Testicular changes during infantile ‘quiescence’ in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy?

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BACKGROUND: Inexplicably, boys treated with some therapies for cancer at age 2–10 years, a time of supposed ‘testicular quiescence’, are at risk of low sperm counts/infertility in adulthood. Our aims were to use the marmoset as a surrogate for man to establish testicular cell function/activity during ‘quiescence’ between the neonatal period and puberty, and to test if any cell activity could be suppressed by prior treatment with a GnRH antagonist.

METHODS AND RESULTS: Based on immunoexpression studies, functional development of Sertoli cells (SGP-2, androgen receptor) and Leydig cells (3β-hydroxysteroid dehydrogenase) was detectable at an age (35 weeks) when the testis is considered to be quiescent, and in advance of the pubertal rise in blood testosterone levels (50–60 weeks). Other changes at 35 weeks were the appearance of focal seminiferous tubule lumens and proliferating germ cells [indicated by immunoexpression of proliferating cell nuclear antigen (PCNA)]. Treatment from 25 to 35 weeks with GnRH antagonist largely (>85%) prevented these changes. However, the PCNA-labelling index of spermatogonia in GnRH antagonist-treated animals did not differ from controls (41.3 versus 43.6%) though total spermatogonia volume per testis was reduced by 41%. Some protein markers (inhibin-α, estrogen receptor-β) showed little change with age or treatment. Beyond 35 weeks, GnRH antagonist-treated animals showed a delay in the pubertal rise in plasma testosterone levels. CONCLUSIONS: These findings reinforce the view that the ‘childhood’ testis is not quiescent. This may explain the damaging effects of some cancer therapies on subsequent fertility of boys and raises the issue of protective intervention. The present studies suggest that GnRH antagonist-based intervention might be only partially successful. Identification of the factors regulating spermatogonial development in the infant marmoset may aid in the design of such strategies.

Key words: germ cells/GnRH antagonist/Leydig cell/Sertoli cell/spermatogonia

Introduction

In excess of 70% of children who are treated for cancer now survive to grow to adulthood, thanks to improvements in therapeutic drugs and their administration regimens (Grundy et al., 2001). One result of this improvement is that the quality of life of childhood cancer survivors has become an increasingly important issue. Infertility or subfertility may occur after successful treatment in both males and females (Bath et al., 1998), and there is increasing interest in strategies to protect or restore fertility in affected individuals or in children ‘at risk’ (Howell and Shalet, 1999; Grundy et al., 2001). As most cancer therapies target rapidly dividing cells, it is understandable how such treatments can affect spermatogenesis adversely in an adult man in whom spermatogonial cell divisions are occurring incessantly. However, it is puzzling why some cancer treatments administered to prepubertal boys should also cause infertility/low sperm counts in adulthood, as the testis in boys aged 2–10 years is generally considered to be quiescent (Chemes, 2001). In seeking solutions to such puzzles, it has been argued that the prepubertal human testis is ‘quietly active’ as opposed to quiescent (Chemes, 2001), and there are data that support such a view (Wu et al., 1990, 1991, 1996; Clark et al., 1997; Chemes, 2001). If this interpretation is correct, it raises the possibility that intervention treatment to truly suppress the testis at the time of cancer therapy might be one way of preserving future fertility. Such interventions have never been tested in prepubertal humans or animals, though induction of testicular quiescence in adult
rodents has been shown to be effective in protecting spermatogenesis (Meistrich et al., 1997, 2000) as has suppression of intratesticular testosterone levels after cytotoxic insult (Shetty et al., 2000).

A major problem in addressing the issues just raised is the paucity of data on the testis itself during human childhood and a virtual absence of data on the functional status of its component cell types. This information is necessary in order to establish which cell types, if any, are active, and thus potentially at risk from therapeutic damage. Because of the obvious difficulties in obtaining such data in the human, and in particular of establishing whether any testicular cell activity can be suppressed by experimental intervention, we have turned to a non-human primate, the marmoset monkey. This primate was chosen as it exhibits the same phases of testis development as the human (Lunn et al., 1994, 1997; Sharpe et al., 2001b), but also on other features such as Sertoli cell proliferation and testicular morphology (Sharpe et al., 2000, and unpublished data). The period in which some animals were administered a potent GnRH antagonist in the present studies is also indicated.

