Testis morphology in patients with idiopathic hypogonadotrophic hypogonadism

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BACKGROUND: Adult patients with idiopathic hypogonadotrophic hypogonadism (IHH) typically present with absent puberty and therefore have prepubertal testes. IHH is recognized as one of the few curable causes of male infertility and is often effectively treated with either gonadotropins or pulsatile GnRH therapy. The objective of this study was to determine the structure of the testis prior to initiation of treatment. METHODS AND RESULTS: Eight adult IHH patients with prepubertal testes (<4 ml), with no previous gonadotropin therapy and with no history of cryptorchidism underwent open bilateral testicular biopsy prior to the initiation of hormonal treatment. The testes of all patients showed seminiferous cords separated by interstitium composed of blood vessels, connective tissue cells and collagen fibres but typical adult Leydig cells were absent. The cords contained only Sertoli cells and early type A spermatogonia. The spermatogonia mostly resided in the centre of the cords and were often large, typical of gonocytes. Sertoli cells appeared immature with ovoid nuclei devoid of infoldings and cytoplasm that lacked polarity. Tight junctional complexes commonly found connecting adult Sertoli cells were lacking. CONCLUSIONS: These results demonstrate that the immature testes from patients with the severe form of IHH possess early spermatogonia that could possibly reinitiate spermatogenesis with appropriate hormone stimulation. Therefore, the immature testis of this IHH subset resembles those of prepubertal boys and may provide important biologic and genetic insights into testicular development.

Key words: hormonal therapy/idiopathic hypogonadotrophic hypogonadism/Sertoli cells/spermatogonia/testis

Introduction

Idiopathic hypogonadotrophic hypogonadism (IHH) is an important disease model in the human male. Men suffering from this disorder classically display a number of clinical symptoms including absent pubertal development, lack of secondary sexual characteristics and infertility. Microadenomas with functional significance (producing hormone or leading to hypopituitarism) exclude the diagnosis of IHH. However, radiologic anomalies of the pituitary gland without functional significance is occasionally seen in IHH as well as in a small proportion of normal healthy adults (i.e. nonfunctional microadenoma or empty sella) (Bolu et al., 2004). This is confirmed by a nearly uniform response to physiological regimens of exogenous GnRH-replacement therapy (Hoffman and Crowley, 1982; Spratt et al., 1987). A small subset of patients with this disorder presents with a partial form of GnRH deficiency as assessed by some degree of testicular growth despite hypogonadal testosterone levels. We have also documented a rare form of IHH, the adult onset or acquired variant of IHH (Nachtigall et al., 1997). In contrast to most other causes of male infertility, IHH is typically curable (Crowley et al., 1985) in many patients. Indeed, long-term GnRH therapy or gonadotropins successfully induce spermatogenesis in the majority of these patients. However, 20–30% of IHH with the most severe form of GnRH deficiency remain azoospermic. (Crowley et al., 1985, 1991; Finkelstein et al., 1989; Pitteloud et al., 2002). This latter observation has attracted the attention of many clinicians and researchers to focus their attention on the management of IHH.

Many of the IHH patients have a testis that resembles the prepubertal testis. Precocious puberty can be induced in immature monkeys following various hormonal regimens, and in these experiments it was demonstrated that there was an increased proliferation of Sertoli cells accompanied by germ cell proliferation (Marshall and Plant, 1996; Ramaswamy et al., 2000). While testicular biopsy was accepted as a diagnostic tool for the assessment of infertility in the 1970s, the evolution of
assisted reproduction techniques (i.e. IVF and ICSI), has popularized the usage of testicular biopsies during the course of treatment of infertile men (Amelar and Dubin, 1973; Matsumiya et al., 1994). Additionally, testicular biopsies have been used in previous studies to rule out Sertoli cell-only syndrome prior to starting therapy in IHH men as well as to monitor the efficacy in inducing spermatogenesis (Hoffman and Crowley, 1982; Tachiki et al., 1998). These histological studies revealed that most IHH men had prepubertal testes. However, these studies included few subjects and focused mostly on seminiferous tubule diameter, basement membrane thickness and the ratio of germ cells to Sertoli cells.

