Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice

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The role of the gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development was examined using normal mice and hypogonadal (*hpg*) mice, which lack circulating gonadotrophins. The disector method was used to determine the number of cells from day 16 of gestation until adulthood. The numbers of Leydig cells did not change significantly between day 16 of gestation and day 5 after parturition in normal mice and were not significantly different from numbers in *hpg* mice at any age up to day 5 after parturition. There was a 16-fold increase in the number of Leydig cells in normal mice between day 5 and day 20 after parturition, followed by a further doubling of number of cells between day 20 and adulthood. The number of Leydig cells in *hpg* testes did not change between day 5 and day 20 after parturition but doubled between day 20 and adulthood so that the number of cells was about 10% of normal values from day 20 onwards. Leydig cell volume was constant in normal animals from birth up to day 20 and then showed a 2.5-fold increase in adult animals. Leydig cell volume was normal in *hpg* testes at birth but decreased thereafter and was about 20% of normal volume in adult mice. The number of Sertoli cells increased continuously from day 16 of gestation to day 20 after gestation in normal mice and then

remained static until adulthood. The number of Sertoli cells in *hpg* testes was normal throughout fetal life but was reduced by about 30% on day 1 (day of parturition). Thereafter, Sertoli cells proliferated at a slower rate but over a longer period in the *hpg* testis so that on day 20 after parturition the number of Sertoli cells was about 50% of normal values, whereas in adult mice the number was 65% of normal. The number of gonocytes did not change between day 16 of gestation and day 1 and did not differ between normal and *hpg* testes. The number of gonocytes increased nine-fold in normal testes but only three-fold in *hpg* testes between day 1 and day 5 after parturition. Gonocytes differentiated into spermatogonia in both normal and *hpg* testes between day 5 and day 20 after parturition. These results show: (i) that fetal development of both Sertoli and Leydig cells is independent of gonadotrophins; (ii) that normal differentiation and proliferation of the adult Leydig cell population (starting about day 10 after parturition) is dependent on the presence of gonadotrophins; and (iii) that the number of Sertoli cells after birth is regulated by gonadotrophins, although proliferation will continue, at a lower rate and for longer, in the absence of gonadotrophins.

Introduction

The first critical steps in testicular development from the indifferent gonad and subsequent masculinization of the fetus are differentiation of the Sertoli cells and Leydig cells (Byskov, 1986; Magre and Jost, 1991). Resultant formation of the male reproductive tract and degeneration of the female tract depends on normal development and function of these cell populations (Jost *et al.*, 1973). Currently, we lack a full understanding of the factors that normally regulate the numbers of Sertoli and Leydig cells during fetal and neonatal life, although it is clear that this is a critical period for subsequent development of normal reproductive function (Orth *et al.*, 1988; Lejeune *et al.*, 1998).

Gonadotrophins regulate the activity of pubertal and

post-pubertal populations of Sertoli cells and Leydig cells (Griswold, 1993; Lejeune *et al.*, 1998), although the role of these hormones in determining the number of testicular cells during the fetal and neonatal periods remains unclear. Evidence from earlier studies in rats and sheep indicates that proliferation of Sertoli cells is at least partially gonadotrophin-dependent during fetal life (Orth, 1984; Thomas *et al.*, 1994), although the stage at which dependency begins is not known. In contrast, there have been no studies to determine whether Leydig cell proliferation and differentiation are gonadotrophin-dependent during the fetal and neonatal period, although O'Shaughnessy *et al.* (1998) showed that Leydig cell function is independent of pituitary control during this time.

The aim of the present study was to measure Leydig cell, Sertoli cell and gonocyte numbers during fetal and postnatal development in normal mice and in hypogonadal (*hpg*) mice, which lack GnRH, and, therefore, endogenous

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circulating gonadotrophins (Cattanach *et al.*, 1977). These data demonstrate the stages of development at which proliferation of each cell type becomes gonadotrophin-dependent and the role of the gonadotrophins in determining the number of cells at puberty.

