The differentiation of mouse gonads \textit{in vitro}: a light and electron microscopical study

SARAH MACKAY and ROBERT A. SMITH

Department of Anatomy, University of Glasgow, Glasgow G12 8QQ, UK

SUMMARY

Indifferent urogenital complexes were excised from mouse foetuses assessed by developmental criteria as day 10-5 or 11. After 4 or 6-7 days in culture, complexes were fixed and examined by light and electron microscopy. The effect of culturing sexed complexes in mixed sex groups was investigated. The effect of the presence or absence of foetal calf serum in the culture medium was considered. No evidence of inhibition of one sex by the other was found. Ovaries developed further in cultures than testes.

INTRODUCTION

\textit{In vitro} culture techniques have recently been used in studies of the differentiation of gonads of mouse (Byskov & Saxén, 1976; Taketo & Koide, 1981; McLaren, 1983) and rat (Stein & Anderson, 1981; Agelopoulou, Magre, Patsavoudi & Jost, 1984). The morphological differentiation \textit{in vitro} of mouse gonads has, however, only been determined previously by light microscopical analysis of $5 \mu m$ paraffin sections, in which structural detail was not adequate for completely satisfactory assessment. Ultrastructural detail is available for the rat ovary, but only for explants removed from day 15 foetuses when morphological differentiation has already occurred (Stein & Anderson, 1981). The present study set out to monitor morphological differentiation by means of epoxy-resin-embedded material so that cultures could be assessed at a more discriminating level using electron microscopy.

Many workers failed to determine the genetic sex of the foetuses from which indifferent complexes were taken (Byskov & Saxén, 1976; Stein & Anderson, 1981; Taketo & Koide, 1981), whilst those who have emphasized the desirability of this simple procedure omitted the precise determination of developmental foetal age, thus grouping together material with potentially as much as a half day gestational age difference, over what is a most critical period of gonadal development (Agelopoulou \textit{et al}. 1984). We believe it is essential to age the foetus accurately by developmental criteria at the time of obtaining the explant in order to assess the subsequent extent of development \textit{in vitro}. Experience has shown that in the strain of mouse used by us (CBA), the chronological age in days \textit{post coitum} and the developmental age may be out of register by 0.5 to 1 day. Our method

Key words: mouse gonads, \textit{in vitro}, urogenital complex, ovary, testis.
therefore included the determination of developmental age of each foetus at the time when the explant was removed. We consider this an important refinement of previous methods since the extent and direction of development \textit{in vitro} can only be assessed accurately from a known developmental stage at the onset of the culture period.

Recently it has been claimed that exogenous serum may exert an inhibitory effect on testicular development in the rat, preventing cord formation yet producing healthier cultures after four days \textit{in vitro} (Agelopoulou \textit{et al.} 1984). This possibility has not been explored in the mouse and so some explants were maintained in medium without serum.

A preliminary report on this study has been made (Mackay & Smith, 1985).

\textbf{MATERIALS AND METHODS}

\textit{Determination of foetal age and sex}

CBA mice from an inbred colony maintained on a light reversal regime were mated between 12.00 noon and 15.00 (at onset of the 9h dark period) and females were checked for vaginal plugs; the day of finding a plug was designated day 0 of pregnancy. Pregnant animals were sacrificed by cervical dislocation on day 10 and day 11 of pregnancy and the uterine horns were dissected. Foetuses enclosed within their membranes were removed to Hanks' buffer and all further dissections were carried out under sterile conditions in a laminar flow cabinet.

The developmental stage of each individual foetus was precisely determined by a method adapted from Theiler (1972). Foetuses were scored with respect to the number of somites and the presence and form of the limb buds (see Table 1). All groups studied were collected on the basis of these staging criteria, thus eliminating error through discrepancies between chronological and developmental age. This ensured uniformity of developmental age of complexes within a group at the onset of the culture experiment. In several experiments foetuses were also sexed by the chromatin test of Farias, Kajii & Gardner (1967). A piece of amnion was stained with 1% acetic orcein and examined for the presence of Barr bodies by one author while the other removed the urogenital complex under a dissecting microscope. The explant consisted of the right and left urogenital complex with a small piece of dorsal mesentery between. Each urogenital complex included the mesonephros and the genital ridge.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Day} & \textbf{Number of somites} & \textbf{Forelimb} & \textbf{Hindlimb} \\
\hline
10 & 30–34 & Present & Hindlimb bud appears \\
10–5 & 35–39 & Forms a projecting appendage & Forms a projecting appendage \\
11 & 40–45 & Handplate forming & Hindlimb bud undivided \\
12 & Over 45 & Handplate with angular contour & Footplate forming \\
14 & & Fingers separate distally & Footplate indented between developing toes \\
15 & & Fingers separate but divergent & Toes separate but divergent \\
17 & & Fingers parallel & Toes parallel \\
\hline
\end{tabular}
\caption{Criteria for determining precise developmental age*}
\end{table}

