The molecular basis of cryptorchidism*

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Cryptorchidism is the commonest malady to affect newborn male infants. Until recently, the molecular aetiology of this syndrome was unclear. Cryptorchidism may be part of a broader testicular dysgenesis syndrome, wherein a disturbance in steroid hormone metabolism, possibly through a perturbed hypothalamic–pituitary–gonadal axis could be involved. Disturbance may be genetic, or extrinsic through endocrine disruptors. Recently, the role of insulin-like factor-3 (INSL3; alternatively called relaxin-like factor) has been highlighted through the cryptorchid phenotype of mice where genes for either INSL3 or its receptor have been ablated. INSL3 is produced by Leydig cells of the fetal testis and acts upon the gubernacular ligament to retain the gonad in the inguinal region, from which it later passes into the scrotum. INSL3 expression in fetal testis is inhibited by maternal exposure to estrogens. Although to date no mutations have been found in the human INSL3 gene responsible for cryptorchidism, one causative mutation in the INSL3 receptor (LGR8 or GREAT) has been reported. Studies on developmental transcription factors, such as Hoxa-10 in mice, suggest that other specific molecular cascades could also lead to a cryptorchid phenotype. Considering its frequency in newborn children, and the severity of the untreated condition (infertility and often testicular cancer) these new findings should generate new information on possible causes and treatments.

Key words: cryptorchidism/Hoxa-10/INSL3/RLF/steroids/testicular dysgenesis syndrome

Introduction

Cryptorchidism is the failure of the testes to descend into the scrotal sacs. In humans, testicular descent normally begins at about the 28th week of pregnancy, and therefore most cases of cryptorchidism are detected at birth or in early infancy. The timing of testicular descent, like most other biological events, follows a normal distribution, so that for many infants cryptorchid at birth, a large proportion (~50%) may spontaneously correct themselves by 3 months of age (Scorer, 1964; Anonymous, 1992; Gill and Kogan, 1997; Toppari and Kaleva, 1999; Toppari et al., 2001). Nevertheless, cryptorchidism is the most common ailment affecting newborn male children, with a frequency of ~1–4% of live male births. As a consequence, the surgical correction of cryptorchidism (orchidopexy) is the most common operation to be carried out on all males (young and old). Because testicular descent generally occurs before birth, premature infants will show a considerably higher frequency of cryptorchidism at birth than those carried to term. According to the most comprehensive studies (Scorer, 1964; Anonymous, 1992), 21–23% of infants with a birthweight <2.5 kg are cryptorchid, compared to only 3–4% with a birthweight >2.5 kg.

The aetiology of cryptorchidism is mostly idiopathic and multifactorial. As suggested from the statistics given above, many cases of cryptorchidism can probably be attributed to disturbances in developmental equilibrium. However, cryptorchidism does not present with a unified symptomatic. According to a recent study by Giannopoulos et al. (2001), of 4000 boys with indicated cryptorchidism, 8.9% presented with non-palpable testes (presumably abdominal or missing), ~41% had testes located at some position within the inguinal canal, and ~50% had sliding or retractile testes, which moved between the scrotum and inguinal canal. Many cases of cryptorchidism are unilateral, though a large number, especially with non-palpable testes, are bilateral. From the Greek study, it was reported that approximately two-thirds of the cases with testes in the inguinal canal were able to respond positively to a stimulation of the hypothalamic–pituitary–gonadal (HPG) axis with hCG and/or GnRH. This implies that an androgen deficiency, or some other Leydig cell function, might in part have been responsible for the cryptorchidism in these patients. Nevertheless, 30–40% of patients in this study with inguinal testes, and >85% of those with abdominal testes, were non-responsive to stimulation of the HPG axis.

