Follistatin is a binding protein for the activin and inhibin family of hormones, regulating their biological activity. In the male reproductive tract, the interaction of these factors is likely to be involved in the regulation of the proliferation of several cell types. We have investigated the presence of follistatin and activin A in seminal plasma using specific immunoassays and have localized follistatin and activin/ inhibin subunits in the adult human testis, prostate and seminal vesicle to establish their likely sources. High concentrations of immunoreactive follistatin were present in seminal plasma in normal men (mean 97.9 ng/ml; 1.43 ng/ml in peripheral plasma) and were similar in men with oligo/azoospermia and following vasectomy. Follistatin immunoreactivity was localized to both Leydig and Sertoli cells of the testis, and to epithelial cells of the prostate gland and seminal vesicle, which are likely to be the predominant sources of the hormone in seminal plasma. Activin A was also present in seminal plasma in normal men but was undetectable following vasectomy, thus deriving from the testis. Consistent with this finding, the βA-subunit was immunolocalized in Sertoli and Leydig cells but was not present in seminal vesicle or prostate gland. The functional significance of the high concentrations of follistatin secreted into seminal plasma by the prostate gland and/or seminal vesicle is uncertain, but they may regulate the biological activity of testis-derived activin A and inhibin B. 

Key words: activin/follistatin/prostate seminal plasma/ seminal vesicle/testis

Introduction

Follistatin is a monomeric glycosylated polypeptide originally isolated from follicular fluid as an inhibitor of follicle stimulat-
Group 1 – Normal men: Semen samples were obtained from 20 normal men, mean age 34 years, range 27–45, recruited from antenatal parentcraft classes. They had thus fathered ongoing pregnancies 20–37 weeks previously. Clinical history, physical examination and semen quality, assessed by WHO criteria (World Health Organization, 1992), were normal in all cases. Samples of peripheral blood were obtained simultaneously, centrifuged, and the plasma stored at –20°C until assay.

Group 2 – Post-vasectomy: Samples were obtained from 20 men, mean age 36 years, range 26–45, submitting routine semen samples 12 weeks after vasectomy. All samples were determined to be azoospermic.

Group 3 – Infertile men: Samples were obtained from 20 oligo/azoospermic (sperm concentration <5×10⁶/ml) men, mean age 33 years, range 26–44, attending an infertility clinic. Conventional semen analysis was performed according to WHO criteria.

Semen samples were produced by masturbation after 3–7 days abstinence, except in the case of post-vasectomy samples where abstinence was not specified. Samples were centrifuged at 3000 g for 5 min, and the seminal plasma was stored at –20°C until assay. This study had the approval of the Reproductive Medicine Subcommittee of the Lothian Research Ethics Committee.

Follistatin assay
Follistatin immunoreactivity was measured using a specific enzyme-linked immunosorbent assay (ELISA) as reported previously (Evans et al., 1998) using recombinant (rh) follistatin-288 as a standard. Standards and samples were treated similarly throughout and were diluted as appropriate with dissociating solution [84 mM sodium deoxycholate, 3.4% Tween 20 (v/v), 1% bovine serum albumin (BSA) (w/v), 5% mouse serum (v/v); McFarlane et al., 1996]. Plates (96 wells; Nunc Maxisorb; Life Technologies Ltd, Paisley, UK) were pre-coated with a monoclonal capture antibody (29/9) raised against rh-follistatin-288 and stored dry using dry coating reagent (Bionostics Ltd, Wyboston, UK). Duplicate standards and samples were added to the plates and incubated for 17 h at 23°C. The plate was washed and the second detection antibody (Fab fragment of clone 17/2) coupled to alkaline phosphatase was applied and incubated for 2 h at 23°C. After further washes, this alkaline phosphatase activity was detected using an alkaline phosphatase detection kit (Life Technologies Ltd, according to the manufacturer’s instructions). The absorbance was measured at 490 nm using a microplate reader (Thermomax; Molecular Devices, Menlo Park, CA, USA) and calculations were performed using dedicated software (Softmax; Molecular Devices). Cross-reactivities with activins and dimeric inhibin isoforms have been previously reported as 0.3%, with significant cross-reaction with follistatin-315 (9.9%; Evans et al., 1998). The limit of detection was <19 pg/ml and intra- and inter-plate coefficients of variation were 7 and 12% respectively. Serial dilution of seminal plasma gave dose–response curves parallel to the standard (Figure 1) and recovery of added standard to seminal plasma was quantitative (109 ± 4%, n = 6).