Materials and Methods

Animals

Normal and *hpg* mice were bred at the University of Glasgow Veterinary School from stock derived originally from the Oxford breeding colony. Animals were maintained as required under United Kingdom Home Office regulations as applied to the use of experimental animals. For timing of fetal development, males were caged overnight with females and the morning was designated as day 0.5 of gestation. For studies on post-natal animals the day of birth was designated as day 1. Adult animals were aged between 70 days and 120 days. Normal and *hpg* mice were distinguished before puberty by PCR as described by Lang (1995).

Fixation and processing

For cell counting using the physical disector method (see below) testes were fixed by immersion in 2.5% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 0.1% (w/v) picric acid in 0.1 mol cacodylate buffer l⁻¹ (pH 7.4). After osmication and dehydration, each testis was embedded in Technovit 7100 (Kulzer and Co, GmbH, Wehrheim) and cut into sections (2 µm thickness). Every tenth or twentieth pair of sections was mounted and stained with toluidine blue in 1% (w/v) borax. For cell counting using an optical disector (see below) testes were fixed in Bouin's fluid overnight, placed in 70% (v/v) alcohol and embedded in Technovit 7100 resin. The testes were cut into sections (20 µm thickness) and stained with Harris' haematoxylin.

Sterology

Two methods of cell counting were used. Most testes were analysed using the physical disector method, but towards the end of the study equipment and software were obtained to allow the optical disector to be used. The two methods provide comparable data (Wreford, 1995 and the present study) but the optical disector is considerably faster. Total testis volume was estimated for both methods using the Cavalieri principle (Mayhew, 1992) and the slides used to estimate the number of cells were also used to estimate testis volume to avoid any requirement for correction factors due to tissue shrinkage. A computer running AutoCADlt97 software (Autodesk Inc, San Rafael, CA) and a digitizing tablet were used to estimate the surface area of selected sections (every tenth or twentieth section). The total surface area of these selected sections is designated SA. The thickness of each section is known (*h*), as is the distance between the sections (*d*), and so total testis volume

(*T_v*) can be calculated by $T_v = SA \times h \times d$. For the physical disector the method used was based on previous descriptions by Sterio (1984) and Gundersen (1986). A Leitz Laborlux S microscope with an attached drawing tube was used to view randomly selected areas of testis with a × 100 oil immersion objective. The drawing tube allowed the outline of the nuclei present to be drawn onto a disector counting frame enclosing an area of 4500 µm². The same area was located in the adjacent section and the nuclei were drawn onto an acetate sheet. Nuclei present in one image but not in both were then counted. A running mean of the number of nuclei present in each pair of drawings was calculated and cell counting was continued until the standard deviation coefficient of the means of the last 50 measurements was < 5%. The optical disector technique (Wreford, 1995) was used to count the number of Leydig and Sertoli cells in the testes of normal and *hpg* adult mice. The numerical density of each cell type was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge) and Stereologer software (Systems Planning Analysis, Alexandria, VA).

The volume density of Leydig cells was determined by the point-counting method using the Stereologer program. Testis sections were cut (2 µm thickness) and the software firstly selected the areas of tissue to be counted and then superimposed a 121 point grid over a video image taken with a × 100 objective lens. At least 100 grids were counted for each testis measured. The mean Leydig cell volume was determined by multiplying the volume density by the total testis volume and dividing by the total number of Leydig cells.

In all studies, gonocytes, Sertoli cells and Leydig cells were identified as described by Hardy *et al.* (1989), Vergouwen *et al.* (1991) and Duckett *et al.* (1997).

Statistical analysis

Results were analysed by two-way ANOVA followed by comparison of individual means using *t* tests.

