METHODS FOR ISOLATION AND CULTURE OF STAGED SPERMATOGENIC LOBULES AND STAGED SERTOLI CELLS FROM DOGFISH TESTIS (Squalus acanthias)

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Introduction

Although it has been known for many years that high intratesticular levels of testosterone are essential for normal spermatogenesis, fundamental questions remain unanswered (e.g. which stages of development are hormone-dependent; how the functions of germ cells, Sertoli cells and Leydig cells are coordinated through the spermatogenic progression). Advances in these areas have been hampered primarily by the structural complexity of the testis of common laboratory animals and man. In these species, a single testicular cross-section has all germ cell stages and associated endocrine elements, making it technically difficult to obtain discrete stages for biochemical analysis or for manipulation of the hormonal milieu.

The spiny dogfish and other selachians are useful alternative models for studying steroid regulation of spermatogenesis. In these species, the spermatogenic wave involves the whole testis rather than a discrete tubular segment, resulting in a topographical separation of different germ cell stages and a readily visible zonation under the dissecting microscope (Callard et al., Endocrinology 117:1328-1331, 1985). Moreover, a single Sertoli cell "nurtures" an isogenetic clone of synchronously developing germ cells (spermatogenic cyst), and multiple cysts from the same stage are grouped into anatomically distinct spermatogenic lobules. An additional advantage of Squalus is that Leydig cells are undeveloped at this phylogenetic level, leaving Sertoli cells as the primary steroidogenic elements of the testis (Pudney and Callard, Anat Rec 209:311-321; and 323-330, 1984). In this report we describe methods for isolating and staging spermatogenic lobules and for maintaining these in vitro as intact three-dimensional "organ cultures". In addition, procedures were devised for preparing monolayer cultures of Sertoli cells from staged lobules.

Materials and Methods

Testes from 1-3 animals (25-75 g) were sectioned transversely and, under a dissecting microscope, each cross-section (approx. 5 mm) was separated into three zones, based on color, opacity, position relative to the mass of epigonal tissue and size of the spherical lobules visible on the cut surface as described previously (Callard et al., Endocrinology 117:1328-1335, 1985). Light microscopic observations verify designation of zones as follows: I, stem cells and spermatogonia (premeiotic stages); II, primary and secondary spermatocytes (meiotic stages); III, spermatids and mature spermatozoa (postmeiotic spermiogenic stages). Tissues from each zone were minced using a McIlwain chopper and fragments (0.5 mm^3) shaken vigorously for 30 min at 4°C in Ca++Mg++-free Hank's Balanced Salt Solution adjusted for urea and NaCl (350 and 280 mM, final concentration, CMFH). Lobules were separated from remaining tissue fragments by

filtration through a double layer of cheesecloth, allowed to sediment for 10 min and washed extensively with large volumes of CMFH (plus 0.5% BSA) to remove single cells and small cell clusters. To obtain Sertoli cells, lobules from Zones I-III were incubated overnight at 4°C in CMFH containing collagenase (1 mg/ml, Sigma Type V) and DNAase (100 ug/ml, Sigma Type IV), washed extensively with large volumes of enzyme-free CMFH and then agitated gently by hand. This treatment digests the acellular basal lamina and releases most of the germ cells; Sertoli cells remain as large multicellular aggregates and can be separated from germ cells by centrifugation for 60 sec at high speed. Lobules or Sertoli cells were resuspended in elasmobranch culture medium (CM) comprised of Leibovitz L-15, 20 mM HEPES, 10% fetal bovine serum (charcoal-stripped to remove steroids), penicillin (1 U/ml), streptomycin (1 ug/ml) and adjusted for urea and NaCl (350 and 280 mM, final concentration) and maintained in multiwell plates (Falcon) in air at 15 or 22 C.

To pinpoint the exact stage of cell development in a given lobule, the specimen was placed on a slide with a drop of medium and positioned on the stage of an inverted phase-contrast microscope. While observing the sample, it was cover-slipped and excess fluid drawn out with lens tissue until a flattened monolayer revealing all cells was obtained but before any cell breakage occurred. This squash preparation was then frozen on carbon dioxide ice, the coverslip removed while still frozen and the slide air-dried and stained (Giesma, Harris' haematoxylin, methylene blue, Oil Red O) or first fixed in methanol:acetic acid (3:1).

To determine whether cells in cultured lobules were able to synthesize DNA, [^{3}H]thymidine (specific activity, 40 Ci/mmol) was added to culture medium (1 uCi/ml) for 1-24 hr. Lobule squashes were prepared, fixed and air-dried as described above. Slides were dipped in Kodak NTB-2 emulsion and exposed at -20°C for 7 days, developed, fixed and stained.