Materials and methods

Animals and treatments

Animals were captive-bred common marmoset monkeys (Callithrix jacchus), maintained in a colony which has been self-sustaining since 1973. For the main part of the present studies, a total of 21 male marmosets aged 25 weeks of age were treated weekly with either vehicle (controls; n = 9) or with 10 mg/kg of a potent long-acting GnRH antagonist (Antarelix; Europeptides, Argentueil, France) (n = 12) until 35 weeks of age. The GnRH antagonist treatment, or treatment with a comparable GnRH antagonist, has been shown previously to completely suppress the neonatal testosterone surge in male marmosets (Lunn et al., 1994, 1997), to suppress FSH-dependent Sertoli cell proliferation during this same period in marmosets and rats (Sharpe et al., 1999, 2000) and to suppress FSH levels to the limit of detection in prepubertal rats (Sharpe et al., 1999). Treatments were administered as weekly s.c. injections in propylene glycol:water (1:1, v:v). To minimize variation between individuals in parameters related to testis size and development, the present study partly used infantile male co-twins (n = 6) with one co-twin being administered vehicle as a control and the other co-twin being administered the GnRH antagonist. This design enabled pair-wise comparison of data for each control and treated co-twin at 35 weeks of age (n = 4 co-twins), thus minimizing the number of animals required for study. A total of six control (all co-twins) and six GnRH antagonist-treated animals (n = 4 co-twins, n = 2 singletons) were killed at 35 weeks of age whilst, in the remaining animals (five control, six GnRH antagonist-treated; including two sets of co-twins), treatment was stopped at week 35 and the animals were allowed to progress through puberty into adulthood. Blood was taken from all animals at week 25 prior to treatment, at week 30 during treatment and at week 35 (end of treatment). In animals that were allowed to grow to adulthood, blood was also taken at weeks 40, 50, 60, 70 and 100 to monitor progress through puberty, as indicated by blood testosterone levels (Lunn et al., 1994, 1997; McKinnell et al., 2001b). Blood samples were collected from the femoral vein using a (heparinized) syringe and needle. It was not necessary to sedate animals for this procedure. These studies were approved by the local ethical committee for studies in primates and were performed according to the Animal Scientific Procedures (UK) Act (1986) under Project Licence approval by the UK Home Office.

To place findings for some testicular cell functional parameters at 35 weeks into perspective, the same endpoints were evaluated in the testes of control marmosets of various ages. The primary use of the latter animals was as controls for other studies unconnected with those presented here.

Tissue collection and processing

Animals were killed via i.p. injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK). Testes with epididymides attached were dissected free of connective tissue and immersion-fixed for 5.5 h in Bouin’s fluid after which the testis was dissected away from the epididymis and weighed; fixed testes were then processed overnight in a Shandon processor and embedded in paraffin.

Immunohistochemical markers of testicular cell development

Several markers were used in order to gauge the status of testicular cell development. Some of these were tests cell-specific, for example sulphated glycoprotein-2 (SGP-2) and anti-müllerian hormone (AMH) for Sertoli cells and 3β-hydroxysteroid dehydrogenase (3β-HSD) for Leydig cells, whereas others (e.g. androgen receptor, inhibin-α) were expressed in more than one cell type. SGP-2 is one of the major
secretory proteins of the Sertoli cells in the testis of the rat and human (O’Bryan et al., 1994; McKinnell et al., 1995) and has also been identified in the marmoset testis (O’Bryan et al., 1994); its precise role(s) in the testis remains unclear. AMH is secreted by the fetal Sertoli cell during sexual differentiation and is responsible for inducing regression of the müllerian ducts (Josso et al., 1998); its role in the postnatal testis is unclear, though a role in suppressing development of the adult generation of Leydig cells has been postulated (Josso et al., 1998; Racine et al., 1998). β-HSD is one of the enzymes required for the biosynthesis of testosterone and is widely recognized as being a specific marker of Leydig cells/Leydig cell precursors (Ge et al., 1996). Inhibin-α is one of the subunits of the dimeric protein, inhibin-B, which is secreted by Sertoli cells and possibly by Leydig cells in the testis, though various forms of the free α subunit may also be secreted (Winters and Plant, 1999; Anderson and Sharpe, 2000). Based on studies in the rat, androgen receptors (AR) are expressed in Sertoli cells just prior to puberty in the rat and in other somatic testicular cells, but not germ cells, at all ages (Bremner et al., 1994). Finally, estrogen receptor-β (ERβ) was used as our studies in various species have shown that it is expressed in most cell types in the testis, including most germ cells, at all ages and its expression appears to be more or less constant in widely differing situations (McKinnell et al., 2001b; Saunders et al., 2001). Finally, proliferating cell nuclear antigen (PCNA), which is expressed widely during the cell cycle, and is thus a marker of ‘non-quiescent’ cells, was used to investigate cell proliferation; PCNA has been used for similar purposes in many previous studies including in the rat (Schlatt and Weinbauer, 1994), marmoset (Millator et al., 2000), thersus monkey (Schlatt and Weinbauer, 1994) and human (Steger et al., 1998). Specific antibodies were used to investigate the immunohistochemical expression of these various target proteins in testicular sections from marmosets. Details of the antibodies and their use are given below.

