells are positive for vimentin (E). In sections of unfixed tissue, CK 19 (H) but no CK 18 (LE65) (G) is detected in the gonad. Wolffian ducts are stained with anti-CK 8 (B, F), the broad-range CK (C, D) and anti-CK 18 (G) antibodies, but not with anti-CK 19 antibody (H). With all anti-CK antibodies used, mesonephric tubules (mt) are negative. ×180.](image)

**Table 1. Cytokeratin monoclonal antibodies and experimental conditions used during immunofluorescence study**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Specificity</th>
<th>Dilution</th>
<th>4% paraformaldehyde-fixed tissue</th>
<th>Unfixed tissue</th>
</tr>
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<tr>
<td>LE41</td>
<td>CK 8</td>
<td>1/5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LE65</td>
<td>CK 18</td>
<td>undiluted</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>RGE53</td>
<td>CK 18</td>
<td>1/5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>LP2K</td>
<td>CK 19</td>
<td>1/5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>lu-5</td>
<td>broad-range</td>
<td>1/5</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Positive reaction.
- Negative reaction.

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Fig. 3. Male (A-D) and female (E-H) differentiating gonads. Immunofluorescence photographs of frozen sections of unfixed tissues in the cranial part of gonads from 13.5-day old fetuses. In male gonads, differentiating seminiferous cords (SC) appear as fibronectin-negative structures (A) which show positive reactions with anti-CK 8 (B), anti-CK 18 (LE65) (C) and anti-CK 19 (D) antibodies. Outside the seminiferous cords (SC), cells are negative for anti-CK 18 (C). Most of these cells, in particular under the surface epithelium, are positive for CK 8 (B) and for CK 19 (D). A-D are serial sections. In females, the gonadal blastema (Go) which does not show epithelial organization is stained for fibronectin (E), CK 8 (F) and CK 19 (H). No significant reactivity is observed with anti-CK 18 antibody (LE65) (G). Wolffian ducts (W) are positive with anti-CK 8 antibody (B, F) and 18 (C, G) antibodies, and negative with anti-CK 19 (D, H) antibody. Mesonephric tubules (mt) are negative with the three anti-CK antibodies. ×180.

range CK antibody, anti-CK 8 antibody and anti-CK 18 antibodies (Fig. 7B for RGE53). The reaction was strongly positive in the basal part of Sertoli cells. Lateral cytoplasmic processes extending to the center of the cord between germ cells were immunostained more faintly (see Figs 5A, 7B). As previously shown (Paranko et al., 1986), no CKs were detected in Sertoli cells two weeks after birth.

In all developmental stages of the gonads, Sertoli cells and other somatic cells were stained with anti-vimentin antibody, and the germ cells were negative for cytokeratin and vimentin. From 14.5 days on, the Wolffian duct, mesonephric tubules and rete testis were positive for all anti-CK antibodies tested.

Differentiating ovary

At 13.5 days of gestation, the female gonad had the same appearance in the upper and lower parts of the genital tract. Neither epithelial organization nor any continuous basement membrane was visible. Fibronectin (Fig. 3E) and laminin deposits were seen throughout the gonad. Anti-CK 8 (Fig. 3F) and anti-CK 19 (Fig. 3H) antibodies stained the gonadal blastema, but no significant reactivity was observed with anti-CK 18 antibodies (LE65) (G). In contrast, the two anti-CK 18 monoclonal antibodies, whereas growing follicles, which were positive with these two antibodies one week after birth, became negative two weeks after birth (Fig. 9D).

Regardless of the developmental stage, all gonadal cells except germ cells, were stained for vimentin. From 14.5 days on, Müllerian duct, regressing Wolffian duct and rete ovarii were positive for all anti-CK antibodies used.

Immunohistochemistry

Two-dimensional immunoblotting was performed on gonadal cytoskeletal preparations from both 19.5- and 20.5-day old male and female fetuses. These stages were chosen since, judging from the immunohistochemical results, the pattern of acidic CK expression was clearly different in each sex. Two-dimensional immunoblotting performed on male (Fig. 10A) and female fetal gonadal cytoskeletal preparations showed that LE41 (anti-CK 8) reacted with two specific isoforms of CK 8 polypeptide. LP2K (anti-CK 19) reacted only on ovary cytoskeletal preparations, and the spot of reactivity confirmed its specificity (Fig. 10B). Anti-CK 18 antibodies (LE65 and RGE53) never gave positive reactions regardless of the sex, not even under overloaded gel conditions (up to 40 tests).

