Structural proteins in sexual differentiation of embryonic gonads

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ABSTRACT. Sexual differentiation of embryonic gonads was studied by immunocytochemical analysis of cytoskeleton, basement membrane and extracellular matrix. The epithelial cells of the prospective gonadal region in both sexes contained vimentin and desmin intermediate filament proteins but not cytokeratin. Basement membrane components laminin, collagen types IV and V, heparan sulfate proteoglycan, and fibronectin were seen in an unorganized form in the extracellular space. The development of the gonads started by proliferation of the pregonadal epithelial cells, which formed separate clusters and loose mesenchyme. In the male gonad the clusters joined together into elongated cords, outlined by basement membrane components. The cord cells became polarized epithelial cells, and cytokeratin appeared with the disappearance of desmin in their cytoplasm. Desmin and vimentin remained in the interstitial cells. In the female gonad the clusters were smaller, and the cords were irregular in shape and size. Desmin disappeared from the cord cells and cytokeratin appeared, but more slowly and less well polarized than in the testis. The present results show that after common early development, the sexual differences in gonads emerge as different organization of the internal epithelial tissue with different timing of changes in intra- and extracellular components.

KEY WORDS. *Embryo, testis, ovary, differentiation, protein*

Introduction

Individuals of different sexes have different genetic constitutions from the moment of conception, but differences are not manifest in the phenotype in the early stages of development. Sexual differences emerge in successive steps, where the previous step provides the regulatory signals for the development of the next step. The first step in this sequence is gonadal differentiation, which is guided by genetic factors (Fig. 1). Gonadal hormones, then, regulate the further development of reproductive and other organ systems, and subsequently sexual behavior and reproductive functions. The details of the regulatory mechanisms in this developmental chain have been largely unknown, but it is obvious that sexual differentiation of the gonad into testis or ovary is a crucial step (Jost *et al.*, 1973; Pelliniemi and Dym, 1980). We have therefore concentrated our research on gonadal differentiation and related embryonic events.

Our approach has been to study the cell and tissue biological processes in the differentiation of the epithelial cords, interstitial cells, and extracellular matrix in the testis and ovary of rat, pig, and human embryos and fetuses. For this review we have chosen our studies of intermediate filament proteins: vimentin, desmin and different subtypes of cytokeratin as indicators of cytoplasmic differentiation in rat embryonic gonads. The matrix components studied are laminin, collagen types IV and V, heparan sulfate proteoglycan, and fibronectin, which are associated with basement membranes, and collagen types I and III as overall matrix proteins. The methods used in this endeavor are conventional and immunocytochemical light and electron microscopy together with protein analysis and morphometry.

Extracellular and cytoskeletal proteins in differentiating rat gonads

The pregonadal stage

The somatic components of the gonads derive from the mesonephros (Fig. 1), and the germ cells migrate to the gonad from the yolk sac endoderm via the mesentery. The most prominent structures in the pregonadal mesonephros are the mesonephric duct and tubuli. The proximal ends of the tubuli are in the vicinity of the ventral mesonephric surface epithelium, and the mesenchyme in that region is very scanty. The simple surface epithelium in the prospective gonadal region of the mesonephros is devoid of a proper basement membrane although basement membrane components are found by immunocytochemistry and electron microscopy in the subepithelial mesonephric mesenchyme. Transient reactions for cytokeratins, vimentin and desmin are seen in the different compartments of the mesonephros, but the prospective gonadal region contains only desmin and vimentin immediately before the onset of gonadal differentiation (Fröjdman *et al.*, 1988).

