Role of compensatory meiosis mechanisms in human spermatogenesis

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Abstract

Disturbances of checkpoints in distinct stages of spermatogenesis (mitosis, meiosis, and spermiogenesis) contribute to impaired spermatogenesis; however, the efficiency of meiotic entry has not been investigated in more detail. In this study, we analyzed azoospermic patients with defined spermatogenic defects by the use of octamer-binding protein 2 for type A spermatogonia, sarcoma antigen 1 for mitosis–meiosis transition and SMAD3 for pachytene spermatocytes. Especially patients with maturation arrest (MA) at the level of primary spermatocytes showed significantly reduced numbers of spermatogonia compared with patients with histologically intact spermatogenesis or patients with hypospermatogenesis (Hyp). For a detailed individual classification of the patients, we distinguished between ‘high efficiency of meiotic entry’ (high numbers of pachytene spermatocytes) and ‘low efficiency of meiotic entry’ (low numbers of pachytene spermatocytes). Only patients with histologically normal spermatogenesis (Nsp) and patients with Hyp showed normal numbers of spermatogonia and a high efficiency of meiotic entry. Of note, only patients with histologically Nsp or patients with Hyp could compensate low numbers of spermatogonia with a high efficiency of meiotic entry. In contrast, patients with MA always showed a low efficiency of meiotic entry. This is the first report on patients with impaired spermatogenesis, showing that half of the patients with Hyp but all patients with MA cannot compensate reduced numbers in spermatogonia with a highly efficient meiosis. Thus, we suggest that compensatory meiosis mechanisms in human spermatogenesis exist.

Reproduction (2014) **148** 315–320

Introduction

In about half of infertile couples, male infertility plays a role and almost 30% are caused by a male factor alone (Brugh & Lipshultz 2004). Besides analysis of semen parameters, a histological evaluation is indicated especially to confirm obstructive azoospermia in men with testes of normal size and normal levels of reproductive hormones (Dohle *et al.* 2012).

The dynamics of spermatogenesis are crucial for elucidation of impaired spermatogenesis, which is caused by many factors such as X-ray, chemotherapy, infections, chromosomal defects, diabetes mellitus, or alcohol abuses (Cerilli *et al.* 2010). Although a lot of aberrations during mitosis (Steger *et al.* 1998, Bar-Shira Maymon *et al.* 2003) and meiosis (Gonsalves *et al.* 2004, Egozcue *et al.* 2005) have been described, only recently it has been shown that also reduced numbers of Sertoli cells or spermatogonia (including the stem cells) contribute to impaired spermatogenesis (Hentrich *et al.* 2011). However, in humans the stem cell niche is still poorly characterized, but A_{dark} as well as A_{pale}

spermatogonia seem to contain subpopulations of spermatogonial stem cells (Dym *et al.* 2009). Of note, in primates, the A_{pale} spermatogonia also contain few spermatogonial stem cells (Hermann *et al.* 2010).

The testicular stem cells in rat and mouse are localized nearly exclusively in a stem cell niche adjacent to the interstitium (Chiarini-Garcia *et al.* 2001, 2003). The stem cell niche is characterized by a microenvironment which is influenced also by Leydig cells and blood vessels (Shetty & Meistrich 2007, Yoshida *et al.* 2007). Furthermore, also Sertoli cells and peritubular cells contribute to the self-renewal of the spermatogonial stem cells (Dadoune 2007, de Rooij 2009).

Especially in patients with maturation arrest (MA) a significant reduction in dividing spermatogonia during mitosis was observed (Steger *et al.* 1998, Bar-Shira Maymon *et al.* 2003), whereas in hypospermatogenesis (Hyp)-accelerated apoptosis rather than proliferative dysfunction was suggested to be responsible for reduced spermatogonial cell numbers (Takagi *et al.* 2001). In addition, in cases with MA reduced recombination rates (up to 50%) were found (Gonsalves *et al.* 2004, Egozcue

Table 1 Antibodies used for quantification of distinct germ cell types.