Results

As determined by trypan blue exclusion and motility of advanced germ cell stages, cultured lobules remained viable up to two weeks in culture. Depending on stage, they varied in size (50-200 mu dia) and appearance under the phase contrast microscope. In a squash preparation all germ cells in a given lobule were in the same stage of differentiation. Lobules containing cells in meiotic (Zone II) or post-meiotic (Zone III) stages were never labeled with [^{3}H]thymidine; however, some labeled cells were seen in lobules from Zone I (premeiotic stages) 1-2 hr after addition of thymidine (Fig. 1), and the percentage labeling increased after longer periods of thymidine exposure. Isolated Sertoli cells began attaching immediately after seeding (85-90% viability) and by 3-5 days had formed a confluent monolayer (Zone I, Fig. 2) or small colonies (Zone II) and single attached cells (Zone III) (not shown). There were stage-related differences in cell size and shape, nuclear size and shape, and abundance of cellular inclusions, presumably lipid droplets. Poly-l-lysine or collagen coating did not improve attachment or significantly alter appearance of the cultures.

Cells seeded on Matrigel rapidly attached, frequently forming branching tubule-like strands of cells, and appeared to enter the gel after 2 days. Germ cells contaminating the Sertoli cell fraction did not attach directly to plastic and most were readily removed when the medium was changed. Round germ cells, but not elongated spermatids, frequently "bound" to the monolayer. When this occurred, germ cells appeared viable throughout the culture period, as indicated by their phase-bright appearance and firm attachment, and these often were seen in multicellular clusters. Sertoli cell cultures were viable for at least 30 days but only Zone I cells seemed to proliferate. Thymidine incorporation was not tested with Sertoli cultures.

Conclusions

In previous studies using *Squalus* testis, we described stage-dependent variations in several steroidogenic enzymes, steroid receptors and a steroid binding protein (Callard *et al.*, Endocrinology 117:1328-1335, 1985; Callard and Mak, PNAS 82:1336-1340, 1985; Ruh *et al.*, Endocrinology 118:811-818, 1986; Mak and Callard, Gen Comp Endocrinol 68:104-112, 1987). Results presented here demonstrate the feasibility of culturing staged spermatogenic lobules (intact Sertoli/germ cell units) or isolated, staged Sertoli cells from *Squalus* testis and open new possibilities for studying the spermatogenic progression, Sertoli-germ cell interactions, and direct effects of steroids under defined conditions *in vitro*. (Supported by NIH grant HD16715.)

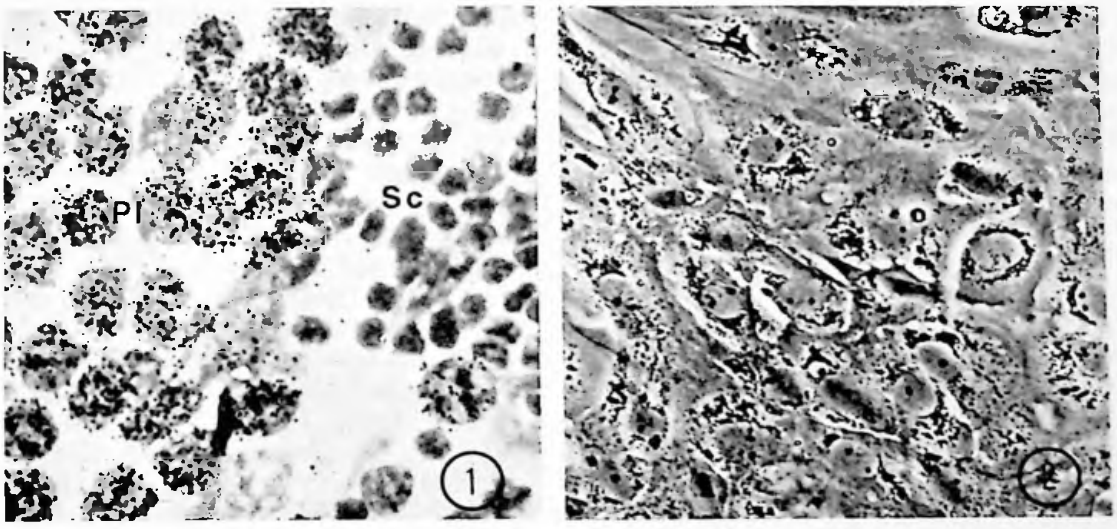

Figure 1. Light micrograph of a methylene blue-stained squash preparation showing a Zone I lobule (premeiotic) on left and Zone III lobule (postmeiotic) on right (x2600). Preleptotene spermatocytes (Pl) are [^{3}H]thymidine-labeled, whereas nuclei of early spermatids (Sp) are unlabeled. Figure 2. Phase contrast micrograph of a Sertoli cell monolayer culture (Zone I) showing large ovoid Sertoli nuclei with one or two prominent nucleoli (x1000).