On two-dimensional electrophoresis gels stained by Coomassie blue, it was impossible to visualize the presence of CKs. Vimentin, however, was observed (not shown).

Discussion

In agreement with previous studies on the rat (Paranko et al., 1986) and on the human (Stosiek et al., 1990a), our immunohistochemical results show the presence of both CK 8 and CK 18 as well as vimentin in fetal and neonatal Sertoli cells. The presence of CK 8 was confirmed by immunoblotting with LE41 antibody which, characterized originally on human material (Lane, 1982), reacted in our model with a single CK of characteristic 2D-gel electrophoretic coordinates corresponding to that of the human CK 8 protein (Moll et al., 1982). In contrast, the two anti-CK 18 monoclonal antibodies LE65 and RGE53 did not give positive reactions in immunoblotting. The epitopes detected by these two antibodies are perhaps not preserved. It is known that LE65 reacts moderately in immunoblotting (Lane, personal communication). However, it is possible that the amount of CK 18 in fetal testicular tissue is insufficient to permit the detection. CK 19 expression, in addition to CKs 8 and 18, was observed in Sertoli cells in early stages of testicular differentiation, up to 16.5-17.5 days of gestation. CK 19 was also detected in undifferentiated gonads and in ovaries up to two weeks after birth. The specificity of LP2K antibody, previously characterized in human tissue (Stasiak et al., 1990), was confirmed by immunoblotting in rat fetal
Fig. 4. Testes from 15.5-day old fetuses. Immunofluorescence detection of fibronectin on frozen section of 4% paraformaldehyde-fixed tissue (A) and electron micrograph (B). Seminiferous cords (SC) are outlined by a basement membrane positive for fibronectin (A) and visible in electron microscopy (B, arrows). They are formed by Sertoli cells with clear cytoplasm and irregular nucleus and germ cells (g) with dark cytoplasm and roundish nucleus. Outside the seminiferous cords, mesenchymal tissue is formed by small dark cells. A, x150; B, x2600.

ovaries. In undifferentiated gonads and differentiating ovaries, no significant staining was observed with the two anti-CK 18 antibodies. As these antibodies failed to give results in immunoblotting, we cannot be certain of the absence of CK 18. The epitopes are perhaps masked in tissue sections. It might be hypothesized that, during Sertoli cell differentiation, CK 18 epitopes are modified and thus become detectable. Another example of differences in the CK expression patterns according to the developmental stage and the sex of the gonad was observed in the reactivity of the broad-range antibody lu-5 on fixed tissues. A positive reaction was obtained in
Fig. 5. Male (A-C) and female (D-F) gonads from 15.5-day old fetuses. Immunofluorescence photographs of frozen sections of unfixed tissue. Inside the seminiferous cords (SC), Sertoli cells show a positive reaction with anti-CK 8 (A), anti-CK 18 (LE65) (B) and anti-CK 19 (C) antibodies. Germ cells in the center of the cords are negative. A-C are serial sections. Female gonads show an intense staining with anti-CK 8 (D) and anti-CK 19 (F) antibodies. The anti-CK 18 antibody (LE65) does not stain gonadal cells significantly (E) but gives a positive reaction in the mesonephric tubules (mt). x180.

Sertoli cells, whereas no significant staining was observed in undifferentiated blastema and ovaries. Lu-5, however, stained gonads of each sex on unfixed tissue. Although the conformation-dependent CK antibody Lu-5 has been described as resistant to treatment with formaldehyde (Franke et al., 1987) it cannot be ruled out that, in our model, the fixation procedure induces destruction or masking of the specific epitope.