Results

Testis volume

Changes in testis volume during development in normal and *hpg* mice are shown (Fig. 1). There was no difference in testis size between the two groups until birth. After birth, testis volume in normal mice continued to increase rapidly, in contrast to the *hpg* mice, which showed a much slower growth rate. By adulthood the volume of the *hpg* testis was about 1.5% of that of normal mice.

Leydig cells

Fetal Leydig cells were identified by their rounded nuclei and distinct, darkly stained cytoplasm, which sometimes contained lipid droplets (Fig. 2a–d). Up to day 1 (day of parturition) the cells appeared singly or in small groups and

Fig. 1. Testis volume during development in normal (■) and hypogonadal (*hpg*) (□) mice. Values are mean $\pm$ SEM.

there were no clear differences in morphology between normal and *hpg* mice. On day 5 after birth, larger clusters of Leydig cells began to appear in normal animals and on day 20 clusters of cells with a more variable nuclear morphology and less distinct cytoplasmic boundaries were observed (Fig. 2e,g). These cells frequently contained small lipid droplets (Fig. 2g) and had a similar morphology to cells in adult mice (not shown). In *hpg* testes the Leydig cells after day 5 were characterized by the presence of large lipid droplets as described by O'Shaughnessy and Sheffield (1990) (Fig. 2h,i). Leydig cells usually appeared singly in *hpg* testes after day 5 (Fig. 2h) except near the rete testis where clusters of Leydig cells were observed (Fig. 2i).

Leydig cell volume did not change in normal mice between birth and day 20 but then increased approximately 2.5-fold to adulthood (Fig. 3). Leydig cell volume in *hpg* mice was normal at birth but decreased to about 50% of normal values by day 20 after parturition. There was no hypertrophy of Leydig cells in adult *hpg* mice and the volume of these cells was about 20% of normal volume in adult mice.

There was little or no change in the number of Leydig cells during late fetal or early neonatal life in normal mice (Fig. 4). However, between day 5 and day 20 after birth, the number of cells increased 16-fold followed by a further doubling between day 20 and adulthood (Fig. 2). In *hpg* mice, the number of Leydig cells was not significantly different from that in normal mice throughout fetal life and up to day 5 after birth. After day 5, the number of Leydig cells increased slightly in *hpg* mice up to day 20 and then doubled between day 20 and adulthood so that on day 20 and in adult mice the number of Leydig cells in *hpg* mice was about 10% of normal values.

Sertoli cells

During fetal development the Sertoli cells were clearly identifiable within the developing tubules of normal and *hpg* mice. The nuclei were of variable sizes and shapes and were located towards the periphery of the tubules (Fig. 2a–d). In early neonatal life, the Sertoli cell nuclei tended to

become more central and by day 20 after birth they had assumed an adult morphology in normal mice (Fig. 1e). There were no clear morphological differences between Sertoli cells from normal and *hpg* mice.

The number of Sertoli cells in normal mice increased rapidly throughout late fetal life and early post-natal life with a 6.5-fold increase in number between day 16 of gestation and birth and a four-fold increase between birth and day 5 (Fig. 4). After day 5, the rate of proliferation decreased and there was a 1.9-fold increase in the number of Sertoli cells between day 5 and day 20, with no further increase thereafter. The number of Sertoli cells was normal in *hpg* mice during fetal life up to day 18 of gestation but significantly lower at birth (Fig. 4). After birth, the number of Sertoli cells continued to increase in the *hpg* mice but at a slower rate than in normal mice, so that by day 5 and day 20 after birth the numbers of Sertoli cells in *hpg* mice were 51% and 48% of normal values, respectively (Fig. 4). However, proliferation of Sertoli cells in the *hpg* testes continued beyond day 20 after birth, so that by adulthood the number of Sertoli cells was about 65% of normal values (Fig. 4).