Activin A and activin AB assays
Activin A and activin AB were measured according to methods described previously (Knight et al., 1996; Evans et al., 1997) with some minor modifications. The standard used was a partially immunopurified human follicular fluid preparation. This was then titrated and expressed in terms of recombinant human activin A or activin AB by calibration with the appropriate recombinant preparation (Genentech Inc., San Francisco, CA, USA) in a range between 10 and 0.156 ng/ml. Standards and samples were treated similarly by diluting in phosphate-buffered saline containing 5% BSA (Sigma) then adding sodium dodecyl sulphate solution (5% final volume). To eliminate false-positive results, dissociate complexes and modify the β-subunits to improve antibody interactions, samples were placed in boiling water for 3 min and allowed to cool before addition of H₂O₂ (2% final volume) for 30 min at 23°C (Knight and Mutukrishna, 1994). For both ELISA for activin A and activin AB, duplicate samples were dispensed on to plates (Nunc Maxisorb; Life Technologies Ltd) passively adsorbed with a monoclonal capture antibody specific to the βA-subunit (E4; stored dry as for follistatin plates) and incubated for 3 h at 23°C. To confer the specificity to activin A or activin AB, either a biotinylated βA- (E4) or βB- (12/13) antibody was applied for 16 h at 23°C. To detect this complex, streptavidin coupled to alkaline phosphatase was added and alkaline phosphatase activity was measured using the same detection system as in the follistatin assay. Cross-reactivities of these assays have been reported in detail previously (Knight et al., 1996; Evans et al., 1997). The detection limit was <0.156 ng/ml for both the activin A and AB assays. Intra- and inter-plate coefficients of variation were 4.9 and 9.1%, and 6.2 and 11.4% for the activin A and AB assays respectively. Recovery of added standard to seminal plasma was quantitative (activin A: 100 ± 16%, n = 10).

Tissue samples
Testicular biopsies were obtained from four young adult men (aged 26–34 years) undergoing investigation for infertility who had serum FSH concentrations in the normal range. All biopsies had normal testicular morphology and azoospermia due to epididymal obstruction. Samples of prostate gland and seminal vesicle that were assessed as having normal morphology were also examined. Tissues were fixed in Bouin’s solution (testis) or 10% neutral buffered formalin (prostate gland and seminal vesicle), washed in ethanol, dehydrated and embedded in paraffin.

Localization of follistatin and β-subunit forms by immunohistochemistry
Follistatin was localized using immunohistochemical techniques described previously (Majdic et al., 1997) with minor adaptations. In brief, sections (5 μm) were dewaxed and rehydrated and endogenous peroxidase was quenched (2% v/v H₂O₂ for 30 min). Antigenic sites were blocked by incubation in a solution of 1% hydrogen peroxide and 2% normal goat serum in phosphate-buffered saline (PBS). Sections were then covered with a primary antibody to follistatin (rabbit polyclonal) or βA subunit (goat polyclonal) diluted 1:300 and 1:600 respectively in PBS containing 1% normal goat serum. For βA subunit, the secondary antibody was a biotinylated antibody to goat IgG (diluted 1:1000 in PBS). For follistatin, the secondary antibody was a biotinylated antibody to rabbit IgG (diluted 1:1000 in PBS). Secondary antibodies were detected with a biotinylated antibody to rabbit IgG and a streptavidin–alkaline phosphatase solution. Reaction products were visualized using an alkaline phosphatase detection kit (Promega, Madison, WI) and sections were counterstained with Mayer’s haematoxylin before coverslipping.
elsewhere (Majdic et al., 1997; Anderson et al., 1998); follistatin: 17/2, as used in the ELISA (concentration range of 5 to 35 µg/ml); βA-subunit: E4 (0.7 to 15 µg/ml); βB-subunit: 12/13 (0.12 to 0.36 µg/ml). Sections were washed and specific binding was detected using a biotinylated horse anti-mouse antibody and an avidin peroxidase complex (according to manufacturer’s instructions; Vector Labs, Burlingame, CA, USA) with 3,3′-diaminobenzidine as chromagen. Sections were counterstained, dehydrated, mounted and visualized by light microscopy.