*Criteria based on Theiler, 1972.
Mouse gonadal differentiation in vitro

**Culturing**

Explants were cleaned free of adjacent tissues and preincubated for 20 min in buffered 0.1-125% collagenase. They were then transferred to the culture medium (Williams E medium supplemented with 2 mM-glutamine, 0.5% gentamicin and 10% foetal calf serum); several complexes were cultured together in each Nunc Petri dish. Some explants were cultured in medium without foetal calf serum.

Cultures were maintained for intervals of 3-4 and 6-7 days at 37°C in 5% CO2/95% air, and their condition was monitored by phase contrast microscopy. Explants became adherent to the bottom of the culture dish within 24 h, except when foetal calf serum was absent from the medium, in which case attachment was poor. Cultures were assessed as unhealthy if necrotic centres were found.

**Fixation, embedding and sectioning**

At the end of the culture periods the complexes were fixed for 30 min in 3% glutaraldehyde in 0.1 M-cacodylate buffer with 3% glucose, washed in buffer and postfixed in 1% osmium tetroxide. They were dehydrated through a graded series of ethanol and embedded in Spurr's epoxy resin. Polymerization was carried out at 70°C. Sections 1 μm thick were cut on a Reichert ultratome and stained with toluidine blue before study by light microscopy. Sample sections were taken throughout the specimen; sections chosen for illustration were representative of the whole. 60-80 nm sections were taken from representative blocks, stained with lead citrate and uranyl acetate and examined on a Jeol JEM 100S electron microscope.

Gonads were removed from control foetuses of determined developmental age (12-17 days) for comparison with cultured material.

**RESULTS**

**Complexes cultured for 3-4 days**

Right and left urogenital complexes were removed from eight foetuses assessed as developmental age 10-5 days. These were not sexed and were cultured in groups of four to a dish. Foetal calf serum was a component of the culture medium. After 3-4 days in culture the material was fixed to assess development by comparison with that in vivo. Of the eight pairs of complexes, two failed to develop, three developed to the indifferent gonad stage, while one developed to the equivalent of each of day 13 ovaries, day 14 ovaries and day 13 testes.

Four pairs of complexes were removed from four foetuses of developmental age 11 days and cultured together, with foetal calf serum as a component of the medium. After 4 days in culture two pairs of complexes showed no development while two had developed to the indifferent gonad stage.

**Complexes cultured for 6-7 days**

When the genetic sex of indifferent complexes was determined before cultures were established, it was found that the sex ratio of differentiated complexes was comparable to that of explants on removal, showing that there was no obvious inhibitory effect of one sex on the other in cocultures.

Sixteen pairs of day 11 complexes were cultured for 6-7 days (see Table 2). Eleven pairs developed to gonads equivalent to those from controls of developmental age day 14 or over, with three pairs of ovaries equivalent to day 17 and a pair of testes reaching a similar stage. Six paired complexes were cultured without
foetal calf serum; four pairs developed to gonads equivalent to controls aged day 14 or over.

Assessment of semithin and ultrathin sections

Fig. 1A shows the excised complex at the time of removal and assessed as developmental age day 11. According to Upadhyay, Luciani & Zamboni (1979), the genital ridge area at this stage comprises the coelomic epithelium, the newly arrived primordial germ cells, and somatic cells contributed from the mesonephric blastema. Cultured complexes were assessed as day 12 equivalents if light microscopy showed the indifferent gonad as a larger compact mass of germ cells and somatic cells covered by a single-layered coelomic epithelium.

Ovarian differentiation

By day 14 the ovary protrudes into the coelomic cavity and comprises large pale germ cells and small darker somatic cells. Fig. 1B shows a control ovary at day 14 for comparison with a day 11 complex after 7 days in culture, shown in Fig. 1C (assessed as equivalent to a day 14 ovary in vivo).