**Antibodies**

Immunolocalization of AR utilized a rabbit polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) raised against an epitope at the N-terminus of human AR, and was used at a dilution of 1:2000. ERβ was immunolocalized using an affinity-purified, polyclonal anti-peptide IgG raised in sheep against a specific peptide in the hinge (D) domain of human ERβ, as previously described in detail (Saunders et al., 2000); it was used at a dilution of 1:1000. Rigorous evaluation of the specificity of the antibodies used, based on Western analyses and pre-absorption, has been detailed in our previous studies (Saunders et al., 2000; Williams et al., 2000; McKinnell et al., 2001a). SGP-2 was immunolocalized using a polyclonal antibody raised in sheep against human SGP-2 (gift from Dr. J. McRae), used at a dilution of 1:3000. Immunolocalization of β-HSD and AMH utilized rabbit polyclonal antibodies against the human proteins (gifts from Professors I.Mason and N.Josso respectively); they were used at a dilution of 1:2000 and 1:1000 respectively. Proliferating cell nuclear antigen (PCNA) was immunolocalyzed utilizing a monoclonal antibody (PC10; Dako) used at a dilution of 1:100. Immunolocalization of inhibin-α utilized a monoclonal antibody (173/9K) described previously (Groome et al., 1990; Majdic et al., 1996), and used at a dilution of 1:1000.

**Immunohistochemistry**

Unless otherwise stated, all incubations were performed at room temperature. Sections were deparaffinized in Histoclear (National Diagnostics, Hull, UK), rehydrated in graded ethanol and washed in water. For AR, ERβ, AMH and inhibin-α, a temperature-induced antigen retrieval step (Norton et al., 1994) was used prior to immunohistochemistry. The method was optimized for each antibody and therefore varied slightly according to the antibody used. Thus, sections were subjected to antigen retrieval in either 0.01 mol/l citrate buffer, pH 6.0 (for AR, AMH and inhibin-α), or 0.05 mol/l glycine, pH 3.5, and 0.01% (w/v) EDTA (for ERβ). After pressure cooking for 5 min at full pressure, sections were left to stand, undisturbed, for 20 min, then cooled under running tapwater before being washed twice (5 min each) in Tris-buffered saline (TBS; 0.05 mol/l Tris–HCl, pH 7.4, 0.85% w/v NaCl). Endogenous peroxidase activity was blocked by immersing all sections in 3% (v/v) H2O2 in methanol (both from BDH Laboratory Supplies, Poole, Dorset, UK) for 30 min, which was followed by two 5 min washes in TBS. To block non-specific binding sites, sections were incubated for 30 min with the appropriate normal serum diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin (BSA; Sigma, Poole, Dorset, UK). For AR, β-HSD and AMH, normal swine serum (NSW) was used and for ERβ, SGP-2, PCNA and inhibin-α, normal rabbit serum (NRS) was used (both from Diagnostics Scotland, Carluke, UK). Primary antibodies were added to the sections at the appropriate dilution in either NSW/TBS/BSA (for AR, β-HSD and AMH) or NRS/TBS/BSA (for ERβ, SGP-2, PCNA and inhibin-α) and incubated overnight at 4°C in a humidified chamber, followed by two 5 min washes in TBS. For AR, β-HSD, AMH and inhibin-α, sections were then incubated for 30 min with horseradish peroxidase (HRP)-labelled polymer (Envision; Dako). For other antibodies, sections were incubated for 30 min with either biotinylated rabbit anti-sheep IgG (Vector Laboratories, Peterborough, UK) in the case of ERβ and SGP-2, or biotinylated rabbit anti-mouse IgG (Dako) in the case of PCNA, then washed twice (5 min each) in TBS, and incubated for a further 30 min with avidin–biotin conjugated to horseradish peroxidase (Dako) diluted in 0.05 mol/l Tris–HCl, pH 7.4, according to the manufacturer’s instructions. All sections were then washed twice (5 min each) in TBS, and immunostaining was developed using Liquid DAB-plus (Dako) until staining was optimal, when the reaction was stopped by immersing sections in distilled water. All sections were then lightly counterstained with haematoxylin, dehydrated in graded ethanol, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). As negative controls, slides were processed as above except that the appropriate normal serum was substituted for the primary antibody. To ensure the reproducibility of findings, tissue sections from four or five animals at each age or treatment group were evaluated, and this was performed on at least two separate occasions.

Immunostained sections were examined and photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a digital camera (DCS330; Eastman Kodak, Rochester, NY, USA). Captured images were transferred to a computer (G4; Apple Computer Inc., Cupertino, CA, USA) and compiled using Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA, USA) before being printed using an Epson Stylus 870 colour printer (Seiko Epson Corp., Nagano, Japan).

