CLINICAL STUDY

Serum activin A and follistatin in disorders of spermatogenesis in men

S Muttukrishna, A Farouk¹, S Sharma¹, L Evans², N Groome², W Ledger and M Sathanandan¹

Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK, ¹Department of Obstetrics and Gynaecology, Harlowood Hospital, Essex RM3 OBE, UK and ²School of Biological and Molecular Sciences, Oxford Brookes University, Headington, Oxford, UK

(Correspondence should be addressed to S Muttukrishna, Department of Obstetrics and Gynaecology, Royal Free–UCL Medical School, 86–96 Chenies Mews, London WC1E 6HX, UK; Email: s.muttukrishna@ucl.ac.uk)

Abstract

Objective: Inhibin, activin and follistatin are glycoprotein hormones produced by the gonads. Recent studies have shown that inhibin B is the predominant form of inhibin in the circulation in men. The objective of this study was to investigate circulating levels of activin A and follistatin in disorders of spermatogenesis in men and their relationship with FSH and inhibin B.

Design and method: Serum from five different groups of men was prospectively collected and stored at -20°C . The groups were men with: (i) proven fertility (controls) ($n = 20$), (ii) primary testicular failure ($n = 15$), (iii) obstructive azoospermia ($n = 10$), (iv) oligospermia ($n = 10$) and (v) miscellaneous sperm dysfunction ($n = 40$). WHO criteria (1992) were used for semen characterisation. Serum concentrations of 'total' activin A, follistatin, FSH and inhibin B were measured using specific two-site enzyme immunoassays.

Results: Activin A levels were significantly lower than in the controls in the obstructive azoospermia group and higher in the miscellaneous sperm dysfunction group. Serum follistatin levels did not significantly vary in any group compared with the controls. Circulating levels of FSH were higher than in the controls in the primary testicular failure and obstructive azoospermic group. Levels of inhibin B were lower than in the controls in all disorders of spermatogenesis studied.

Conclusion: This study demonstrates that activin A and follistatin are in the circulation in males and activin A levels are significantly lower in obstructive azoospermia and higher in miscellaneous sperm dysfunction than in controls. The mechanism involved in altering the levels of activin A in these conditions is not clear. However, high follistatin:activin A molar ratios (>2.5) in all groups suggests that all activin A in the circulation is bound to follistatin in males.

European Journal of Endocrinology 144 425–429

Introduction

Inhibins are gonadal glycoprotein hormones consisting of alpha and beta subunits linked by disulphide bridges. Inhibin A is an $\alpha\text{-}\beta_{\text{A}}$ dimer and inhibin B is an $\alpha\text{-}\beta_{\text{B}}$ dimer. Activins are homodimers of beta subunits linked by disulphide bridges. Activin A is a $\beta_{\text{A}}\text{-}\beta_{\text{A}}$ dimer, activin AB is a $\beta_{\text{A}}\text{-}\beta_{\text{B}}$ dimer and activin B is a $\beta_{\text{B}}\text{-}\beta_{\text{B}}$ dimer. Follistatin is a single chain glycoprotein.

Inhibins, activins and follistatin have been implicated in the endocrine regulation of pituitary follicle-stimulating hormone (FSH). Inhibins and follistatin have an inhibitory effect on pituitary FSH secretion, while activins have a stimulatory effect. Follistatin is also a high-affinity activin-binding protein that regulates the bio-availability of activins.

Development of specific enzyme immunoassays (EIAs) for inhibin A (1, 2), inhibin B (3), activin A

(4), activin AB (5) and follistatin (6) has facilitated the measurement of different molecular forms of inhibins, activins and follistatin. Recent studies have shown that inhibin B is the major circulating form of inhibin in men, while inhibin A is not detected in male serum. In a previous study, close negative correlation was found between inhibin B and FSH concentrations in semen donors, infertile men and men with elevated FSH, but not in a group of healthy volunteers (7).