Cryptorchidism is often associated with other developmental deficits in the male reproductive organs. This has led Skakkebaek et al. (2001) to define a testicular dysgenesis syndrome (TDS). The origin of TDS is presumed to occur either through the influence of genetic mutations (e.g. 45,X/46,XY mosaicism or point mutations), or through endocrine disruption at the time of sex determination. This can then lead to disturbed Sertoli and/or Leydig cell function. If the former, then germ cell defects can arise, e.g. reduced semen quality, or carcinoma in situ (CIS); if the latter, then there would as a result be at least an androgen deficiency, and hence a disturbance of androgen-dependent differentiation of the external genitalia (cryptorchidism, hypospadia, microenpen). Nevertheless, cryptorchidism is often found as a unique symptom, not associated with the other listed defects, strongly suggesting that androgen deficiency alone is not a sufficient explanation. Indeed, in one study of 48 cryptorchid men there was no association found between cryptorchidism and androgen receptor defects (Suzuki et al., 2001).

The embryology of testicular descent

Prior to gonadal differentiation, the undetermined gonad lies in a perirenal position, and is loosely held in place by dorsal and ventral ligaments (Figure 1). The dorsal ligament is referred to as the cranial...
The role of androgens in testicular descent

Since stimulation of the HPG axis appears to be effective for some cases of cryptorchidism (Giannopoulos et al., 2001), what role do androgens play in the embryology of the descending testis? Firstly, cryptorchidism is a common associated symptom in many severe and mild forms of hypogonadal hypogonadism (e.g. Pitteloud et al., 2002) and TDS. Patients with defects in androgen production or metabolism show varied forms of reduced virilization, including abdominal, inguinal and scrotal testes (Sultan et al., 2001). Others with partial androgen insensitivity syndrome, with mutations in the androgen receptor, show variable development of the Wolffian duct, and may have micropenis, hypospadias and cryptorchidism (Brinkmann, 2001; Sultan et al., 2001). The natural fnm (testicular feminization) mutant mouse, which has no functional androgen receptor, exhibits all of the above symptoms (Adham et al., 2000). Finally, patients with partially inactivating mutations in the LH receptor show a similar pattern of developmental symptoms ranging from pseudohermaphroditism through to each and all of the symptoms of TDS (Latronico, 2000). Again, there are mouse models for this. In the LuRKO mouse, where the LH receptor has been ablated, there is very low testicular and circulating testosterone, and full pseudohermaphroditism (Zhang et al., 2001). Similar symptoms are evident for the hpg (hypogonadal) mouse, where the GnRH gene is mutated (Charlton et al., 1983). However, the exact role of androgens in testicular descent is still quite unclear. During intra-abdominal descent, androgens at least contribute to the regression of the CSL. Gubernacular development, in contrast, appears to be independent of androgens, since it occurs in androgen-resistant mice and humans normally, being able to keep the testes close to the inguinal region. The second migration step, from the groin to the scrotum, is assumed to be more dependent on androgens (Hutson et al., 1997), reflected by the success rates of hormonal treatment: roughly, the higher the position of the testes, the less effective is stimulation of the HPG axis to induce descent (Giannopoulos et al., 2001). It should be noted, however, that increasing LH would not only cause an elevation in androgens, but probably also other differentiation-dependent Leydig cell products, such as INSL3. In mice, the whole process of male phenotype differentiation apparently requires fewer androgens than in humans (Mahendroo et al., 2001; Pakarinen et al., 2002). The phenotype of
mice with inactive LH receptors is less dramatic than in humans (Zhang et al., 2001). Similarly, 5α-reductase deficiency in mice is not essential for development of the male urogenital region (Mahendroo et al., 2001), unlike in humans. It has recently been shown for rats, that INSL3 and DHT can synergize to increase gubernacular development in vitro (Kubota et al., 2002).

**Müllerian inhibiting substance**

MIS (also known as the anti-Müllerian hormone, AMH) is produced by immature Sertoli cells as a secreted, circulating peptide hormone, and is responsible for the involution of the Müllerian tract in male embryos (Josso et al., 2001). It has also long been considered as a possible candidate in the context of testicular descent and cryptorchidism. While there is still no data on the situation in humans, in a possible candidate in the context of testicular descent and cryptorchidism. While there is still no data on the situation in humans, in a recent study on the MIS-receptor knockout mouse there appears to be no effect at all by the mutation on gubernacular size and development (Bartlett et al., 2002). In a further recent study on factors influencing the growth of the rat gubernaculum in explant cultures, MIS was found to have only a very weak effect on the growth of the gubernaculum, acting through MIS type 2 receptors (Kubota et al., 2002). Whilst there may be no effect of MIS directly on the gubernaculum, at least adult-type Leydig cells are also known to express the type 2 MIS receptor. If fetal Leydig cells are similar, this might offer a second, reinforcing mechanism to encourage testicular descent.