The ovigerous cords appear by day 15 when septa of vascular connective tissue break up the compact mass. Similar cords demarcated by connective tissue elements were seen in cultured gonads assessed as equivalent to day 15 ovaries. By day 17 meiosis is in progress. Some cultured complexes reached this stage (Fig. 2B and Table 2). Electron microscopy of cultured complexes (see Fig. 2C) provides evidence of meiosis: synaptonemal complexes seen as tripartite ribbons in the nuclei of germ cells show that they are in the pachytene stage of the first meiotic prophase. The processes of somatic cells which begin to separate the germ cells of the ovigerous cords as a preliminary to follicle formation are also clearly shown. This stage of mouse ovarian differentiation has not previously been observed in vitro.

Table 2. Number of paired day 11 complexes maintained for 6–7 days in vitro and equivalent stage of development reached at end of culture period

<table>
<thead>
<tr>
<th>Number of complexes</th>
<th>Culture conditions</th>
<th>Indifferent gonad (day 12)</th>
<th>Stage of development reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Indifferent gonad (day 12)</td>
<td>Ovary (days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>16</td>
<td>+FCS</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>-FCS</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Only complexes accurately staged as day 11 at the onset of culture were included. The stage of development reached at the end of the culture period was assessed by comparison to control in vivo criteria (see Table 1).

The foetal calf serum (FCS) concentration where present was 10% in Williams E medium.
Necrotic explants have been omitted from the table: two explants from each group.
Fig. 1. (A) Urogenital complex excised on day 11 showing genital ridge (gr), mesonephric duct (md) and mesonephric blastema (mb). (B) Control ovary on day 14 showing large pale germ cells (arrows) and small dark somatic cells. (C) Complex excised on day 11 and equivalent to day 14 ovary after 7 days in culture. Germ cells arrowed. Bars, 25 μm.
Fig. 2. (A) Control ovary on day 17 showing ovigerous cords (oc). Bar, 25 μm. (B) Ovigerous cords (oc) equivalent to those of day 17 control after 7 days' culture of 11 day complex. Bar, 25 μm. (C) Meiotic oocyte from cultured day 11 complex after 7 days in culture, showing tripartite ribbon (small arrow) of synaptonemal complex in the nucleus. Processes of somatic cells (large arrows) beginning to separate the oocytes. Bar, 1 μm.
**Testicular differentiation**

Fig. 3A illustrates an *in vivo* control testis on day 14 of development, when seminiferous cords form, composed of a mixture of germ cells and somatic cells destined to become Sertoli cells. A similar stage was achieved in culture (Fig. 3B). The alignment of fibroblasts in layers at the edge of the cultured complex probably represents the first step in the formation of a tunica albuginea. From day 15 onwards control testes show the tunica albuginea formed and seminiferous tubules with germ cells towards the centre of the tubule (Fig. 3C). Fig. 3D illustrates similar features formed in culture, showing the tunica albuginea and dark somatic cells partly delimiting the tubules. Electron microscopy of day 17 control testes shows the formation of a basal lamina around the seminiferous tubule, whereas basal lamina formation was not obvious in cultured complexes (Fig. 4A, B). There is no evidence of meiosis in day 17 control testes and no synaptonemal complexes were seen in testes assessed as day 17 equivalents after 7 days in culture together with female explants.

**DISCUSSION**

The culture system developed by Byskov & Saxén (1976) was designed specifically to test the possibility of an inhibitory effect being exerted on gonads of one sex by those of the other *in vitro*. The method allowed gonads of different developmental stages and sexes to be cultured on either side of a nucleopore filter. This system was adopted by Taketo & Koide (1981) although we assumed that these authors were using the filter as a substrate in order to keep the explant at the air–water interphase. Our method differed from that used by these two earlier studies as collagenase treatment was used to allow slight dispersion of the explanted tissue. Fibroblasts were seen to migrate out from the explant and attach to the bottom of the culture dish, thus aiding attachment of the explant and forming a natural bed for its support; nucleopore filters were not necessary, due to the small size of the explant on day 11.

We believe that it is essential to stage the foetus by developmental criteria at the time of explant removal. Previous studies (Taketo & Koide, 1981; Byskov & Saxén, 1976) used chronological age only, and we find that, in the strain of mouse we use, chronological age and developmental age are frequently different. 11 day foetuses were obtained from mice killed on day 11 of pregnancy; only those foetuses assessed as day 11 on developmental criteria were used, inconsistent litter mates were discarded. Some previous studies failed to sex foetuses at the time of explant removal. Agelopoulou *et al.* (1984) sexed foetuses by staining the amnion for Barr bodies but failed accurately to stage foetuses by developmental criteria, relying on a chronological age.