In males, there is evidence for the presence of inhibin/activin subunits in the Leydig and Sertoli cells. Inhibin B is also measurable in seminal plasma, and may reflect the functional state of the seminiferous epithelium (8). Several groups have studied the relationship between inhibin B and FSH in normal men and in men with disorders of spermatogenesis (7–15). There are no studies reporting levels of activin A and follistatin in male circulation and in disorders of

spermatogenesis. In most systems activins have the opposite biological function to that of inhibins. We proposed to explore the possible endocrine role of activin A in the pituitary–gonadal axis of men. Therefore, the objective of this study was to investigate circulating levels of activin A and follistatin in disorders of spermatogenesis in men and their relationship with FSH and inhibin B.

Materials and methods

Patient groups

WHO (1992) criteria were used for normal semen characterisation (27). All groups of men had a semen analysis. Semen analysis was carried out manually by one person for all research patients. Group 1 were normal control men, subjects with proven fertility ($n = 20$); group 2 were patients with primary testicular failure, i.e. they had both azoospermia and bilateral small testes with raised FSH ($n = 15$); group 3 had obstructive azoospermia, i.e. normal testes and normal FSH with radiologically demonstrated bilateral obstruction ($n = 10$); group 4 had oligospermia, i.e. decreased sperm number ($n = 10$) and normal FSH; group 5 had miscellaneous sperm dysfunction, i.e. normal testes, normal FSH and normal sperm number or decreased sperm number with decreased sperm motility ($n = 40$). The ages of the patients are shown in Table 1.

Hormone assays

‘Total’ activin A Serum concentrations of ‘total’ activin A were measured using an EIA specific for ‘total’ activin A as described in detail elsewhere (4). An in-house standard preparation (partially purified human follicular fluid) was standardised against recombinant human (rh) activin A (Genentech, San Francisco, CA, USA) and used as the assay standard. The mean intra- and interassay coefficient of variation (CV) values were 6.5 and 7.7% respectively. The minimum detection limit of the assay was 50 pg/ml.

Follistatin Serum concentrations of ‘total’ follistatin 288 (FS288) were measured in duplicates as previously described (6). Follistatin standard used in the assays was affinity purified (from human follicular fluid) and

standardised against rh follistatin. The minimum detection limit of the assay for rh follistatin standard was 20 pg/ml. The mean intra- and interassay CV values were <10%. The assay cross-reacts 9.9% with FS315.

Inhibin B Serum concentrations of dimeric inhibin B were measured in 100 µl duplicates using an EIA as described in detail elsewhere (3). An in-house standard preparation (partially purified human follicular fluid) was standardised against rh inhibin B (Genentech) and was used as the assay standard. Minimum detection limit of the assay for rh inhibin B was 15 pg/ml. The mean intra- and interassay CV values were 6.2 and 7.2% respectively.

FSH Serum concentrations of FSH were measured using an Immulite-chemiluminescent assay kit (Diagnostic Products Ltd, Los Angeles, CA, USA). The mean intra- and interassay CV values were <10%. The detection range was 0.1–170 mIU/ml.

Statistical analysis

Results given in the text are means $\pm$ S.E.M. Data were log transformed to obtain a normal distribution. Unpaired Student's *t*-tests were carried out to compare the controls with the different groups. Correlation analysis was carried out controlling for age to study the relationship between different hormones measured. Statistical Package for Social Sciences (SPSS) was used for the analysis.

Results

Serum activin A

‘Total’ activin A concentrations in control men ranged from 85 to 240 pg/ml with a mean of 150 ± 8 pg/ml. Activin A levels were significantly lower than in the controls in the obstructive azoospermia group (62.2 ± 5.8 , $P < 0.001$) and higher in the miscellaneous sperm dysfunction group (201.6 ± 5.5 , $P < 0.01$). However, serum activin A did not vary significantly from the controls in the other groups (Fig. 1).

