Absence of anti-Müllerian hormone (AMH) and M2A immunoreactivities in Sertoli cell-only syndrome and maturation arrest with and without AZF microdeletions

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BACKGROUND: Some genes identified in the AZF locus are expressed only in germinal cells; others are ubiquitous. AZF microdeletions seem to occur at the earliest stages of ontogenetic development, and one might therefore assume that Sertoli cells preserve some immature characteristics and that their immunophenotype may be modified by the existence of a molecular defect. MATERIALS AND METHODS: Two immunohistological markers of Sertoli cell immaturity [anti-Müllerian hormone (AMH) and M2A] were tested in two histopathological groups (maturation arrest at spermatocyte I stage and Sertoli cell-only syndrome). We analysed 68 testicular samples obtained from 39 patients with non-obstructive azoospermia associated or not with AZF microdeletions. RESULTS: The absence of M2A and AMH immunoreactivity in adult gonads was observed without any correlation to spermatogenetic impairment or molecular deficit in the AZF region. In the samples of these two series, Sertoli cells showed a mature phenotype for AMH and M2A markers. CONCLUSIONS: In patients with AZF microdeletions, the genotype-phenotype correlations seem to be more complex than has been suggested previously; more detailed characterization of the immunohistochemical phenotype associated with the molecular defect may be useful in understanding the spermatogenic failure mechanism.

Keywords: AMH/azoospermia factor/M2A/male infertility/Sertoli cells

Introduction

Various publications in the last 10 years have confirmed the major role of the AZF region in preserving spermatogenesis. However, in cases of AZF microdeletions, the pathophysiological mechanism of spermatogenic disruption remains unknown, and it is impossible histologically to distinguish severe testiculopathies with Yq microdeletions from those without Yq microdeletions. Some genes identified in the AZF locus are expressed only in germinal cells (DAZ or RBM1); the others, such as DFFRY or DBY, are ubiquitously expressed (Chandley and Cooke, 1994; Menke et al., 1997; Brown et al., 1998; Mazeyrat et al., 1998). The Y chromosome microdeletions may alter the various cell types directly or indirectly.

Thus, the immunohistological phenotype of gonads could be different in infertile patients with AZF deletions. A recent study using the cytokeratin-18 antibody did not show any differences in immunoreactivity in AZF deleted patients (Bar-Shira Maymon et al., 2000).

However, Foresta et al. report that the inhibin B production in patients with Yq deletions was ~70% higher than in nondeleted patients, and the functional relationship between FSH and inhibin B was normally preserved (Foresta et al., 2001).

Other markers may also allow us to detect modifications of gonadal immunohistological phenotype in infertile patients with AZF deletions.

Two potential markers of Sertoli cell functional status [anti-Müllerian hormone (AMH) and M2A] attracted our attention; these markers seem to be expressed independently of cytokeratin-18.

AMH is a functional marker of Sertoli cell differentiation (Tran et al., 1987; Joss et al., 1993). The role of AMH during postnatal gonadal maturation and after puberty remains uncertain. Its implication in germ cell proliferation (Cazorla et al., 1998; Fénelch et al., 1999), as well as its possible effect on the differentiation and function of Leydig cells, were recently suggested (Racine et al., 1998). Conventional immunohistochemical methods cannot detect AMH immunoreactivity in testes of adult patients with normal spermatogenesis, but a renewal of AMH immunoreactivity was described in the Sertoli cells of patients with spermatogenesis arrest or Sertoli cell-only syndrome (Steger et al., 1996).

M2A is an oncofetal marker corresponding to a surface sialoglycoprotein expressed in fetal gonocytes and Sertoli cells (Baumal et al., 1989; Jorgensen et al., 1993, 1995; Marks et al., 1999). This antigen was also identified in several
testicular tumours such as seminomas, carcinomas in situ and teratomas (Bailey et al., 1986; Giwercman et al., 1988; Marks et al., 1999).

