Some histochemical and ultrastructural observations on the early foetal pig testis

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SUMMARY

Testes of foetal pigs between 26 to 35 days post coitum (p.c.) were investigated histochemically and ultrastructurally. Diaphorase and Δ3-3β-hydroxysteroid dehydrogenase activities were studied using, respectively, NADH and pregnenolone and dihydroxy androsterone as substrates.

Ultrastructurally, attention was focused on the development of mesenchymal cells and on the sustentacular cells in the primitive sex cords in an attempt to detect the origin of Leydig cells. Histochemically there is a concentration of activity toward the interstitium with increasing age. Also the reactions increase in intensity. Ultrastructurally no evidence for Leydig cell development from Sertoli cells could be observed. Mesenchymal cells between the sex cords show a development toward Leydig cells. This is absent in mesenchymal cells in the future tunica albuginea.

Before 30 days p.c. no ‘true’ Leydig cells can be observed morphologically. The role of the rough endoplasmic reticulum/mitochondrial complex, which is present in many mesenchymal and sustentacular cells, is discussed.

INTRODUCTION

Gonadal development in the male pig has been studied biochemically, histochemically as well as morphologically (Moon & Raeside, 1972; Moon, Hardy & Raeside, 1973; Moon & Hardy, 1973; van Straaten & Wensing, 1978; Raeside & Middleton, 1979; Ford, Christenson & Maurer, 1980; van Vorstenbosch, Colenbrander & Wensing, 1982, 1984a; van Vorstenbosch, Spek, Colenbrander & Wensing, 1984b).

A large part of the foetal period has been investigated thoroughly but there is still a short but physiologically significant period lasting from 28 days until 35 days post coitum (p.c.) that has been largely neglected in morphological studies. During this period the transient testosterone production starts and peaks (Raeside & Sigman, 1975; Raeside & Middleton, 1979; Ford et al. 1980). Testosterone production in the male gonad at the onset of this period is occurring before the Leydig cells appear at day 30 p.c. (Pelliniemi, 1975a,b, 1979).

Key words: testis, pig foetus, histochemical, ultrastructural, Leydig cells.
Although smooth endoplasmic reticulum (SER) is an important organelle normally involved in steroid synthesis (Christensen, 1975), no SER is found in the differentiating cells in the pig gonad up to 27 days p.c. At 35 days p.c. Leydig cells are well developed and contain appreciable amounts of SER. These cells also contain peculiar complexes of mitochondria and long profiles of rough endoplasmic reticulum (RER) which are in close spatial relationship. These complexes are also found in preSertoli cells of 35 days p.c. and wane in both cell types between 52 and 60 days p.c., coinciding with the decline of testosterone production (van Vorstenbosch et al. 1982, 1984a; Ford et al. 1980). They are already present in most cell types of male and female gonads of 27 days p.c. (Pelliniemi, 1975fc).

This study aimed: (1) to determine possible sites of testosterone production based upon the distribution pattern in the testis of 3\(^\beta\)-hydroxysteroid dehydrogenase (3\(^\beta\)-HSD) from 26 to 35 days post coitum, and (2) to compare morphologically the first detectable Leydig cells with those at the peak of testosterone synthesis at 35 days p.c.

**Materials and Methods**

Foetuses of Yorkshire and Dutch Landrace crossbred pigs have been used. The sampling ages were 26, 28, 30 and 35 days p.c. The day of insemination was precisely known and considered as day 0 post coitum. Numbers and ages of the animals used are recorded in Table 1. The method of collecting the foetuses has been described earlier (van Vorstenbosch et al. 1982).

**Histochemistry**

The gonadal ridge area of animals of 26 days p.c. was excised. The entire testes of foetuses of 28 days p.c. were used, while those of animals of 30 or 35 days p.c. were cut into smaller parts. After dissection, materials were frozen in liquid nitrogen and stored at -70°C. Cryostat sections were used for the histochemical reactions.

The NADH diaphorase reaction to detect Leydig cells was carried out as described by Dierichs, Wrobel & Schilling (1973) and as modified by van Straaten et al. (1978). Pregnenolone and dihydroepiandrosterone (DHA) were used as substrates for \(\Delta^3\)-3\(^\beta\)-hydroxysteroid dehydrogenase (3\(^\beta\)-HSD).

The reactions and the estimation of their intensity were carried out as described by van Straaten et al. (1978).

**Electron Microscopy**

The entire gonad or samples of the gonads were taken at ages 26, 28 and 30 days p.c. according to the method described above. The tissue handling was according to the method described earlier (van Vorstenbosch et al. 1982).
Sex determination
From 28 days onward sex can be determined by histological investigation, as sex cord anlagen are visible in the male foetus. At 26 days p.c. sex is determined by karyotyping. The method consists of trypsinization of the mesonephros and limb buds, and the subsequent culture of the isolated cells for one or two days followed by karyotyping (Bosma, Colenbrander & Wensing, 1973).