While a great deal is known about the histology of the normal adult testes, less is known about prepubertal testes, and there is an even greater paucity of information regarding the histology of IHH testes. The normal adult human testis is composed of seminiferous tubules resting on a tunica propria containing a number of myoid cells layers separated from the seminiferous epithelium by a zone of connective tissue and a basement membrane. The seminiferous epithelium contains the nonproliferating fully mature Sertoli cells and the proliferating and differentiating generations of germ cells, from spermatogonia to fully mature spermatids. The interstitium has clusters of Leydig cells in the angular intervals between the seminiferous tubules. In the prepubertal testis, only immature Sertoli cells and type A spermatogonia are present. The prevailing view is that among the type A spermatogonia resides a stem cell population and that these stem cells must be stimulated directly, or indirectly via Sertoli cells, in order for spermatogenesis to be initiated. It is well established that normal spermatogenic activity in healthy men is due to the interaction of Sertoli cell-produced growth factors and germ cells with the support of testosterone produced by the Leydig cells and other blood-borne substances (Dym, 1983).

A review of the literature reveals few recent publications on the histomorphology of the testis, in general, and the germ cells, in particular, in patients with IHH (de Kretser, 1968). Such data would certainly help in understanding the basis of physiological changes that occur in these patients as a result of the treatment protocol and help determine whether those patients with a testicular size of 4 ml or less represent a population with truly prepubertal morphology, despite their advanced chronological age. The present study aims at defining the morphological features of the testes of patients with the most severe form of IHH, i.e. those with a prepubertal testicular size prior to the commencement of the treatment. This may assist in understanding more about the microenvironment of the testis in these patients that might be best suited for the restoration of fertility.

Materials and methods

The testicular biopsies described are from a cohort of eight men (mean age 24 years, ranging from 19 to 32 years) with IHH prior to the commencement of the treatment protocol. All studies were reviewed and approved by the Partners Human Research Committee prior to the initiation of study procedures. All subjects provided written informed consent. We have chosen strict and uniform clinical inclusion criteria aimed at selecting the most severely affected IHH men (testicular volume <4 ml). Men with a history of cryptorchidism were excluded from the study as were men with prior gonadotropin treatment to avoid confounding factors. All subjects had hypogonadal serum testosterone levels (19 ± 4 ng/dl, normal range 300–1000 ng/dl) with undetectable gonadotropins (LH and FSH < 1.6 IU/l), as assessed by a 12-h overnight frequent blood sampling study, consistent with absent endogenous GnRH secretion. Evaluation of the IHH testicular tissue was made in comparison with histological samples of healthy normal testes. The normal testis tissue was obtained from archival material (age unknown) from our pathology department as well as from a previous NIH funded study where we examined the response of the testis to vasectomy (Jarow et al., 1987).

Testicular biopsies were prepared for histological study using standard procedures as described previously (Cavicchia and Dym, 1978). Immediately upon removal from the patient, the testicular biopsies (measuring approximately 3 × 2 × 2 mm²) were placed in 5% glutaraldehyde buffered in 0.2 M s-collidine. One-millimetre cubes of tissue were cut with a razor blade and then fixed for ∼2 h. The blocks were washed in buffer (several changes—30 min) and placed in a solution containing 2 parts of 2% osmium tetroxide in H₂O and 1 part of 3% potassium ferrocyanide in H₂O for 2 h. The tissue was then dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Epon. One-micron sections for light microscopy were cut with glass knives, mounted on slides and stained with 1% toluidine blue in 1% sodium borate. These sections were examined under the light microscope using low power (×10) and high power (×63) Planapo objectives in a Zeiss Axioplan2. For transmission electron microscopy, ultrathin sections (20–50 nm) were cut with a diamond knife using an ultramicrotome (PowerTome-XL; Boeckeler Instruments, Tucson, Arizona, AZ, USA), mounted on grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi 7600 transmission electron microscope operating at 60 kV.