Serum follistatin

Follistatin levels ranged from 443 to 1591 pg/ml in the control group and the levels did not significantly vary in any group compared with these controls (Fig. 2).

Follistatin:activin A ratio

The mean follistatin:activin A molar ratios for the controls (3.4 ± 0.3), primary testicular failure (4.2 ± 0.4), obstructive azoospermia (19.8 ± 1.3), oligospermia (3.6 ± 0.4) and miscellaneous sperm

Table 1 Means $\pm$ S.E.M. (range) of ages in the different groups of men.

Group	Age
Control	33.3 ± 0.9 (28–46 years)
Primary testicular failure	35.3 ± 1.6 (31–50 years)
Obstructive azoospermia	37.6 ± 0.8 (34–42 years)
Oligospermia	34.8 ± 0.6 (29–38 years)
Miscellaneous sperm dysfunction	35.5 ± 0.8 (28–51 years)

Figure 1 Scatter plot of serum 'total' activin A measurement in controls (○), primary testicular failure (●), obstructive azoospermia (◇), oligospermia (◆) and miscellaneous sperm dysfunction (△). Levels were significantly lower ($P < 0.001$) than in the controls in the obstructive azoospermia group, and higher ($P < 0.01$) in the miscellaneous sperm dysfunction group.

dysfunction (2.7 ± 0.2) were analysed. The ratios were all >2 suggesting that there was a relative abundance of follistatin in all groups of men that could bind to activin A in the circulation.

Serum inhibin B

Serum concentrations of inhibin B in control men ranged from 200 to 892 pg/ml with a mean of 393.8 ± 37.7 pg/ml. Circulating levels of inhibin B were significantly lower than in the controls in men with primary testicular failure (65.5 ± 19.3 pg/ml; $P < 0.001$), obstructive azoospermia (224.1 ± 40.4 pg/ml, $P < 0.01$), oligospermia (266.2 ± 13.0 pg/ml, $P < 0.05$), and miscellaneous sperm dysfunction (277.2 ± 18.9 pg/ml, $P = 0.01$) (Fig. 3).

Serum FSH

Circulating levels of FSH were higher than in the controls (2.73 ± 0.22 mIU/ml) in men with primary testicular failure (19.90 ± 2.65 mIU/ml, $P < 0.001$),

Figure 2 Scatter plot of serum 'total' follistatin measurement in controls (○), primary testicular failure (●), obstructive azoospermia (◇), oligospermia (◆) and miscellaneous sperm dysfunction (△). Levels in any group did not significantly vary from those in controls.

Figure 3 Scatter plot of serum inhibin B measurement in controls (○), primary testicular failure (●), obstructive azoospermia (◇), oligospermia (◆) and miscellaneous sperm dysfunction (△). Levels were significantly lower than in the controls in men with primary testicular failure ($P < 0.001$), obstructive azoospermia ($P < 0.01$), oligospermia ($P < 0.05$), and miscellaneous sperm dysfunction ($P = 0.01$).

obstructive azoospermia (4.84 ± 0.83 mIU/ml, $P < 0.05$), oligospermia (4.89 ± 0.90 mIU/ml, NS) and miscellaneous sperm dysfunction (4.64 ± 0.73 mIU/ml, NS) (Fig. 4).

Relationship between FSH, inhibin B, activin A and follistatin

Activin A and follistatin did not significantly correlate with FSH in any group. Interestingly, follistatin had a significant negative correlation with inhibin B ($r = -0.74$, $P < 0.01$) in the primary testicular failure group and a positive correlation with inhibin B ($r = 0.74$, $P = 0.02$) in the obstructive azoospermic group. Serum follistatin did not significantly correlate with levels of activin A in any group.