Data analysis
Follistatin and activin A concentrations showed a skewed distribution, thus data are presented as geometric mean, range, and interquartile range. Correlations and comparisons between groups were determined after logarithmic transformation, using analysis of variance.

Results
Follistatin in seminal plasma
Concentrations of immunoreactive follistatin in seminal plasma from normal men, following vasectomy, and in infertile men are shown in Figure 2. Follistatin immunoreactivity was detectable in all samples of seminal plasma from normal men, mean concentration 97.9 ng/ml, range 2.7–538 ng/ml, interquartile range 199 ng/ml. Follistatin was present in much lower concentration in peripheral plasma (P < 0.0001), mean 1.43 ng/ml, range 0.86–2.55 ng/ml, interquartile range 0.85 ng/ml. There was no correlation between follistatin concentrations in seminal plasma and peripheral plasma or between follistatin in seminal plasma and sperm concentration. There was also no correlation between follistatin concentration and volume of the ejaculate. Follistatin concentrations were similar in infertile men with severe oligo/azoospermia, although there was a narrower range. The mean concentration in this group was 57.9 ng/ml, range 10.5–165 ng/ml, interquartile range 81.8 ng/ml.

Concentrations of immunoreactive activin A in seminal plasma are shown in Figure 3. Activin A was detectable in seminal plasma from normal and oligo/azoospermic men, but was undetectable in all post-vasectomy samples (Figure 3). The mean concentration in the normal men was 0.73 ng/ml, range 0.32–2.1, interquartile range 0.23 ng/ml. In the oligo/azoospermic group, the mean concentration was 0.48 ng/ml, range 0.1–1.45 ng/ml, interquartile range 0.55 ng/ml. These results were not significantly different. No activin AB was detectable in any sample from any group.

Immunolocalization of follistatin and β-subunits in testis and reproductive tract
Follistatin immunoreactivity was localized in all the testis biopsies examined in the cytoplasm of Sertoli and Leydig cells (Figure 4a and b). Little follistatin immunoreactivity was present in germ cells. At higher concentrations of the antibody, some positive immunostaining was present in the material present in the lumen of the tubule, consistent with follistatin being a secreted product. In the prostate gland, follistatin immunoreactivity was localized predominantly in epithelial cells of the glands, with little specific immunoreactivity local-

Figure 2. Concentration of follistatin in seminal plasma collected from normal (n = 20), post-vasectomy (n = 20) and oligo/azoospermic men (n = 20).

Figure 3. Concentration of activin A in seminal plasma collected from normal (n = 20), post-vasectomy (n = 20) and oligo/azoospermic men (n = 20). The dotted line indicates the detection limit of the assay. All post-vasectomy samples were below this level of detection. n.d. = not detected.

Follistatin immunoreactivity was also detectable in seminal plasma following vasectomy. In 20 azoospermic samples, the mean follistatin concentration was 133 ng/ml, range 27.9–814 ng/ml, interquartile range 320 ng/ml. This was not significantly different from seminal plasma follistatin concentration in normal men, but was significantly higher than in men with oligo/azoospermia (P < 0.01).

There was a weak but statistically significant correlation between immunoreactive follistatin concentration in seminal plasma and age (r = 0.27, P < 0.01, all men). This relationship was present when men were divided into the normal and post-vasectomy groups (r = 0.44, P = 0.05; r = 0.54, P < 0.02 respectively) but not in the oligo/azoospermic group. There were no differences in the age distributions between these three groups.

Activin in seminal plasma
Activin A was detectable in seminal plasma from normal and oligo/azoospermic men, but was undetectable in all post-vasectomy samples (Figure 3). The mean concentration in the normal men was 0.73 ng/ml, range 0.32–2.1, interquartile range 0.23 ng/ml. In the oligo/azoospermic group, the mean concentration was 0.48 ng/ml, range 0.1–1.45 ng/ml, interquartile range 0.55 ng/ml. These results were not significantly different. No activin AB was detectable in any sample from any group.