RESULTS

Histochemistry
The results are shown in Table 2 and Fig. 1.

NADH-diaphorase
At 26 days p.c. NADH showed a weak overall reaction in the gonadal ridge. At 28 days the pattern of distribution of the reaction was still the same, however, the intensity had increased. At 30 days p.c. the pattern had changed; the activity now was clearly limited to the interstitium; in the sex cord anlagen there was no activity at all. At 35 days the pattern was comparable to 30 days p.c. with an increase in the intensity of the reaction.

Δ⁵-3β-hydroxysteroid dehydrogenase
At 26 days p.c. a diffuse 3β-HSD reaction was present all over the gonad with DHA as a substrate. With pregnenolone as a substrate the reaction was negative.

At 28 days p.c., both with DHA and pregnenolone as substrates, the reaction was positive in the area of the cord anlagen. The intensity of the reaction with both substrates was about the same. The localization of this reaction was not as diffuse as the reaction at 26 days p.c.

At 30 days p.c. the reaction after using both substrates was much more intense and located mostly in the interstitium. The reaction with pregnenolone as substrate showed about twice the intensity compared to DHA.

At 35 days p.c. the intensity of the 3β-HSD reaction became less intense with pregnenolone as a substrate in comparison to the reaction at 30 days p.c., but it was clearly located in the interstitium only. Both substrates gave the same intensity of reaction.

Table 2. Results of histochemistry

<table>
<thead>
<tr>
<th>Days post coitum</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH (Diaphorase)</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Pregnenolone (3β-HSD)</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>DHA (3β-HSD)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

The reaction intensity scaled from + (very weak) to ++++ (very intense).
At 26 days p.c. the male as well as the female gonadal anlage showed the same distribution pattern of these reactions. With the method used it is very difficult to pinpoint the exact position until about 35 days p.c. since there seems to be a reaction in both interstitium and cords.

**Electron microscopy**

At 26 and 28 days p.c. no Leydig cells can be observed. In no cells of the gonad of either male or female could any SER be detected. In most cells,
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with the exception of germ cells and mesothelial cells, mitochondria/(R)ough (E)ndoplasmic (R)eticulum complexes can be observed frequently. They consist of a close spatial relationship between RER and mitochondria.

From 28 days p.c. onwards the testis consists of four compartments.

(1) At the periphery a cover of one or some layers of mesothelial cells. This layer does not obviously change between 28 and 30 days p.c.

(2) A peripheral mesenchymal cell layer. At 28 days p.c. this layer is not well developed. At 30 days this compartment is clearly developed and well recognizable as the future tunica albuginea.

(3) The sponge-like sex cord anlage. At 28 days p.c. this anlage is often in very close vicinity to the mesothelial cell layer cover of the testis. At 30 days p.c. the periphery of the anlage has moved centrally.

(4) Spaces within the cord anlage. They will give rise to the future interstitium. It is in this area that at 30 days p.c. Leydig cells can be detected next to the mesenchymal cells and only there. They occur patchwise and still large areas are devoid of them.

The four compartments will be described below at 30 days p.c. If there are differences to 28 days p.c. and 35 days p.c. they will be mentioned explicitly (Fig. 2).

(1) Mesothelial layer

The testis is covered with slightly elongated cuboidal epithelial cells, which are interconnected by tight junction-like structures. Further cell contacts are very loose. Except for a few small projections on the coelomic side, the cell membrane is smooth. Coated pits or signs of pinocytosis are rarely observed. The cells mostly rest upon a well-defined basal lamina. Their nuclei are almost always located in the apical part of the cells. Except for numerous polysomes and free ribosomes their cell organelles are not extensively developed. Among these cells there are many containing lipid-like droplets of various sizes. Mitochondria are slender and long with lamellar cristae. The nuclei are somewhat irregular and do not possess prominent nucleoli.

Especially before 35 days p.c. this layer seems to be very dynamic and frequently cells can be observed that are leaving the layer to migrate downward into the peripheral layer of loose mesenchymal cells. Amongst these migrating cells are not only cells containing lipid-like droplets, but also cells without 'lipid' droplets. We have no clear idea about their fate. After 35 days p.c. the droplet-containing cells are no longer present. (Figs 3A,B, 4A.)

(2) Peripheral mesenchymal layer

The bulk of the mesenchymal cells are slender and have long processes. They often contact each other with short blunt projections showing tight-junction-like connections. The cells often possess a well-developed Golgi apparatus. The cytoplasmic matrix is very lucent. A few long RER profiles, incompletely covered
with ribosomes, can be observed (so-called transitional ER). Mitochondria are mostly elongated and have lamellar cristae. They are not very often in close contact with the RER. (Fig. 4B.)