Serum inhibin B had a significant correlation with FSH levels in the primary testicular failure ($r = -0.67$,

Figure 4 Scatter plot of serum FSH measurement in controls (○), primary testicular failure (●), obstructive azoospermia (◇), oligospermia (◆) and miscellaneous sperm dysfunction (△). Levels were significantly higher than in the controls in men with primary testicular failure ($P < 0.001$) and obstructive azoospermia ($P < 0.05$).

$P < 0.05$) and miscellaneous sperm dysfunction ($r = -0.56$, $P < 0.001$) groups.

Discussion

Activin/inhibin subunits are expressed in the testis early in fetal development and inhibin is present in the circulation in the neonate and during childhood (16). In the adult, inhibin and activin subunits can be identified within both the Sertoli and Leydig cell populations and a paracrine role for these peptides in the regulation of gonadal function has been proposed (17, 18). Epididymis has been also reported as a site of activin subunit expression and protein production in men (19). Activin subunits are also detectable immunohistochemically in the testis, and activin A in seminal plasma disappears after vasectomy, suggesting an epididymal/testicular origin. Follistatin has been detected in both Sertoli and Leydig cells, and is secreted into seminal plasma (8).

Inhibin B in the male circulation is inversely correlated with FSH, as evidenced by a number of studies (7, 9–15). Testosterone treatment suppresses levels of FSH, luteinising hormone and inhibin B. Inhibin B levels recovered slowly after treatment was discontinued, returning to the pre-treatment level weeks later than FSH, but concurrently with the return of spermatogenesis, at which time the inverse relationship with FSH was restored (20). Inhibin B levels in healthy men fall slowly but progressively with ageing (21), mirroring a gradual rise in FSH, and consistent with the decreased levels of sperm production and serum testosterone seen in elderly men. In this study there was no significant difference in age between the different groups of men (Table 1). Therefore, the relationship between activin A and age in men is unclear.

While inhibin B has received most attention in clinical studies of testicular function in health and disease, activin A is also present in the seminal plasma (8) and may reflect spermatogenic competence. In the present study we have measured circulating levels of activin A and follistatin in a number of clinically defined groups of fertile and infertile males, and investigated the inter-relationship between these peptides, inhibin B and FSH.

Levels of activin A were lower in the obstructive azoospermic group and higher in the miscellaneous sperm dysfunction group. Follistatin did not appear to reflect spermatogenic failure, with levels of follistatin in the groups of infertile males not differing from controls. FS315 is reported to be the major follistatin form in the human circulation (22). The follistatin assay used in this study essentially detects FS288 and considerably cross-reacts with FS315 (9.9%), suggesting that levels of follistatin could be much higher (up to 9-fold) in these samples. In this study, follistatin:activin A molar ratios in all groups of the male serum are >2

suggesting that there is no 'free' activin A available to exert an endocrine effect on the pituitary. However, there may be other molecular forms of activins and inhibins that could be bound to follistatin, indicating the possibility of 'free' activin A in the circulation. Nevertheless, the lack of relationship between FSH and activin A would also support the speculation that all activin A in the male circulation is bound to follistatin.

Consistent with other reported studies, of the four groups of infertile males, those with primary testicular failure exhibited the lowest levels of inhibin B, whereas those with obstructive azoospermia, oligospermia (with normal motility) and miscellaneous sperm dysfunction (impaired motility, but normal sperm count or decreased sperm count) had higher levels, although the values for all groups were significantly lower than those seen in normal controls.

As demonstrated previously, there is a correlation between FSH and inhibin B in the males with spermatogenic disorders. This correlation was not observed for activin A. Significant negative correlation between follistatin and inhibin B ($r = -0.74$, $P < 0.01$) in the primary testicular failure group and a positive correlation ($r = 0.75$, $P = 0.02$) between follistatin and inhibin B in the obstructive azoospermic group are not explainable at present. Men with primary testicular failure had the highest levels of FSH and those with other types of spermatogenic dysfunction also had levels that were elevated above those seen in controls.

