Cytoplasmic filaments in fetal and neonatal pig testis

Camillo J. A. H. V. van Vorstenbosch, Ben Colenbrander, Cees J. G. Wensing
Department of Anatomy, School of Veterinary Medicine, State University of Utrecht/The Netherlands

Frans C. S. Ramackers, G. Peter Vooijs
Department of Pathology, University of Nijmegen/The Netherlands

Received December 15, 1983
Accepted March 12, 1984

Intermediate filament — vimentin — pig Leydig cells

Leydig cells in developing fetal pig testis contained during the fetal regressive phase large accumulations of intermediate filaments. Before and after this period these filaments were arranged in a criss-cross fashion. In the pig as well as in the dog testis these filaments have been characterized as vimentin. Within the vimentin aggregates occasionally a weak positive actin reaction was seen in pig but not in dog Leydig cells. Microfilaments were hardly observed.

Most Sertoli cells contained a layer of actin microfilaments close to the basal cell membrane. In the lower cell compartment and around the nucleus (intermediate) vimentin filaments could be observed in a criss-cross configuration.

Introduction

Cytoplasmic filaments are observed in interstitial (Leydig) cells in the testes of man [7, 12], guinea pig [3], rat [6, 13], dog [32, 5] and pig [1, 2, 9, 37, 38]. Based on their organization and diameters (varying between 5 and 12 nm) they are considered to be actin, actin-like and intermediate filaments. A spatial relationship has been observed between the filaments and the mitochondrial and nuclear outer membranes [1, 6, 18, 37].

In the fetal and neonatal pig Leydig cells huge amounts of cytoplasmic filaments are present which are frequently organized in whirls. The size of these whirls approaches that of a nucleus. They have been observed from approximately 50 days post coitum until shortly after birth after which they decrease in number and size [37, 38]. A similar whirl-like organization of cytoplasmic filaments has been described in Leydig cells of adult dogs [5].

The nature of these filaments in pig Leydig cells is still under discussion. In fetal and neonatal Leydig cells they are described as intermediate filaments [37, 38], in Leydig cells of adults as 7 nm filaments [2], but also as actin-like filaments [1].

Intermediate filaments in vertebrate cells of mesenchymal origin consist specifically of vimentin, while prekeratin-intermediate filaments are restricted to true epithelial cells. The lack of prekeratin and the presence of vimentin in Sertoli cells is indicative for their mesenchymal origin [13].

The aim of this study was to describe the filamentous system in fetal and neonatal pig Leydig and Sertoli cells with and without gonadotrophic stimulation and to investigate their chemical nature. The chemical nature of the filamentous whirls in the adult dog Leydig cells was also investigated.

Materials and methods

Pigs, crossbreds of Yorkshire and Dutch Landrace were used. The age of the animals ranged from 35 days post coitum until one month after birth. Normal and decapitated fetuses were used. Decapitation in utero was carried out at 42 days post coitum according to the method of [31]. The 2 animals used for the immunofluorescence technique were of different litters and 52 days p.c. of age. After caesarian section tests were removed quickly and parts were used for electron microscopy and immunohistochemistry. Data on the number of animals used, sample data and the number of litters are given in Table 1. Dog testis material was derived from a 9-year-old mongrel dog.

### Table 1. Experimental design: the number of animals observed in each group.

<table>
<thead>
<tr>
<th>Age in days (± 1 day)</th>
<th>Post coitum</th>
<th>After birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>55</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>62</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>75</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>80</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>90</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Term</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>12</td>
</tr>
</tbody>
</table>

- Decapitated animals
- Controls
- Normal (+ untreated) animals
- Litter
- 1 Day variation in the sampling date, the age of the animals was precisely known.

1) Dr. Camillo J. A. H. V. van Vorstenbosch, Vakgroep Funktionele Morfologie, Faculteit der Diergeneeskunde, Rijksuniversiteit Utrecht, Yalelaan 1, 3508 TD Utrecht/The Netherlands.
Fig. 1a. Leydig cell 52 days p.c. decapitated pig. — Note the bundles of intermediate filaments which run in different directions. The other organelles are dislocated to the cell periphery. — Bar 1 μm. — 18 500 x. — b. Enlargement of the encadred area in a. — Note cross-sections of transversely cut SER tubules. — Bar 0.1 μm. — 37 000 x.
Electron microscopy

Materials were handled and processed as reported elsewhere [37]. In brief, a double fixation in 2.5% glutaraldehyde followed by 1% OsO4 according to [29] was succeeded by blockstaining in 2% uranylacetate prior to dehydration in graded series of acetone. Materials were cleared in propyleneoxide and embedded in either DER (Dow Chemical) or in Durcupan ACM (Fluka). Semithin sections were stained with either paragon or toluidine blue. Thin sections were stained with lead citrate [35]. A Philips EM 201 G microscope was used at 80 kV with a 0.30 μm objective aperture.

