Spermatogonial proliferation patterns in men with azoospermia of different etiologies

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Objective: To demonstrate the pattern(s) of spermatogonial proliferation in different spermatogenic disorders.

Design: Retrospective case-control study.

Setting: Teaching hospital.

Patient(s): Azoospermic men who underwent testicular biopsy for sperm recovery and preparation for intracytoplasmic sperm injection.

Intervention(s): Testicular biopsy evaluation by quantitative immunohistochemistry for proliferating cell nuclear antigen (PCNA).

Main Outcome Measure(s): The expression of PCNA in spermatogonia as an index of proliferating activity in testes with focal spermatogenesis, spermatocyte maturation arrest, or normal spermatogenesis.

Result(s): In biopsies with focal spermatogenesis (11 men), there was a statistically significant reduction of PCNA-labeled spermatogonia in seminiferous tubules showing spermatocyte arrest compared with the expression in adjacent tubules with advanced spermatogenic stage or in normal spermatogenesis (obstructive azoospermia, six men). However, PCNA expression in tubules of the group with complete maturation arrest (six men) was significantly higher compared with the same spermatogenic defect—spermatocyte arrest—within focal spermatogenesis biopsies.

Conclusion(s): Different causes underlie the spermatogenic disorders reported in this study. In focal spermatogenesis, the disorder is associated with the presence of mitotic inactive spermatogonia. The detection of normal active spermatogonia in the spermatocyte arrest group indicates that the spermatogenic defect, which is accompanied by meiosis impairment, is not related to a malfunction of spermatogonial proliferation.

Key Words: Spermatogonia, proliferating cell nuclear antigen (PCNA), nonobstructive azoospermia, meiosis impairment, immunohistochemistry

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Controlled cell proliferation of the germ line is of fundamental importance during normal spermatogenesis, assuming a highly coordinated mechanism between the somatic Sertoli cells and the germ cells undergoing mitosis and meiosis (1). Efficiency of the spermatogenetic process depends on the mitotic activity of spermatogonia and loss of germ cells during meiosis and spermiogenesis (2, 3).

Mitotic activity of cells in human biopsies can be adequately evaluated by detecting the expression of S-phase-related proteins such as proliferating cell nuclear antigen (PCNA) (4–7). An auxiliary protein to DNA polymerase-δ, PCNA is involved in nucleotide excision repair mechanisms (5, 7) and has a biological half-life of approximately 20 hours (5). It is expressed in the cell nuclei, with a maximum expression in the S and G2 phases (6). In the normal seminiferous epithelium, PCNA is prominently expressed in the nuclei of mitotic active spermatogonia and occurs in the nuclei of primary spermatocytes as well (8, 9).

The appearance of a heterogeneous phenotype is a common histologic finding in testicular biopsies taken from nonobstructive azoospermic men; this condition involves a variable degree of spermatogenic impairment.
in adjacent seminiferous tubules, ranging from normal spermatogenesis to absence of germ cells (Sertoli cell–only) and complete atrophy of adjacent tubules (10–12). It is also referred to as mixed atrophy (13), and the etiologic mechanism of its underlying pathogenesis has yet to be elucidated.

Compared with those of mixed atrophy, testicular biopsies that are characterized by a homogenous phenotype of spermatocyte maturation arrest in all the apparent tubules of samples obtained from both testicles are relatively rare. Using a less strict definition of spermatocyte arrest, maturation arrest was observed in 18% of testicular biopsy specimens from men with a clinical diagnosis of azoospermia (14). It is widely accepted that a chromosome-pairing impairment during meiosis is the etiologic factor underlying maturation arrest at the spermatocyte stage (15–20).

Spermatogonia are particularly important when studying spermatogenic impairment because they are the precursors of the spermatogenesis process. With the use of an antibody against PCNA, the current study aimed to investigate the pattern(s) of spermatogonial proliferation within three defined groups of azoospermic men who differed by their testicular histopathologic characteristics and the presence of testicular spermatozoa in multiple biopsies of both testes. These groups had also been previously defined for meiotic impairment, as judged by the bivalent formation of chromosomes X, Y, and 18 in their spermatocytes (19, 20). Evaluation of spermatogonial proliferation in such a histologic subdivision has not been reported previously.

