CLINICAL STUDY

Inhibin B plasma concentrations in infertile patients with *DAZ* gene deletions treated with FSH

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Abstract

Objective: The *DAZ* (deleted in azoospermia) gene family on the Y chromosome long arm is the major candidate for the *AZFc* (azoospermia factor *c*) phenotype of male infertility and it is expressed only in germ cells. The aim of the study was to assess Sertoli cell function in subjects with *AZFc* deletions.

Design: Case-control, prospective study.

Methods: We have studied six severely oligozoospermic subjects with *AZFc-DAZ* deletions, and looked whether they responded in terms of inhibin B production to a 1 month FSH treatment. These patients were compared with three groups of patients affected by different spermatogenic alterations not related to deletions on the Y chromosome.

Results: Although affected by severe testiculopathy, patients with *AZFc-DAZ* deletions had only slightly elevated FSH, and normal inhibin B plasma concentrations. Inhibin B responded normally during FSH treatment, supporting the hypothesis that Sertoli cells are not altered. On the contrary, other severe testiculopathies not related to Y chromosome deletions showed high FSH and low inhibin B concentrations, with no response to FSH treatment. In these cases the cause of the spermatogenic defect probably damaged both germ and Sertoli cells. Finally, idiopathic patients with a hormonal status similar to Y-deleted patients (slightly elevated FSH and normal inhibin B concentrations) did not respond to FSH treatment, suggesting that Sertoli cells were already at their maximal functional capability.

Conclusions: These data confirm that Sertoli cell function is not damaged in patients with *AZFc-DAZ* deletions and that the strong reduction of germ cells does not affect the FSH–inhibin B feedback loop.

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Introduction

Microdeletions of the Y chromosome long arm (Yq) involving the so-called *azoospermia factor* (*AZF*) *a*, *b* and *c* are an important cause of severe male infertility (for review see (1)). The spermatogenic disruption caused by such genetic alteration is due to the absence of genes expressed either in germ cells only or in many tissues other than the testis. The former group includes the *DAZ* (deleted in azoospermia) and the *RBMY1* (RNA-binding motif on the Y) genes, which are considered the major *AZFc* and *AZFb* candidates respectively (2, 3). The second group includes the *USP9Y* (ubiquitin-specific protease 9, Y chromosome) and the *DBY* (DEAD box on the Y) genes, which are both *AZFa* candidates (4–6). Deletions in *AZF* regions cause azoospermia or severe oligozoospermia and most frequently involve the *AZFc-DAZ* region (5).

Patients with Yq microdeletions represent an interesting *in vivo* experimental model to study the

relationship between Sertoli and germ cells, especially when germ cell-specific genes are deleted. In fact, in such cases the testicular damage results from a primary defect intrinsic to germ cells, while Sertoli cells are not directly affected (7). On the contrary, other testiculopathies, such as those caused by cryptorchidism, testicular trauma or orchitis, may be associated with dysfunction also of the Sertoli cells, and probably in these cases the primary alteration is a combined damage to both Sertoli and germ cells. We have recently supported these hypotheses by comparing Sertoli cell function, as evaluated by inhibin B plasma concentration, in severely infertile patients with and without Yq microdeletions (7). We showed that inhibin B plasma levels were significantly higher in patients with Yq deletions with respect to those without deletions and this behaviour was even more evident when germ cell-specific genes (such as *DAZ*) were deleted. These data suggested that Sertoli cell function in patients with Yq deletions is only partially altered and

that inhibin B could be used as a valid marker to distinguish severe testiculopathies related to this genetic alteration from those related to other causes.

Inhibin B is produced by Sertoli cells under stimulation by endogenous or exogenous follicle-stimulating hormone (FSH) and in infertile patients treated with FSH an increase in inhibin B production is evident only when pretreatment basal FSH and inhibin B plasma concentrations are in the normal range, while no response is seen when pretreatment FSH plasma levels are elevated (8, 9). Therefore, a normal response of inhibin B to FSH denotes that Sertoli cells are functioning normally. However, inhibin B production reflects also interactions between Sertoli and neighbouring germ cells (10). To analyse better Sertoli cell function and their relationship with germ cells we used the *in vivo* model represented by patients with Yq microdeletions in which the primary defect involves only germ cells and not Sertoli cells. We studied six severely infertile men with *AZFc-DAZ* deletion and a testicular picture of severe hypospermatogenesis, and looked whether they responded to FSH treatment in terms of inhibin B production. The results of this study further elucidate the relationship between germ and Sertoli cells and strengthen the hypothesis that Sertoli cells are not affected in Yq-deleted patients.

Subjects and methods

Subjects

The study was approved by the Hospital Ethical Committee and informed consent was obtained from each subject.