Results

Light Microscopy

Since the biopsy tissue was fixed in glutaraldehyde and embedded in Epon, greater resolution is possible compared to routine embedding in paraffin. Examination of favourably oriented one-micron sections of Epon embedded tissue in the normal adult human testis reveals that the Sertoli cell is a tall columnar cell extending from the base of the seminiferous epithelium to the tubule lumen (Dym, 1983). The prepubertal testis contains seminiferous cords measuring an average 50–80 micra in diameter, as compared with fully differentiated seminiferous tubules in the adult, which are over 200 micra. Testicular biopsies from the IHH patients with no prior pubertal development before commencement of hormone therapy revealed small seminiferous cords with no lumens (Figure 1A). The tunica propria surrounding the cords showed a thickening of the basement membrane of varying degrees (Figure 1B, arrows) and the myoid cells within the tunica were flattened and had a normal morphology (Figure 1B, arrowheads). The interstitial area exhibited fibroblasts, other connective tissue cells, and blood vessels. Normal-looking adult-type Leydig cells were absent. The prepubertal testis is also marked by the absence of Leydig cells, which make their appearance during puberty. The intertubular region of the normal adult testis contains many large Leydig cells, blood vessels, collagen fibrils and various connective tissue type cells. In several of the patients, there

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appeared to be an increase in the connective tissue in the interstitial areas (Figure 1A). The seminiferous cords were composed of immature Sertoli cells and primitive type A spermatagonia (Figure 1B). The Sertoli cells possessed ovoid nuclei but lacked the characteristic nuclear infoldings and cytoplasmic polarity exhibited by mature Sertoli cells (Figure 2, white circles). The Sertoli cells were resting on the basement membrane. Spermatogonial cells (Figure 2, asterisks) were concentrated mostly in the middle of the cords. Some of the spermatogonia were extraordinarily large with few organelles in the cytoplasm. The appearance of these immature spermatagonia varied greatly but no typical dark type A (A_dark) or pale type A (A_pale) was noted. No spermatocytes or spermatids were present in most of these patients. Using the software in the Axioplan2 Zeiss microscope, the diameters of the cords (40 cords were measured in each patient) were measured and they ranged from 97 to 114 micra (Table I) compared to about 200 micra in a normal adult testis. Table I outlines the morphological characteristics of the testis in eight of the pretreatment IHH patients examined thus far.

Electron Microscopy

Low power electron microscopic observations confirmed and extended the light microscopic images. The seminiferous cords were composed of only spermatogonia and immature Sertoli cells. In the Sertoli cells, perhaps the most characteristic feature viewed with the electron microscope is the immature appearance of the cells—they are not tall columnar as in the normal adult human nor are there the typical Sertoli–Sertoli tight junctional complexes. The nuclei are round to slightly oblong with a finely granular chromatin and few clumps (Figure 3); the Sertoli cells of the IHH patients do not exhibit the infoldings that are characteristic of the more mature Sertoli cell. Nucleoli are observed but not in most sections (Figure 4). Numerous rod-shaped mitochondria, some lipid droplets, cisterns of endoplasmic reticulum, free ribosomes and Golgi profiles are commonly observed (Figure 4). Initial formation of the Sertoli–Sertoli junctions is occasionally found and a single cistern of endoplasmic reticulum close to the plasma membrane is noted—this is typical of these junctional complexes (Figure 5). In a few IHH patients, the basement membrane is 10–15 times the normal thickness (Figure 6).

Perhaps, the most distinguishing feature of the spermatogonia is that in most IHH patients large areas of cytoplasm were found which contained only ribosomes either free or clustered as polyribosomes (Figure 7). Profiles of smooth endoplasmic reticulum were scattered throughout the cell. A small Golgi apparatus with associated vesicles was often noted near the nucleus. Oblong mitochondria with transverse cristae were located singly or in small perinuclear groups (Figures 7 and 8). Lysosomes, dense granules and multivesicular bodies were occasionally noted, but not more than 1 or 2 per section. The nuclei of the spermatogonia were spherical to ovoid and possessed chromatin that was uniformly dispersed as fine granules. Several nucleoli were visible in many of the cells and these were mostly located adjacent to the nuclear envelope (Figure 8). Although many of the spermatogonia were found in the centre of the seminiferous cords, cytoplasmic projections were noted emanating from the spermatogonia towards the basement membrane (Figures 7 and 8).

The tunica propria of the normal human seminiferous tubules is markedly different from that found in rodents. Whereas in the rodents one or two layers of flattened peritubular myoid cells surround the seminiferous tubules, in the human three to five circumferentially arrayed cells, each one overlapping the other but separated by abundant type I collagen, surround the tubules. The Sertoli cells and the spermatogonia at the base of the seminiferous tubules rest on a thin basement membrane, under which are the collagen fibres and the myoid cells. In the IHH patients, the tunica is also composed of multiple layers of peritubular myoid cells separated by variable amount of type I collagen. The seminiferous epithelium sits on a basement membrane adjacent to which is the innermost
peritubular myoid cell. In some patients, the basement membrane is much thicker than normal (Figure 6).