**Determination of PCNA-labelled germ cell volume per testis and the spermatogonial labelling index**

The volume per testis of PCNA-labelled cells was determined by standard point-counting. Cross-sections of testes from all marmosets in each treatment group were examined under oil-immersion using a Leitz x63 plan apo objective fitted to a Leitz laborlux microscope and a 121-point eyepiece graticule. Using a systematic clock-face sampling pattern from a random starting point, 16 fields were counted. Points falling over the nuclei of PCNA-positive cells were scored and expressed as a percentage of the total points counted. For each animal, the values for percentage nuclear volume were converted to absolute nuclear volumes per testis by reference to testis volume (= weight) as shrinkage was minimal, i.e. testis weights before and...
Figure 2. General testicular morphology and Sertoli cell functional status at age 35 weeks in marmoset co-twins treated for the previous 10 weeks with either vehicle (control) or a GnRH antagonist (GnRHa). Functional status of the Sertoli cells was assessed by immunexpression of sulphated glycoprotein-2 (SGP-2) and androgen receptor. Note in controls, but not in GnRHa-treated animals, the presence of ‘vacuoles’ (asterisks) in the cytoplasm of Sertoli cells, thought to reflect focal lumen formation and the accumulation of seminiferous tubule fluid (see also Figure 4). Scale bar = 100 µm.

after fixation were comparable in each treatment group. All PCNA-positive cells detected within the seminiferous tubules in this study were identified as being germ cells; no labelled Sertoli cells were observed.

Point-counting enabled comparative determination of the relative proportions of non-quiescent cells in the testes of control and GnRH antagonist-treated marmosets, but did not discriminate between germ cell types (some spermatogonia and some spermatocytes were PCNA-labelled). As it is the proliferating spermatogonia that are thought to be most at risk from toxic effects of cancer therapy, the PCNA-labelling index of spermatogonia was determined separately. The method used the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc., Alexandria, VA, USA) and utilized an Olympus BHS microscope fitted with an automatic stage (Applied Scientific Instrumentation Inc, Eugene, OR, USA). The area fraction probe places a grid in the frame and the Object Area fraction is determined by clicking each ‘×’ that touches the object of interest (in this case, the cytoplasm or nucleus of a spermatogonium). Each spermatogonium was classed as PCNA-positive or -negative. Spermatogonia that were in contact with the basement membrane of the seminiferous tubule were classified as germ cells. Spermatogonial nuclei were easily distinguished from Sertoli cell nuclei but it was decided that no attempt would be made to exclude preleptotene spermatocytes (which may still be in contact with the basement membrane) from this analysis as these cells (especially unlabelled) could not be reliably identified under the present circumstances. The PCNA-labelling index of spermatogonia was calculated as a percentage of the total of labelled + unlabelled cells.

Determination of Leydig cell volume per testis
Leydig cell volume per testis was determined by point counting on sections of Bouin-fixed testes which had been immunostained for 3β-HSD using modifications of methods described previously (Sharpe et al., 1998, 2000). The method used the Area Fraction Probe in the Stereologer software programme as described above. Once completed, Stereologer automatically displays the results including area fraction and coefficient of error. The values for area fraction were then converted to absolute volumes per testis by reference to testis volume (= weight).

Plasma levels of testosterone
Levels of testosterone in plasma were measured using an enzyme-linked immunosorbent assay adapted from an earlier radioimmunoassay method (Corker and Davidson, 1981) as detailed elsewhere (Atanassova et al., 1999). The limit of detection was ~12 pg/ml.

Statistical analysis
Comparison of data from control and GnRH antagonist-treated marmosets used either analysis of variance or Student’s t-test. Where comparison of endpoints was made for co-twin marmosets (n = 4) at week 35, the paired t-test was used. In all of the latter comparisons, as well as for plasma testosterone levels during progression through
puberty, data were log-transformed prior to analysis as there were unequal variances in control and GnRH antagonist-treated groups.

**Results**

**Effect of GnRH antagonist treatment on testis weight and gross morphology at 35 weeks of age**

Mean testis weight in GnRH antagonist-treated males (52 ± 6 mg, mean ± SD, n = 6) at 35 weeks was reduced by 34% ($P < 0.05$) compared with vehicle-treated controls (78 ± 19 mg, n = 4). In both control and GnRH antagonist-treated marmosets, the Sertoli cells still appeared immature, based on their nuclear size and morphology and on their positional arrangement within the seminiferous epithelium (Figure 2). In other respects (e.g. numbers of germ cells), testes from GnRH antagonist-treated males appeared less developed than controls, though there was considerable variation between individual controls in the degree of their development. For this reason, subsequent comparisons of specific endpoints at 35 weeks in control and GnRH antagonist-treated animals focused on co-twin comparisons, though it is emphasized that both of the singleton GnRH antagonist-treated marmosets studied at 35 weeks also showed delay in testis development comparable with that shown by GnRH antagonist-treated co-twin males.