In this compartment other cell types can be observed, firstly lipid-containing mesothelial-like cells which apparently have migrated from the mesothelial layer. They can be found deep in the mesenchymal layer. Especially before 35 days p.c. it is often difficult to interpret the spatial configuration of the basal lamina of the mesothelial layer. In a number of cases this structure cannot only be found underneath the mesothelial layer, but also underneath mesenchymal cells just

Fig. 2. 28 days p.c. Spaces as shown in the sex cord anlage are the future interstitium. m, mesothelial layer; p, peripheral mesenchymal layer; s, sex cord anlage. ×2500. Bar, 10 μm.
Fig. 3. (A) 28 days p.c. Between normal mesothelial cells (n), cells can be observed containing lysosome-like bodies and lipid droplets. Ribosome-rich cells (r) positioned below the lipid containing cells, can also be found between the mesothelial cells. Basal lamina (arrowheads). ×8000. (B) 28 days p.c. enlargement from Fig. 1A. Note the well-developed Golgi apparatus and the thick bundle of actin-like material (arrow). Note also the discontinuity of the basal lamina under the cell marked (b) (asterisk). Arrowheads under basal lamina. ×15000. Bars, 1 μm.
below this layer. A second cell type can be distinguished which is also mesenchymal but differs from the bulk of the mesenchymal cells in possessing a huge amount of free or clustered ribosomes and short cell projections. (Fig. 3A,B.)

Fig. 4. (A) 28 days p.c. Mesothelial cells. Note the lipid droplet in a mesothelial cell and a cell containing many lipid droplets which is positioned below the normal cell layer. ×8000. (B) 28 days p.c. Normal mesenchymal cells in the peripheral layer. Tight junction-like cell connections (arrowheads). ×15000. Bars, 1 μm.
The sex cord anlage

The sex cord anlage comprises an anastomosing system of cell plates or cords. At 28 days p.c. they are still in close proximity to the mesothelial cell layer, but at 30 days their periphery has clearly moved more centrally. This anlage consists of two cell types: Sertoli cells and germ cells. The latter are spread diffusely through the cord. We did not study their morphology.

The cords are surrounded by a basal lamina, which is sometimes weakly developed. The Sertoli cells (sustentacular cells), are wedge shaped. They are interconnected by loose cell contacts and the width of their intercellular spaces varies, but frequently their cell membranes run more or less parallel. Coated pits are rare. The Golgi apparatus is well developed. The most characteristic feature is the very large number of mitochondria in close vicinity with long RER profiles, which have few ribosomes. The cisterns are always filled with flocculent material. Small RER profiles are seldom seen. Clusters of ribosomes are present. The cell matrix of the majority of the cells is dark. There are no signs of the lipid droplets in the basal compartment of the cell, which are so characteristic of later stages of development. A modest number of membrane-bound electron-dense bodies are present. The nucleus is somewhat irregular and shows no prominent nucleoli. Between 28 and 35 days the organization of these cells does not markedly change. (Fig. 5A,B.)

The interstitium between the developing cords

Inside this interstitial area mesenchymal cells show an electron-dense matrix. The cell membrane is smooth except for some small protrusions. There are two cell types that differ from the cells in the peripheral layer. The first type can be followed morphologically until they have developed into young Leydig cells. These young Leydig cells are intermingled between undifferentiated interstitial cells. The RER profiles are small and transitional but no true SER can be found. Mitochondria are elongated or ovoid. Some tubular cristae can be found. Mitochondrial/RER complexes are frequently present. The nucleus is often ovoid and shows a prominent nucleolus. Gap junctions or epitheloid cell connections are sometimes seen. Inside the cell a well-developed Golgi apparatus can be seen. The second type of mesenchymal cells shows some very long RER profiles in intimate relationship with mitochondria. The cell matrix is rather dense. The cellular organization does not fit the type mentioned before. (Figs 6B, 7.)

In the interstitium differentiated single or clustered Leydig cells are present in patches from 30 days onwards. The clustered cells are epitheloidally connected with some interdigitations and gap junctions. SER in tubular form is abundantly present. Mitochondria have mixed lamellar and tubular cristae, but sometimes only tubular cristae. The cisterns of the SER can be slightly swollen, giving the cells a rather characteristic appearance. The ER is distributed all over the cell. RER/mitochondrial complexes are present but are far less obvious than in most other mesenchymal cells in this compartment. RER long profiles are rather sparse,
but small profiles are common and display mostly the transitional form. Lipid droplets can be observed occasionally. The nucleus is mostly ovoid and possesses a well-developed nucleolus. The cells are mostly surrounded by a basal lamina. (Figs 8, 9.)
Fig. 6. (A) 28 days p.c. Mesenchymal cell from the interstitial compartment between sex cord anlagen. Note the intimate RER mitochondrial relationship. ×18000. (B) 28 days p.c. Mesenchymal cell from the interstitial compartment between the sex cord anlagen; from this form of mesenchymal cells in the interstitium a line can be followed towards Leydig cells. ×18000. Bars, 1 μm.
The main difference between Leydig cells of 30 days p.c. and of 35 days p.c. of age is the increase in number, and an increase in branched tubular SER. (Fig. 10A,B,C.)

