The role of insulin 3, testosterone, Müllerian inhibiting substance and relaxin in rat gubernacular growth

Y.Kubota1*, C.Temelcos1*, R.A.D.Bathgate2, K.J.Smith2, D.Scott2, C.Zhao2 and J.M.Hutson1,3

1F.Douglas Stephens Surgical Research Laboratory, Murdoch Children’s Research Institute and 2The Howard Florey Institute, University of Melbourne, Victoria, Australia
3To whom correspondence should be addressed at: Department of General Surgery, Royal Children’s Hospital, Parkville, Victoria 3052, Australia. E-mail: hutsonj@cryptic.rch.unimelb.edu.au

Transabdominal testicular descent is influenced by various anatomical and hormonal factors and is mediated by gubernacular enlargement and regression of the cranial suspensory ligament, but its mechanism remains controversial. The aim of this study was to determine which hormones have a direct effect on the proliferation of cells in the day 17 fetal rat gubernaculum in vitro, using an organ culture system. The effects of synthetic rat insulin 3 (INSL3), inactive INSL3, dihydrotestosterone (DHT), DHT + INSL3, human Müllerian inhibiting substance (hMIS), hMIS + INSL3 and human gene 2 relaxin were tested, together with co-culture with fetal rat testis. Cell proliferation was assessed using a bromodeoxyuridine labelling index. The results showed that MIS and relaxin have a mild effect on gubernacular growth, whilst INSL3 and DHT have a more marked effect. The combination of MIS + INSL3 showed an effect close to that of co-culture with testis. However, the most pronounced effect was caused by DHT + INSL3. RT–PCR analysis indicated that the fetal rat gubernaculum strongly expresses putative INSL3 receptors, weakly expresses MIS type II receptors and does not express relaxin receptors. In conclusion, a number of different hormones directly influence growth of the gubernaculum in vitro, including the recently reported hormone INSL3. INSL3 shows a direct stimulatory effect on the swelling reaction, while DHT and MIS may have roles in augmenting this growth.

Key words: gubernaculum/INSL3-relaxin/testis descent

Introduction

Although the precise anatomical and hormonal mechanisms involved in testicular descent remain elusive, a two stage model has been proposed (Hutson et al., 1997). During the first or ‘transabdominal’ phase, caudal enlargement of the gubernaculum (the ‘swelling reaction’) occurs, holding the testis near the inguinal region during abdominal growth. The cranial suspensory ligament also regresses. In the second or ‘inguinoscrotal’ phase, the testes descends into the scrotum, under androgenic control.

The factors involved in the first phase of descent remain controversial. The swelling reaction occurs normally in both mice and humans with complete androgen resistance and in flutamide-treated fetal rats, suggesting no role for androgen (Hutson et al., 1997). Some evidence from animal models and human inter-sex conditions supports a role for Müllerian inhibiting substance (MIS) (Scott, 1987; Hutson et al., 1990, 1997). In persisting Müllerian duct syndrome, where there is a genetic defect in the MIS gene or its receptor, the testes are undescended and the gubernaculum is thin and elongated, suggesting failure of the gubernacular swelling reaction (Guerrier et al., 1989; Hutson and Baker, 1994; Hutson et al., 1994). However, these observations have not been translated into direct evidence for a role for MIS experimentally (Tran et al., 1986; Fentener van Vlissingen et al., 1988).

Involvement of a third testicular factor has been hypothesized for some time (Fentener van Vlissingen et al., 1988; Levy and Husmann, 1995). Recently, a mouse mutant for insulin 3 (INSL3) (also called Leydig insulin-like hormone or relaxin-like factor) was found to have intra-abdominal undescended testes and deficient gubernacula (Nef and Parada, 1999; Zimmermann et al., 1999). Furthermore, a study by Emmen et al. revealed an essential role for both androgen and INSL3 in the gubernacular swelling reaction using an organ culture of fetal rat gubernacula with the testes from INSL3 or MIS mutant mice (Emmen et al., 2000). In addition, synthetic INSL3 has been shown to induce cell growth in primary cultures of post-natal rat gubernaculum (Boockfor et al., 2001). More recently, over-expression of INSL3 in female mice has been shown to cause gubernacular swelling and ovarian descent in the whole animal (Adham et al., 2002).