**Sertoli cell development**

Four protein markers of Sertoli cell development were used, AMH, inhibin-α, AR and SGP-2, as our studies of the marmoset testis during development have indicated that these show very different temporal patterns of immunoexpression (McKinnell et al., 2001b; C.McKinnell et al., unpublished data). All of these protein markers were detectable immunohistochemically in Sertoli cells at week 35 in the testes of all four control marmosets, AR in the nucleus and the other three proteins in the cytoplasm. In GnRH antagonist-treated animals, the most dramatic change, compared with controls, was in SGP-2 immunoexpression which was either non-detectable or was just detectable in small focal patches (Figure 2). AR immunoexpression was also consistently reduced in intensity in Sertoli cell nuclei of GnRH antagonist-treated males, but the difference from control was not as marked as for SGP-2 (Figure 2). AMH and inhibin-α were both immunoexpressed at only low intensity in control males at 35 weeks of age and GnRH antagonist-treated showed either no consistent change (AMH) or a small but consistent increase in intensity of immunoexpression (inhibit-α; not shown). To place the GnRH antagonist-induced change in immunoexpression of SGP-2 in context, the pattern of this marker was compared in the testes of marmosets from birth through to adulthood (Figure 3). At birth (not shown) and throughout the neonatal period and early...
infancy (15–25 weeks), SGP-2 expression was non-detectable and first became detectable at week 35; thereafter, immunexpression increased markedly by late puberty and in adulthood. Based on these observations, SGP-2 appears to be an indicator of adult Sertoli cell function, and suppression of its expression in GnRH antagonist-treated males is therefore taken as evidence of delayed maturational development of the Sertoli cells.

Variable numbers and sizes of ‘vacuoles’ were evident in the central portions of the cytoplasm of Sertoli cells in all control animals at 35 weeks (Figure 2), but not at younger ages (Figure 3). These ‘vacuoles’ were far less evident or were completely absent from GnRH antagonist-treated males (Figure 2). These vacuoles were considered to represent the very early stages of lumen formation/semiferous tubule fluid (STF) production, as formation of the vacuoles was closely associated with SGP-2 and AMH immunoexpression; the ‘vacuoles’ may reflect focal formation of inter-Sertoli cell tight junctions.

Germ cell development

Control twins had more numerous and more advanced germ cell types (some early spermatocytes were apparent) in their seminiferous tubules than did their GnRH antagonist-treated co-twins, though there was considerable variability between individual control males (Figure 4). To highlight this difference and to provide an indication of cell proliferation, immunostaining with PCNA was applied (Figure 4). Virtually all cells within the seminiferous tubules that labelled with PCNA were germ cells and these were evidently more numerous in control twins than in their GnRH antagonist-treated co-twin brothers. Most germ cells, as well as Sertoli cells, immunoexpressed ERβ in their nuclei and the intensity of this expression did not change detectably in GnRH antagonist-treated compared with control marmosets (Figure 4).

Leydig (3β-HSD positive) cell development

Leydig cells were evident in the interstitium of all control 35 week males though there was variation between males in the volume per testis of these cells (Figure 4). It was also evident that these Leydig cells had very scanty cytoplasm. In contrast, Leydig (3β-HSD positive) cells were virtually non-detectable in GnRH antagonist-treated males and only an occasional, isolated 3β-HSD positive cell was evident (Figure 4). To place Leydig cell development in 35 week control marmosets in context, two analyses were undertaken. First, immunoexpression of 3β-HSD was compared in marmosets from neonatal through to adult life (Figure 5). This showed that 3β-HSD immunexpression was relatively intense neonatally (4 weeks) then declined at 20 and 35 weeks before increasing markedly at puberty and through to adulthood. Second, Leydig cell nuclear volume (approximates to the number of cells) and cytoplasmic volume (approximates to steroidogenic capacity) at 35 weeks in control marmosets was compared with marmosets of later ages. This comparison revealed that, based on nuclear volume of 3β-HSD positive cells, the number of Leydig cells increased ~5-fold between 35 weeks and puberty/adulthood whereas cytoplasmic volume per testis increased by up to 10-fold in the same period (Figure 6).