**MATERIALS AND METHODS**

**Patients**

A total of 23 azoospermic men aged 21 to 36 years were evaluated for primary infertility. They underwent repeated semen analyses, the results of which were recorded. In all cases, azoospermia was reconfirmed before the testicular sperm extraction (TESE) procedure according to the protocol described previously by Hauser et al. (21). Testicular sperm extraction was performed by multiple testicular sampling for the intracytoplasmic sperm injection (ICSI) procedure.

All patients provided written informed consent to undergo genetic evaluation. The study was approved by the local institutional review board committee in accordance with the Helsinki Declaration of 1975.

Patients had been previously evaluated for meiosis impairment as described elsewhere (19, 20). In addition, the Y-chromosome microdeletion test was performed by the multiplex polymerase chain reaction with genomic DNA isolation from peripheral blood samples (22). Only patients with no Y-chromosome microdeletions were included in the study. Chromosome karyotyping was performed on peripheral lymphocytes with G-banding. All the study patients had a 46,XY complement of chromosomes.

**Testicular Sampling and Biopsy Evaluation**

Multiple testicular sampling was performed in both testes using the method previously described by Hauser et al. (21). Three biopsies were performed in each testicle. One of the biopsies from each testicle was divided into two pieces, one small piece for histopathologic investigation and the other for evaluating meiosis by fluorescence in situ hybridization (FISH). The main portion of this biopsy as well as the two other biopsies from other locations in the testes were minced for extracting spermatozoa to be used in the ICSI procedure (21). Whenever spermatozoa were found, they were cryopreserved or used for ICSI while they were still fresh, and the excess of spermatozoa were cryopreserved and used later (23).

The biopsies for histopathologic evaluation were fixed in routine Bouin’s fixative solution (Sigma Chemical Co., St. Louis, MO) for approximately 4 hours and embedded in Paraplast (Oxford Labware, St. Louis, MO). Histologic analysis of spermatogenesis was performed on hematoxylin and eosin stained thin sections. At least 20 seminiferous tubules in each section were scored.

**Classification of the Studied Groups**

According to the results of the histologic study performed as described later, patients were classified into three groups. In group A (11 men), focal spermatogenesis was characterized by mixed tubular atrophy and the presence of spermatozoa as identified by histologic examination or during exploration of the minced testicular tissue in the different locations. This group was characterized by a high rate of chromosome bivalent formation in the spermatocytes’ nuclei (19, 20).

Group B (six men) had a homogenous appearance of maturation arrest at the spermatocyte stage and complete absence of spermatozoa in all six biopsies taken from the different sites of both testes. This group was characterized by a dramatically low rate of chromosome bivalents formation in the spermatocytes (19, 20).

In group C (obstructive azoospermia, six men), normal spermatogenesis was classified according to the definition of Silber and Rodriguez-Rigau (24). This group, considered the control, was characterized by a high rate of chromosomal bivalent formation in spermatocytes similar to that of group A (19, 20).

**Immunohistochemistry**

Immunohistochemistry was performed using monoclonal antibody against PCNA (Clone:PC10, Zymed, San Francisco, CA). Briefly, 3-μm sections were mounted on SuperFrost Plus glass microscopic slides (Menzel, Glazer, Braunschweig, Germany) and processed by the labeled streptavidin-biotin (LAB-SA) method using a HISTOSTAIN plus kit (Zymed, San Francisco, CA).

The sections were treated with 3% H2O2 for 5 minutes, followed by a 10-minute incubation with a universal blocker,
CAS Block (Zymed, San Francisco, CA), for reducing non-specific immunolabeling. Sections were incubated for 1 hour with 1:40 dilution of PCNA antibody. Negative-control incubations were performed by substituting nonimmune serum for the primary antibody (these control sections were entirely negative).