However, it remains to be determined whether or not INSL3 acts directly or indirectly on the fetal gubernaculum. Furthermore, the potential direct roles of other testicular factors, either alone or in combination with INSL3, have not been studied. In this study, we examined the direct effects of INSL3 and other hormones and factors on the proliferation of cells in the gubernaculum in vitro. We also aimed to confirm, by Northern blotting, that INSL3 mRNA is expressed in day 17 fetal rat testes. Additionally, the recent identification of the receptor for relaxin (leucine-rich repeat-containing, G-protein-coupled receptor 7, LGR7) and a putative receptor for INSL3 (LGR8) (Hsu et al., 2002) has allowed us to examine the expression of these receptors in the gubernaculum.

*These authors contributed equally to this work.
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Materials and methods

Animals

Sprague–Dawley rats maintained at the Royal Children’s Hospital, Melbourne were used. Male and female adult rats were mated overnight and the morning a vaginal plug was found was designated as embryonic day 0 of pregnancy. Dams were killed by CO2 inhalation on day 17 and the rat fetuses were immediately removed and placed in ice until dissection.

Organ cultures

Gubernacula were harvested from each male fetus with the aid of a dissecting microscope and placed in sterile phosphate-buffered saline (PBS). The organ culture system used consisted of 2% molten Agar placed on a stainless-steel grid, which was suspended over the inner well of a Falcon 3010 organ culture plate (Becton Dickinson Labware, Lincoln Park, New Jersey, USA). Each pair of gubernacula was placed on the cut surface of the Agar. The outer well contained 2 ml of sterile PBS. The inner well was filled with 0.7 ml Iscoves modified Dulbecco’s medium (Gibco, Grand Island, NY, USA) supplemented with penicillin, streptomycin, 10% fetal calf serum, L-glutamine (1 mg/ml), non-essential amino acids (10 µg/ml; Sigma Chemical Co., Castle Hill, NSW, Australia), nucleosides (10 µg/ml; Sigma), lipids (10 µg/ml; Gibco), transferrin (0.33 µg/ml; Boehringer Mannheim, Castle Hill, NSW, Australia) and insulin (10 µg/ml; Sigma).

The gubernacula were incubated over standard medium (control) or medium mixed with: separate rat INSL3 A and B chains (10–2 mol/l), rat INSL3 (10–2 mol/l), dihydrotestosterone (DHT) (10–3 mol/l; Sigma), human MIS (1 µg/ml), human gene 2 relaxin (10–3 mol/l), INSL3 (10–3 mol/l) + DHT (10–3 mol/l) or INSL3 (10–5 mol/l) + MIS (1 µg/ml). The synthesis of rat INSL3 A and B chains and subsequent chain combination to yield rat INSL3 peptide (A/B heterodimer disulphide linked) has been described previously (Smith et al., 2001). Separate rat INSL3 A and B chains were used as it was expected, as for the related hormone relaxin (Tan et al., 1998), that the A and B chains alone would be inactive. The investigators were initially blinded to the identity of the rat INSL3 and the separate A and B chains to control for the methodology, but at the same time the aim was to clearly demonstrate that the A and B chains alone were inactive. Finally, some of the gubernacula were co-cultured with testes of day 17 rat fetuses, which were harvested concurrently. The culture medium in these dishes contained no added hormones. The organ culture system was maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% O2 for 2 days.

Cell proliferation in the fetal rat gubernacula was assessed using bromodeoxyuridine (BrdU). This is one of the halopyrimidines and, like thymidine, is incorporated into the DNA of the nuclei during the S-phase of the cell cycle. yuridine (BrdU). This is one of the halopyrimidines and, like thymidine, is

BrdU staining

After culture, 2% Agar was added on the gubernacula, and they were then fixed in 10% buffered formalin for 2 h at room temperature. The tissue was processed, embedded in paraffin, cut into 5 µm sections and placed on silane-coated slides. The BrdU staining was performed according to previously described protocols (Ramasamy et al., 2001).