The development of the indifferent gonad

The development of the gonads starts with a proliferative thickening of the surface epithelium in both sexes at the same time. The epithelium is still devoid of a proper basement membrane, but is underlined by fibronectin (Paranko *et al.*, 1983), laminin, and type I and III collagen (Paranko, 1987). The extracellular matrix components are reorganized according to the cells which form the transient tissue of the gonadal blastema (Pelliniemi and Dym, 1980). During this early phase of gonadal development the number of blastema cells increases and clus-

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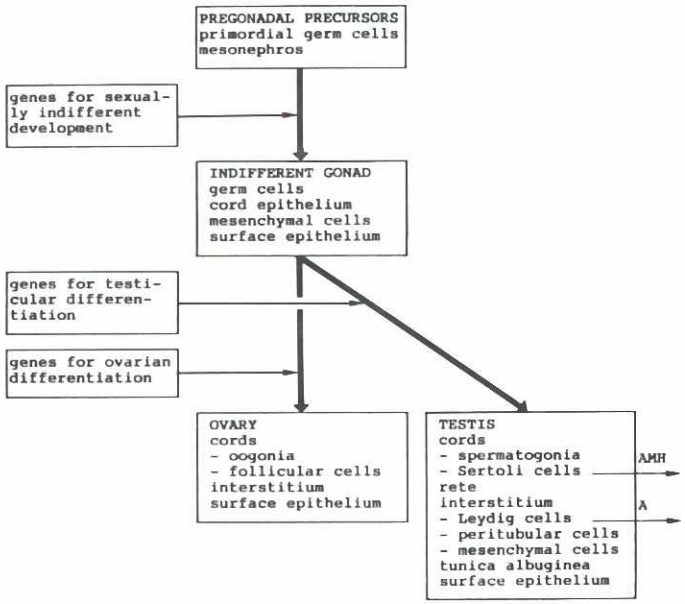

Fig. 1. *Regulatory factors (thin arrows) acting at successive stages in early embryonic differentiation of rat gonads (thick arrows), and the development of the different gonadal components as described in the text. The male genetic factor/s triggers the differentiation of the gonad into a testis, which by male hormones (A, AMH) masculinizes the extragonadal organs. In the absence of the proposed male factor/s an ovary and other female organs develop according to their own program. A, androgens; AMH, anti-Müllerian hormone.*

ters of closely associated cells appear in the vicinity of, and in continuity with, the surface epithelium. The reactions for fibronectin (Paranko *et al.*, 1983) and laminin (Pelliniemi *et al.*, 1984) are negative inside these cell clusters in the gonads of both sexes. The clusters become surrounded by a layer of basement membrane components. Vimentin and desmin are found in the cells of the developing gonad and cytokeratin appears now for the first time in the cytoplasm of the cluster cells in both sexes as a sign of epithelial cell differentiation (Fröjdman *et al.*, 1986, 1988). Following steps in gonadal development bring out histologically, ultrastructurally and immunocytochemically detectable differences between male and female gonads.

Testis

In the male gonad the cell clusters (Paranko *et al.*, 1983) grow and make contact with each other and form elongated cords (Fröjdman *et al.*, 1988) which also include the germ cells. At the same time the cords become surrounded by a thin layer of extracellular matrix proteins: collagen type III (Paranko, 1987), IV and V, laminin (Fig. 2), fibronectin and heparan sulfate proteoglycan (Pelliniemi *et al.*, 1984). Subepithelial accumulations of collagen fibers and a differentiating basement membrane are also observed by electron microscopy. The formation of the cords is accompanied by disappearance of desmin from the somatic cord, or Sertoli cells, and by a sex-specific distribution of their cytokeratin (Fig. 3) and vimentin (Fröjdman *et al.*, 1988). Vimentin- and desmin-containing cells remain, however, in the interstitium throughout the fetal period. Accumulations of cytokeratin and Vimentin are found in the basal cytoplasm of the Sertoli cells in the periphery of the cords (Fröjdman *et al.*, 1986; Paranko *et al.*, 1986). The differentiation of the cytokeratin-containing Sertoli cells proceeds in a sex-specific manner, and differences in the expression of the different cytokeratin subclasses are noticed in the cells of the cords and the rete. Cytokeratins appear gradually in the flattened surface epithelial cells and the amount of subepithelial basement membrane components increases. This is accompanied by the formation of the tunica albuginea, which separates the cords from the surface epithelium of the testis. Functional differentiation of the Sertoli cells as producers of the anti-Müllerian hormone (Fig. 1) is demonstrated by the regression of the paramesonephric ducts (Josso *et al.*, 1977; Paranko *et al.*, 1984).