Semen analysis, bilateral testicular fine needle aspiration cytology (FNAC) (11) and PCR analysis of Yq euchromatin using 40 sequence-tagged sites (5, 12) allowed us to select, among the infertile patients previously reported (7), six subjects who satisfied the following criteria: (i) they were affected by either azoospermia or severe oligozoospermia (sperm count $< 2 \times 10^6$ /ml) with a testicular cytological picture of severe hypospermatogenesis (thus excluding Sertoli cell-only syndrome and spermatogenic arrest); and (ii) they presented a deletion confined to the *AZFc* region (subinterval 6C–6E of the Y chromosome), therefore removing only the *DAZ* gene cluster.

Semen samples were examined on two different occasions, separated by a 3 week interval, with 3 days of sexual abstinence, following WHO guidelines (13). Azoospermia was confirmed after sperm centrifugation. All subjects carried a normal 46, XY karyotype and PCR analysis of Yq was performed as previously described (5, 7, 14), and *DAZ* deletions were eventually confirmed by Southern blotting (12, 14, 15). Details of the testicular FNAC and analysis

have been previously given (11, 16, 17); briefly, hypospermatogenesis is characterised by a quantitative reduction in the absolute number of germ cells, which are, however, in normal relative proportions, i.e. no maturation disturbances are present. Sertoli index (SEI), which represents the proportion of Sertoli cells to the total number of germ cells (number of Sertoli cells/number of spermatogenic cells $\times 100$) (11, 16, 17), is used to distinguish mild, moderate and severe forms of hypospermatogenesis (SEI 100–300, 300–600 and > 600 respectively, with the normal value below 100).

These patients and their response to FSH treatment were compared with infertile patients without Y chromosome microdeletions selected among subjects previously reported (9). In particular three groups of patients were studied: (i) group A included 15 patients affected by mild oligozoospermia (sperm count $5\text{--}10 \times 10^6$ /ml) with a testicular cytological picture of mild hypospermatogenesis (SEI 100–300); (ii) group B included 18 patients affected by moderate oligozoospermia (sperm count $2\text{--}5 \times 10^6$ /ml) and a testicular cytological picture of moderate hypospermatogenesis (SEI 300–600); (iii) group C included 25 patients affected by severe oligozoospermia (sperm count $< 2 \times 10^6$ /ml) and a testicular cytological picture of severe hypospermatogenesis (SEI > 600). These three groups identified patients also on the basis of inhibin B and FSH plasma concentrations (9), as discussed below.

Fifty normal fertile subjects were considered as controls for seminal and basal hormonal plasma concentrations.

Hormone assays

FSH and luteinising hormone (LH) plasma concentrations were measured in each subject by RIA using ^{125}I -labelled FSH and LH (Ares-Serono, Milan, Italy). Intra- and interassay coefficients of variations were 2.6 and 3.6%, and 3.7 and 2.8% respectively. Testosterone was measured with a double antibody RIA utilising commercial kits (Radim, Rome, Italy). Intra- and interassay coefficients of variations were 7.8 and 7.0% respectively. Oestradiol was measured by RIA using commercial kits (Radim). Intra- and interassay coefficients of variations were 7.5 and 9.8% respectively. Plasma concentrations of inhibin B were measured by a solid-phase sandwich ELISA specific for the dimeric inhibin B form (Serotec, Oxford, UK) (18, 19). The first antibody is directed to the βB -subunit and the second antibody to the α -subunit and conjugated to alkaline phosphatase. The assay has $< 0.1\%$ cross-reactivity with activin forms and approximately 1% with inhibin A. Assay sensitivity was 15 pg/ml and the inter- and intra-plate variation coefficients were 6.4 and 6.8% respectively.

Table 1 Seminal, testicular cytological and hormonal data (means±s.d.) of the patients studied and inhibin B response during FSH treatment with 75 IU i.m. on alternate days.

	<i>n</i>	Sperm count (× 10 ⁶ /ml)	Testicular cytology	FSH (IU/l)	Inhibin B (pg/ml)	Inhibin B response to FSH treatment
Group A	15	5–10	Mild hypospermatogenesis (SEI 100–300)	3.5±1.9	182.3±96.4	Yes
Group B	18	2–5	Moderate hypospermatogenesis (SEI 300–600)	9.2±3.8*	175.1±87.5	No
Group C	25	<2	Severe hypospermatogenesis (SEI > 600)	15.8±7.3°	50.1±22.1°	No
DAZ-deletion	6	<2	Severe hypospermatogenesis (SEI > 600)	7.9±4.8*	194.1±135.2	Yes
Controls	50	>20	n.e.	2.8±1.3	229.7±81.6	n.e.

n.e. not examined.

* $P < 0.05$, ° $P < 0.01$ vs controls.

