Müllerian inhibiting substance production and testicular migration and descent in the pouch young of a marsupial

JOHN. M. HUTSON1, GEOFFREY SHAW2, WAI SUM O4, ROGER V. SHORT2,3 and MARILYN B. RENFREE2

1Department of Paediatrics, Royal Children's Hospital, University of Melbourne, Parkville, Victoria 3052, Australia
2Departments of Anatomy and Physiology, Monash University, Melbourne, Victoria 3168, Australia
4Department of Anatomy, University of Hong Kong, Li Shu Fan Building, 5 Sassoon Road, Hong Kong

Summary

The ontogeny of Müllerian inhibiting substance (MIS) production by the developing testis of an Australian marsupial, the tammar wallaby (Macropus eugenii), was determined during pouch life using an organ-culture bioassay of mouse fetal urogenital ridge. This information was related to the morphological events during testicular migration and descent. MIS biological activity was found in testes (but not ovaries or liver) of pouch young from 2 to 85 days of age. MIS production had commenced by day 2, which is within a day of the first gross morphological signs of testicular differentiation. Müllerian duct regression occurred between 10 and 30 days, which partly coincided with testicular migration to the inguinal region and enlargement of the gubernacular bulb (15 to 30 days). These observations are consistent with the hypothesis that MIS may be involved in testicular transabdominal migration. The epididymis commenced development and growth only after the testis had descended through the inguinal ring. This provides no support for the suggestion that the epididymis is involved in testicular descent into the scrotum. The basic sequence of events in post-testicular sexual differentiation in the wallaby is sufficiently similar to that seen in eutherian mammals to make it an excellent experimental model for future studies of testicular differentiation, migration and descent.

Key words: Müllerian inhibitory substance, testicular descent, tammar wallaby (Macropus eugenii), marsupial mammal, pouch young.

Introduction

Müllerian inhibitory substance (MIS) in eutherian mammals, including the rat, calf and human (Donahoe et al. 1982) causes regression of the Müllerian (or paramesonephric) duct, the anlage of the Fallopian tube, uterus, cervix and upper vagina (Josso & Picard, 1986). The hormone has been purified from fetal (Picard & Josso, 1984) and neonatal (Budzik et al. 1985) calf testes, and is a glycoprotein of about 140x10^3 M_r. Recently the bovine and human genes for MIS have been isolated (Cate et al. 1986). Apart from Müllerian duct regression, there are no other proven functions for MIS, although there are some data that suggest it causes inhibition of meiosis in the ovary (Takahashi et al. 1986) and may initiate testicular differentiation (Vigier et al. 1987) and transabdominal migration of the testis from the posterior abdominal wall to the internal inguinal ring (Hutson & Donahoe, 1986). Studies of testicular migration and descent in the pig (Wensing & Colenbrander, 1986), dog (Baumans et al. 1983) & mouse (Hutson, 1986) suggest that a nonandrogenic factor, such as MIS, may control this early phase of testicular migration. Subsequent descent of the testis down the inguinal canal into the scrotum appears to be under androgenic (testosterone) control, since it usually fails to occur in humans (Hutson, 1986), rats (Bardin & Catterall, 1981), mice (Hutson, 1985), raccoon, dogs (Fentener van Vlissingen et al. 1984), sheep (Bruere et al. 1969) & cattle (Nes, 1966) with complete androgen resistance.

The mechanics of testicular descent remain poorly understood, although in recent years the importance
of the gubernaculum has been appreciated. The gubernaculum is a cord of mesenchyme that connects the inguinal region to the primitive gonad and mesonephros. With sexual differentiation and descent of the testis, the male gubernaculum undergoes specific changes that are absent in the female (Backhouse, 1964). During transabdominal migration of the testis, the caudal end of the gubernaculum initially enlarges, only to regress again during testicular descent into the scrotum (Wensing, 1973). This initial gubernacular swelling may be controlled by MIS, since it is abolished in male mice fetuses exposed to diethylstilbestrol (DES), which also prevents regression of their Müllerian ducts (Raynaud, 1958).