Histological assessment

The incorporation of BrdU into newly synthesized DNA served as an indication of cell division. The nuclei of gubernacular cells containing unequivocal dark brown diaminobenzidine deposits were scored as BrdU-labelled cells; purple haematoxylin-stained nuclei were scored as unlabelled cells. The number of BrdU-labelled nuclei and total nuclei were counted per field in four areas, both centrally and peripherally in the gubernaculun, using a light microscope at high magnification (×400).

Statistical analysis

The proliferation of the rat gubernaculum was estimated by BrdU labelling index (% labelled cells/total cells counted ×100). Counting was confirmed by two independent observers (Y.K. and C.T.). The data were expressed as mean ± SEM and statistical significance was assessed using Student’s two-tailed t-test. P < 0.01 and P < 0.05 were defined as significant and mild difference respectively.

RNA preparation, cloning of probe and Northern blotting

RNA preparation and RT–PCR

Liver and testis were collected from male, and late pregnant ovary from female, adult Sprague–Dawley rats killed with an overdose of isoflurane (Abbott Australasia Pty Ltd, Cronulla, NSW, Australia), and day 17 gubernaculum (and fetal rat testes were collected from animals being used for gubernacular preparations. Total RNA was extracted and RT was performed as previously described (Bathgate et al., 2002). RT–PCR was then performed for the various targets using the appropriate primer combinations outlined below. The full rat INSL3 cDNA coding region (386 bp) was amplified using primers based on the published sequence (Spiess et al., 1999). A specific rat relaxin 1 fragment was amplified using primers equivalent to nucleotides 203–221 and 579–598 of the rat relaxin 1 cDNA (GenBank Acc. No. NM_013413). The expression of the rat MIS type II receptor was studied using primers corresponding to nucleotides 10–30 and 219–239 of the rat MIS type II receptor cDNA sequence (X71916). Sequences for rat LGR7 and LGR8 were obtained by BLAST searching of the incomplete high throughput genome sequence (NCBI) using the human LGR7 (AF190500) and LGR8 (AF403384) cDNA sequences. Rat equivalents were identified in BAC clone sequences (AC098607 and AC009890) which showed high homology to human LGR7 (85%) and LGR8 (81%) respectively, but poor homology between each other or to any other LGR sequence (<50%). These sequences were used to design primers for RT–PCR, the LGR7 primers (sense 5’-GTTGATCTCTTTTGCCTTTAGG-3’; antisense 5’-GAAATAAGTTAGTCTAGTGAC-3’) and LGR8 primers (sense 5’-GATGGGCGTTACGGCTGTC-3’; antisense 5’-AGTACAGA-GGTGTGTTACTT-3’) generating products of 736 and 803 bp respectively. The expression of GAPDH was used as a control for the integrity of cDNA synthesis and was amplified using primers equivalent to nucleotides 789–808 and 1017–1036 of the rat GAPDH cDNA sequence (NM_017008). All the primers were designed to cross intron–exon boundaries and hence control for genomic DNA contamination. The RT–PCR reactions, subsequent isolation of products and sequencing on both strands were performed as previously described (Bathgate et al., 2002).