AMH and M2A are sequential markers of immature or de-differentiated Sertoli cell status: AMH expression seems to be arrested later than that of M2A (Steger et al., 1999). AZF microdeletions are supposed to appear at very early ontogenetic developmental stages, so that Sertoli cells may keep some immature characteristics. At the same time, progressive decrease of spermatogenesis marked by partial reversion of Sertoli cell immunophenotype may be observed in infertile patients. To verify these hypotheses, we tested the two immunohistochemical markers (AMH and M2A) of immature Sertoli cells and examined the possibility of differential AMH and/or M2A immunoexpression in patients with two well characterized spermatogenic disorders associated or not with AZF microdeletions.

Materials and methods
This study included 68 testicular samples from 39 patients with idiopathic azoospermia aged 28–48 years: in 29 patients, biopsy was bilateral. Thirty-one samples with non-obstructive infertility were obtained from an archive of diagnostic testicular biopsies and eight samples from an attempt to isolate testicular sperm for ICSI. Six samples were from patients with AZF microdeletions. All the samples were divided into two histological series. In group I (n = 25), biopsies with a Sertoli cell-only syndrome, five biopsies had an AZF deletion, four AZF deletion (DFRRY) (Blagosklonova et al., 2000) and one AZFb/c deletion (sY114–Y160; karyotype: 45,X/46.X del(Y)(q11-qter)). In group II (n = 14), biopsies with maturation arrest at meiosis I, one sample had an AZFb deletion (sY114–RBM1).

Two fetal testes (18 and 20 weeks) and two postnatal testicular biopsies (2 months and 4 years) were used as positive controls. Two testicular biopsies of patients with active spermatogenesis and without AZF microdeletions were used as a reference.

Immunohistochemistry was performed using D2-40 mouse monoclonal antibodies against the M2A antigen (courtesy of Professor A.Marks, Department of Medical Research, University of Toronto, Ontario, Canada) and rabbit polyclonal antibodies against recombinant human AMH (courtesy of Dr R.Rey, Unité de Recherches sur l’Endocrinologie du Développement, Montrouge, France).

M2A immunohistochemistry
All biopsy samples were fixed in a Bouin mixture, paraffin embedded. The sections (7 µm thick) were deparaffinized and rehydrated. The slides were incubated in Target Retrieval Solution (Dako, Glostrup, Denmark) at 80°C for 20 min. They were then left in the above solution for 20 min at room temperature (RT) and immersed in phosphate-buffered saline solution (PBS), pH 7.4, for 10 min. Aspecific sites were blocked with PBS containing 10% bovine serum albumin (BSA) for 30 min at RT. The sections were incubated overnight at 4°C with the primary antibody diluted in PBS containing 0.3% Triton X-100, 10% lactoproteins and 1% BSA. The titre of the M2A antibody was 1:4000. After being washed three times for 5 min each in PBS, the sections were incubated for 1 h at RT with CYTM3-conjugated AffiniPure donkey anti-mouse IgG (Jackson Immuno Research, West Grove, USA).

AMH immunohistochemistry
The sections were treated as previously described before blocking endogenous peroxidase activity by treatment with 3% H2O2 for 10 min. The slides were then incubated overnight at 4°C with the primary antibody diluted 1:1000 in PBS. The sections were then incubated for 1 h at RT with the secondary antibody, unlabelled goat anti-rabbit IgG (P.A.R.I.S., Compiegne, France) diluted 1:50 in the same solution that was used for the primary antibody. Finally, the sections were incubated with rabbit PAP (peroxidase-anti-peroxidase) (Dako). The reaction was revealed by diaminobenzidin (DAB).

Controls
All tests were performed on fetal, postnatal and adult control testicular tissue. The negative control was not treated with the primary antibody.

Results
Six biopsies with AZF deletions were completely negative with anti-M2A and anti-AMH antibodies. All the samples with Sertoli cell-only syndrome and maturation arrest were completely M2A negative. A few AMH-marked Sertoli cells were found in only two testes of the group I patients without deletions. This finding was unilateral in both cases (Figure 1).

The possibility of using AMH and M2A antibodies on the Bouin fixed and paraffin embedded material was confirmed on control tissues. Two markers of immature Sertoli cells were strongly expressed in fetal testes.