The role of inhibin measurement in the clinical management of male infertility has been explored since the development of the 'Monash' RIA for inhibin. However, results with this method proved disappointing, with no obvious differences between fertile and infertile men, excluding those with idiopathic hypogonadotrophic hypogonadism (23, 24).

This study, for the first time, has explored the potential of activin A and follistatin as discriminatory biochemical markers of spermatogenic disorders. Data suggest that 'total' activin A may be useful in predicting obstructive spermatogenic disorder. However, larger studies have to be carried out to confirm this observation and to have the statistical power to analyse the predictive statistics. It will also be interesting to compare a group of castrate men to confirm the testicular contribution of activin A to the male systemic circulation. There seems little value in serum measurement of follistatin in predicting spermatogenic disorders.

In contrast to the largely reproductive tract origin of the inhibins, activins are ubiquitous, being synthesised by a large number of tissues (25) and are viewed as paracrine rather than endocrine factors (26). The function of follistatin in serum appears to be to bind and neutralise circulating activin, thereby preserving activin's ability to act as a locally acting factor without interference from a high background level in the

circulation. However, the activin/follistatin system may play a significant autocrine/paracrine role in the testis.

In summary, this study has explored the relationship between serum activin A, follistatin, FSH and inhibin B in men with spermatogenic disorders. Activin A may prove to be useful in predicting obstructive azoospermia. The follistatin:activin A molar ratio confirms that all activin A in the circulation is bound to follistatin, suggesting that activin A cannot have an endocrine role at the pituitary. This is supported by the lack of a significant relationship between FSH and activin A in the circulation.

Acknowledgements

The authors thank Joan Bellenger (Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital) for help with the FSH assays. We thank Genentech Inc. for the human recombinant activin A and inhibin B standards.