An event concomitant with the formation of the testicular cords is the remarkable growth and differentiation of the interstitium. This includes the differentiation of the prospective myoid cells (Paranko *et al.*, 1982), and the appearance of collagen type I in the interstitium (Paranko, 1987). The interstitium is characterized by the differentiation of Leydig cells and their active androgen production (Fig. 1, Niemi and Ikonen, 1961; Tapanainen *et al.*, 1984). The fetal Leydig cells appear as single cells or in irregularly-outlined groups (Roosen-Runge and Andersson, 1959; Kuopio *et al.*, 1989). We observed that the Leydig cells are surrounded by a laminin and collagen type IV-containing discontinuous basement membrane, which later also surrounded the islet-like Leydig cell clusters. Small patches of basement membrane components are found on early Leydig cell precursors also, which suggests that changes in cell surface take place during the differentiation of the Leydig cells (Kuopio and Pelliniemi, 1988). It is possible that the aggregation and clustering of the Leydig cells are mediated by their cell surface components and associated extracellular matrix. The basement membrane and distinct clustering of the fetal Leydig cells give direct support to the earlier observations of the epithelial nature of the testicular Leydig cells (Hooker, 1970).

Ovary

The main components of the growing ovary are clusters of epithelial and germ cells, interstitium and a surface epithelium. The histological organization resembles first that of the testis at the same age. The cells in the ovarian clusters are also devoid of laminin and fibronectin on their surfaces inside the clusters, which are smaller and more evenly distributed than those in the testis (Paranko *et al.*, 1983). The developing clusters join into an irregular meshwork of cords, (Fig. 4). Although basement membrane components are found around the ovarian cords, the ultrastructurally-detectable basement membrane is less advanced than that of the corresponding testis (Grund and Pelliniemi, 1987). The basement membrane around the cords is interrupted at multiple sites where the cells of the interstitium are in contact with the epithelial cord cells (Paranko, 1987). The disappearance of desmin in the more loosely organized ovarian cords is slower than in the testis, and the polarization and colocalization of vimentin and cytokeratin (Fig. 5) in the somatic cells of the cords is less pronounced (Fröjdman *et al.*, 1988). In addition, a sex-specific reorganization of the cytokeratin subclasses in the cord cells is noticed. The cortical cell clusters and smaller cords of the ovary are still continuous with the cuboidal surface epithelium, and there is no sign of a tunica in the ventral part of the gonad (Paranko *et al.*, 1983; Paranko, 1987). Larger clusters of cord cells occupy the medulla of the