Discussion
In normal adult men, there are two populations of type A spermatogonial stem cells, the type $A_{\text{dark}}$ and type $A_{\text{pale}}$ (Clermont, 1963; Heller and Clermont, 1964). The type $A_{\text{pale}}$ is considered to be a renewing type of stem cell and upon division, it can give rise to other type $A_{\text{pale}}$ cells as well as to more differentiated type B spermatogonia (Heller and Clermont, 1963). The type $A_{\text{dark}}$ is a reserve type of stem cell and this cell type may be called upon to replenish the spermatogonial population after injury or other insult (Dym and Clermont, 1970). This pattern

Figure 2. Light micrograph of 1 μm toluidine blue stained sections of human seminiferous tubules from an idiopathic hypogonadotropic hypogonadism patient showing seminiferous cords containing only Sertoli cells and spermatogonia. The asterisks highlight the spermatogonial nuclei and the white circles delineate the Sertoli cell nuclei. Panel A demonstrates the abundant interstitial tissue separating the cords (above the cord). The arrows in Panel B show the thickened basement membrane. Several of the spermatogonia are particularly large especially the one seen in Panel C, right middle cord. Note the pseudopod in this large spermatogonium that seems to be projecting towards the basement membrane (arrow). The basement membrane surrounding these cords does not appear to be thickened. Panel D demonstrates other cords showing several spermatogonia and Sertoli cell nuclei. Panels A to D, ×1000.
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Table 1. Morphological characteristics of the testis in eight of the pretreatment idiopathic hypogonadotropic hypogonadism patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>SC/SPGA only</th>
<th>Thickened basement membrane</th>
<th>Leydig cells</th>
<th>Increased interstitial fibrosis</th>
<th>Sertoli cell polarity</th>
<th>Tubule diameter (micra)</th>
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<tr>
<td>164-66-815</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>110 ± 5.9</td>
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<tr>
<td>164-66-831</td>
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<td>No</td>
<td>No</td>
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<td>Yes</td>
<td>114 ± 3.2</td>
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<tr>
<td>164-66-849</td>
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<td>No</td>
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<td>No</td>
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<td>183-50-868</td>
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<td>No</td>
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<td>No</td>
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<td>190-57-652</td>
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<td>No</td>
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<tr>
<td>192-64-191</td>
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<td>No</td>
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<td>No</td>
<td>112 ± 7.0</td>
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<tr>
<td>193-69-073</td>
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<td>No</td>
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<td>105 ± 6.4</td>
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<tr>
<td>196-73-649</td>
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<td>No</td>
<td>Yes</td>
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<td>97 ± 5.5</td>
</tr>
</tbody>
</table>

SC, sertoli cell; SPGA, spermatogonia.
Data are presented as mean ± SD.

of spermatogonial renewal has been evaluated in nonhuman primates and is generally similar to that observed in man (Clermont, 1967, 1969). However, very little is known about the kinetics of the spermatogonial population in men with immature testes including IHH patients. Fukuda and colleagues have described three types of early gonocytes/spermatogonia in fetal testis, namely gonocytes, intermediate cells and prespermatogonia (Fukuda et al., 1975); more recently, an immunohistochemical profiling of these cells was performed and the three types were classified depending upon the presence of OCT4, C-KIT and MAGE-A4 (Gaskell et al., 2004). We found a great deal of variation among the early germ cells in the IHH cohort described here. However, it was not possible to correlate our findings with the previous reports, as immunocytochemistry was not performed on these biopsies. Of interest, the transcription factor-activator protein AP-2γ is expressed in the early gonocytes and then down-regulated during germ cell differentiation (Hoei-Hansen et al., 2004). Therefore, this transcription factor may be involved in the subsequent differentiation of the gonocytes after hormonal stimulation in the IHH men.