Treatment with FSH

Patients were treated for 1 month with highly purified human FSH (Metrodin HP; Serono, Milan, Italy) at a dose of 75 IU i.m. on alternate days. This treatment regimen has been previously shown to be able to stimulate inhibin B and sperm production (8, 9). Inhibin B, FSH, LH, testosterone and oestradiol plasma concentrations were measured every 2 weeks.

Statistical analysis

Statistical analysis was performed with the S-Plus statistical package (20), and using ANOVA. The results are given as means±s.d. $P < 0.05$ and < 0.01 were regarded as statistically significant and highly significant respectively.

Results

In Table 1 are summarised the seminal, testicular cytological and hormonal data of the patients. Patients of group A, affected by mild oligozoospermia associated with mild hypospermatogenesis have been previously shown to respond to FSH treatment in terms of both inhibin B production and sperm number increase (9). They were characterised by normal FSH and inhibin B plasma concentration (3.5±1.9 IU/l and 182.3±96.4 pg/ml respectively). Patients of group B, affected by a more severe testiculopathy and previously shown to be non-responders to FSH treatment (9), have slightly increased FSH values with respect to controls (9.2±3.8 vs 2.8±1.3 IU/l, $P < 0.05$), but inhibin B concentrations still in the normal range (175.1±87.5 vs 229.7±81.6 pg/ml). Patients of group C were affected by the most severe testicular damage as reflected by the very high levels of FSH (15.8±7.3 IU/l, $P < 0.01$ vs controls) with reduction of inhibin B (50.1±22.1 pg/ml, $P < 0.01$ vs controls). Also these patients have been previously shown to be non-responders to FSH treatment (9). Patients with *AZF_c-DAZ* deletions were characterised by a severe testiculopathy similar to patients of group C, but had only slightly increased FSH (7.9±4.8 IU/l, $P < 0.05$ vs

controls) and normal inhibin B (194.1±135.2 pg/ml) concentrations (Table 2). Therefore, patients with *AZF_c-deletions* are comparable with patients of group B with regard to hormonal data, and to patients of group C with regard to testicular histological damage. LH, testosterone and oestradiol plasma concentrations were not different between the groups and they were in the normal range (data not shown).

Treatment for 1 month with highly purified FSH at the dose of 75 IU i.m. on alternate days produced a rise in serum FSH during the study period with the peak after 2 weeks of treatment in all subjects (data not shown), confirming the validity of this treatment protocol. In patients of group A and in patients with *AZF_c-DAZ* deletions, inhibin B plasma levels increased significantly in study weeks 2 and 4 ($P < 0.01$ vs basal levels). In groups B and C, inhibin B plasma levels remained unchanged throughout the study period. Figure 1 shows the inhibin B plasma concentrations during the treatment in the four groups of patients. No modifications in LH, testosterone and oestradiol plasma concentrations occurred during the treatment with FSH (data not shown).

Discussion

Inhibin B is produced by Sertoli cells under stimulation by FSH and in turn it is the major regulator of the negative feedback loop controlling FSH secretion. However, its production is regulated also by neighbouring germ cells (10), even if the exact spermatogenic cell type exerting this effect is not known. Inhibin B is therefore

Table 2 Individual hormonal parameters of *DAZ*-deleted patients.

Patient number	FSH (IU/l)	Inhibin B (pg/ml)
1	9.9	108
2	7.2	102
3	7.0	151
4	1.6	385
5	5.6	346
6	16.0	73

produced by Sertoli cells only if these cells are normally functioning and germ cells may exert their effect. Sertoli cells are essential in the mechanisms triggering and regulating the process of spermatogenesis and disruption of their function is generally expressed as a reduction in the production of inhibin B (for review see (21)). Alternatively, low inhibin B plasma concentrations may result from an altered control by germ cells. Several pathological conditions, such as varicocele, cryptorchidism, orchitis or testicular trauma, affect spermatogenesis leading to oligozoospermia or azoospermia. The relative involvement of Sertoli and germ cells caused by such conditions is largely unknown, but it can be speculated that some testiculopathies may be associated with dysfunction of both these cellular compartments whereas in other cases the primary defect is intrinsic to germ cells without altering Sertoli cells. We have recently supported the latter hypothesis by analysing an important and unique model, such as that represented by patients with microdeletions in Yq (7). In fact, we demonstrated that Sertoli cell function in Yq-deleted patients is only partially altered, as demonstrated by significantly higher plasma concentrations of inhibin B in these patients with respect to subjects affected by similar spermatogenic damage but without Yq deletions. Furthermore, patients with deletions involving genes expressed exclusively in germ cells (such as *DAZ*), showed the highest concentrations of inhibin B, while this hormone was strongly reduced in patients with larger deletions involving also ubiquitously expressed genes (7). Patients without Yq deletions invariably

had lower inhibin B concentrations, suggesting that in such cases the cause of the testiculopathy may have damaged both Sertoli and germ cells.