We observed membrane and sometimes perinuclear M2A immunostaining in spermatogonia and in the Sertoli cells of fetal and 2 month old male gonads (Figure 1). However, M2A immunostaining was not observed in the testes of a 4 year old boy and in two adult reference tissues.

The use of anti-AMH antibodies yielded intense cytoplasmic immunostaining in the Sertoli cells of both fetal and child testes (Figure 1). On the other hand, the Sertoli cells were AMH negative in adult reference tissues.

Discussion
The results showed that M2A and AMH immunoexpression was not modified by spermatogenic impairment associated or not with molecular deficit in the AZF region. The hypothesis that some Sertoli cells in AZF deleted men had not matured, and thereby preserved immunoreactivity to M2A or to AMH (later maturation marker), was not confirmed by this preliminary study. Two sequential Sertoli cell maturation markers revealed the same immunohistochemical pattern. In both groups of patients, AMH and M2A immunophenotypes of Sertoli cell were found to be matured, with no correlation to both existence of Y chromosome microdeletions and to histological phenotype (Sertoli cell-only syndrome or maturation arrest at spermatocyte I stage).

M2A negativity could mean either an irreversible loss of the expression of this marker after puberty, or a very low immunoreactivity level. Steger et al. suggest an abolition of M2A immunoexpression at puberty with a possible resumption in the case of a neoplastic process (Steger et al., 1999).

The situation with AMH is more complex or even contradictory. Fetal testes present important AMH immunoreactivity, but the production of this substance decreases successively after birth and especially at puberty. In
Figure 1. Immunohistochemical staining for M2A antigen (A, B, C) and anti-Müllerian hormone (D, E, F). (A) M2A immunostaining in fetal seminiferous cords (original magnification ×400). (B and C) Testicular biopsy with Sertoli cell-only syndrome and biopsy with active spermatogenesis are M2A negative (original magnification ×100 and ×400). (D) Fetal Sertoli cells show strong immunoreactivity for AMH (original magnification ×200). (E) Specimen of an AZFb/c-deleted man with SCO syndrome. Sertoli cells are AMH negative (original magnification ×200). (F) Biopsy with Sertoli cell-only syndrome (no deletions of the Y chromosome were detected in the patient). A single tubule is AMH positive (original magnification ×400). (Scale bars: A = 30 μm, B = 100 μm, C = 30 μm, D = 60 μm, E = 60 μm, F = 30 μm).

Adult subjects with qualitatively normal spermatogenesis, immunohistochemistry methods cannot detect the presence of AMH in Sertoli cells (Steger et al., 1996, 1999). A possible renewal of AMH immunoreactivity in cases of seriously impaired spermatogenesis (Steger et al., 1996, 1999) seems to be in apparent contradiction with the recent
work, suggesting that the lowest spermatogenic concentrations of AMH correspond to particular severity of the spermatogenic disorder (Fenichel et al., 1999). Immunoactivity to AMH in situ does not necessarily correspond to an increased level of this hormone in seminal fluid, but perhaps could be explained by retention (altered release) of the AMH by altered Sertoli cells. On the other hand, the absence of AMH in situ immunoreactivity (without taking into account the sensitivity of the technique) may be explained by either a complete release of this hormone into the seminiferous lumen, or a general decrease of the hormone production level.

Our study and similar ones include patients having histological syndromes whose aetiology is not clear. The results of the studies mentioned above are difficult to compare because of possible aetiological heterogeneity and the different sensitivity of the immunodetection techniques applied. Combined use of both techniques (biopsy immunostaining and ELISA) may be useful in assessing the data available. An absence of AMH immunoreactivity as in our case proves that the immature and/or dedifferentiated phenotype of Sertoli cells is rather rare in infertile patients.

A recent study attempting to evaluate cytokeratine-18 expression in the Sertoli cells of patients with non-obstructive infertility did not demonstrate a difference between the immunoactivity pattern in subjects with and without AZF microdeletions (Bar-Shira Maymon et al., 2000). In our study, we did not observe any immunohistological differences between samples with and those without Y deletion. In the cases of deletions in the AZF region, neither tested marker was discriminating. AZF microdeletions do not appear to interfere with immunoexpression of AMH and/or oncocfetal M2A antigen.

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