**The importance of insulin-like peptide 3 in testicular descent**

INSL3 was discovered as a novel gene product of the Leydig cells in 1993 (Adham et al., 1993). Since then it has been shown to be a major secretory hormone of the testis in all mammalian species so far examined (Ivell and Bathgate, 2002). In its structure very like the peptide hormones relaxin or insulin, INSL3 is expressed in both fetal and mature adult-type Leydig cells in a differentiation-dependent manner (Balvers et al., 1998). Most importantly, in mice in which the INSL3 gene has been averted exhibit a clear cryptorchid phenotype in the male, but otherwise no obvious defects to the male reproductive organs (Nef and Parada, 1999; Zimmermann et al., 1999). Early surgical correction of the cryptorchidism in these mice leads to a normal fertile male phenotype. These are important findings, since they reflect the phenotype most commonly observed in classical cryptorchidism in the human.

Most recently, a novel G-protein-coupled receptor (LGR8) has been cloned which specifically binds and responds to INSL3/RLF in transfected cells (Hsu et al., 2002; Kumagai et al., 2002), with a Kd for the hormone in the low nanomolar range. Moreover, mutations in this gene (also called GREAT) in the mouse can lead to cryptorchidism (Overbeek et al., 2001) and have also been linked to a cryptorchidism case in the human (Gorlov et al., 2002). Treatment of rat gubernaculum explants from embryonic day 18 with INSL3/RLF leads to rapid and massive growth of the ligament in culture, an effect which synergizes with DHT treatment, though the latter alone only has a mild effect (Kubota et al., 2002). The same authors have shown that these gubernaculum explants do express the LGR8 receptor (Kubota et al., 2002).

Although INSL3/RLF appears to be a good candidate for a gene to be mutated in cases of cryptorchidism, to date no causative mutations have been discovered in the human INSL3 gene, though numerous studies have been carried out, and a few polymorphisms have been described (Krausz et al., 2000; Tomboc et al., 2000; Koskimies et al., 2000; Lim et al., 2001; Marin et al., 2001; Takahashi et al., 2001; Baker et al., 2002). However, given the very high expression levels of the INSL3 gene in Leydig cells, it is unlikely that a sporadic heterozygous mutation would give rise to cryptorchidism. A random screening of spontaneously cryptorchid patients is therefore probably not an appropriate experimental design. Rather, rare cases of familial cryptorchidism (e.g. brothers) should be selected where there is a higher chance of finding a homozygous mutant state.

Because INSL3/RLF is expressed in a differentiation-dependent manner, any factor which influences Leydig cell differentiation may also affect INSL3/RLF expression and hence cause cryptorchidism. Whilst it is generally accepted that fetal Leydig cells differentiate independently of the HPG axis, at least in rodents, these Leydig cells nevertheless possess LH receptors in the perinatal period, and hence could be influenced by gonadotrophins. In the hpg mouse, the fetal population of Leydig cells at birth appear to express high levels of INSL3/RLF mRNA and protein, although the animals are cryptorchid (Grocock et al., 1988; Baker et al., 1997; Balvers et al., 1998). This would suggest that some aspect of the HPG axis is still required either to cause secretion and action of INSL3/RLF, or because HPG-induced androgens are required for other discrete functions within testicular descent (e.g. involution of the CSL). Kubota et al. (2002) have recently shown for the rat that DHT synergizes with INSL3/RLF in LGR8-dependent gubernacular growth.

