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GAMETE BIOLOGY

Increased apoptosis of germ cells in patients with AZFc deletions

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Abstract
Purpose AZFc deletions are associated with variable testicular histology ranging from the Sertoli cell only to spermatogenic arrest and hypospermatogenesis. Such variable phenotypes may be explained by progressive germ cell regression over time. Increased apoptosis is likely responsible for progressive regression of spermatogenic potential. This study evaluated germ cell apoptosis as a cause of the progressive decrease in the number of germ cells in patients with AZFc deletions.
Methods This study evaluated germ cell apoptosis in patients with AZFc deletions. A total of 151 patients who were diagnosed with either severe oligozoospermia or non-obstructive azoospermia were screened for Y chromosome microdeletions. Germ cell apoptosis was examined using terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) on formalin-fixed 5-μm sections of testicular specimens.
Results Seven out of 117 (6.0%) patients with azoospermia and 4 of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions. The percentage of apoptotic germ cells in the testes of patients with AZFc deletions were significantly increased compared to those of patients without AZFc deletions.
Conclusions These results suggest that increased apoptosis of germ cells is responsible for the progressive decline of spermatogenic potential in patients with AZFc deletions.

Keywords Apoptosis · AZF genes · Germ cells · Inhibin B · Microdeletions

Capsule Males carrying AZFc deletions exhibit diminished sperm cell numbers due to an enhanced incidence of apoptosis.

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Introduction

AZFc deletions are the most frequent genetic cause of male infertility, observed with a prevalence of 10–15% in patients with severe oligozoospermia and azoospermia [1]. The DAZ gene family is thought to be the major candidate responsible for the AZFc phenotype. The DAZ gene encodes a protein with an RNA-binding domain that is expressed exclusively in germ cells [2]. The natural RNA substrates of DAZ proteins remain undefined, and the biological function of DAZ has not yet been elucidated.

AZFc deletions are associated with variable testicular histology, ranging from the Sertoli cell only to spermatogenic arrest and hypospermatogenesis. A possible explanation for such variable phenotypes is the progressive germ cell regression over time, which has been reported in patients with AZFc deletions [3–8].

The control of germ cell apoptosis plays an important role during normal spermatogenesis [9–12]. Increased apoptosis can induce a progressive decrease in the number of germ cells. No studies have thus far assessed the apoptosis of germ cells in patients with AZFc deletions. Therefore, the current study evaluated germ cell apoptosis as one of the causes of the progressive decrease in the number of germ cells in patients with AZFc deletions.

Materials and methods

Patients

A total of 151 patients who were diagnosed with severe oligozoospermia (sperm concentration of less than 1×10^6 per ml) or non-obstructive azoospermia were screened for Y chromosome microdeletions. Among these, 117 were azoospermics and 34 were oligzoospermics. Patients with iatrogenic azoospermia, varicocele or cryptochidism were excluded from this study. As controls, testicular samples were obtained from five patients with obstructive azoospermia who had normal spermatogenesis.

Specimens of bilateral testicular tissue were obtained by open biopsy. The biopsies were classified according to McLachlan *et al*. [13] as follows: hypospermatogenesis, all stages of spermatogenesis are present but reduced to a varying degree; germ cell arrest, the total arrest at a particular stage; Sertoli cell-only, no tubules containing germ cells. This study was approved by the hospital's Institutional Review Board and informed consent was obtained from all patients.

Y chromosome microdeletion assay

Genomic DNA was isolated from peripheral blood lymphocytes using standard procedures. Y chromosome microdeletions were evaluated using polymerase chain reaction of Y chromosome-specific STS markers. The STS markers used were as follows: AZFa: sY83, sY95, sY105; AZFb: sY118, sY126, sY136; AZFc: sY152, sY254, sY255, sY283.

In situ end labeling of testicular tissue sections

In order to detect apoptosis, terminal deoxy-nucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (TUNEL) was performed on formalin-fixed 5-μm tissue sections of specimens using an In Situ Apoptosis Detection Kit (Takara Bio Inc., Shiga, Japan). In brief, each section was deparaffinized and rehydrated. After incubation with 20 μg/ml Proteinase K (Boeheringer Mannheim, Mannheim, Germany), endogenous peroxidase were blocked with 2% H_2O_2 in methanol for 30 min. TdT enzyme was dropped on the sections and incubated at 37°C for 60 min. Then antifluorescein isothiocyanate horseradish peroxidase conjugate was placed on the sections and incubated at 37°C for 30 min. Slides were washed three times in PBS, developed with 0.05% diaminobenzidine (DAB), and stained for 10–15 min at room temperature. The specimens were then washed three times in distilled water, dehydrated and mounted. For quantitative evaluation, the percentage of labeled cells per total 200 cells of germ cells was evaluated for each patient.

Hormone assays

Semen samples were centrifuged (3000× g; 5 min) and the seminal plasma was stored at –20°C within one hr after ejaculation. Inhibin B was measured by two-site enzyme-linked immunoassay (Serotec Ltd., Oxford, UK).