References

- Groome NP, Illingworth PJ, O'Brien M, Cooke I, Ganesan TS, Baird DT *et al.* Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay. *Clinical Endocrinology* 1994 **40** 717–723.
- Muttukrishna S, Fowler PA, Groome N, Mitchell GG, Robertson WR & Knight PG. Serum concentrations of dimeric inhibin during the spontaneous human menstrual cycle and after treatment with exogenous gonadotrophin. *Human Reproduction* 1994 **9** 1634–1642.
- Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather J *et al.* Measurement of dimeric inhibin-B throughout the human menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 1401–1405.
- Knight PG, Muttukrishna S & Groome NP. Development and application of a two-site enzyme immunoassay for the determination of total activin-A concentrations in serum and follicular fluid. *Journal of Endocrinology* 1996 **148** 267–279.
- Evans LW, Muttukrishna S, Knight PG & Groome NP. Development, validation and application of a two-site enzyme-linked immunosorbent assay for activin-AB. *Journal of Endocrinology* 1997 **153** 221–230.
- Evans LW, Muttukrishna S & Groome N. Development, validation and application of an ultra sensitive two-site enzyme immunoassay for human follistatin. *Journal of Endocrinology* 1998 **156** 275–282.
- Illingworth PJ, Groome NP, Byrd W, Rainey WE, McNeilly AS, Mather JP *et al.* Inhibin B: a likely candidate for the physiologically important form of inhibin in men. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 1321–1325.
- Anderson RA, Evans LW, Irvine DS, McIntyre MA, Groome NP & Riley SC. Follistatin and activin A production by the male reproductive tract. *Human Reproduction* 1998 **12** 3319–3325.
- Anawalt BD, Bebb RA, Matsumoto AM, Groome NP, Illingworth PJ, McNeilly AS *et al.* Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *Journal of Clinical Endocrinology and Metabolism* 1996 **81** 3341–3345.
- Jensen TK, Andersson AM, Hjollund NH, Scheike T, Kolstad H, Giwercman A *et al.* Inhibin B as a serum marker of spermatogenesis: correlation to differences in sperm concentration and follicle-stimulating hormone levels. A study of 349 Danish men. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 4059–4063.
- Klingmuller D & Haidl G. Inhibin B in men with normal and disturbed spermatogenesis. *Human Reproduction* 1997 **12** 2376–2378.
- Pierik FH, Vreeburg JT, Stijnen T, De Jong FH & Weber RF. Serum inhibin B as a marker of spermatogenesis. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 3110–3114.
- Mahmoud AM, Comhaire FH & Depuydt CE. The clinical and biological significance of serum inhibins in subfertile men. *Reproductive Toxicology* 1998 **12** 591–599.
- von-Eckardstein S, Simoni M, Bergmann M, Weinbauer GF, Gassner P, Schepers AG *et al.* Serum inhibin B in combination with serum follicle-stimulating hormone (FSH) is a more sensitive marker than serum FSH alone for impaired spermatogenesis in men, but cannot predict the presence of sperm in testicular tissue samples. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 2496–2501.
- Bohring C & Krause W. Serum levels of inhibin B in men with different causes of spermatogenic failure. *Andrologia* 1999 **31** 137–141.
- Andersson AM, Juul A, Petersen JH, Muller J, Groome NP & Skakkebaek NE. Serum inhibin B in healthy pubertal and adolescent boys: relation to age, stage of puberty, and follicle stimulating hormone, luteinising hormone, testosterone and oestradiol levels. *Journal of Clinical Endocrinology and Metabolism* 1997 **82** 3976–3981.
- Wada M, Shintani Y, Kosaka M, Sano T, Hizawa K & Saito S. Immunohistochemical localization of activin A and follistatin in human tissues. *Endocrine Journal* 1996 **43** 375–385.
- Majdic G, McNeilly AS, Sharpe RM, Evans LR, Groome NP & Saunders PT. Testicular expression of inhibin and activin subunits and follistatin in the rat and human fetus and neonate and during postnatal development in the rat. *Endocrinology* 1997 **138** 2136–2147.
- Risbridger GP & Cancilla B. Role of activins in the male reproductive tract. *Reviews of Reproduction* 2000 **5** 99–104.
- Anderson RA, Wallace EM, Groome NP, Bellis AJ & Wu FC. Physiological relationships between inhibin B, follicle stimulating hormone secretion and spermatogenesis in normal men and response to gonadotrophin suppression by exogenous testosterone. *Human Reproduction* 1997 **12** 746–751.
- Byrd W, Bennett MJ, Carr BR, Dong Y, Wians F & Rainey W. Regulation of biologically active dimeric inhibin A and B from infancy to adulthood in the male. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2849–2854.
- Schneyer AL, Hall HA, Lambert-Messerlian GM, Wang QF, Sluss P & Crowley WF. Follistatin activin complexes in human serum and follicular fluid differ immunologically and biochemically. *Endocrinology* 1996 **137** 240–247.
- Lambert-Messerlian GM, Steinhoff M, Zheng W, Canick JA, Gajewski WH, Seifer DB *et al.* Multiple immunoreactive inhibin proteins in serum from postmenopausal women with epithelial ovarian cancer. *Gynecological Oncology* 1997 **65** 512–516.
- Shekter CB, McLachlan RI, Tenover JS, Matsumoto AM, Burger HG, de Kretser DM *et al.* Stimulation of serum inhibin concentrations by gonadotropin-releasing hormone in men with idiopathic hypogonadotropic hypogonadism. *Journal of Clinical Endocrinology and Metabolism* 1988 **67** 1221–1224.
- Ying SY, Zhang Z & Huang G. Expression and localization of inhibin/activin subunits and activin receptors in the normal rat prostate. *Life Science* 1997 **60** 397–401.
- Welt CK & Crowley WF Jr. Activin: an endocrine or paracrine agent? *European Journal of Endocrinology* 1998 **139** 469–471.
- WHO Criteria. *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction* 1992, edn 3, pp 3–26. Cambridge University Press.

Received 23 August 2000

Accepted 21 December 2000