Knockout mice have proved very useful in dissecting which aspects of testicular descent are regulated by different hormones. In the INSL3 knockout, the testes are abdominal, but only loosely connected in the peritoneal cavity (Nef and Parada, 1999; Zimmermann et al., 1999). The reason for this is that these mice express androgens, and therefore the involution of the CSL occurs as in the wild type. Only the gubernaculum fails to develop. In the tfm mouse, which lacks a functional androgen receptor, INSL3/RLF would be expressed normally. Therefore the gubernaculum would develop as in the wildtype, retaining the testis in the inguinal region. However, the lack of an androgenic effect caused by the mutation means that the CSL does not involute, but remains developed, causing the testis to be held in the peritoneal cavity as if on a taut bowstring (Adham et al., 2000). If the tfm mouse is crossed with the INSL3 knockout mouse, then the testes in the male progeny adopt an ovarian location: the CSL is well developed, and the gubernaculum, in the absence of INSL3/RLF, involutes (Adham et al., 2000). Two recent studies have used transgenics to induce expression of INSL3/RLF in fetal female mice (Adham et al., 2002; Koskimies et al., 2003). Of particular interest here, besides the movement of the ovary to an inguinal location, is the observation that there is also an increased incidence of inguinal hernia in the female mice, suggesting that INSL3/RLF may also play a role in the passage of the testes from the abdominal cavity through the body wall into the scrotum.

**Estrogens and cryptorchidism**

Back in the 1950s, pregnant mothers were being treated with diethylstilbestrol as a hormonal support to pregnancy. The practise was abandoned when it was found that it led to a high rate of cryptorchidism and other genital defects (Gill et al., 1979; Stilman, 1982). Experimental treatment of pregnant rats with estrogens has reinforced the finding, that exposure of male fetuses to estrogens leads to a failure of the testes to descend (Grocock et al., 1988; Toppari and Skakkebaek, 1998; McLachlan et al., 2001). This has also been linked to the consistent increase in cryptorchidism in humans in recent decades, and the possible role in this of xenoestrogen exposure (Toppari and Skakkebaek, 1998). In a recent study, mice were treated with diethylstilbestrol in pregnancy, sufficient to cause cryptorchidism in the newborn males (Emmen et al., 2000; Nef et al., 2000). Examination of the male fetuses showed that in the treated animals on embryonic days 16 and 18, there had been complete suppression of INSL3/RLF mRNA in the fetal testes, compared with the strong
hybridization signals seen in control mice at this time. Interestingly, in the same study, it was shown that the transcription factor SF-1 was not affected (Emmen et al., 2000). This is important, since it has been shown elsewhere that SF-1 is a major transcription factor involved in the expression of the INSL3 gene, with three putative binding sites for this transcription factor in the proximal promoters of the mouse, rat and bovine genes (Koskimies et al., 1997, 2002; Zimmermann et al., 1998; Spiess et al., 1999; R.Ivell, N.Walther and H.Sadeghian, unpublished). None of these promoters indicate specific responsive elements for estrogen receptors, so that the effect of estrogens on this gene are likely to be indirect through as yet unknown pathways.

A situation similar to treatment of the mothers with estrogens has been generated by overexpressing a constitutive transgene in male mice (AROM+), encoding the enzyme aromatase (Li et al., 2001). All the AROM+ males were ‘cryptorchid, with the testes located in the bottom of the abdominal cavity’, and the ‘weights of the testes, epididymis, seminal vesicles, and prostate lobes were significantly reduced’ (Li et al., 2001). In these mice, two things have occurred: firstly, excess estrogens in the male fetuses will have led to a suppression of INSL3/RLF expression; secondly, the excess estrogens have probably caused a feedback inhibition of the HPG axis, leading to hypoandrogenaemia. Whether estrogens are similarly effective in the human is not known, but it seems likely that there could be estrogenic action both at the level of the testis as well as via a desensitization of the HPG axis.

Deletion of the transcription factor Hoxa-10 leads to cryptorchidism

The transcription factor Hoxa-10 has been implicated in the early embryonic development of the reproductive system. Particularly in the female, ablation of the Hoxa-10 gene leads to a lack of development of the uterus and oviduct. The male knockout mice are viable but infertile (Rijli et al., 1995; Satokata et al., 1995). While they are normally virilized, implying normal androgen production, they are bilaterally cryptorchid with only vestigial gubernacula. In-situ hybridization for Hoxa-10 transcripts in wild type mice shows that these are expressed predominantly in the gubernaculum, as well as in the kidney, but not in other organs and tissues of the male reproductive system. Thus, while we have no information on the role of this transcription factor in any other species besides the mouse, it is definitely another candidate gene which deserves attention in the context of cryptorchidism. In particular, it would be interesting to determine its mode of regulation as well as its target genes in the gubernaculum. A further gene which may be involved in testicular descent is that for the DNA-binding protein Desr (Lahoud et al., 2001). Whilst having a general growth retardation, mice with this gene ablated specifically show defects in the development of the male reproductive tract and are cryptorchid.