Statistical analysis

The Mann-Whitney U test was used for statistical analyses using the StatView 5.0 statistical analysis program (Abacus Concepts, Berkeley, CA, USA). Statistically significant differences were confirmed for p values less than 0.05.

Results

Seven out of 117 (6.0%) patients with azoospermia and 4 of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions (Table 1). AZFa, AZFb and AZFc were deleted in two azoospermic patients. AZFb and AZFc were deleted in one azoospermic patient. AZFc was deleted in four azoospermic patients and in four severe oligozoospermic patients. All patients with AZFa+b+c and AZFb+c deletions had a complete absence of spermatozoa upon testicular biopsy. Of the 8 patients with AZFc deletions, 6 had spermatozoa within the testis or ejaculate.

Serum and seminal plasma Inhibin B were undetectable in patients who lacked testicular spermatozoa. The seminal plasma Inhibin B level was greater than 15 pg/ml in all patients who had spermatozoa in testes or ejaculate (Table 2). Sequential seminological data was available in two patients with AZFc deletions. Patient 4 showed a

Table 1 Summary of DNA analysis of the twelve patients with Yq microdeletions

Markers	Patients										
	1	2	3	4	5	6	7	8	9	10	11
sY83	+	+	+	+	+	+	+	+	+	+	+
sY95	–	+	+	+	+	+	+	+	+	+	+
sY105	–	+	+	+	+	+	+	+	+	+	+
sY118	–	–	+	+	+	+	+	+	+	+	+
sY126	–	–	–	+	+	+	+	+	+	+	+
sY136	–	–	–	+	+	+	+	+	+	+	+
sY152	–	–	–	–	–	–	–	–	–	–	–
sY254	–	–	–	–	–	–	–	–	–	–	–
sY255	–	–	–	–	–	–	–	–	–	–	–
sY283	–	–	–	–	–	–	–	–	–	–	–
sY166	+	+	+	+	+	+	+	+	+	+	+

Table 2 Hormone values and clinical details of the ten patients with Yq microdeletions

Patients											
	1	2	3	4	5	6	7	8	9	10	11
Age (years)	45	44	43	42	35	36	36	55	46	34	48
Testicular volume (ml) right/left	5/8	8/10	4/3	17/15	7/7	17/16	14/13	18/11	10/9	8/7	5/5
Sperm count ($X10^6$/ml)	0	0	0	0.7	0.06	0	0.2	1.9	0	0	0
Deleted AZF regions	a,b,c	b,c	b,c	c	c	c	c	c	c	c	c
Inhibin B (pg/ml)											
Serum	<15	<15	<15	195	42	300	100	90	<15	<15	<15
Seminal plasma	<15	<15	<15	107	30	108	28	660	110	<15	<15
FSH (mIU/ml)	40.3	12.6	60.1	4.2	28.8	5.7	16.3	8.7	21.5	10.3	31.9
Histology	SCO	SCO	GA	GA	GA	HYPO			GA	GA	GA
Sperm recovery	–	–	–	+	+	+	+	+	+	–	–
Percentage of apoptotic cells (%)				2.5	5.0	4.0			7.5		7.0

SCO Sertoli cell-only, *GA* germ cell arrest, *HYPO* hypospermatogenesis

decline in the total sperm concentration from an average of 0.7 x 10^6 per ml to 0.02 x 10^6 per ml over 25 months. The serum and seminal plasma Inhibin B levels decreased from 195 pg/ml and 107 pg/ml to 35 pg/ml and 32 pg/ml, respectively. Patient 5 showed a decline in total sperm concentration from 0.06×10^6 per ml to azoospermia over 34 months. Serum and seminal plasma Inhibin B levels decreased from 42 pg/ml and 30 pg/ml to 18 pg/ml and 15 pg/ml, respectively.

Apoptosis was evaluated in the testes of 5 patients with AZFc deletions (patient 4, 5, 6, 9 and 11). Fifteen patients without AZFc deletions whose testicular histology were hypospermatogensis (3patients) or germ cell maturation arrest (12 patients) were also evaluated for apoptosis in testes. There was no significant difference in the testicular histology between these two groups.

The percentage of apoptotic germ cells in the testes of patients with AZFc deletions were significantly increased compared to those of patients without AZFc deletions and patients with obstructive azoospermia (5.2% vs. 2.1%, $p<0.01$; 5.2% vs. 1.0%, $p=0.01$; Table 3).