For the estimation of the filament diameters a negatively stained catalase crystal standard was used which showed the 8.12 nm periodicity (Polyscience, Warrington, PA 18976/USA).

Immunohistochemistry

Small, fresh blocks of testis were quickly frozen in liquid nitrogen immediately after removal of the testis and stored at -70°C and again transferred to liquid nitrogen for transport to the laboratory for immunohistochemistry. Thin (4-7 μm) sections were cut on a cryostat and air dried. Until use for the indirect immunofluorescence technique these sections were fixed in cold methanol (-20°C) for 10 min and kept at -40°C. Shortly before incubation with the first antiserum the sections were subsequently dipped in acetone at room temperature, rinsed with phosphate buffered saline, pH 7.4 (PBS) and incubated with the first antiserum for the indirect immunofluorescence technique as described previously [26]. Incubation periods lasted for 45 min at room temperature.

The following antisera were used:

1. A rabbit antiserum directed against human epidermal keratin isolated from foot calluses as essentially described previously [26, 27]. Dilution was 1:5 to 1:20.

2. An antiserum raised against vimentin isolated from calf lenses using preparative gel electrophoresis as described before [26]. For specificity of reactions of vimentin antiserum and immunoblotting, see also [17, 20, 22]. The vimentin antibodies are used in a dilution of 1:10 to 1:20.

3. A rabbit antiserum directed against chicken gizzard muscle desmin, isolated by preparative gel electrophoresis. The preparation of this antiserum has been previously described [28]. The serum was diluted 1:40 to 1:80 for immunofluorescence.

4. A rabbit antiserum directed against denatured chicken gizzard actin, kindly provided by Dr. M. Osborn (Göttingen, W. Germany). This antibody has been described previously [23].

5. A rabbit antiserum directed against bovine muscle actin, kindly provided by Dr. H. Geerzen (Amsterdam, The Netherlands). This antibody was diluted 1:5 in the indirect immunofluorescence assay.

Incubation procedures, use of second antibodies and embedding in the indirect immunofluorescence technique have been described previously [26].

Results

Electron microscopy

In the Leydig cells at 35 days post coitum a rather large amount of criss-cross arranged 8 to 12 nm filaments was observed without a special topographical preference. At 52 days post coitum the number of intermediate filaments had increased dramatically. A great deal of the cytoplasm of Leydig cells was occupied by these filaments (Fig. 1). They were mostly arranged in large thick bundles or in whirls. Often these whirles were composed of several entwined bundles as could be seen in sections through a whirl. It could not be excluded that a whirl consists of one twisted bundle. The filaments are always arranged strictly in parallel. Whirls frequently showed an open center in which cytoplasmic components, such as mitochondria, endoplasmic reticulum or lipid droplets could be observed (Fig. 2). The intermingling of intermediate filaments and smooth endoplasmic reticulum sometimes showed a regular interspacing. Frequently filaments seemed to originate from ribosomes on the outer nuclear membrane or from the outer membrane of mitochondria (Figs. 3a, b). The amount of these filaments in Leydig cells was most remarkable. Other interstitial cells never showed intermediate filaments in such huge quantities.

From 52 days post coitum until birth whirls of intermediate filaments were a constant finding. However from 80 days onwards a new generation of Leydig cells developed most likely originating from the peritubular compartment. In cells of this new generation whirs were found in a much lower frequency. Shortly after birth the number of Leydig cells containing whirs or clusters of intermediate filaments dropped sharply. In these cells again a criss-cross arrangement of intermediate filaments became dominant. The tight packing of an extremely well developed smooth endoplasmic reticulum made the observation difficult. Decapitation did not effect the arrangement of filaments in whirs.

The sexcords differed greatly in appearance between 35 days p.c. and 52 days p.c. but between 52 days p.c. and 30 days post partem they changed little. They remained immature and showed no signs of lumen formation or spermatogenesis. The distribution of the intermediate filaments did not differ throughout the observation period. Intermediate filaments are observed in low frequency in the basal compartment of Sertoli cells. In the middle and apical compartment they were hardly observed at all. If present in the basal compartment they showed a criss-cross configuration and incidentally a weak clustering. Attachments of filaments to nuclear and mitochondrial outer membranes were frequently seen (Figs. 4a, b).

In gonocytes and spermatogonia no intermediate filaments were observed. Anastomosing bundles of microfilaments run parallel to the basal lamina and were so tightly packed that reliable measurements were impossible. Short microfilaments were attached to junctions between gonocytes and Sertoli cells.

Immunocytochemistry

The whirls consisting of intermediate filaments were easily identified as intensely fluorescent dots by fluorescence microscopy in fetal pig and adult dog Leydig cells with antibodies to vimentin (Figs. 4a, d, e). The vimentin-positive dots and the nuclei were in close proximity (Fig. 4e).

In other interstitial cell types in the fetal pig a modest vimentin-positive reaction could be observed, while in the dog this reaction was relatively stronger (Figs. 4a, b). Sertoli cells in the seminiferous tubules of pig and dog showed a weak vimentin-positive reaction (Figs. 4a, b). A strong reaction was observed in the endothelium of blood vessels and in smooth muscle cells of arterial walls. The
myoid cells in the dog testis were also positive for vimentin.