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Figure 4. Localization of follistatin immunoreactivity (identified by brown chromagen) in: (a and b) normal adult human testis predominantly in the cytoplasm of Leydig (L) and Sertoli (S) cells; (c) prostate gland predominantly in epithelial cells of the glands; (d) in seminal vesicle in glandular epithelial cells; and (f) in the transitional epithelium on the surface of the prostate gland.
(e) Immunolocalization of βA-subunit in testis in Leydig cells and within the seminiferous tubule predominantly in Sertoli cells.
(g) Representative tissue control section demonstrating no specific follistatin immunoreactivity in prostate gland. Scale bars: a, b, e, f and g = 50 μm; c and d = 100 μm.

In stromal tissues (Figure 4c). Furthermore, there was intense follistatin immunoreactivity in the cells on the outer surface of the transitional epithelial layer that lies on the surface of the gland (Figure 4f). In the seminal vesicle, follistatin immunoreactivity was localized in epithelial cells in the glands (Figure 4d), and also in the outer layers of cells of transitional epithelium (not shown).

Immunoreactive βA-subunit was localized in the testis where it intensely stained the cytoplasm of Leydig cells (Figure 4e). Within the tubule of the testis, weaker βA immunoreactivity was present predominantly in the cytoplasm of Sertoli cells, but not germ cells, although because of the close proximity of these cell types, we are not able to completely rule out immunostaining in all germ cells. The βA-subunit was also immunolocalized in the material in the lumen of the tubules, indicating that it is likely to be a secreted product. Specific immunoreactivity for either the βA- or βB-subunit was not clearly observed in the prostate gland or seminal vesicle at the low antibody concentrations that exhibit minimal non-specific binding (not shown). In all appropriate parallel negative-control sections, no specific immunoreactivity was observed (Figure 4g).
Discussion

Activins and inhibins are members of the TGF-β superfamily, and the proliferative and anti-proliferative effects of these factors have been described in a variety of reproductive and non-reproductive tissues (Hedger et al., 1989; Mather et al., 1990; Xu et al., 1995; McPherson et al., 1997), which may be regulated by follistatin (Michel et al., 1993; Moore et al., 1994). Seminal plasma contained high concentrations of follistatin immunoreactivity, approximately 100-fold higher than in matched peripheral plasma controls and there was no relationship between follistatin concentrations in the two biological fluids. This is consistent with the demonstration in the ram that the testis does not contribute greatly to circulating follistatin concentrations (Tilbrook et al., 1996) and with the concept of follistatin as a local regulator of activin. Similar high concentrations have been found in follicular fluid (250 ng/ml), but not in other biological fluids so far examined (Evans et al., 1998). The concentration of immunoreactive follistatin in seminal plasma was similar in normal men and following vasectomy, indicating that the testis is not the major source.

Follistatin immunoreactivity and mRNA have been recently demonstrated in the prostate gland, and were localized to the basal epithelial cells and the fibroblastic stroma in non-malignant regions of prostate cancer specimens (Thomas et al., 1997). The present results demonstrate a similar distribution in normal human prostate, and the intense follistatin immunostaining in the basal epithelial cells of the prostate gland and seminal vesicle suggests that these tissues are likely to be the major sources of the follistatin in seminal plasma. The distribution of follistatin immunoreactivity in the prostate was noted to be different when detected by two antibodies in the study of Thomas et al. (1997), with stromal protein not detected using the same monoclonal antibody as used in the present study. Two main forms of follistatin of 288 and 315 amino acids have been described, resulting from alternate splicing (Shimasaki et al., 1988; Michel et al., 1990; Inouye et al., 1991). The antibody used here does not recognize all isoforms with equal affinity, predominantly recognizing the follistatin-288 isoform but it also has a significant cross-reactivity with the follistatin-315 isoform (Evans et al., 1998). These results therefore suggest that the prostatic epithelium and stroma may differentially produce the two isoforms of follistatin. Differential production of the two isoforms has been reported in prostatic carcinoma cell lines, with the expression of mRNA for the 288 isoform correlating with resistance to the growth-inhibiting effects of activin on these cells (McPherson et al., 1997).

Seminal plasma follistatin concentrations demonstrated here exhibited a significant positive relationship with age in normal men and in the post-vasectomy group. Further studies, using assays specific to the different isoforms, are required to assess the possible value of seminal plasma follistatin measurement in this clinical context. While there was no relationship between the volume of the ejaculate and follistatin concentration, the possible influence of abstinence was not addressed directly. It is also possible, in view of the broad-ranging effects of activin, that the very high concentrations of follistatin in the ejaculate may have a role in the female reproductive tract (Draper et al., 1997).