Protein	Source	Catalog number	Species	Clonality	Dilution
OCT2	Novocastra (Wetzlar, Germany)	NCL-OCT-207	Mouse	Monoclonal	1:75
SAGE1	Sigma-Aldrich (St. Louis, USA)	HPA003033	Rabbit	Polyclonal	1:2000
SMAD3	EnoGene Biotech (Frankfurt, Germany)	E021324	Rabbit	Polyclonal	1:300

et al. 2005) and an increased frequency of genetic abnormalities (Weedin *et al.* 2011).

In this study, we analyzed germ cell numbers in the early phase of spermatogenesis with an emphasis on mitosis–meiosis transition. Furthermore, we classified the deficiencies individually by distinguishing between high and low efficiency of meiosis. Our main finding in this study was that cases with histologically normal spermatogenesis (Nsp) and cases with Hyp could compensate reduced numbers of spermatogonia with a highly efficient meiosis suggesting a compensatory mechanism.

Materials and methods

Patients

Testicular biopsies were obtained from 90 patients between 1998 and 2012 and were indicated because of normogonadotropic obstructive or non-obstructive azoospermia. After written informed consent, biopsies were taken under general anesthesia. The Ethics Committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany approved the study (75/00 and 56/05). After fixation in Bouins solution and embedding in paraffin, 5 µm sections were stained with hematoxylin and eosin and spermatogenesis was evaluated histologically according to the scoring system of Bergmann & Kliesch (1998, 2010). The patients were classified into histologically Nsp ($n=30$; median age 39, range 16–56; mean score 9.93, range 9–10), Hyp ($n=30$; median age 37, range 18–63; mean score 8.43, range 6–10), and MA at the level of spermatocytes (MA, $n=30$; median age 34.5, range 17–69; mean score 0).

Antibodies

To classify especially early stages of spermatogenesis (spermatogonia and mitosis–meiosis transition), we identified several highly robust markers with a good signal-to-noise ratio and distinct expression pattern in human germ cells. For a detailed analysis of impaired spermatogenesis, three markers were chosen (Table 1), octamer-binding protein 2 (OCT2 (POU2F2)) for early spermatogonia (Lim *et al.* 2011), sarcoma antigen 1 (SAGE1) for dividing and differentiating spermatogonia (Lim *et al.* 2011), and mitosis–meiosis transition (Looijenga 2011), and SMAD3 for pachytene spermatocytes (Hentrich *et al.* 2011).

Immunohistochemistry

Immunohistochemistry was carried out on 5 µm sections of bouin-fixed, paraffin-embedded specimen, as published previously (Konrad *et al.* 2007). The Envision System from Dako (Hamburg, Germany) combined with DAB staining was used

according to the manufacturer's instructions. Counterstaining was done with hematoxylin. The antibodies used for detection are given in Table 1. Digital images were obtained with the inverse microscope FSX100 (Olympus) using the Olympus FSX-BSW Software. The images were processed with Adobe Photoshop 7.0.

Quantification of germ cells

The numbers of germ cells were obtained from 26.63 ± 12.58 tubular cross sections per specimen representing Nsp, Hyp, and MA for each staining. As published recently (Hentrich *et al.* 2011) and also shown in this study, it was sufficient to use ~15–35 nearly round cross sections for quantification; we never observed outliers.

There is no consensus on how quantification of testicular biopsies with an emphasis on impaired spermatogenesis should be performed. The number of Sertoli cells is different in patients

Figure 1 Immunohistochemical detection of OCT2 (A, D and G), SAGE1 (B, E and H), and SMAD3 (C, F and I) in biopsies from patients with histologically normal spermatogenesis (A, B and C), patients with hypospermatogenesis (D, E and F), and cases with maturation arrest (G, H and I). OCT2 was found mainly in A_{dark} spermatogonia (Ad) and to a lesser extent in A_{pale} spermatogonia (Ap). Also few OCT2-negative A_{dark} spermatogonia ($Ad(-)$) could be identified. SAGE1 was expressed in A_{dark} spermatogonia up to type B spermatogonia (B), but also in some (pre)-leptotene spermatocytes (pl). In contrast, SMAD3 was localized primarily in the cytoplasm of pachytene spermatocytes (p). The arrows point to the regions provided enlarged in the insets. Scale bar = 25 µm (A, B and C), the same magnification was used in (D, E, F, G, H and I). Counterstaining was done with hematoxylin.