It may thus be assumed that, as already mentioned for the reactivity of CK 18 antibodies, differentiation of Sertoli cells provokes a change in the conformation of CKs which renders the lu-5 epitope detectable despite fixation. On the other hand, the synthesis of a new CK whose conformation would permit the lu-5 epitope to be accessible in fixed tissues may well be suggested. This hypothesis cannot be investigated as the lu-5
Fig. 6. Male (A-C) and female (D-F) gonads from 15.5-day old fetuses. Immunofluorescence photographs of frozen sections of unfixed tissue (A, B, D, E) and of 4% paraformaldehyde-fixed tissue (C, F). Sertoli cells are stained by the anti-CK 18 antibody RGE53 (A) and the broad-range CK antibody (lu-5) (B, C). Note that the lu-5 reactivity is more intense in unfixed (B) than in fixed tissues (C). In female, the anti-CK 18 antibody RGE53 (D) stains the Wolffian duct (W) but does not show significant reactivity in the gonad (Go). The broad-range CK antibody gives an intense positive reaction in the gonad on unfixed tissue (E), and very few deposits on fixed tissue (F). ×180.

epitope is not reactive after SDS-PAGE (Franke et al., 1987). The use of other broad-range and specific CK antibodies should improve our analysis.

The expression of CKs in ovigerous cords has already been described in the rat fetal ovary (Fröjdman et al., 1989), yet no precision regarding the CK polypeptide types was mentioned. In granulosa cells of the human adult ovary, both CKs 8 and 18, as well as the presence of desmosomes, are observed (Czernobilsky et al., 1985; van Niekerk et al., 1991). On the contrary, in the rat adult ovary (Czernobilsky et al., 1985) granulosa cells do not express CKs, and intercellular junctions are of the adherens type. Our results show that CKs 8 and 19 are detected first in primary and growing follicles but progressively disappear in granulosa cells of growing follicles. This study will have to be carried out on the
Fig. 7. Male (A–C) and female (D–F) gonads from 18.5-day old fetuses. Immunofluorescence photographs of frozen sections of 4% paraformaldehyde-fixed tissue (A) and unfixed tissue (B–F). Seminiferous cords (SC) and ovigerous cords (OC) appear as structures negative for fibronectin (A and D, respectively). In the male gonad, Sertoli cells show a strong reactivity with the anti-CK 18 antibody (RGE53) (B) but very few, if any, deposits with the anti-CK 19 antibody (C). On the contrary, anti-CK 19 (F) but not anti-CK 18 (RGE53) (E) antibodies stain the somatic cells of ovigerous cords.

Regardless of the sex of the gonad, outside the cords the tissue is positive for fibronectin and negative for CKs. Epithelium (e) at the surface of albuginea (alb) is stained strongly for CK 19 (C) and very weakly for CK 18 (B). ×180.

adult rat ovary in order to investigate if CK expression continues to be observed in primary follicles or is not at all detected, as reported by Czernobilsky et al. (1985). CKs were found in the gonadal blastema before sexual differentiation occurs. Somatic cells that are not organized in epithelial structures are stained for CKs and vimentin, whereas mesenchymal cells of the mesonephros are stained only for vimentin. This expression of CKs recalls the unexpected locations of CKs reported in non-epithelial structures during early embryogenesis (Erickson et al., 1987; Page, 1989; Viebahn et al., 1988). It has been suggested that such an expression in mesenchymal cells could be a feature of an undifferentiated mesenchyme in which cell mi-
Fig. 8. Female gonads from 15.5-day old fetuses. Immunodetection of fibronectin on a frozen section of 4% paraformaldehyde-fixed tissue (A) and electron micrograph (B). Cellular nests negative for fibronectin (A) are differentiating into the blastema. They are formed by germ cells (g) and somatic cells (B). Outside these nests, the mesenchymal tissue positive for fibronectin (A) is composed of elongated cells (B). A, ×150; B, ×2600.

Grations occur (Viebahn et al., 1988). This expression could also indicate the initiation of differentiative events towards an "epithelialization" (Page, 1989). In the undifferentiated gonads, these two suggestions can be taken into account, since germ cells are still migrating at this stage and since an epithelial morphogenesis, i.e. seminiferous or ovigerous cord formation, will take place.

The first CK proteins present during embryonic life are CK 8 and CK 18 (Jackson et al., 1980; Läne et al., 1983; Lehtonen et al., 1983), which are generally regarded as the characteristic CK pair expressed in
simple epithelia (Franke et al., 1981; Moll et al., 1982). In the first stages of gonadal differentiation and in ovaries, the acidic CK detected was CK 19 rather than CK 18. The acidic CK 19, found in both simple and stratified epithelia (Moll et al., 1982; Stasiak et al., 1989), is the smallest CK with no functional C-terminal, nonhelical domain (Bader et al., 1986). It has been suggested that CK 19 may be expressed in order to redress an unbalanced production of acidic and basic CKs (Savtchenko et al., 1988; Bader and Franke, 1990).