Tammar wallabies (Macropus eugenii) weigh only 440 mg on the day of birth. At this stage, the gonads of males and females are still attached to the functional mesonephric kidney high in the abdomen (Tyndale-Biscoe & Renfree, 1987) and are not morphologically different by light microscopy and morphometry (O et al. 1988). Testicular morphology changes rapidly after birth and seminiferous tubules are clearly seen in the gonads of males by day 2 postpartum (Short et al. 1988). Thus while the young are easily accessible within the pouch, testicular differentiation, migration and descent occur (Tyndale-Biscoe & Renfree, 1987). Since the timing of these events in marsupials differs from the common eutherian pattern, the aims of this study were to provide preliminary data on the timing of MIS production by the testes, and to relate this to the development of the testis and gubernaculum and testicular migration and descent.

Materials and methods

Animals

62 pouch young of known age and sex determined by phenotypic appearance and/or karyotype using the method described by O et al. (1988) were obtained from the breeding colony of tammar wallabies (Macropus eugenii) maintained at Monash University. The day of birth is designated as day 0.

Morphology

The young tammars were removed from the pouch and transferred in an insulated warm container to the laboratory, where they were killed by decapitation. The abdominal cavity was opened under sterile conditions and the right gonad removed for MIS bioassay. Care was taken to preserve the normal anatomy on the left side of the animal. The caudal half of the pouch young was fixed in Bouin’s solution for at least one week and stored in 70% ethanol. After trimming the specimens, they were embedded in paraffin blocks and serial coronal or transverse sections were cut at 6 μm and stained with haematoxylin and eosin or Masson’s trichrome.

Sections were examined under a dissecting microscope and drawn with the aid of a camera lucida or by microprojector, at magnifications ranging from ×30 to ×100.

Bioassay

Gonads and liver from pouch young of different ages (2–91 days old) were weighed and then incubated in an organ-culture system to detect MIS biological activity (Donahoe et al. 1977). The standard MIS bioassay was modified to use the 134-day fetal mouse (instead of rat) urogenital ridge, which was cocultured with the wallaby tissue for 72 h on an agar-coated grid over 0.7 ml of CMRL medium (Gibco, NY, USA) with 100 i.u. penicillin and 200 μg streptomycin (Gibco, NY, USA) and 10% fetal calf serum (Gibco, NY, USA). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, following which the tissue was embedded in 2% agar. The specimens were then fixed in Bouin’s solution, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Serial sections (8 μm) were stained with haematoxylin and eosin. The degree of regression of each mouse Müllerian duct was scored subjectively from zero (no regression) to 5 (complete regression) by two independent observers after examination of sections spanning the entire duct length. In early regression (grade 1), the duct is smaller than normal and the basement membrane has begun to dissolve. The mesenchyme around the duct forms a loose whorl. With increasing regression, the diameter of the duct shrinks further to about half the normal size (grade 2). The lumen then decreases in size (grade 3) or becomes obliterated (grade 4), leading to total regression (grade 5).

MIS antibody preparation

Antiserum to MIS was raised in three New Zealand white rabbits against purified bovine MIS donated by Dr P. K. Donahoe (Massachusetts General Hospital, Boston). The initial immunization was performed with Freund’s complete adjuvant and subsequent boosts were given with Freund’s incomplete adjuvant. All injections were subcutaneous. Immunoglobulin-G was isolated from the combined MIS antiserum by incubation with protein-A-sepharose (Sigma) followed by elution with citrate buffer (0.1 M, pH 3.5). This IgG fraction was dialysed against 10 mM phosphate buffer containing 0.15 M NaCl (PBS, pH 7.4) and SDS-PAGE was performed to check the purity of the total IgG fraction. Either 25 μl or 250 μl of concentrated IgG was added to the organ-culture medium, following preliminary studies showing that 250 μl was able to inhibit MIS activity from bovine and mouse testes.

Results

MIS production

MIS biological activity was found in almost all testes examined, which were from pouch young 2 to 85 days old (Fig. 1). In contrast, the ovaries (3–91 days) and pieces of liver from males or females contained no measurable MIS (results not shown). Rabbit polyclonal antibodies raised against bovine MIS were
tested for their ability to inhibit the biological activity of wallaby MIS after concentration of the IgG fraction of the antiserum to 25-3 mg ml⁻¹. When 25 μl of the concentrated IgG was added to the bioassay medium (0-7 ml), no significant inhibition of MIS activity was found. When 250 μl was added, however, MIS activity was abolished in seven out of eight assays (Fig. 2) (P < 0.01, Wilcoxon rank-sum test).