Table 2 Numbers of distinct germ cells per cross-section in impaired spermatogenesis

	<i>n</i>	Mean (S.E.M.)	<i>P</i> values		
			Nsp vs Hyp	Nsp vs MA	Hyp vs MA
OCT2: Early spermatogonia			ns	0.01	0.001
Nsp	30	2.09 (0.25)			
Hyp	30	2.05 (0.23)			
MA	30	0.96 (0.16)			
SAGE1: dividing/differentiating spermatogonia			0.01	0.001	ns
Nsp	30	15.62 (0.82)			
Hyp	30	11.16 (0.61)			
MA	30	9.10 (0.77)			
SMAD3: pachytene spermatocytes			0.001	0.001	0.001
Nsp	30	16.35 (0.92)			
Hyp	30	8.64 (0.56)			
MA	30	1.53 (0.36)			

Nsp, normal spermatogenesis; Hyp, hypospermatogenesis; MA, maturation arrest; ns, not significant.

with MA or Hyp (Hentrich *et al.* 2011). Thus, the Sertoli cell to germ cell ratio is not applicable for the quantification of germ cell numbers.

Statistical analysis

Values from each experiment were used for calculation of the means (per cross section) and the respective S.E.M. Differences between the groups were calculated with the test from Mann–Whitney. The *P* values below 0.05 were considered statistically significant.

Cut off values to distinguish between normal germ cell numbers vs reduced germ cell numbers were determined with receiver operating characteristic-curves and area under the curve. Besides sensitivity and specificity also the positive likelihood ratio was calculated. For all statistical analyses GraphPad Prism 6 (San Diego, CA, USA) was used.

Results

Identification of germ cell markers for early spermatogenesis

We identified the expression of OCT2 mainly in spermatogonia A_{dark}, but also in very few spermatogonia

A_{pale} (Fig. 1A, D and G). Often the OCT2-positive spermatogonia were localized in small groups in close proximity to interstitial arterioles or venules, which is in accordance to the characteristics of the stem cell niche in murine testis (Shetty & Meistrich 2007, Yoshida *et al.* 2007) and thus have been also termed as reserve stem cells (Dym *et al.* 2009). However, we found also OCT2-negative A_{dark} and A_{pale} spermatogonia.

We identified SAGE1 in very few spermatogonia A_{dark}, but in nearly all A_{pale} and type B spermatogonia as well as in (pre)-leptotene spermatocytes and round spermatids (Fig. 1B, E and H), thus representing mitotic spermatogonia and transition of mitosis into meiosis. For characterization of pachytene spermatocytes, we used SMAD3 (Fig. 1C, F and I).

Analysis of meiosis efficiency

All three markers were used for the quantification of germ cell numbers (early spermatogenesis) per cross section and thus also for individual classification of meiosis efficiency in cases of impaired spermatogenesis. Quantification of OCT2-positive spermatogonia showed nearly identical values for Nsp and Hyp, but cases with MA demonstrated a significant reduction of ~50% (Table 2). Of note, quantification of SAGE1-positive germ cells (spermatogonia up to (pre)-leptotene spermatocytes) revealed strongly and significantly reduced cell numbers in Hyp and in patients with MA compared with the controls with histologically Nsp (Table 2). Similarly, SMAD3-positive pachytene spermatocytes were reduced by 47% in Hyp and by 91% in cases with MA compared with Nsp (Table 2).

Individual classification of patients with impaired spermatogenesis

For a detailed individual classification of the patients, we determined the cut off values for all three markers to distinguish between normal and lower germ cell numbers (Table 3). Furthermore, we classified spermatogenesis into ‘high efficiency of meiotic entry’ (high numbers of SMAD3-positive spermatocytes, Fig. 2A) and ‘low efficiency of meiotic entry’ (low numbers of SMAD3-positive spermatocytes, Fig. 2B). Both groups could be further divided into four subgroups according

Table 3 Statistical analysis of the three markers used for individual classification.