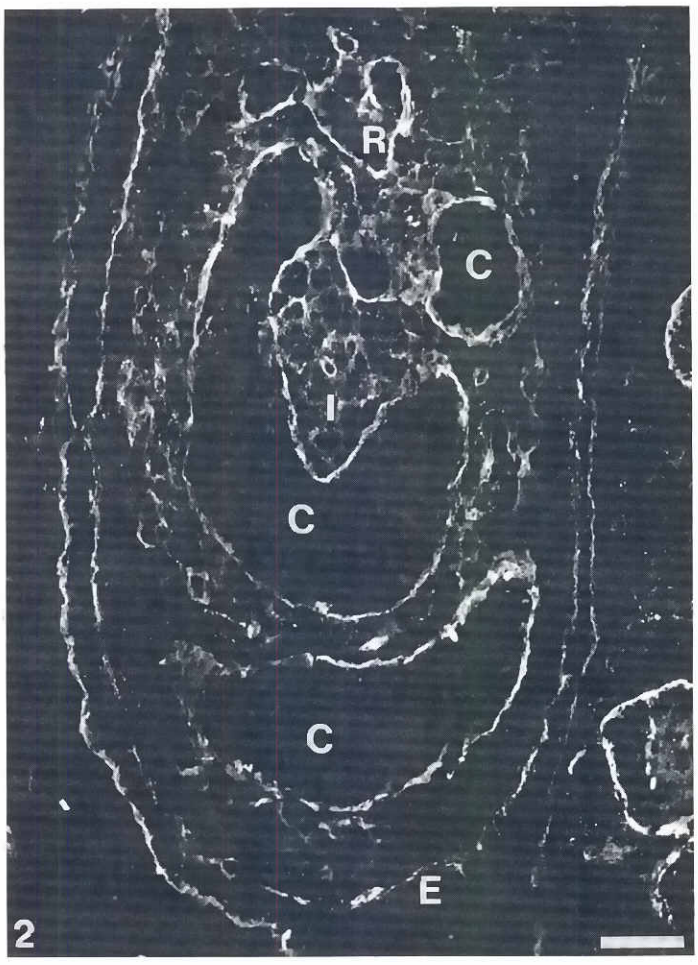

Fig. 2. *Laminin immunofluorescence of the embryonic testis shows reaction around the testicular cords (C), under the surface epithelium (E) and in multiple sites in the interstitium (I). (R) rete. Age 15 days. Scale bar 40 μm.*

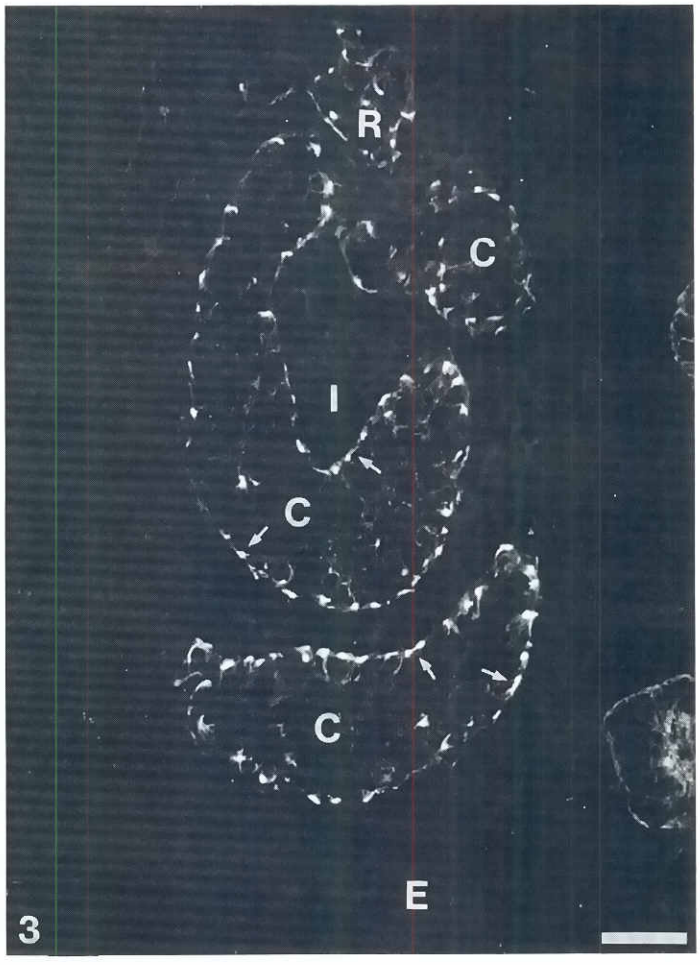

Fig. 3. *Double immnofluorescence of the same section as in Fig. 2 shows cytokeratin in the epithelial cells of the cords (C) and rete (R). Accumulations of cytokeratins are seen in the basal cytoplasm of the basal Sertoli cells (arrows), but also in other parts of the cytoplasm as fine filament bundles. The interstitium (I) is negative for cytokeratins. (E) surface epithelium. Age 15 days. Scale bar 40 μm.*

ovary and no proper rete is found in the ovary before the onset of folliculogenesis.