Northern blotting

Total RNA (10 µg) from liver and testis and two separate pooled samples of day 17 fetal testis were separated on standard 3%–[N-morpholino]propane sulphonatic acid/formaldehyde gels. RNA from late pregnant rat ovary was run on a separate gel. Equal loading of RNA was confirmed by ethidium bromide staining of the 18S and 28S ribosomal RNA bands. The gel was photographed before the RNA was transferred to optimized Hybond-NX membranes (Amer sham Pharmacia, Rydalmere, NSW, Australia) and probed for INSL3 and relaxin 1 mRNA expression with random primed [32P]-labelled probes corresponding to the PCR products generated above. The membrane was hybridized at 65°C overnight in 0.25 mol/l (NaH2PO4-Na2HPO4), pH 7.2, 1 mmol/l EDTA, 20% sodium dodecyl sulphate (SDS), followed by three washes for 5 min in 2 X standard saline citrate (SSC), 0.1% SDS at room temperature, and then a 30 min wash at 65°C in 0.1 X SSC, 0.1% SDS. Membranes were first exposed to a phosphoimager plate for 48 h at room temperature before being analysed in a FujiX 2000 Phosphoimagier (Fuji Photo Company, Japan) and then exposed to BioMAX MS film (Integrated Sciences, Melbourne, Victoria, Australia) together with a Hyperscreen (Amer sham) at ~80°C.

Results

In total, between 22 and 24 gubernacula were analysed in each section. Between 209 and 501 cells were counted for each gubernaculum. Examination under light microscopy revealed minimal necrosis (Figure 1). No difference was detected between the central and peripheral aspect of each gubernaculum. The BrdU labelling index of the controls was 27.2 ± 0.62% and the testis co-culture 35.6 ± 0.65%, and this difference was significant (P < 0.001) (Figure 2). In terms of the hormones, there was a significant difference in the index between separate rat INSL3 A and B chains and intact rat INSL3 (26.6 ± 0.55 versus 32.6 ± 0.84%; P < 0.001). These results prove...
Figure 1. Histological features of gubernacula labelled with BrdU after different treatments. (A) Control ×1000, (B) inactive INSL3 (peptide X) ×1000, (C) INSL3 (peptide Y) ×1000, (D) INSL3 + DHT ×1000. Arrow = darkly stained BrdU-labelled cell. Arrowhead = unlabelled cell.

Figure 2. Cell proliferation in the day 17 fetal rat gubernacular organ culture as assessed using bromodeoxyuridine (BrdU) labelling. Control, media alone; A/B chain, separate A and B chains of INSL3 (10⁻⁷ mol/l); INSL3, rat INSL3 (10⁻⁷ mol/l); DHT, dihydrotestosterone (10⁻⁸ mol/l); MIS, human MIS (1 µg/ml); relaxin, human gene 2 relaxin (10⁻⁷ mol/l); INSL3 (10⁻⁷ mol/l) + DHT (10⁻⁸ mol/l) + MIS (1 µg/ml); fetal testis, co-culture with day 17 fetal rat testis. Between 22 and 24 gubernacula were analysed in each section. *P < 0.05 and **P < 0.001 versus control; #P < 0.01 and ##P < 0.001 versus INSL3. NS = not significant.

mRNA expression of INSL3 and relaxin 1

The full coding region of the rat INSL3 cDNA and a partial sequence of the rat relaxin 1 cDNA were cloned for use in Northern blot analysis. Sequencing of both strands from multiple subclones confirmed that both sequences were identical to those published (Hudson et al., 1981; Spiess et al., 1999). Northern blotting confirmed that INSL3 mRNA is expressed in the day 17 fetal rat testis at a similar level to that of adult testis (Figure 3). As expected, there was no INSL3 mRNA expression in the liver, and the mRNA size (~0.8 kb) was exactly the same as previously published (Spiess et al., 1999). In contrast, rat relaxin 1 was not expressed in the fetal or adult testis, although it was expressed at high levels in the pregnant ovary (Figure 3) at a size (~1 kb) consistent with that published previously (Hudson et al., 1981).

mRNA expression of LGR7, LGR8 and MIS type II receptors

RT-PCR was used to study the expression of the rat LGR7, LGR8 and MIS type II receptors in the fetal rat gubernaculum (Figure 4). Strong GAPDH products were amplified for each cDNA but not with the H₂O control, indicating that the cDNA syntheses were all successful and there was no cDNA contamination across the samples. MIS type II receptors were strongly expressed in the adult and fetal testis and moderate expression was found in the gubernaculum. The LGR7 receptor was strongly expressed in the cerebral cortex and moderately expressed in the testis, but was not expressed in the gubernaculum. In contrast, the LGR8 receptor was only expressed in the gubernaculum. All the PCR products outlined above were sequenced and shown to correspond to the expected sequences.
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Figure 3. Northern blot analysis of relaxin and INSL3 mRNA expression in fetal rat testis. Pregnant rat ovary and adult rat testis were used as positive controls for relaxin and INSL3 expression respectively. Equal loading and RNA quality are indicated by the 18S RNA hybridization. Note the specific hybridizing signals for INSL3 in the adult and fetal rat testis, whereas a specific hybridizing signal is only seen for relaxin in the pregnant ovary.