Quantification of testis cell development and its retardation by GnRH antagonist treatment

To confirm the changes in immunoeexpression profiles in GnRH antagonist-treated co-twins described above, three of the endpoints described were quantified, one each relating to Sertoli cells, germ cells and Leydig cells. As an indicator of Sertoli cell maturational development, lumen (‘vacuole’) volume per testis was quantified for each of the four sets of control and GnRH antagonist-treated co-twins (Figure 7). This analysis showed that lumen volume was reduced by an average of 78% (range 57–97%) in GnRH antagonist-treated males when compared with their co-twin controls. Similarly, Leydig cell volume was reduced by an average of 97% (range 95–98%) following GnRH antagonist treatment (Figure 7). The volume of PCNA-positive (germ) cells per testis was also reduced in GnRH antagonist-treated males by 88% (range 80–94%) but separate analysis of the spermatogonial PCNA-labelling index failed to show any significant difference between control and GnRH antagonist-treated co-twins, though overall numbers of spermatogonia per testis were consistently reduced (Figure 8). It was also evident from Figure 7 that variation between individual control males in the parameters measured showed consistency, at least for the comparison of PCNA-positive cells and Leydig cell volume, probably indicating an inter-relationship between the endpoints (e.g. between Leydig cell development and germ cell proliferation/development).

Effect of GnRH antagonist-treatment from week 25 to week 35 on the pubertal rise in testosterone levels

Plasma testosterone levels in control marmosets remained low or at the limit of detection (<0.12 ng/ml) from week 25 to week 35, then showed some evidence of a minor rise in some animals at 40–50 weeks and by weeks 60–70 had achieved adult levels (Figure 9). In GnRH antagonist-treated males, testosterone levels remained consistently low during weeks 25–50 and thereafter showed a delayed increase at 60–70 weeks in most but not in all animals (see Figure 9), with the result that mean levels for the treatment group were significantly less than those of controls at 60 and 70 weeks of age. The timing of increase in testosterone levels in GnRH antagonist-treated males varied considerably between individuals, as illustrated by comparison of the data for the two sets of co-twins that went through puberty. For the second of these, the GnRH antagonist-treated co-twin still had baseline testosterone levels at 60 and 70 weeks of age when the control twin had adult levels of testosterone (Figure 9). In contrast, the other GnRH antagonist-treated co-twin had levels of testosterone within the normal adult range by 70 weeks. At week 100, all GnRH antagonist-treated males had testosterone levels that were within the normal adult range (Figure 9).

Discussion

The present studies had two specific aims. First, to establish if there was any detectable evidence for functional activation
Infantile primate testis

Figure 4. Immunoexpression of PCNA, ERβ and 3β-HSD at age 35 weeks in the testes of marmoset co-twins treated for the previous 10 weeks with either vehicle (control) or a GnRH antagonist (GnRHa). Note that immunoexpression of PCNA within seminiferous tubules is confined to the nuclei of germ cells whereas expression of 3β-HSD is confined to the cytoplasm of Leydig cells. In contrast, ERβ is immunoexpressed in the nuclei of both of these cell types as well as in Sertoli cells (arrows). Asterisks indicate the presence of ‘vacules’ in the cytoplasm of Sertoli cells in controls, that are thought to reflect focal lumen formation. Scale bars = 100 µm.

of testicular cells during the period of ‘childhood quiescence’ in the male marmoset. Second, to establish if this presumed activation could be suppressed by inhibiting gonadotrophin secretion by the administration of a potent, long-acting GnRH antagonist for the preceding 10 weeks. It was hoped that this information would cast light on the puzzling susceptibility of the childhood human testis to adverse effects of some cancer therapies and demonstrate whether or not induction of testicular quiescence, as a strategy for protecting spermatogenic potential, is a realistic possibility in such situations. On the assumption that our findings in the marmoset can be transposed to the corresponding situation in boys, our results provide important new information on the functional activation of the testis during infancy and childhood, offer a potential explanation for the puzzle referred to above, and raise important issues concerning the feasibility of reducing/preventing this activation therapeutically.

There are various data which together suggest that the prepubertal human testis may be ‘quietly active’ as opposed to truly quiescent (Wu et al., 1990, 1991, 1996; Clark et al., 1997; Chemes, 2001). For example, germ cell number per testis increases ~3-fold during childhood along with a significant increase in testis volume/weight (Muller and Skakkebaek, 1983; Paniagua and Nistal, 1984), and increases in Leydig cell development (Nistal et al., 1986) and in the intratesticular (Chemes, 1996) and spermatic vein levels of testosterone...
Figure 5. Changes in Leydig cell immunoexpression of 3β-hydroxysteroid dehydrogenase from week 35 to adulthood in the marmoset testis. Images are representative of three to six animals in each age range. Scale bar = 100 µm.

Figure 6. Changes in Leydig cell (3β-hydroxysteroid dehydrogenase-positive) nuclear and cytoplasmic volume per testis from week 35 through puberty (58–64 weeks) into adulthood in the marmoset testis. Values are mean ± SEM for three or four animals per age group. *P < 0.05, **P < 0.01, in comparison with respective value for week 35 (Student’s t-test comparison after logarithmic transformation of the data).