In the early stage of ovarian differentiation, the interstitium is scarce in contrast to that of the testis, and the lower growth rate of the ovarian interstitium is apparently the main reason for the differences in size between the testis and the ovary. In comparison with the testis, collagen type I appears later in the growing ovarian interstitium, and no special mesenchymal cell differentiation is detected (Paranko, 1987). Both vimentin and desmin are found in the insterstitial cells throughout the embryonic development (Fröjdman *et al.*, 1988).

Concluding remarks

All the intermediate filament antibodies we have used give a similar reaction in the epithelial cell clusters of early gonads in both sexes, and no sex-specific differences are observed by ultrastructural methods. This is in agreement with the present concept of an indifferent gonadal stage (Pelliniemi and Dym, 1980). Sexual differentiation of the gonads is made evident by specific changes in the type and distribution of cytoskeletal and extracellular proteins in and around the epithelial gonadal cords. Accumulation of cytokeratin and vimentin in the basal cytoplasm of the somatic cord cells and the disappearance of desmin in a sex-dependent manner correlates with the epithelial polarization, and with the formation of the basement membrane. The present observations suggest that the expression and distribution of different intermediate filaments are in dynamic interaction with the cell functions, the stage of temporal and sexual differentiation, polarization, and environment.

Much of the effort in the study of the gonadal sexual dimorphism has been focused on the development and formation of the epithelial cords in both sexes. However, an active role for the intestitium in gonadal differentiation has also been suggested (Pelliniemi, 1975). This is supported by Tung *et al.* (1984) who show the importance of the interaction between epithelial Sertoli cells and interstitial myoid cells in testicular cord formation. The present results indicate an important, sex-dependent role for the interstitial cells, not only in their own dif-

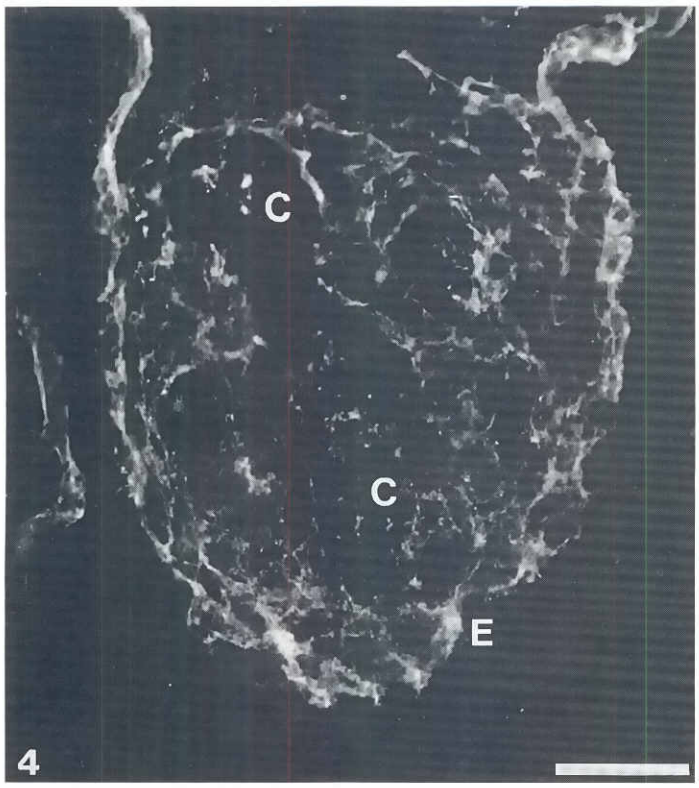

Fig. 4. *Immunocytochemical localization of laminin in the embryonic female ovary shows reaction around the larger medullary cords and smaller cortical cords and cell clusters (C) which in part are continuous with the surface epithelium (E). The scarce interstitium contains laminin. Age 14 days. Scale bar 40 μm.*