Figure 4. RT–PCR for MIS type II receptor (229 bp), putative INSL3 receptor (LGR8; 803 bp), relaxin receptor (LGR7; 736 bp) and GAPDH (247 bp) to control for cDNA quality. Note the specific expression of the MIS type II receptor in the testis and gubernaculum, of the LGR7 receptor in the cerebral cortex and adult testis, and of the LGR8 receptor in the gubernaculum only. The size of the DNA markers (bp) are indicated.

Discussion

This study, using an organ culture system, has allowed us to directly investigate which hormones affect growth of the fetal gubernaculum. We found that DNA synthesis in the rat gubernaculum is increased significantly by rat INSL3, DHT and MIS+INSL3, with the latter combination resulting in a BrdU labelling index approaching that of co-culture with testis. MIS and relaxin, individually, were also active to a lesser extent. The combination of DHT+INSL3 had the greatest effect, with a BrdU index of almost 40% compared with 27% in the controls. Rat INSL3 A and B chains alone showed no effect, indicating that native INSL3 is likely to have a heterodimeric A/B structure, such as in relaxin. The expression of INSL3 mRNA was confirmed in day 17 fetal rat testis by Northern blotting. Previous studies indicated that there was very little expression of INSL3 mRNA at this time (Spiess et al., 1999; Parry et al., 2001). However, the results of the current study confirm that the fetal testes do in fact express significant amounts of INSL3 mRNA at this critical stage of gubernacular development. In contrast, no expression of relaxin 1 was observed in the testis of either day 17 fetal or adult rat testis.

INSL3, or relaxin-like factor, is the newest hormone demonstrated to be involved in testicular descent. It is a member of the insulin-like hormone superfamily and was first recognized by cloning projects using testicular tissue, hence its original name of Leydig insulin-like peptide (Adham et al., 1993; Burkhardt et al., 1994). Although recognized for some time, its function was unknown until 1999 when two groups investigating mice mutants for INSL3 found bilateral cryptorchidism and developmental abnormalities of the gubernaculum (Neef and Parada, 1999; Zimmermann et al., 1999). INSL3 transcripts were also present in the developing testis, but not in the gubernacular bulb or other neighbouring tissue. Further detailed analysis of this model confirmed that in the homozygous mutants, testes remained high in the abdomen and the gubernacula were feminized with a deficient mesenchymal core (Kubota et al., 2001).

What was not known was whether INSL3 acted on the gubernaculum directly, which we have investigated in this present study. Emmen et al., also using an organ culture system, showed that rat gubernaculum co-cultured with mouse gonads appeared to require both androgen and INSL3 (Emmen et al., 2000). Neither of these hormones alone was sufficient to induce gubernacular growth to the extent seen in normal testes. More recently, it was shown that synthetic INSL3 induced growth of primary cultures of post-natal rat gubernaculum (Boockfor et al., 2001). These results are consistent with our own findings; however, we have extended these findings to confirm the direct actions of these hormones, including MIS, on the fetal gubernaculum.