Gonocytes

The gonocytes in normal and *hpg* testes were clearly identifiable in the fetal testes with large round nuclei, often surrounded by a distinct cytoplasm, and situated within the lumen of the tubules. The gonocytes were clearly distinguishable from the nuclei of the Sertoli cells (Fig. 2). Some cells had migrated to the basement membrane of the tubules by day 5 after birth and by day 20 the gonocytes had differentiated into spermatogonia in both normal and *hpg* testes (Fig. 2e,f). Further differentiation and development along the spermatogenic pathway was significantly greater at day 20 in normal mice compared with *hpg* mice (Fig. 2e,f).

Gonocyte numbers did not change between day 16 of gestation and birth in normal mice (Fig. 4). There was a nine-fold increase in the number of gonocytes in normal testes between day 1 and day 5. In the *hpg* mice, gonocyte numbers were not significantly different from normal values during fetal life. There was an increase in gonocyte numbers in *hpg* mice between day 1 and day 5 after birth, but the rate of proliferation was lower than in normal mice and gonocyte numbers were reduced significantly on day 5.

Discussion

Normal masculinization of the fetus and fertility in adult life depend on differentiation and development of Leydig cells and Sertoli cells during fetal and neonatal life. The results of the present study show that, during the fetal period, differentiation and proliferation of both Sertoli cells and Leydig cells are largely independent of endogenous gonadotrophins. After birth, normal development of the number of cells becomes gonadotrophin-dependent in both cell types

Fig. 2. Photomicrographs of testicular tissue from mice of different ages stained with toluidine blue. (a) Normal mouse on day 1 (day of birth); (b) hypogonadal (*hpg*) mouse on day 1; (c) normal mouse on day 5; (d) *hpg* mouse on day 5; (e) normal mouse on day 20; (f) *hpg* mouse on day 20; (g) normal mouse on day 20; (h) *hpg* mouse on day 20; and (i) *hpg*

Fig. 3. Leydig cell volume during post-natal development in normal (■) and hypogonadal (*hpg*) (□) mice. Values are mean $\pm$ SEM. *Indicates that there is a significant difference in the number of cells between normal and *hpg* mice at that age ($P < 0.05$).

but by day 20 the effect of gonadotrophin withdrawal is considerably greater in Leydig cells than in Sertoli cells.

The number of Leydig cells in normal mice did not change significantly between day 16 of gestation and day 5 after birth, and then increased markedly to 6×10^5 cells per testis at day 20 after birth. Vergouwen *et al.* (1991, 1993) showed that labelling of Leydig cells by [^3H]thymidine was very low through fetal life from day 14 of gestation, and that the number of Leydig cells remained constant after birth up to day 10 before increasing significantly to 5×10^5 cells per testis at day 18. Together, the results of the present study and Vergouwen *et al.* (1991, 1993) indicate that after initial differentiation and proliferation of fetal Leydig cells at about day 12 of gestation (Gondos, 1980) there is little further change in the size of the fetal Leydig cell population. In mice, as in other species, a separate adult population of Leydig cells arises before puberty with adult cells first detectable in the mouse about days 7–10 (Baker *et al.*, 1999; Nef *et al.*, 2000). Therefore, it is highly likely that the increase in the number of Leydig cells after day 10 is due to differentiation and proliferation of the adult population. The normal number of Leydig cells observed in fetal/neonatal *hpg* testes demonstrates that the number of fetal Leydig cells is not dependent on gonadotrophins. This finding is in agreement with studies in sheep in which fetal hypophysectomy did not alter the number of fetal Leydig cells (Hochereau-de Reviers *et al.*, 1995). Currently, little is known about the factors that govern differentiation and early proliferation of fetal Leydig cell populations, although *Sry* may be involved in regulating proliferation of the pre-Leydig cell lineage (Schmahl *et al.*, 2000). Lack of *Wnt-4* expression appears to be essential for fetal Leydig cell differentiation (Vainio *et al.*, 1999). Animals lacking Desert hedgehog (*Dhh*) are feminized at birth, indicating a role for *Dhh* in fetal Leydig cell differentiation or function (Clark *et al.*, 2000).