Table 3 Analysis of apoptosis in germ cells of testes

	Percentages of apoptotic cells (mean±SD)
Patients with AZFc deletions ($n=5$)	5.2±2.0[a,b]
Patients without AZFc deletions ($n=15$)	2.1±0.9
Obstructive asoospermic patients ($n=5$)	1.0±0.7

[a] Significantly different from patients without AZFc deletions ($P<0.01$)

[b] Significantly different from obstructive asoospermic patients ($P=0.01$)

Discussion

In this study, seven out of 117 (6.0%) patients with azoospermia and 4 out of 34 (11.8%) patients with severe oligozoospermia had Y chromosome microdeletions. These findings were consistent with previous reports of microdeletion frequencies between 6.2 and 25.9% in Japanese males [14, 15]. In the present study population, the frequency of Y chromosome microdeletions was lower in azoospermic patients than in oligozoospermic patients. Other Japanese studies [14] also reported a low frequency of Y chromosome microdeletions in azoospermic patients (4.2%) in comparison to oligozoospermic patients (15.9%). Nagata *et al.* [16] reported that the sperm retrieval rate by testicular sperm extraction in Japanese azoospermic patients was low in comparison to other studies. Other common genetic causes may exist in Japanese azoospermic patients. Eight out of 11 patients with Y chromosome microdeletions had complete AZFc deletions (b2/b4 deletion). The seminal phenotype of patients with complete AZFc deletions varied from azoospermia to severe oligozoospermia. Progressive regression of the germinal epithelium over a period of time has been reported which may be an explanation for such variable phenotypes [5]. However, Oates *et al.* [17] reported that 4 patients with AZFc deletions had stable sperm production over time. The discrepancies between the studies may have been due to the small number of patients.

In this study, 2 patients with AZFc deletions were followed over 2 years. Both patients exhibited a decline in total sperm concentration over 2 to 3 years, associated with a decrease in serum and seminal plasma Inhibin B levels. This finding supports a hypothesis of progressive depletion of the seminiferous epithelium. There is an association between serum Inhibin B levels and testicular pathology in

patients with AZFc deletions [18]. The current study also suggested that Inhibin B is a good marker for spermatogenic potential in patients with AZFc deletions. However, further studies with a greater number of study patients will be required to confirm the progressive decline of spermatogenic potential in patients with AZFc deletions and the utility of Inhibin B as a marker of spermatogenesis.

Mammalian spermatogenesis is a highly regulated process, and apoptosis appears to play an essential role in maintaining an appropriate number of germ cells that can be adequately supported and matured by the Sertoli cells [19]. Several authors have reported accelerated apoptosis of germ cells in infertile men with impaired spermatogenesis [9–12]. In the present study, the percentages of apoptotic germ cells were comparable to those reported in other studies. Only Tesarik et al. [9] reported much higher percentages of apoptotic germ cells in patients with incomplete spermatogenesis. The discrepancy between the studies might have been due to the method of apoptosis detection. Tesarik et al. examined the germ cell apoptosis by analyzing cell smears from mechanically disintegrated testicular tissues and used a FITC-labeled nucleotide to detect DNA fragmentation.

The mechanisms of the germ cell apoptotic process underling spermatogenesis impairment are poorly understood. In the current study, increased germ cell apoptosis was observed in patients with AZFc deletions in comparison to patients without AZFc deletions and patients with obstructive azoospermia. This increase in apoptosis may be responsible for the progressive loss in spermatogenic potential. Rajpurkar *et al.* [20] demonstrated that chronic cigarette smoke induced apoptosis in rat testis. They concluded that increased apoptosis might be one of the pathogenic mechanisms responsible for defective spermatogenesis in the rat following chronic cigarette smoking. A varicocele has a progressively toxic effect on the testes that may ultimately result in irreversible infertitity [21]. Hassan et al. [22] reported that the percentage of apoptotic cells in seminiferous tubules of infertile patients with varicocele was significantly higher than in patients with obstructive azoospermia (6.29% vs. 2.71%). These percentages of apoptotic germ cells were comparable to those reported herein.

AZFc contains five protein-coding gene families (BPY2, CDY, DAZ, CSPG4LY and GOLGA2LY), which are all transcribed in testicular tissue [23]. These genes are thought to be associated with spermatogenesis, but their function is unknown. The best-characterized gene family in the AZFc region is the DAZ gene. The DAZ gene family encodes a protein with an RNA-binding motif, suggesting a functional role in mRNA stability or in the translational regulation of its target RNA. The CDC25 family has been recognized as the downstream target of DAZL, which is the autosomal DAZ family gene [24, 25]. CDC25 phosphatases play a key role in cell cycle progression by controlling the activation of cyclin-dependent kinases [26]. Of the CDC25 family, CDC25A is expressed at a high level in the testis, suggesting that CDC25A plays a crucial role in the mitotic or meiotic regulation of spermatogenesis [27, 28]. Inactivation of CDC25 induces cell cycle arrest and apoptosis of hepatocellular carcinoma cells [29]. The inhibition of the CDC25 function, owing to a loss of DAZ genes, may contribute to the accelerated germ cell apoptosis observed in patients with AZFc deletions.

This is the first paper reporting increased apoptosis of germ cells in patients with AZFc deletions. Further studies with a larger population are needed to confirm these results.

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