Morphological differentiation was monitored by assessment of plastic-embedded material by semithin and ultrathin sections using both light and electron microscopy. This allowed a more discriminating evaluation of the cultured material. For example, Taketo & Koide (1981) showed functional differentiation
Fig. 3. (A) Seminiferous cords (sc) developing in a day 14 control testis. (B) Complex excised on day 11 and cultured for 7 days showing seminiferous cords (sc) and the beginning of tunica albuginea (ta) formation. (C) Seminiferous tubules from a day 15 control testis with germ cells towards the centre of the tubules. (D) Complex excised on day 11 and equivalent to a day 17 control testis, showing a well-formed tunica albuginea (ta), dark somatic cells delimiting tubules and germ cells towards the centre of the tubules. Bars, 25 μm.
Mouse gonadal differentiation in vitro

Fig. 4. (A) Day 17 control testis showing the formation of the basal lamina (bf) outside the seminiferous tubule. (B) Cultured testis equivalent to day 17 control. The basal lamina is not evident. Bars, 5 μm.

of the developing ovary in culture by acetoorcein staining of meiotic oocytes, but their light micrographs do not allow the anatomical differentiation of the cultured ovary to be assessed. Electron microscopy allows the relationship between differentiating oocytes and somatic cells to be examined. In the present study cultured mouse ovaries were shown, for the first time in vitro, to have achieved the stage of organization and the beginning of the stage of compartmentalization (Upadhyay et al. 1979). The synaptonemal complexes seen by electron microscopy provided evidence that this morphological differentiation was matched by functional differentiation of the cultured ovaries. Stein & Anderson (1981) used electron microscopy in this way to study the development of rat gonads in vitro but report only on ovaries cultured after differentiation has occurred.

The work of Byskov & Saxén (1976) indicated that the differentiation of the female gonad is inhibited when it is cocultured with a testis, and also that 11 day testicular germ cells can be triggered to enter meiosis when cocultured with a day 14 ovary. Byskov & Saxén showed that meiosis could be induced in male gonads not only when filter systems were used that allowed cell contact but also when filter systems were used that prevented it, and also in the case of single gonads of different sexes cultured in the same dish. However, they reported a reduced
expression of this triggering effect in systems where the two sexes were not in close apposition and suggest that there may be a limited radius of activity of the postulated diffusible substance involved. This suggestion could explain the absence of triggering and inhibiting effects in our cultures. In no instances were meiotic stages observed in the testes cultured in the presence of female gonads. Stein & Anderson (1981) found that in the rat the separation of filter pairs by a collagen matrix prevented the expression of diffusible meiosis-regulating substances. Presumably any inhibitory factors present in our system were of such localized effect that they were not expressed. Ovaries developed further in cultures than testes; in the presence of foetal calf serum 60% of ovaries reached day 17 equivalents compared to 17% of testes. McLaren (1983) also found that germ cell survival was better in the ovary than in the testis where preSertoli cells appeared to proliferate at the expense of the germ cells.

Taketo & Koide (1981) used 10% horse serum in their culture medium and showed that it allowed development of seminiferous cords in the mouse. Our results support their findings although we found little evidence of a basement membrane with electron microscopy. Magre (1985) reported electron microscopical observations of rat gonads cultured in vitro, showing that no basement membrane was formed in the presence of serum whereas it was distinct in testes cultured without foetal calf serum. This possible sign of disruption of normal differentiation in the present study warrants further consideration. Chartrain, Magre, Maingourd & Jost (1984) used 15% foetal calf serum as a component of their culture medium and found that in the rat it prevents the differentiation of seminiferous cords.

Our results support those of previous studies by Taketo & Koide (1981) and by McLaren (1983), extending their light microscopical findings with ultrastructural observations. Our method takes account of possible discrepancies between developmental and chronological age to improve the accuracy of estimations of the extent of development in vitro. Further studies are in progress to explore the possibility of a ‘serum effect’ in the mouse; it would be interesting to explore cellular differentiation in the presence of serum by testing for antiMullerian hormone production by Sertoli cells.

We thank Professor R. J. Scothorne for his most helpful criticisms and advice during the preparation of this manuscript. Miss M. Hughes assisted with the photography and Miss B. Robinson typed the final manuscript, for which we are grateful.

REFERENCES


Mouse gonadal differentiation in vitro


(Accepted 24 April 1986)