(Santoro et al., 1981) have been reported between 2 and 4 and between 6 and 8 years of age. The latter changes may coincide with night-time activation of pulsatile LH secretion (Wu et al., 1990, 1991, 1996; Hayes and Crowley, 1998). There is also evidence that formation of spermatocytes, and even occasional spermatids, is a normal feature of the childhood testis (Muller and Skakkebaek, 1983; Paniagua and Nistal, 1984; Rey et al., 1993; Chemes, 2001), but all of these cells degenerate because of the lack of appropriate maturation or functional support from the Sertoli and Leydig cells (Chemes, 2001). The latter observations imply that germ cell proliferation and differentiation are more widespread than is revealed simply by counting the numerical increase in germ cells. All of these described changes occur at least 4–5 years prior to the onset of puberty (Chemes, 2001), and although they are of small magnitude when compared with the change in the same parameters that occur during puberty, it may be these changes that are responsible for the susceptibility of the testis of boys to damage by some cancer therapies.

The present studies in the marmoset reinforce the impression gained from the limited human studies by demonstrating that significant testicular cell activity is detectable at an age (35 weeks) when the testis is considered to be ‘quiescent’ and which is considerably in advance of the first signs of normal puberty in our colony of marmosets (50–60 weeks), based on the blood levels of testosterone (Lunn et al., 1994, 1997; McKinnell et al., 2001b; this study). This activity extended to Sertoli, Leydig and germ cells based on various functional markers of their maturational development and/or proliferation. Moreover, our studies show that this cellular activation is largely, if not completely, dependent on gonadotrophin stimulation as the functional cell changes were almost completely inhibited by treatment for 10 weeks with a GnRH antagonist.
Infantile primate testis

Figure 7. Effect of treatment of marmoset co-twins with either vehicle (controls) or a GnRH antagonist from weeks 25–35 on seminiferous tubule (ST) lumen volume, the volume of proliferating cell nuclear antigen (PCNA)-positive cells (= germ cells) and 3β-hydroxysteroid dehydrogenase positive (Leydig) cells per testis at 35 weeks. Each bar in the main panel shows data for a single co-twin whereas bars in the panel to the right show the group means. *P*-Values shown were derived using the paired *t*-test and log-transformed data.

With the exception of expression of low levels of 3β-HSD in Leydig/precursor cells, the marmoset testis at age 20–25 weeks shows none of the cellular activity found at 35 weeks (this study plus our unpublished data). This suggests that development of the testis towards its adult status and function is initiated much earlier than ‘at puberty’. Moreover, the early changes occurring during the ‘quiescent’ phase are without any observable manifestation outside of the testis itself, as has been suggested (Chemes, 2001). Indeed, even within the testis, the clear impression morphologically was that Sertoli cells were still immature, yet evaluation of their functional status via SGP-2 indicated that their functional differentiation was already under way. If such findings in the marmoset are predictive of the human, then they imply that functional development of testicular cells is likely to be initiated at some point during childhood. It is not possible to predict accurately in what age range such changes might occur because of the considerably greater length of human childhood compared with the equivalent period in the marmoset. However, other data (Wu et al., 1990, 1991, 1996) suggest that this could be by mid-childhood. As the present studies have identified a number of protein markers of testicular cell functional development, application of these to human testicular autopsy specimens should enable us to establish if, and when, comparable changes in expression of these markers occurs during human testis development. Such studies are in progress.

The potential importance and usefulness of the present findings depends on the extent to which the marmoset provides an appropriate model for development of the human testis during childhood. As this period has been poorly studied thus far, it is difficult to draw definitive conclusions. In most other respects the marmoset does appear to be a reasonable model for the human. Thus the marmoset descends its testes into the scrotum by birth, exhibits neonatal proliferation of Sertoli cells and a testosterone surge followed by infantile ‘quiescence’, a pattern of events comparable with those in the human male (Lunn et al., 1994, 1997; Sharpe et al., 2000; McKinnell et al., 2001b). Finally, in adulthood the organization of spermatogenen-
Figure 9. Effect of treatment of marmosets with vehicle or a GnRH antagonist (GnRHa) from weeks 25–35 on plasma testosterone levels from pretreatment through to adulthood. Top panel shows mean ± SEM data for all animals (n = 11–12 per group up to age 35 weeks and n = 5–6 at later ages) and the lower two panels show data for two sets of co-twins to illustrate the variation between animals in delay of the pubertal testosterone rise after GnRHa treatment. Note that the low testosterone level at 100 weeks in control twin 3 is not unusual and is probably the result of the natural episodic variation in testosterone levels. *P < 0.05, in comparison with control (Student’s t-test using log-transformed data).

esis in the marmoset shows many of the features typical of the human (Millar et al., 2000; Sharpe et al., 2000). Other non-human primates, for example the Cebus monkey, show comparable changes with the marmoset and human (Rey et al., 1993), though the more commonly used Rhesus monkey shows some notable differences in the timing of testicular descent and neonatal Sertoli cell proliferation (Sharpe et al., 2000), but does show a neonatal testosterone surge (Mann et al., 1997; Weinbauer and Nieschlag, 1999) as in the human (Andersson et al., 1998) and marmoset (Lunn et al., 1994; Weinbauer and Nieschlag, 1999; Sharpe et al., 2000; McKinnell et al., 2001b).