In most tissues analysed in this study, there seems to be an adjustment between expression of the two acidic CKs 18 and 19. Thus, CK 19 but no CK 18 was observed in the undifferentiated gonads and ovary. In contrast, CK 18 but no CK 19 was found in Wolffian duct at 12.5-13.5 days of gestation and in Sertoli cells in the prenatal and postnatal periods. In some cases, simultaneous expression of CKs 18 and 19 was noticed as in rete testis, rete ovarii and differentiating Sertoli cells between 13.5 and 16.5-17.5 days of gestation. In the

Fig. 9. Postnatal ovaries from 7- (A-C) and 15- (D) day old females. Immunofluorescence detection of fibronectin (A) and CK 8 (B-D) on frozen sections of unfixed tissue. A and B are serial sections. Primary follicles (pf) and growing follicles (gf) are negative for fibronectin (A). A strong reactivity for CK 8 is observed in primary follicles (B-D). In growing follicles, the reactivity is weak at 7 days after birth (B, C) and is no longer observed at 15 days after birth (D). ov, oviduct. A, B, ×80; C, D, ×200.
Fig. 10. Analysis of CKs present in 19.5-day old male (A) and female (B) gonads by two-dimensional immunoblotting. Cytoskeletal proteins were separated by NEPHGE in the first dimension, followed by SDS-PAGE. (A) Immunodetection of two isoelectric variants of CK 8 with antibody LE41; (B) immunodetection of CK 19 with antibody LP2K. The positions of vimentin (V) and reference proteins, actin (Ac), bovine serum albumin (BSA), phosphoglucom kinase (PGK), were visualized by Ponceau S staining of nitrocellulose sheets before immunodetection of cytokeratins.

In the latter case, it is possible that expression of CK 19 is a "residual" expression from somatic cells differentiating in Sertoli cells. Presence of CK 19 has been associated with either early stages of differentiation (Stasiak et al., 1989; Stosiek et al., 1990b) or a labile state of differentiation (Stasiak et al., 1989). It has been suggested that CK 19 could behave as a "neutral CK in terms of differentiation" (Stasiak et al., 1989); for instance, in progenitor cells of stratified epithelia, CK 19 would polymerize with type II CK pending the synthesis of the tissue-specific type I CK (Stasiak et al., 1989). Does CK 19 play this role of "switch" CK (Stasiak et al., 1989) during sexual differentiation of the gonads? It seems that this might be possible during testis differentiation, since CK 19 disappears progressively while CK 18 begins to be expressed simultaneously with the differentiation of Sertoli cells. Concerning the ovary, no such shift in the expression of CKs has been observed. The present study must be extended to analyze latter stages and to look for expression of other CK polypeptides. Nevertheless, even for the ovary, the presence of CK 19 might be correlated to a certain labile state of differentiation. Indeed, ovaries of 14.5-day old fetuses (but not of 20.5-day old fetuses (Vigier et al., 1987)) are able to undergo modifications in the sense of a morphological masculinization if they are cultured in vitro, either in the presence of the fetal testicular hormone AMH (Vigier et al., 1987) or in the presence of fetal testes (Charpentier and Magre, 1990). Preliminary results have shown that masculinization is accompanied by changes in CK expression; the CK broad-range lu-5 antibody stains positively masculinized ovaries and does not stain control ovaries (Charpentier and Magre, 1990). It remains yet to be verified if this difference in the staining by lu-5 antibody is equivalent to the changing CK expression pattern observed during the testicular differentiation.

The results of the present study show the existence of a differential immunofluorescence expression of CK 18 and CK 19 according to the developmental stage and the sex of the gonad. The first step of further studies on the mechanisms responsible for such a differential expression will be to determine whether the immunofluorescence negative results observed (i.e., the absence of CK 18 in the ovary and the progressive disappearance of CK 19 in the testis) may be due to epitope-masking phenomena according to the differentiation state of the gonad or to the real absence of the proteins. In the latter case, the level(s) of regulation (transcriptional, post-transcriptional or post-translational) will have to be determined.

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References


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