Until recently, the receptors for both INSL3 and relaxin had not been discovered. During the preparation of this manuscript, two orphan receptors, LGR7 and LGR8, were shown to be responsive to relaxin (Hsu et al., 2002). It was clearly shown that LGR7 was a relaxin receptor; however, although the LGR8 receptor responded to relaxin stimulation, it is more likely to be a receptor for INSL3 (Hsu et al., 2002). We have investigated the expression of both of these receptors, as well as the MIS type II receptor, in the fetal rat gubernaculum. As expected, the MIS type II receptor is highly expressed in the fetal and adult testis (Teixeira et al., 2001), but is also expressed in the fetal gubernaculum. The LGR7 receptor is expressed in large amounts in the cerebral cortex, as expected by the presence of relaxin binding sites in this tissue (Tan et al., 1999), but is not expressed in the fetal gubernaculum. In contrast, the LGR8 receptor is expressed only in the fetal gubernaculum. The absence of LGR8 PCR products in the cerebral cortex, where relaxin binding sites are found, provides further evidence that LGR8 is not a receptor for relaxin. Furthermore, the demonstration of INSL3 binding sites in crude rat gubernacular membranes (Boockfor et al., 2001), together with the demonstration in this study of LGR8 but not LGR7 transcripts in the fetal rat gubernaculum, is strong evidence that LGR8 is in fact an INSL3 receptor.

The role of MIS in testicular descent remains controversial. A number of observations have implied that MIS is involved in the first phase of descent; however, several experimental studies have precluded a role (Tran et al., 1986; Fentener van Vlissingen et al., 1988; Josso et al., 1997). Our study suggests that MIS may indeed have a role, with MIS alone stimulating DNA synthesis mildly, but more so when combined with INSL3. A potential role for MIS is further implicated by the demonstration of MIS type II receptor expression in day 17 gubernaculum. Although the recent report of gubernacular swelling in INSL3 over-expressing female mice suggests that MIS is not required (Adham et al., 2002), detailed histological studies of the gubernacular ontogeny were not performed. Further
studies are required to determine if INSL3 over-expression can produce a completely 'masculine' swelling reaction, both qualitatively and quantitatively.

It is generally agreed that the second, but not the first, phase of testicular descent is androgen-dependent (Hutson and Donahoe, 1986; Hutson et al., 1997). In contrast, our study revealed that DHT stimulates gubernacular growth in vitro, and if combined with INSL3 there is a significant difference not only from the controls, but also from the testis co-culture. Emmen et al. found a similar effect using a synthetic androgen (Emmen et al., 2000). In comparison, two earlier in-vitro studies using isolated porcine gubernaculum cells failed to show an effect of androgen (Fentener van Vlissingen et al., 1988; Visser and Heyns, 1995). The exact role of androgen is unclear. Undoubtedly the gubernaculum is sensitive to androgen, but the exact inter-relationship between these hormones requires further studies.

The effect of relaxin on gubernacular growth was initially surprising, even though it is a peptide of the same family. Relaxin knockout mice have normally descended testes, although gubernacular development has not been studied (Zhao et al., 1999). However, a recent study has shown that relaxin may be involved in testicular descent in the rat (Parry et al., 2001). We have shown that the fetal rat testis does not express relaxin 1 mRNA or the LGR7 receptor, but both gonadotropin (Neff and Parada, 1999; Levy and Husmann, 1995; Hutson et al., 1997). It would be anticipated that mutations of INSL3 or its receptor would be found in only a small percentage of patients with cryptorchidism.

In conclusion, we investigated the direct effects of a number of hormones on gubernacular growth in vitro, as assessed by DNA synthesis. Our results reveal that rat INSL3, DHT, human MIS and relaxin influence growth of the gubernaculum. These results correlate with the expression of MIS type II and putative INSL3 receptors in the fetal gubernaculum. Further studies are needed to determine the exact inter-relationship between these hormones.

Acknowledgements
The authors thank Professor G.W.Tregear for his advice and support, Dr J.D.Wade for his assistance with the synthesis of rat INSL3 and Sharon Layfield for her assistance with the PCR. This work was supported by a grant from the NHMRC (no. 149210). C.T. would like to acknowledge support from the Bruce Pearson Fellowship of the Australasian Urological Foundation. Work carried out at the Howard Florey Institute was supported by an Institute Block grant (reg. key #983001) from the NHMRC.

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Submitted on April 19, 2002; accepted on July 22, 2002