The genitofemoral nerve and role of calcitonin gene related peptide (CGRP)

In addition to the factors outlined above, there appears to be a very important function which can be attributed to the genitofemoral nerve and its principal neurotransmitter calcitonin gene related peptide (CGRP). Severence of this nerve either in pathology (spina bifida) or in experimental animals also leads to cryptorchidism, which appears to be partly alleviated by application of CGRP (Hutson et al., 1997). This aspect reflects the morphology of the gubernaculum, which additionally comprises smooth muscle cells presumed to be the targets for CGRP action.

The consequences of cryptorchidism

Since the testes are not really required until puberty, why is it so important that the testes of neonates are surgically corrected as soon as possible after birth? There is considerable information in the literature to show that cryptorchid patients have a higher risk of developing CIS and hence testicular cancer (seminomas) (Dieckmann and Skakkebaek, 1999). They also have a very high risk of becoming irreversibly infertile. Interestingly, there are even defects in the descended testes of unilaterally cryptorchid patients. Almost all of these symptoms can be ameliorated by early orchidopexy soon after birth. Normal scrotal temperature in men is ~4–5°C lower than abdominal temperature. Both experimental studies in rats and other species, as well as clinical observations in humans, indicate five major points where abdominal temperature can negatively influence male reproductive function (Setchell, 1998). Firstly, and most importantly, is the differentiation step from the early gonocyte stem cells to form the A dark spermatogonia (adult stem cells). This reserve of stem cells is essential if spermatogenesis is to occur effectively at puberty. It has been shown that a delay in orchidopexy beyond 1 or 2 years of age can drastically reduce the number of A dark spermatogonia available for later spermatogenesis (Hadziselimovic and Herzog, 2001; Huff et al., 2001). A disturbance at this very early differentiation step is also associated with the transformation of gonocytes into CIS cells, and hence the seeds for later testicular cancer.

The second heat-sensitive step in spermatogenesis is in the transition from pachytene spermatocytes to early round spermatids. This is unlikely to be relevant for human cryptorchidism, but has been demonstrated in various experimental models, and may be relevant where testicular overheating is possible for occupational reasons. In an elegant study by Mu et al. (2000) in the rhesus monkey, it was shown that mild heating caused an induction of the p53 transcription factor in spermatocytes, leading to an induction of p21, and hence activated retinoblastoma (Rb) protein. This, in turn, leads to a massive down-regulation of the master transcription factor TR2, which plays an important role in the regulation of post-meiotic genes in spermatocytes. Sertoli cells also appear to be temperature sensitive, though the mechanisms involved are not clear. An increase in temperature also increases the risk of oxidative damage to DNA in the developing spermatids, and hence also to embryo survival in the later offspring of affected males (Setchell, 1998).

Finally, it is worth pointing out that the coolest area of the scrotum is in fact that where the cauda epididymis is located. The role of the epididymis in the human is not fully clear, but in all other species examined it is essential for maturation and storage of sperm once these have left the germinal epithelium. This function depends to a large part on specific proteins secreted from the epididymal epithelium. In a series of studies on the epididymides of cryptorchid animals and on primary epithelial cell cultures, it has been shown that some of these epididymal gene products are exquisitely sensitive to small changes in temperature (Pera et al., 1996; Kirchhoff et al., 2000). There is no information as to whether epididymal function, and hence sperm maturation, is irreversibly compromised in adult humans who have been treated for cryptorchidism as children.

Is testicular descent influenced by environmental factors?