	OCT2 (Cut off=1.06)			SAGE1 (Cut off=10.84)			SMAD3 (Cut off=8.6)		
	Sensitivity	Specificity	LR+	Sensitivity	Specificity	LR+	Sensitivity	Specificity	LR+
Nsp/Hyp	23	87	1.8	40	90	4.0	47	97	14
Nsp/MA	57	87	4.3	77	90	7.7	100	97	30
Hyp/MA	57	77	2.4	77	60	1.9	100	53	2.1

Values for sensitivity and specificity are given in %; LR+, positive likelihood ratio; Nsp, normal spermatogenesis; Hyp, hypospermatogenesis; MA, maturation arrest.

to the cut off values for early spermatogonia and dividing/differentiating spermatogonia (Fig. 2). The individual evaluation of each patient revealed that patients with histologically Nsp ($n=29$) or patients with Hyp ($n=14$) showed a high efficiency of meiotic entry and normal numbers of early spermatogonia or dividing/differentiating spermatogonia (Fig. 2A). Remarkably, only patients with histologically Nsp (seven from seven) as well as patients with Hyp (seven

from 14) could compensate low numbers of spermatogonia with a high efficiency of meiotic entry (Fig. 2). In contrast, patients with MA always showed a low efficiency of meiotic entry and also revealed defects more often in the numbers of dividing/differentiating spermatogonia compared with defects in early spermatogonia (Fig. 2B). Especially subgroup 5 indicates missing compensatory mechanisms in meiosis as patients with normal numbers of spermatogonia showed low efficiency of meiosis (Fig. 2B).

Figure 2 Classification of patients into high meiosis efficiency (A) and low meiosis efficiency (B). Both groups consist of four subgroups with normal numbers of early spermatogonia (OCT2⁺) and dividing/differentiating spermatogonia (SAGE1⁺) or reduced numbers of early spermatogonia (OCT2⁻) and dividing/differentiating spermatogonia (SAGE1⁻). The cut off values from Table 3 were used for classification. Only cases with normal spermatogenesis (Nsp) or hypospermatogenesis (Hyp) demonstrated high meiosis efficiency (A). Interestingly, only Nsp or Hyp could compensate low numbers of early spermatogonia (OCT2⁻) and/or dividing/differentiating spermatogonia (SAGE1⁻) with a highly efficient meiosis (A). In contrast, all cases with maturation arrest (MA) showed low meiosis efficiency besides defects in numbers of early spermatogonia or dividing/differentiating spermatogonia (B).

Discussion

Besides the identification and evaluation of markers for early spermatogenesis, the primary aim of this study was the individual classification of defects in the early phase of spermatogenesis in cases of impaired spermatogenesis.

Intriguingly, the expression of OCT2 was found primarily in clusters of human A_{dark} spermatogonia in a stem cell niche most often adjacent to the interstitial vasculature. This suggests that OCT2 is expressed very early in human spermatogenesis, most likely in stem cells as described by Lim *et al.* However, as also shown by Lim *et al.* (2011), as well as in this study, only a subgroup of A_{dark} spermatogonia is OCT2-positive. In this study, identification of few OCT2-positive A_{pale} spermatogonia is in accordance to results obtained in monkeys (Hermann *et al.* 2010), which showed that this subgroup also consists of undifferentiated spermatogonia, possibly stem cells. Thus, we believe like Dym *et al.* (2009) and (Hermann *et al.* 2010) that both A_{dark} and A_{pale} spermatogonia contain spermatogonial stem cells. In contrast, Ehmecke *et al.* (2006) described that only the A_{dark} spermatogonia comprise spermatogonial stem cells.

In line with our previous findings (Hentrich *et al.* 2011), we found reduced numbers of spermatogonia in cases with MA. Moreover, we observed low numbers of early spermatogonia in cases with Hyp as well as in MA, indicating deficiencies already in the early phase of spermatogenesis. Thus, without A_{dark} spermatogonia, which contribute to stem cell renewal, spermatogenesis is severely impaired (Holstein 1999).

In addition to stem cell markers, we were also interested in identification of marker(s) for dividing and differentiating spermatogonia. We observed SAGE1 expression in very few A_{dark} spermatogonia, but mainly in dividing spermatogonia and (pre)-leptotene spermatocytes indicating entry into meiosis. Similarly Chen *et al.* (2011) and Lim *et al.* (2011) found SAGE1 in dividing spermatogonia and meiotic entry. Interestingly, SAGE1 expression was not detectable before puberty (Lim *et al.* 2011), thus further corroborating the importance of SAGE1 for differentiation of spermatogonia.