Fig. 4. Changes in numbers of (a) Leydig cells, (b) Sertoli cells and (c) gonocytes in the testes of normal (■) and hypogonadal (*hpg*) (□) mice during development. Values are mean $\pm$ SEM ($n = 3-6$ mice). *Indicates that there is a significant difference in the number of cells between normal and *hpg* mice at that age ($P < 0.05$).

Unlike the fetal Leydig cell population, failure of normal numbers of adult Leydig cells to develop after day 5 in *hpg* mice shows that the adult Leydig cell population is critically dependent on gonadotrophins for establishment of a normal population size at this time. This finding is

mouse on day 20, in the region of the rete testis. Representative Sertoli cells (S), Leydig cells (L), gonocytes (G), spermatocytes (Sp) and peritubular cells (P) are labelled, as are lipid droplets (Lp) visible within some Leydig cells. Scale bars represent 10 μm .

consistent with recent studies on LH receptor knockout mice which indicate that the numbers of Leydig cells are reduced in adult animals (Lei *et al.*, 2001; Zhang *et al.*, 2001), although the cells were not counted in these studies and comparisons cannot be made with the results reported here. Dependence upon gonadotrophins for Leydig cell proliferation after day 5 correlates with what is known about control of fetal and adult Leydig cell function. During fetal development, testosterone concentrations and expression of steroidogenic enzymes are normal in the testes of *hpg* mice (O'Shaughnessy *et al.*, 1998). However, after birth there is a rapid decrease in intratesticular testosterone concentrations in the testes of *hpg* mice so that by day 5 testosterone is largely undetectable and remains undetectable into adulthood (Scott *et al.*, 1990; O'Shaughnessy *et al.*, 1998). Thus, maintenance of fetal Leydig cell function becomes gonadotrophin-dependent shortly after birth but as there is little or no Leydig cell proliferation at this time in normal mice, no effect of gonadotrophin deficiency on the numbers of cells is observed.

A study by Ariyaratne *et al.* (2000) has indicated that initial functional differentiation of adult Leydig cell precursors in rats might be independent of LH. In mice, as in rats, this will occur between day 10 and day 20, a period during which there is little change in the number of Leydig cells in *hpg* testis. This finding indicates that LH is either required for proliferation of differentiated adult Leydig cells during this period or early functional differentiation of precursors to pre-Leydig cells may be LH-independent, whereas later development of these Leydig cells is LH-dependent. Interestingly, in *hpg* mice there is an approximate doubling of the number of Leydig cells between day 20 and adulthood, about the same percentage increase as in normal mice. This late increase in the number of Leydig cells may be partly due to LH-independent differentiation of Leydig cells in both normal and *hpg* mice or may be due to induction of Leydig cell proliferation by factors such as anti-Müllerian hormone and thyroxine (Behringer *et al.*, 1994; Mendis-Handagama *et al.*, 1998; Racine *et al.*, 1998; Teerds *et al.*, 1998). In addition, studies on *Dhh*-null mice have indicated that *Dhh* may be a crucial regulator of adult Leydig cell and precursor cell differentiation (Nef *et al.*, 2000).

At birth, Leydig cell volumes were normal in the testes of *hpg* mice, which is a further indication that function in these cells is also normal at this time. Early adult Leydig cell volume in normal animals was the same as fetal Leydig cell volume but there was a marked hypertrophy of the cells after puberty. Leydig cell volume decreased in the *hpg* testes after birth and remained low in the adult mice, thereby indicating that adult hypertrophy in normal mice is gonadotrophin-dependent.