Testicular migration and descent
In the day 2 postpartum male tammar, the testis was recognizable as such by the formation of primitive seminiferous cords. The testis and mesonephros were located within the abdominal cavity, with the 0-5 mm diameter testis on the medial side of the larger (0.75×2.5 mm) mesonephros. The lower pole of the testis and the caudal end of the mesonephros were attached to the inguinal region by a 0-1 mm thick gubernacular cord, 1 mm long (Fig. 3A). Two scrotal bulges were easily identified on the ventral body wall, anterior to the pubis. The gubernaculum extended through the developing body wall into the scrotal bulges, with the apex of the processus vaginalis within the gubernaculum extending about halfway from the inguinal canal to the scrotum. Distally, the processus was a narrow slit, but near the external oblique muscle the space was wider and filled with the gubernaculum proper.

By day 10 after birth, the testis had well-formed seminiferous tubules and it had increased to about 1 mm in length while the mesonephros remained unchanged in size. Although the caudal end of the Müllerian duct had not reached the urogenital sinus by day 10, the cranial root showed early signs of regression, including decrease in size, breakdown of the basement membrane and a loose whorl of surrounding mesenchyme. The intra-abdominal cord of the gubernaculum was still 1 mm long (Figs 3B, 5A), although the extra-abdominal gubernaculum extended into the fused scrotal bulges. The apex of the processus vaginalis invaded the centre of the gubernaculum down to the neck of the scrotum (Fig. 4A). The gubernaculum proper within the processus had enlarged to twice its diameter at 2 days, forming an extra-abdominal ‘bulb’. The peripheral (vaginal) part of the gubernaculum contained myoblasts differentiating into the cremaster muscle.

By day 15 to 17, the testis had elongated to 1.3-5 mm, but its lower pole remained about 1 mm from the inguinal canal (Fig. 3C). The excretory tubules of the mesonephros had begun to involute and the mesonephros now was a similar size to that of the testis (0.5×1.5 mm). The Müllerian ducts were in advanced regression. The two scrotal swellings were completely fused and now formed a bulbous projection anterior to the pubis. The gubernacular mesenchyme was enlarging, with the extra-abdominal bulb now about four times bigger (0.25×0.6 mm) than at day 2 (Fig. 4B).

By day 29, the testis (0.75×1.25 mm) had moved from its intra-abdominal position through the inguinal canal into the extra-abdominal space above the neck of the scrotum (Fig. 3D), which was a well-formed structure 1 mm in diameter. The excretory tubules of the mesonephros had involuted completely, leaving the mesonephric ducts, which had not yet enlarged by elongation and convolution to form the epididymis. The Müllerian duct had almost completely regressed. The processus vaginalis extended into the midscrotal gubernacular mesenchyme, while the bulb of the gubernaculum had enlarged still...
Fig. 2. Mouse urogenital cords cocultured with wallaby gonads. (A) An ovary from a 9-day-old pouch young induces no regression of the Müllerian duct (Md) (grade 0 regression). The Wolffian duct has regressed in the absence of androgen support. (B) A testis from a 19-day-old pouch young causes grade 2–3+ Müllerian duct regression, and also stimulates the Wolffian duct (Wd). (C) In the presence of 25 μL of rabbit anti-bovine-MIS IgG concentrate a testis from a 19-day-old pouch young causes Müllerian duct regression grade 2+. (D) 250 μL of IgG is sufficient to block the MIS activity of a testis from a 19-day-old pouch young (grade 0 Müllerian duct regression). Slides are stained with haematoxylin and eosin. Bar, 0.1 mm.

further to 0.3×0.6 mm (Figs 4C, 5B).

The testis had reached the neck of the scrotum by day 44. By this time, the scrotum had grown to 3 mm in diameter, the apex of the processus reached to the bottom of it, and the cremaster muscle was well developed. The bulb of the gubernaculum had begun to shrink, now measuring 0.4 mm in diameter (Fig. 4D), with its total length from scrotal attachment to the developing epididymis being 1.5 mm.

Descent of the testis was essentially complete by day 64–68, when it was located near the bottom of the scrotum. Further testicular growth had occurred, since it now measured 1×1.5 mm. The caput of the epididymis was enlarging rapidly by elongation of the mesonephric ducts. The vaginal layer of the gubernaculum had condensed around the processus vaginalis, especially cranial to the testis, to form a tight fascial sheath for the spermatic cord. The diameter of the
processus was less than at the time of testicular migration. The gubernaculum was shorter than previously observed and was oriented differently, the testis now hanging down from the involuting gubernaculum rather than being cranial to it.