No reaction with keratin antibodies was seen in seminiferous tubules and in the interstitium. A strong reaction, however, was seen in the epithelial cells lining the rete testis.

The only positive reaction to desmin was observed in vascular smooth muscle cells.

Incubation of fetal pig testis with antibodies to actin usually gave a negative reaction in seminiferous tubules and interstitium. In some of the vimentin whips in Leydig cells a faint reaction could be observed. Only a few scattered Sertoli cells showed a weak reaction. Myoid cells showed a strong reaction with the actin antibodies.

Discussion

Pig Leydig cell development in the fetal and neonatal period takes place in two distinct waves: the first wave lasts from approximately 30 until 60 days post coitum with a maximum at 35 days post coitum, the second starts at approximately 70 days and lasts until one month after birth, with a maximum at approximately 10 days post partem.

Fig. 2. Leydig cell control pig 52 days p.c. A large concentric whirl of intermediate filaments, intermingled with SER profiles (asterisk). In the center of the whirl normal mitochondria, an aberrant circular mitochondrion and some RER profiles. — Bar 1 μm. — 20 000 x.
Between the two waves massive Leydig cell regression takes place. The first wave is pituitary independent [37], but the second is clearly under control of pituitary hormones [4, 37]. During the summits a criss-cross arrangement of the intermediate filaments can be observed [9, 24, 36, 37, 38], but during the decline of the first developmental wave huge filamentous whirls develop. From fetal decapitation experiments it is evident that the intermediate filament accumulations are not under pituitary control [37, 38]. Even after cytology the intermediate filaments remain easily identifiable. Similar intermediate filament accumulations have been described in adult dog Leydig cells [5, 32]. The strongly vimentin-positive dots, as observed by fluorescence techniques, correlate well with the electron microscopical data and indicate the vimentin nature of these intermediate filaments.

In most Sertoli cells action-like microfilaments were observed but with the immunofluorescent technique only a weak or negative reaction was noted in some of these cells. The discrepancy between the morphological and immunological observations are probably due to the low packing density of the filaments rather than to the lack of specificity of the serum, since myoid cells in the dog tests reacted very strongly with this serum. The observations on vimentin and actin in Sertoli cells confirm earlier observations [13, 33].

Intermediate filaments of the vimentin type have been observed in many cell types [34], usually uniformly distributed throughout the cytoplasm [16]. Occasionally, they are found in higher concentrations around the nucleus. Especially in cultured cells the wavy pattern of these cytoskeletal structures becomes apparent. Accumulations of intermediate filaments can occur under certain physiological conditions, for example during mitosis [15, 19, 21]. Under certain pathological conditions these intermediate filament accumulations also become prominent [8, 25]. Some drugs and heat shock seem to be able to destroy the integrity of the intermediate filamentous networks either directly or indirectly resulting in an accumulation of the filaments [14, 25]. Similar developments have been described in declining breast carcinomas cell cultures [34].

The accumulation of vimentin-intermediate filaments in declining Leydig cells of the first developmental wave.
seems to be a part of this regressive process. In adult pigs similar effects have been observed after administration of a Zn⁺⁺ deficient diet. Under these conditions the SER in Leydig cells involutes and subsequently large whorls of intermediate filaments develop [18]. The question arises whether the vimentin accumulations in adult dog Leydig cells also have to be considered a sign of a regression process or an expression of a normal functional morphology.

The faint actin reaction in some Leydig cells in fetal pigs in areas with a strong vimentin reaction could mean a colocalization of both actin and vimentin (the latter representing the bulk of the filaments). Such a colocalization has been described in adult dog Leydig cells [32], but it is doubtful if this is a common phenomenon in dog Leydig cells [5].

Although we have indications that vimentin intermediate filament accumulation is correlated to a regressive process in Leydig cell development, the biochemical background of this phenomenon remains to be solved. Whether or not hormonal stimuli can influence cytoskeletal organization in testicular Leydig cells is an intriguing question to be dealt with in future studies.

Acknowledgements. The authors are indebted to Professor Dr. W. A. de Voogd van der Straaten for helpful discussion and critical reading of the manuscript and also to Dr. Vera Baurmann, Dr. A. A. Maedonald and Dr. J. Meyer as locum tenens for a number of necessary sections. The authors are grateful to the following for their personal involvement and the quality of their work: Mr. H. van Dijk, Mr. G. Hol, Mr. J. Nokkert (animal care); Mr. E. Spek (E. M. tissue processing and part of the electron microscopy); Miss Anita Huysmans (immunohistochemistry and fluorography); Mrs. L. Michielsen (typing work); Mr. H. Otter and Mr. O. van der Veen (printing of the electron micrographs), Medical Dept. of Photography of the University of Nijmegen (printing of the fluorographs).

References