The numbers of Sertoli cells in normal mice increased throughout fetal and neonatal development up to day 20. This finding is in agreement with a number of earlier studies in various species which have shown that Sertoli cell proliferation occurs mainly during fetal and prepubertal life

(Steinberger and Steinberger, 1971; Curtis and Amann, 1981; Orth, 1982, 1984; Johnson *et al.*, 1984; Kluin *et al.*, 1984; Bortolussi *et al.*, 1990; Vergouwen *et al.*, 1991, 1993; Hochereau-de Reviers *et al.*, 1995). The number of Sertoli cells determined in the present study at day 20 after birth and in adult mice (approximately 2.3×10^6 cells per testis) is in good agreement with the number reported by Vergouwen *et al.* (1993) after day 18 (approximately 1.8×10^6 cells per testis). A number of studies have shown that in rats a reduction of circulating gonadotrophin concentrations during the neonatal period leads to a reduction of about 45% in the number of Sertoli cells (van den Dungen *et al.*, 1990; Atanassova *et al.*, 1999; Sharpe *et al.*, 1999, 2000). The results of the present study now extend this finding to show that, in mice, fetal development is largely gonadotrophin-independent and that a requirement for gonadotrophins is not established until late fetal life. This finding is consistent with earlier studies by Orth (1984) showing that late decapitation of rat fetuses or injection of FSH antiserum at day 18 of gestation reduced [^3H]thymidine incorporation into Sertoli cells. After birth, the rate of proliferation of Sertoli cells in the testes of *hpg* mice was reduced significantly, which is consistent with data described above for neonatal rats and with studies showing that treatment of intact neonatal rats or neonatal *hpg* mice with FSH will increase the number of Sertoli cells (Meachem *et al.*, 1996; Singh and Handelsman, 1996). Interestingly, proliferation continued in the *hpg* testes after day 20, which may indicate that gonadotrophins are required to stop Sertoli cell proliferation at the start of puberty.

The gonadotrophin-independent Sertoli cell proliferation during fetal life and the continued proliferation after birth in *hpg* mice, albeit at a lower rate, highlights the importance of other factors in regulating the number of Sertoli cells. Early proliferation of Sertoli cells, before sex cord formation, appears to be stimulated by *Sry* (Schmahl *et al.*, 2000) and there is clear evidence that thyroxine acts to inhibit Sertoli cell proliferation in the neonatal period (van Haaster *et al.*, 1992, 1993). Regulation of the fetal growth of the number of Sertoli cells remains unclear, although *Fmr1*, glial cell line-derived neurotrophic factor and transforming growth factor α , may all be involved (Slegtenhorst-Eegdean *et al.*, 1998; Hu *et al.*, 1999; Levine *et al.*, 2000; Petersen *et al.*, 2000). The role of these and other factors in determining the final numbers of Sertoli cells in normal testes remains to be elucidated.

The testes of *hpg* mice have very low testosterone concentrations after birth (O'Shaughnessy and Sheffield, 1990; O'Shaughnessy *et al.*, 1998) and the reduced Sertoli cell proliferation in the neonatal period may be due to lack of androgen stimulation rather than direct FSH stimulation. However, Singh and Handelsman (1996) have shown that neonatal treatment of *hpg* mice with testosterone does not change subsequent numbers of Sertoli cells in the adult mice. As testosterone concentrations are normal in *hpg* testes in the fetal period (O'Shaughnessy *et al.*, 1998) it

is likely that the reduced number of Sertoli cells in neonatal *hpg* testes is due directly to lack of FSH.

Primordial germ cells divide mitotically in developing fetal mouse testes until about day 13.5 of gestation and then arrest until just after birth when they resume mitosis (McLaren, 1984). As the number of gonocytes present during fetal life is determined by day 13.5 it is to be expected that gonocyte numbers would be normal in *hpg* testes. After birth, gonocytes in both normal and *hpg* testes re-entered mitosis but from a measure of the number of germ cells present on day 5 it is clear that the rate of proliferation is slower in the absence of circulating gonadotrophins. This may be a reflection of the lower rate of Sertoli cell proliferation at this time or a decrease in Sertoli cell activity through loss of FSH and testosterone stimulation (O'Shaughnessy *et al.*, 1998).

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