We found that SAGE1-positive spermatogonia were severely reduced in patients with Hyp and MA, but only

rarely in patients with histologically Nsp. Thus, defects in the early phase of spermatogenesis, especially during mitosis, contribute also to impaired spermatogenesis. Similarly Bar-Shira Maymon *et al.* (2003) found a significant reduction in dividing spermatogonia in patients with spermatogenic defects, which was also described for Hyp and patients with MA (Steger *et al.* 1998). However, because reduced numbers of early spermatogonia might contribute to reduced numbers of mitotic spermatogonia, thus, any study on mitotic defects must also include quantification of early spermatogonia in order to discriminate properly between mitotic defects or reduced numbers of spermatogonia before meiosis or a combination of both.

SMAD3, which is also a key mediator of activin-induced proliferation of Sertoli cells (Itman *et al.* 2011), is mainly expressed in pachytene spermatocytes in rat (Xu *et al.* 2003), mouse (Itman *et al.* 2011), and human testis (Hentrich *et al.* 2011). The reduced SMAD3-positive pachytene spermatocyte numbers in patients with Hyp or MA (Hentrich *et al.* 2011) was also described without marker expression analysis in cases with reduced sperm cell numbers (Roosen-Runge *et al.* 1957, Zukerman *et al.* 1978). In contrast to the retained SMAD3 protein expression in cases with impaired spermatogenesis, the BOULE protein (expressed in human leptotene to pachytene spermatocytes in Nsp) was completely absent in cases with MA (Luetjens *et al.* 2004). Furthermore, alternative splicing of the BOULE gene was supposed to serve as a predictive marker for meiotic efficiency (Kostova *et al.* 2007).

The individual classification of patients with impaired spermatogenesis by categorizing meiotic efficiency in the early phase of spermatogenesis is a promising extension of the classification system reported by Bergmann & Kliesch (1998, 2010). Although studies are lacking to assess the early phase of spermatogenesis up to pachytene, earlier studies showed up to 50% reduced recombination rates in cases with MA (Gonsalves *et al.* 2004, Egozcue *et al.* 2005). Similarly, a sixfold increased frequency of genetic abnormalities was observed in cases of MA compared with Hyp (Weedin *et al.* 2011).

In this study, we found that patients with histologically Nsp showed the lowest and patients with MA the highest numbers of defects in the early phase of spermatogenesis as well as in the meiotic phase. Especially in the subgroups 2–4 (Fig. 2A), a high meiotic efficiency but low numbers of spermatogonia, meiotic compensatory mechanisms must exist to ameliorate these defects. Obviously, all cases with MA are lacking this mechanism in addition to severely reduced numbers of spermatogonia. Interestingly, in cases with MA, reduction in early spermatogonia numbers (54%) is nearly similar to the reduced numbers of dividing/differentiating spermatogonia (42%), suggesting that loss of early spermatogonia might be primarily causative.

Of note, in cases of Hyp, which is the most heterogeneous group (Takagi *et al.* 2001), we could identify both groups of patients with high or low efficiency of meiosis in almost all combinations with reduced numbers of early spermatogonia or reduced numbers of dividing/differentiating spermatogonia. However, in contrast to cases with MA, the frequency of low efficiency of meiosis was clearly lower in cases of Hyp.

In summary, this study revealed the efficiency of meiotic entry as a factor for male infertility and allows for a more detailed analysis of individual cases. We provide a refinement of the established classification system launched by Bergmann & Kliesch (1998, 2010). In line with the diagnosis of MA, all cases demonstrated reduced numbers of spermatocytes; however, the majority additionally experienced reduced numbers of spermatogonia. Furthermore, we could provide evidence that in cases with Nsp as well as in cases with Hyp, compensatory meiosis mechanisms might exist to attenuate the loss of spermatogonia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to L Konrad (KFO 181/2 P2).

Acknowledgements

The authors thank Cornelia Hof for excellent technical assistance and Prof. Kliesch (Center of Andrology and Reproductive Medicine, University of Muenster, Germany) for biopsy sampling.

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Received 28 May 2014

First decision 13 June 2014

Revised manuscript received 18 June 2014

Accepted 26 June 2014