Therefore, we have better analysed Sertoli cell function in patients affected by severe testiculopathies related to *DAZ* deletions, by evaluating their inhibin B response to FSH treatment. We compared these patients with three different groups of infertile subjects.

The first group (group A) consisted of patients affected by a less severe spermatogenic damage who have previously shown to respond to FSH treatment in term of both inhibin B and sperm production. These patients were hormonally identical to normozoospermic subjects and their response to FSH treatment demonstrated the full competence of Sertoli cells and the lack of any dysfunction in the FSH–inhibin B loop. This group of patients represented an 'internal control' in order to compare inhibin B response between the other groups of patients.

Patients of the second group (group B) were hormonally similar to patients with *DAZ* deletions (moderately elevated FSH and normal inhibin B) but the oligozoospermia and tubular damage were less severe. As previously demonstrated (9) these patients did not show any increase in inhibin B production during FSH treatment. Our hypothesis is that in these patients the high endogenous plasma FSH concentrations may have induced a maximal functional activation of Sertoli cells which cannot be further stimulated by FSH treatment. These data further suggested that high basal FSH concentrations represent a negative prognostic factor for the inhibin B response and sperm number increase,

Figure 1 Effects of FSH treatment (75 IU i.m. on alternate days) on inhibin B plasma levels (means $\pm$ s.d.) in the four groups of patients. * $P < 0.01$ vs basal levels.

and Sertoli cell function seems to be only partially altered in these patients.

The third group of patients (group C) had a seminal and testicular cytological pattern comparable with patients with *DAZ* deletions, that is an important oligozoospermia due to severe hypospermatogenesis without maturation disturbances of the spermatogenic process. The hormonal profile of these patients resembles that of a severe primary testiculopathy with very high FSH and low inhibin B plasma concentrations. These data suggest that in such cases the cause of the spermatogenic defect may have damaged both Sertoli and germ cells, and the absence of any inhibin B response during FSH treatment supports the hypothesis that the function of Sertoli cells is strongly altered.

Patients with *DAZ* deletions are characterised by severe testiculopathy, but FSH plasma concentrations are only slightly elevated and inhibin B is in the normal range. As previously shown (7), these data suggest that Sertoli cell function in *DAZ*-deleted patients is not altered and that the spermatogenic alteration in these cases results from a primary defect intrinsic to germ cells. It should be noted that the group of *DAZ*-deleted patients included in the present study is highly selected, as they were affected by severe testiculopathy not including SCOS, and they presented normal or only slightly elevated FSH and normal inhibin B concentrations. However, about 50% of patients with *AZF_c* deletions have Sertoli cell-only syndrome (SCOS) with elevated FSH and variable inhibin B concentrations (7). This variability may explain different results obtained by other authors (22).

In a previous study (7) we showed that the relative contribution of germ cells to inhibin B production is about 30%, since this hormone was about 70% higher in *Yq*-deleted patients with respect to non-deleted patients affected by comparable spermatogenic damage. In *DAZ*-deleted patients the normal function of Sertoli cells and the presence of germ cells explain the normal production of inhibin B and the normal response to FSH treatment. A formal proof to this hypothesis could be drawn from the study of the response to FSH in *DAZ*-deleted patients affected by Sertoli cell-only syndrome. However, the Hospital Ethical Committee did not approve this request.

In deleted patients, the high FSH plasma concentrations preserve a normal production of inhibin B, as Sertoli function is not intrinsically damaged. Therefore, subjects of group B and those *DAZ*-deleted apparently have a similar hormonal profile, but this reflects a different functional status. In fact, in the former case a moderate alteration of Sertoli cells exists and the normal values of inhibin B represent the maximal functional capability of Sertoli cells. This is demonstrated also by the absence of increase in inhibin B during FSH treatment. On the contrary, in the latter case the strong quantitative reduction of germ cells determines a new equilibrium between FSH and Sertoli cell

function, which is still preserved. This hypothesis is confirmed by the normal response of Sertoli cells to FSH administration.

This study was designed to investigate Sertoli cell function in infertile patients with *DAZ* deletions and not to study the possible effect of FSH treatment on sperm production in these patients. Analysis of sperm concentrations requires at least 2 months of FSH therapy. At the end of our study period (1 month) there was no modification in sperm parameters in any *DAZ*-deleted patient. The normal basal inhibin B concentrations, the normal response to FSH treatment, the only slightly elevated FSH concentrations and the testicular cytological pattern characterised by hypospermatogenesis only could suggest that these patients may be responsive to treatment in terms of sperm production (9), and a longer survey of these patients is necessary to clarify these aspects.

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