Discussion

The developing testes of tammar pouch young produce MIS; tammar testes caused significant Müllerian duct regression in the mouse bioassay and rabbit polyclonal antibodies raised against purified bovine MIS blocked this biological activity. No MIS bioactivity was found in ovaries of pouch young aged 3–91 days; antral follicular development does not begin in the ovaries until day 110 of pouch life (Tyndale-Biscoe & Renfree, 1987). These results are in accord with previous reports that MIS from a wide variety of mammalian and avian species can cause Müllerian duct regression in the rat or mouse embryo (Donahoe et al. 1982; Josso & Picard, 1986), and suggest that the biochemistry and function of marsupial MIS is suf-
Testis differentiation
MIS secretion
Mullerian regression
Transabdominal migration
Ingunoscrotal descent

Fig. 6. Diagram summarizing the temporal relationship between testicular development, MIS secretion, Mullerian duct regression, and testicular migration and descent in tammar pouch young.

control of a nonandrogenic hormone such as MIS (Baumans et al. 1983; Hutson, 1985).

MIS production has already begun by day 2 post-

ficiently homologous with that of eutherian mammals to make marsupials a useful experimental model.

The testes of wallaby pouch young produced biologically active MIS in all samples examined from day 2 to day 85. The onset of MIS production therefore precedes Mullerian duct regression which occurs between days 10 and 30. This is consistent with the observation in rats that the Mullerian duct is sensitive to MIS only during a short period (Donahoe et al. 1982). The transabdominal migration of the testis and enlargement of the gubernacular bulb occurs at a similar time, between 15 and 30 days (Fig. 6). This is consistent with the hypothesis that MIS is involved in these functions.

Testicular androgen production is probably occurring over this same period, since the Wolffian duct regresses in females but not males between days 10 and 25. However, in eutherians transabdominal migration of the testis and gubernacular outgrowth are not dependent on androgens (Baumans et al. 1983), leading to suggestions that they might be under the
partum, the earliest age tested, when we see the first appearance of seminiferous cords in the developing male gonad (Short et al. 1988). This is comparable to the fetal rat, where MIS production begins at 13 days of gestation, which is at the commencement of morphological differentiation of the testis (Jost, 1972; Tran et al. 1987). If in future studies we can show that the onset of MIS secretion precedes morphological differentiation of the testis, this would support a role for MIS in testicular differentiation (Vigier et al. 1987).

The ontogeny of testicular migration and descent in the tammar wallaby is similar to that seen in pigs (Wensing & Colenbrander, 1986) and humans (Backhouse, 1982). In particular, the anatomical development of the gubernaculum is similar to that in other mammalian species. There is an outgrowth phase, where the extra-abdominal (caudal) gubernaculum shortens. Wensing & Colenbrander (1982) have proposed that the gubernaculum is the mediator of testicular descent and, in the tammar, the gubernacular enlargement is associated with transabdominal migration of the testis over a distance of 1 mm. After the testis passes through the abdominal wall via the inguinal canal, the gubernaculum becomes progressively smaller, which is similar to the ‘regression’ phase described in pigs (Wensing & Colenbrander, 1986).

The development of the wallaby epididymis is different from that in eutherians, particularly the mouse (Hadziselimovic et al. 1978). Because the marsupial mesonephros persists for some time after birth as a functional kidney (Tyndale-Biscoe & Renfree, 1987) the subsequent differentiation of its duct system into an epididymis is delayed, as in chick embryos, where the mesonephros functions until hatching (Romanoff, 1960). This is completely different from the mouse and human where the mesonephros involutes much earlier in fetal development (Hadziselimovic & Kruslin, 1979). Hadziselimovic has proposed that enlargement of the caput of the epididymis may push the testis into the scrotum, thereby being the cause of testicular descent (Hadziselimovic & Kruslin, 1979). This hypothesis cannot hold true for the tammar wallaby, where the epididymis does not even begin to develop until about the time the testis enters the scrotum. In addition, the tammar testis cannot enter the inguinal canal (which has a diameter of about 0.5 mm just before descent) until after the mesonephros has involuted. Inguinal passage of the testis, therefore, appears to be more dependent on mesonephric regression, rather than epididymal enlargement.

The tammar wallaby pouch young offers a unique opportunity to study gonadal differentiation and testicular migration and descent without the need for complex intrauterine surgery and without the confounding variable of an endocrine placenta. In this paper, we have attempted to describe the normal sequence of events; further studies are now in progress to investigate the role of MIS in testicular differentiation, migration and descent.

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References


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