The seminiferous cords of prepubertal boys consist of spermatogonia and immature Sertoli cells. During early puberty, Sertoli cells begin maturing coincident with the establishment of the blood–testis barrier and initiation of spermatogenesis. Subsequently, Sertoli cells reach full maturity. These cells acquire high protein synthesis capacity and support the dramatic increase in meiotic germ cells. These pubertal changes are initiated by the activation of the hypothalamic–pituitary–gonadal axis and are characterized by the appearance of nocturnal, pulsatile GnRH-triggered gonadotropin secretion. In early puberty, GnRH predominantly stimulates FSH release from the pituitary. As puberty advances, LH secretion
predominates in response to GnRH secretion (Grumbach, 2002). In contrast to the normal activation of the hypothalamic–pituitary–gonadal axis during puberty, GnRH-deficient patients examined in this study had no prior gonadotropin exposure and therefore have testes that are very similar to those found in the prepubertal boys.

In the present study, testicular biopsies of IHH patients with no prior puberty demonstrate seminiferous cords rather than tubules. This finding resembles that seen in the normal immature testis. The absence of a tubule lumen also correlates with a lack of Sertoli cell-fluid secretion. Sertoli cell differentiation is associated with the development of the Sertoli–Sertoli tight junctions, also called blood–testis barrier. This allows fluid secretion into the centre of the cords thus establishing a tubule lumen with concomitant initiation of the first wave of spermatogenesis (Vitale et al., 1973). These present findings confirm that immature germ cells are present in the testes of these IHH men and appropriate exogenous gonadotropin stimulation of the testes will likely induce spermatogenesis in the men. A thickened basement membrane and interstitial hyalinization is often a common feature of diseased human testes (Jarow et al., 1987; Haider et al., 1999), and a similar increase in connective tissue components was noted in several of these patients. The thickened basement membrane may interfere with blood-borne substances

**Figure 5.** An electron micrograph of two adjoining Sertoli cells (SCs) from an idiopathic hypogonadotropic hypogonadism patient separated by their plasma membranes (arrows). A Golgi apparatus (G) is seen in the SC at the left. The arrowheads point to endoplasmic reticulum adjacent to the plasma membrane—this is typical of the Sertoli–Sertoli tight junctions found between adult SCs; ×5400.

**Figure 6.** An electron micrograph of the basal portion of a seminiferous cord from an idiopathic hypogonadotropic hypogonadism patient showing the thickened basement membrane (asterisks) that is characteristic of about half of the patients. Type I collagen is noted on the outside of a peritubular myoid cell (arrow); ×5000.

**Figure 7.** An electron micrograph of the basal portion of a seminiferous cord from an idiopathic hypogonadotropic hypogonadism patient showing two large spermatogonia (SPGA) near the basement membrane. Both cells have pseudopods that appear to be touching the basement membrane (arrows). These SPGAs have a cytoplasm mostly devoid of organelles except for a number of mitochondria and free ribosomes. Note that the SPGAs are very much larger than the adjacent Sertoli cells (SC); ×2000.
reaching the seminiferous epithelium, thus interfering with normal germ cell development.

The varied morphology of early spermatogonia in the IHH patients requires further elucidation. Indeed, some of these cells must be spermatogonial stem cells capable of initiating spermatogenesis with the appropriate stimulation. Gonocytes are present in the immature testis and eventually develop into type A-dark and type A-male spermatogonia during puberty. It is possible that the variations in the spermatogonial morphology are due to the heterogeneity in the population in terms of completeness of GnRH deficiency, despite our strict inclusion criteria used to identify the most severely affected IHH patients. In addition, while IHH was previously thought to be solely a hypothalamic defect, advances in the genetics of the disorder have uncovered several genes, which are not only expressed in the hypothalamus but also in the testes (i.e. GPR54, FGFR1 gene). Mutations in those genes may underlie some of the morphologic differences observed in the spermatogonia. Further studies will be required to correlate these findings with the subsequent outcomes of therapy as they relate to testicular growth, seminiferous tubular function, semen quality and fertility.

Acknowledgements

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Figure 8. An electron micrograph of the basal portion of a seminiferous cord from an idiopathic hypogonadotropic hypogonadism patient showing one large spermatogonia (SPGA) near the basement membrane. The cell has a pseudopod that extends towards the basement membrane (short arrow—lower left). Four nucleoli are apparent in this spermatogonial nucleus (arrows). This SPGA also has a cytoplasmic organelle (short arrow—lower left). Note that the SPGAs are very much larger than the adjacent Sertoli cells (SC): ×3000.

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