Biotinylated second antibody was applied for 10 minutes, followed by incubation with horseradish peroxidase-conjugated streptavidin (HRP-SA) for 10 minutes. Following each incubation, the slides were washed thoroughly with Optimax wash buffer (Biogenex, San Ramon, CA). The immunoreaction was visualized by an HRP-based chromogen/substrate system, including DAB (brown) chromogen (Liquid DAB substrate kit; Zymed, San Francisco, CA). The sections were then counterstained with Mayer’s hematoxylin, dehydrated, and mounted for microscopic examination.

**Histomorphometric Analysis**

Quantitative microscopic analysis was performed using an Olympus BX50 microscope at a magnification of ×400. Single seminiferous tubules were analyzed, and the data were summarized according to defined defects such as spermatocytes or spermatogonial maturation arrest. Twenty tubules of each such defined spermatogenic defect were scored in each specimen. The number of immuno-labeled spermatogonia and the total number of spermatogonia were determined in cross-sections of individual seminiferous tubules. The ratio of PCNA-positive spermatogonia per total number of spermatogonia in each tubule was considered the percentage of proliferating spermatogonia. The calculations are presented as mean ± SEM. Student’s t-test was used for significant difference between groups. *P < .05 was considered statistically significant.

**RESULTS**

In the normal seminiferous epithelium, the PCNA antibody provided a nuclear staining in cells of the germ line: prominent staining was demonstrated in the nuclei of the spermatogonia and also in the nuclei of primary spermatocytes up to the pachytene stage (Fig. 1A), whereas the somatic Sertoli cells were negative for the marker’s expression (see Fig. 1A).

Various spermatogenic impairments in adjacent seminiferous tubules were observed in biopsies of azoospermic men with focal spermatogenesis (see Fig. 1B). Given this phenomenon of mixed atrophy, the histomorphometric measurements were performed on individual seminiferous tubules rather than on the whole biopsy to compare immuno-positive spermatogonia in tubules with the same spermatogenic defect. Unlike the heterogeneous phenotype of the focal spermatogenesis group, biopsies of spermatocyte arrest of group B had a homogeneous appearance of maturation arrest at the spermatocyte stage in all the apparent tubules (see Fig. 1C).

Quantitative immunohistomorphometric analysis revealed different patterns of PCNA labeling of spermatogonia for the different spermatogenic disorders found in biopsies with focal spermatogenesis (group A, Table 1). When the data were summarized according to defined defects such as spermatocytes or spermatogonial maturation arrest, there was a statistically significant reduction of PCNA-labeled spermatogonia in tubules showing arrest of spermatogenesis compared with adjacent spermatogenic foci-tubules with full spermatogenesis (group A, see Table 1).

The rate of PCNA-labeled spermatogonia in the group with a homogeneous appearance of spermatocyte maturation arrest (group B) was higher compared with tubules with the same spermatogenic defect (i.e., spermatocyte arrest) that

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**TABLE 1**

Quantification of proliferating cell nuclear antigen (PCNA) expression in testicular biopsies in the groups of azoospermic men revealing focal spermatogenesis (group A), spermatocyte maturation arrest (group B), or normal spermatogenesis (obstructive azoospermia, group C).

<table>
<thead>
<tr>
<th>Study group</th>
<th>Focal spermatogenesis (Group A, n = 11)</th>
<th>Spermatocyte arrest (Group B, n = 6)</th>
<th>Normal spermatogenesis (Group C, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of spermatogenic impairment in individual seminiferous tubules</td>
<td>Spermatogenie foci</td>
<td>Spermatocyte arrest</td>
<td>Spermatogonial arrest</td>
</tr>
<tr>
<td>Percentage of PCNA-labeled spermatogonia</td>
<td>37.28 ± 5.65</td>
<td>24.34 ± 6.51</td>
<td>13.14 ± 4.35</td>
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<td>Note: Values are mean ± SEM.</td>
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<td></td>
<td>*Significantly (P &lt; .005) lower than the other means demonstrated in spermatogenic foci (group A), spermatocyte arrest (group B), and normal spermatogenesis (group C).</td>
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had been found within the biopsies of focal spermatogenesis. The high labeling ratio in group B was similar to that of the spermatogenic foci in group A (see Table 1).