It is logically presumed that the ability of GnRH antagonist administration to suppress testicular cell activation in the marmoset during ‘childhood’ in the present studies results from suppression of gonadotrophin secretion. However, this could not be shown directly because of the continued unavailability of assays for the detection of gonadotrophins in the marmoset. However, this observation demonstrates the feasibility of being able to suppress such testicular cell activity. This is widely considered to be a prerequisite for any method of protecting spermatogenic potential in situ in boys with cancer who are at risk of future infertility because of their cancer therapy (Howell and Shalet, 1999; Grundy et al., 2001). As the present findings demonstrate functional activation of Sertoli and Leydig cells, as well as of germ cells, in the ‘childhood’ marmoset testis, they do not provide an unequivocal answer as to which cell type(s) in the testes of children might confer susceptibility to damage by cancer therapies, though it is presumed that loss of stem spermatogonia is ultimately responsible for the loss of sperm production in adulthood in such children (Howell and Shalet, 1999; Grundy et al., 2001). In this regard, the present data demonstrate that spermatogonial proliferation, based on PCNA-labelling, was apparently not suppressed in 35 week marmosets by GnRH antagonist administration, though the absolute volume per testis of spermatogonia was reduced. These findings suggest that the survival of spermatogonia may be partially gonadotrophin-dependent whereas the proliferation of surviving spermatogonia is largely or completely gonadotrophin-independent. If the latter involves spermatogonial stem cells, then GnRH antagonist therapy is likely to be ineffectual in providing protection against cancer therapies that will affect these cells. Nevertheless, current interest in testicular germ cell transplantation means that understanding of the factors regulating spermatogonial stem cell proliferation and spermatogonial differentiation are moving ahead fast, and this new understanding is likely to open new interventionist possibilities.

For the reasons voiced above, it is considered essential that several key questions are answered sequentially before GnRH agonist/antagonist or other intervention therapies in childhood could be considered clinically as a means of protecting the testis from cytotoxic therapy. First, it has to be demonstrated that such a therapeutic intervention is safe and does not itself induce unacceptable long-lasting problems. We are undertaking such studies in the marmoset by tracking control and GnRH antagonist-treated animals through puberty and into adulthood. Data for the pubertal rise in testosterone levels, and its delay in GnRH antagonist-treated marmosets, are presented in the current study. Fertility of these animals is being assessed and eventually the animals will be subjected to a full autopsy. If these findings do not indicate any major long-term effects of the GnRH antagonist treatment, the logical next question to
ask is whether this treatment really does confer testicular protection against potentially damaging cancer therapy. For this purpose, we would test whether GnRH antagonist treatment, as undertaken in the present studies, is able to protect against local testicular irradiation. However, the present findings indicating continued (and presumably gonadotrophin-independent) proliferation of spermatogonia, based on PCNA-labelling, cast doubt on whether such a strategy would be successful. It therefore appears that improved understanding of the factors regulating spermatogonial proliferation will be necessary before protection strategies for the human testis in childhood can be considered seriously.

Though our findings raise questions about the likelihood of success of GnRH antagonist intervention therapy as a protection for the testis in boys with cancer, they provide important new information that strongly supports the concept that the testis in childhood is quickly active (Chemes, 2001). The present studies have examined this activity in the ‘childhood’ marmoset testis using various protein markers, and we have studies in progress to evaluate immunoexpression of the same markers in the human testis throughout childhood. Combined with more detailed studies of spermatogonial development, these studies should provide much-needed information on the status of cell activity in the human testis in childhood and thus allow determination of the age range when intervention ‘testis-quietening’ therapy is potentially viable. At the very least, such studies should improve our understanding of cell development and cell–cell interactions in the childhood testis and help to elucidate why the testis at this time is susceptible to toxic damage by some cancer therapies.

In summary, the present findings add to the growing impression that the ‘childhood’ testis is not quiescent and that activation of testicular cell function occurs well before the onset of puberty (gonadarche). These changes are largely gonadotrophin-dependent as they can be mostly prevented by GnRH antagonist administration, though an important exception appears to be spermatogonial proliferation. These findings indicate that the regulation of GnRH (gonadotrophin)-independent spermatogonial proliferation requires elucidation, but provide new insight into the unexplained susceptibility of the ‘quiescent’ childhood testis to damage by certain cancer therapies and open the possibility of designing interventionist protection strategies.

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