The present results demonstrate that adult human testis contains follistatin immunoreactivity localized in both Leydig and Sertoli cells. This is in contrast to the situation during fetal life, where a previous study using the same antibody detected no follistatin immunoreactivity (Majdic et al., 1997), and follistatin mRNA expression was also undetectable in the fetal testis (Roberts, 1997). In contrast, activin and inhibin appear to be produced from the early stages of development in both rat and human testis and their expression in Sertoli cells varies with the stage of the seminiferous epithelium (Kaipia et al., 1992; Majdic et al., 1997; Roberts, 1997). As follistatin can prevent many of the biological effects of activin, including paracrine effects within the testis (Mather et al., 1993; Di Simone et al., 1998), the presence of follistatin in the adult but not fetal testis suggests that there is a major developmental change in the functional activity of this paracrine system. The timing and control mechanisms of this change remain to be determined. The close parallels between the human and rat in this respect and the recent demonstration that transgenic mice over-expressing the follistatin gene show Leydig cell hyperplasia, spermatogenic arrest and seminiferous tubular degeneration provide further support for the importance of this paracrine regulator in the testis (Guo et al., 1998).

Activin A was present in seminal plasma but only from intact and non-vasectomized men, indicating that the testis is the major source. Concentrations of activin A did not differ between normal men and those with oligo/azoospermia. This contrasts with the relationship between inhibin B in seminal plasma and sperm concentration (Anderson et al., 1998). The significance of this is unclear, but may reflect the different functions of activin and inhibin within the testis. The presence of activin A in seminal plasma is consistent with the presence of βA-subunit immunoreactivity in the Sertoli cells, making this the likely site of activin A secretion into seminal plasma. The presence of βA-subunit immunoreactivity in the adult human testis demonstrated here confirms a previous report using a less specific antibody (Vleijen et al., 1993) and provides a basis for the production of activin A (and potentially of inhibin A) within the testis, which has been demonstrated using rat Sertoli cells (de Winter et al., 1993). This, together with the limited data available on functional effects within the testis, is consistent with regulatory effects on the interaction between Sertoli cells and developing germ cells and possibly also Leydig cell steroidogenesis (reviewed in Mather et al., 1997). We have demonstrated recently, using specific antibodies, that Sertoli cells also express both immunoreactive βB- and α-subunit proteins and that these cells appear to be the predominant site of inhibin B secretion by the testis into seminal plasma, with no inhibin A being detected (Anderson et al., 1998). Similarly, Leydig cells also express βA-, βB- and α-subunit proteins, and inhibin B and activin A are the predominant family members found in the circulation. These findings indicate that, within both the Leydig and Sertoli cells, the βB-subunit is utilized for inhibin B production, while the βA-subunit is used to form activin A. There was no secretion
of detectable activin AB. However, the development of a specific activin B assay is awaited to determine whether that form is also secreted. The regulatory mechanisms for the formation of the various specific dimers and their respective roles in the paracrine and endocrine control of testis function remain to be elucidated.

Activin is inhibitory to the growth of some human prostatic cancer cell lines, an effect which is blocked by follistatin (McPherson et al., 1997). The mRNA for the β-subunits has been demonstrated in samples from men with benign prostatic hyperplasia, and it has been suggested that changes in the relative production of activin and follistatin may result in a change in the resistance to the antiproliferative effect of activin and be involved in the development of prostate cancer (Thomas et al., 1997). We were unable to detect specific immunostaining for the activin βA-subunit in the prostate or seminal vesicle. These differences may be due to the different antibodies used. It is possible that these tissues contain only low amounts of the βA-protein, or that there is increased expression in hyperplastic prostate tissue. The present data, however, confirm that the normal adult prostate gland will be exposed to activin, whether derived from the testis or produced endogenously.

In conclusion, these results demonstrate the presence of follistatin and activin A in seminal plasma. The high concentrations of follistatin appear to derive from the prostate and seminal vesicle, while activin A derives predominantly from the testis. The adult testis, however, does contain follistatin, in contrast to the fetus, indicating a major change in the function of the follistatin/activin system during development.

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References


Follistatin and activin in seminal plasma


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