The first suggestion that there might be an environmental component in the aetiology of cryptorchidism was published in 1992, when it was shown that for both cryptorchidism and hypospadias there had been a significant increase in reported cases over the last 25 years (Giwercman et al., 1993; Toppatti et al., 2001). This was then linked to the other investigations which showed a negative influence of maternal estrogen exposure on the development of the external
genitalia in male fetuses (see above). Together, these observations raised the very obvious suspicion that environmental agents (endocrine disruptors) might be having an influence on testicular descent. While it must be evident from the experimental findings outlined above that any compound with estrogenic or anti-androgenic effects to which a male fetus is exposed might disturb the dynamics of testicular descent, the experimental studies generally make use of much higher concentrations of substances than those to which a pregnant mother and her fetus may be exposed. This argument does not, however, take account of synergies between several different low-dose xenobiotic compounds, which is a much more realistic situation, and has been shown to occur in an experimental context.

Perhaps the most convincing evidence that xenobiotic compounds in the environment might influence testicular descent comes from a recent analysis of the incidence of cryptorchidism in newborn infants in maternity hospitals in East and West Berlin over the last four decades (Oehme, 2002). It was shown from this study that the frequency of cryptorchidism in newborn infants was between 3 and 4% for the years 1965–1971 in both East and West Berlin. This level was maintained in East Berlin in the subsequent decade, 1972–1983, but declined drastically in West Berlin over the same period to <1%. In the most recent period for which statistics were available (1987–1997), the frequency of cryptorchidism was reduced to <0.5% in West Berlin, and to <2% in East Berlin. The important fact to note here is that there was widespread application of DDT-containing insecticides in both East and West Berlin up until the 1960s and 1970s. DDT was banned from use in West Berlin in 1972, but continued to be used widely in East Berlin until shortly before German unification in 1989. The statistics on the incidence of cryptorchidism in East and West Berlin correlate perfectly with the use of the known xenobiotics DDT, and its metabolite DDE, which have been shown experimentally to have marked estrogenic and/or antiandrogenic effects (Gray et al., 2001). Supporting this assumption, Hosie et al. (2000) have found increased levels of certain organochlorine compounds in patients with cryptorchidism.

Conclusion

Cryptorchidism is a very frequent ailment amongst newborn male children. It needs to be corrected as soon as possible after birth, ideally by ~6 months of age, in order to save the spermatogonial stem cells and reduce the risk of later testicular cancer. While a hormonal approach to descend the testes may be effective, one should also consider what other effects a large and unusual stimulation of the HPG axis at this very early age might have. There has been no long-term follow-up of children treated in this way, and very few experimental studies. Thus surgical orchidopexy remains the method of choice. Only now are we beginning to understand the molecules which underlie this important developmental step. Of these, the new Leydig cell peptide hormone INSL3/RLF and its receptor LGR8 appear to play a key role, although we still have experimental data only for rodents. We still know almost nothing about how these molecules are regulated in the perinatal period, especially in the human, nor how they might be externally influenced in a positive or a negative sense. Unfortunately, this hormone is still not available in anything except the smallest of experimental quantities, so that no clinical studies can be carried out yet. Similarly, we still have very little information on the pharmacology of its receptor, LGR8. The recent study from the Hsueh laboratory (Hsu et al., 2002; Kumagai et al., 2002) suggests that the related peptide hormone relaxin can also activate the LGR8 receptor. We know virtually nothing about this peptide in the context of male development. The very first immunoassays for INSL3/RLF are currently being developed (Büllesbach et al., 1999; R.Ivell, M.Schumacher and S.Hartung, unpublished), so that soon it should be possible to begin tracing the endocrinology of this peptide hormone in humans and experimental animals.

Cryptorchidism is a variable and diverse phenotypic trait and hence is likely to be multifactorial, involving both the HPG axis, as well as specific hormones such as MIS and INSL3/RLF. Additionally, developmental factors such as Hoxa-10 may be implicated in some patients. As a developmental trait, it is more likely that a shift in the dynamic equilibrium of the factors involved is responsible to a greater degree than a single gene mutation, and that quantitative rather than qualitative deficits underlie the disturbance. Considering how many children are affected by cryptorchidism, and the severity of the consequences upon delayed treatment (infertility, testicular carcinoma), then a major research effort should be made to expand our current knowledge in both research and clinical application.

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