Obvious morphological relationships cannot be determined between the Sertoli cells and the Leydig cells. Both cell types show a totally different morphology and intermediate forms are never observed. Indications of Sertoli cells migrating into the interstitium were never observed.

DISCUSSION

Androgen production in the early foetal gonads has been reported in different species such as sheep (Attal, 1969), rabbit (Lipsett & Tullner, 1965; Catt et al. 1975; George, Catt, Neaves & Wilson, 1978), rat (Noumura, Weisz & Lloyd, 1966), pig (Stewart & Raeside, 1976). In the pig, androgen production starts before the appearance of Leydig cells (Moon, Hardy & Raeside, 1973; Ford et al. 1980; Pelliniemi, 1975).

Histochemical investigations of the distribution pattern of 3β-HSD in the early foetal pig gonads (crown–rump–length: 1.5–2 cm) showed a positive reaction in the cord anlagen and a negative reaction in the interstitium for both DHA and pregnenolone as substrates, with a maximum intensity of the reaction even before
the appearance of Leydig cells (Moon & Raeside, 1972). The results of the histochemical study reported in this paper are only partly in agreement with those of Moon & Raeside (1972). 3β-HSD with DHA as a substrate showed a steady increase in intensity, but with pregnenolone as a substrate the reaction peaks at 30 days p.c. Thus before the appearance of Leydig cells, the cord anlage and the interstitium between this anlage shows a positive 3β-HSD reaction. In the sections the precise location of the positive cells could not be identified accurately, but it is remarkable that the RER–mitochondrial complexes which are so evident in both these compartments are hardly present in the cells of the peripheral mesenchymal layer, while in this layer no 3β-HSD activity was noticed.

Fig. 8. 30 days p.c. Young Leydig cells showing an epitheloid arrangement, mitochondria show tubular cristae and small vesicular SER. Note the round nucleus with its prominent nucleolus. ×18000. Bar, 1 μm.
Fig. 9. 30 days p.c. A later stage: young Leydig cell clearly showing well developed SER of the tubular form. The mitochondria contain tubular cristae and a well developed Golgi apparatus can be seen. Circular gap junction (arrowhead). ×18 000. Bar, 1 μm.

The RER-mitochondrial complexes are still present in both Leydig and Sertoli cells at 35 days p.c. Thereafter these complexes vanish in Leydig and Sertoli cells, coinciding with the decrease in testosterone production (van Vorstenbosch et al. 1982, 1984a,b; Ford et al. 1980). It is tempting to suggest that these complexes are the initial sites of testosterone production and that they maintain testosterone secretory capabilities for some time after the appearance of fully differentiated Leydig cells. This could explain the overall reaction in cords and interstitium in the early stage.

Dierichs et al. (1973) and van Straaten et al. (1978) used the NADH-diaphorase reaction as a marker for late foetal and postnatal Leydig cells. We used this reaction to localize Leydig cells at a much earlier age. We did not expect to find...
Fig. 10. (A) 35 days p.c. Leydig cell: abundant SER and many mitochondria with tubular cristae. ×15 000. (B) 35 days p.c. Leydig cell: tubular SER. ×18 000. (C) 35 days p.c. Leydig cell: note the mitochondrial/RER complex. ×18 000. Bars, 1 μm.
any activity before the appearance of Leydig cells. Surprisingly, a positive reaction was already seen at 26 days.

From 28 days Leydig-like cells begin to appear in between the cords and after 30 days fully developed Leydig cells are present. Between 30 and 35 days the increase in the number of Leydig cells coincides with the increasing plasma testosterone concentration (Ford et al. 1980). There is also an increase in SER and a decrease in transitional ER (van Vorstenbosch et al. 1982). We did not find convincing evidence that Leydig cells derive from Sertoli cells as has been suggested by Pelliniemi (1975). The Sertoli cells are already clearly differentiated at 28 days and have a characteristic morphology. They are confined within a basal lamina. Cells ‘breaking through’ this lamina into the interstitium were not observed, making Pelliniemi’s suggestion improbable.

The clear difference in structure between the mesenchymal cells of the peripheral layer and the cord anlage suggests an influence of this anlage on the mesenchymal cells of the latter compartment. This influence might be responsible for the initiation of Leydig cell differentiation.

The lipid-like droplets present in the cells of the mesothelial layer are quite remarkable and apparently are characteristic for the period between 28 and 35 days, as they are not observed earlier (Pelliniemi, 1975) or later.

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