**DISCUSSION**

The current study demonstrates different patterns of spermatogonial proliferation in different spermatogenetic impairments.

In the group with focal spermatogenesis (group A), the highest percentage of PCNA-labeled spermatogonia (indicative for spermatogonial mitotic activity) was detected among the seminiferous tubules that were characterized by full spermatogenesis. A low rate of immuno-labeled spermatogonia could be demonstrated by tubules that showed arrest of spermatogenesis either at the level of spermatogonia or that of primary spermatocytes. These immunohistochemical characteristics of the biopsies indicate that the disorder is associated with the presence of mitotic inactive spermatogonia in focal spermatogenesis histology. Furthermore, these characteristics provide evidence for demonstrating that spermatogonia in the active mitotic state are required for the progression of spermatogenesis in the minute foci; an abnormality of spermatogonial proliferating activity disrupts the progression of full spermatogenesis.

In the group characterized by the homogenous appearance of spermatocyte maturation arrest (group B), the majority of spermatogonia were found to be mitotic active, as indicated by the high percentage of PCNA-labeled spermatogonia. In this group, which is characterized by a dramatically low rate of bivalent formation of the spermatocyte chromosomes (19, 20), spermatogenic failure appeared to be a consequence of meiosis impairment (19, 20) and not due to a malfunction of spermatogonial activity.

The proliferation of spermatogonia in normal and pathological human seminiferous epithelium has been previously studied using the PCNA marker (8, 9). Testicular biopsies characterized by mixed atrophy and hypospermatogenesis were analyzed in the study of Steger et al. (8). Their data demonstrated that the low spermatogenic efficiency in infertile men is not only due to postmeiotic events but also to a decrease in the mitotic activity of spermatogonia (8). Their findings are in agreement with the results of our current

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study, which demonstrated an impairment of spermatogonial mitotic activity in the seminiferous epithelium of biopsies with mixed atrophy and focal spermatogenesis (group A).

The balance between spermatogonial proliferation (indicated by PCNA expression) and apoptosis of spermatogonia (indicated by the TUNEL method) was determined in the study of Takagi et al. (9). Their results indicated that in some of the infertile patients, accelerated apoptosis rather than proliferation dysfunction of spermatogonia may participate in the pathogenesis of idiopathic hypospermatogenesis (9).

Accordingly, it might be assumed that in the biopsies of group B, who demonstrated normal functioning of spermatogonia, complete maturation arrest, and meiotic impairment, a process of accelerated apoptosis of cells of the germ line may partly cause impaired spermatogenesis in addition to the spermatogenic failure that had occurred as a result of meiotic impairment.

The same specimens analyzed in the current study were previously assessed by immunohistochemical analysis for evaluating the maturation status of the somatic component of the seminiferous epithelium, the supportive Sertoli cells (25). That evaluation was performed using the markers for immature Sertoli cell state, cytokeratin 18, and anti-mu (25). That evaluation was performed using the markers for evaluating the maturation status of the somatic component of previously assessed by immunohistochemical analysis for impairment.

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In contrast, Sertoli cells in the group with complete maturation arrest (group B) revealed a mature phenotype (25). In this group, which is characterized by meiotic impairment and normal proliferating functioning of spermatogonia, the spermatogenic defect was intrinsic to the germ line meiotic prophase, without affecting Sertoli cell’s differentiation (25).

The results of the current study taken together with the findings of the earlier study (25) point to various etiologies underlying the failure of spermatogenesis. An abnormality of spermatogonial functioning was shown to disrupt the normal progression of spermatogenesis. When the primary defect was intrinsic to the spermatocytes’ meiotic prophase, however, there was no effect on spermatogonial proliferation.

References
24. Silber SJ, Rodriguez-Rigau L. Quantitative analysis of testicle biopsy: Pathology for their substantial contribution, and Esther Eshkol, M.A., Tel Aviv Sourasky Medical Center, for editorial assistance.