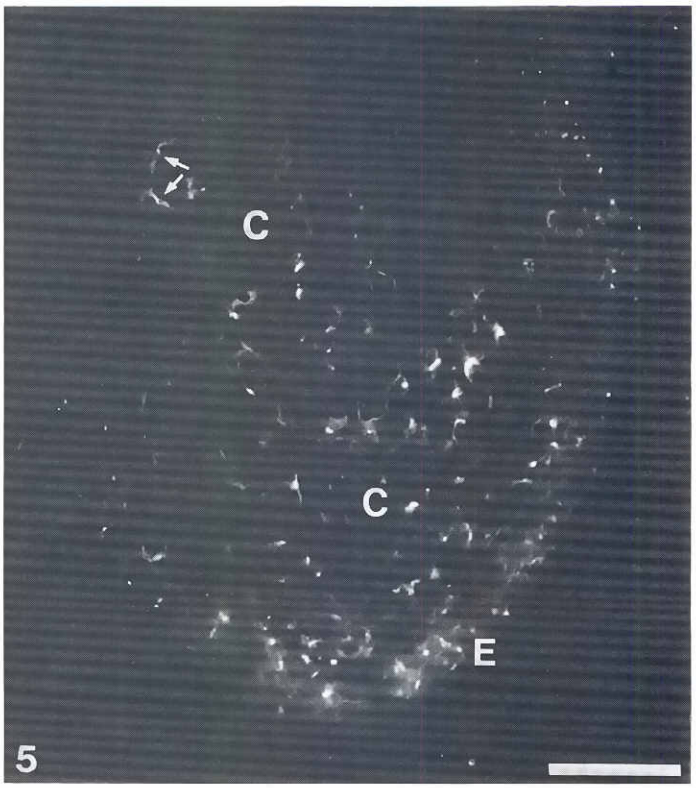

Fig. 5. *Double immunofluorescence of the same section as in Fig. 4 shows cytokeratin in the epithelial cells inside the ovary, and in the surface epithelium (E). The cytokeratin containing cord cells (C) seem to be randomly distributed, but in the larger medullary cords, the peripheral cord cells have basal accumulations of cytokeratins (arrows) like those in the testis. Age 14 days. Scale bar 40 μm.*

ferentiation, but also in the formation of the epithelial cords in the testis and ovary. The differentiation of the gonadal cords seems to proceed, at least in part, in concert with the differentiation of the interstitium, and its regulation most probably involves activation and inhibition of similar (or the same) genes in both males and females, but at different times (Pelliniemi and Lauteala, 1981). One has to emphasize that the embryonic ovarian development is the basic program from which the testicular pathway branches out (Fig. 1) and ovarian development continues under its own regulatory system through epithelial organization and initiation of germ cell meiosis into the folliculogenesis and successive events.

The present immunocytochemical observations of the similar distribution of the structural proteins in the indifferent gonad, and their partial similarities in the later testis and ovary do not imply that their functional organization and molecular constitution are the same in both tissues. We have shown this e.g. for the basement membrane proteins, where the light microscopic reaction is similar in both sexes but there are ultrastructural sex differences in the actual basement membranes. In addition, many antibodies, especially polyclonal, are not able to identify separately all the subclasses of the antigens present in the tissue. Therefore, it is possible that the observed differences e.g. in laminins in the different compartments of the testis and ovary (Leu *et al.*, 1986) are related to the observed differences in the expression of the intermediate filaments during gonadal differentiation (Fröjdman *et al.*, 1988) and to the sexually different organization and timing of basement membrane development.

The analysis of sexual differentiation has traditionally been approached from different disciplinary positions, e.g. morphology (Byskov, 1986), immunology (Ohno, 1980; Wachtel, 1983) and genetics (Page *et al.*, 1987). Today, the increasingly wider possibilities of specific identification and localization of genes and their products calls for a holistic approach and increasing cooperation between scientists from different fields. Against this background one should realize that the differentiation of the reproductive organs is not only a matter of unfolding the sex differences, but also includes the basic events of general genetic regulation of growth and differentiation.

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