The proliferation of spermatogonia in normal and pathological human seminiferous epithelium: an immunohistochemical study using monoclonal antibodies against Ki-67 protein and proliferating cell nuclear antigen

Klaus Steger¹, Ines Aleithe¹, Hermann Behre² and Martin Bergmann¹,³

¹Institute of Anatomy and Cell Biology, University of Halle, Große Steinstraße 52, D-06097 Halle (Saale) and ²Institute of Reproductive Medicine, University of Münster, Germany
³To whom correspondence should be addressed

The quantitative distribution pattern of Ki-67 protein and proliferating cell nuclear antigen (PCNA) immunoreactivity was studied in human testis biopsies. In normal seminiferous epithelium Ki-67 is expressed in nuclei of spermatogonia, while PCNA additionally occurs in nuclei of primary spermatocytes. The staining pattern of spermatogonia is as follows (Ki-67-positive/PCNA-positive): 26.6 ± 12.4%/46.3 ± 9.5%. No stage-dependent differences were found. Biopsies with mixed atrophy (score = 7) showed a significant (P < 0.05) decrease of immunopositive spermatogonia to 19.9 ± 3.0%/31.4 ± 5.7% (score 1) with minimal variation between different samples (score 7 to 1). Associated with defined histological defects such as hypospermatogenesis (hyp), spermatogenic arrest at the level of spermatids (sda), spermatocytes (sca) or spermatogonia (sga), however, there was a significant (P < 0.05) decrease of Ki-67 staining in tubules showing hyp (28.6 ± 8.8%), sda (25.6 ± 9.3%), sca (23.7 ± 9.3%) and sga (16.2 ± 6.0%) and of PCNA staining in sca (32.2 ± 11.8%) and sga (20.0 ± 9.5%), respectively. The decrease of immunoreactive spermatogonia did not correspond to elevation of follicle stimulating hormone (FSH). These data demonstrate that the low spermatogenic efficiency in infertile men is not only due to postmeiotic events, but also to a decrease in the meiotic activity of spermatogonia, and is not related to serum FSH.

Key words: human testis/immunohistochemistry/Ki-67 protein/PCNA/spermatogonial proliferation

Introduction

During normal spermatogenesis controlled cell proliferation is of fundamental importance, assuming highly coordinated mechanisms between the mitotically inactive Sertoli cells and the germ cells undergoing mitosis and meiosis. Efficiency of spermatogenesis depends on (i) the proliferative activity of spermatogonia and (ii) the loss of germ cells during meiosis and spermiogenesis. In the human, spermatogenic efficiency is low compared to other species and mainly due to the loss of spermatocytes (Johnson et al., 1992). In the human and primate testis, the population of type A spermatogonia can be differentiated into type A_pale and type A_dark depending on nuclear morphology (Clermont, 1963; Fouquet and Dadoune, 1986). It is still a matter of debate how these cells are involved in proliferative activity. On the basis of [³H]thymidine labelling in humans (Chowdhury and Steinberger, 1977) and in non-human primates (Clermont and Antar, 1973), A_dark spermatogonia were regarded as non-proliferating cells (for review see Meistrich and van Beek, 1993). For the primates this was later confirmed by Schlatt and Weinbauer (1994) immunohistochemically using antibodies against the proliferating cell nuclear antigen (PCNA). In the human, however, Paniagua et al. (1987) demonstrated that, in the normal seminiferous epithelium, A_pale and A_dark spermatogonia were able to replicate their DNA.

In testicular biopsies from oligozoospermic men, various patterns of spermatogenic impairment are found in adjacent tubules, a phenomenon called mixed atrophy (Sigg, 1979). Because spermatogonia are the stem cells of spermatogenesis, this cell type is of special interest when studying spermatogonial impairment. Chaturvedi and Johnson (1993) suggested an involvement of differences of early spermatogonial divisions in men with low and high daily sperm production, and recent data from Werner et al. (1997) demonstrated that spermatogenic defects are associated with a decreasing cytoplasmic heat shock protein (hsp)60 immunoreactivity in spermatogonia. Because of the fact that hsp60 is necessary for normally functioning mitochondria, one can also assume negative effects on the mitotic proliferation of spermatogonia.

Using monoclonal antibodies against the two proliferation markers PCNA and Ki-67 protein, we studied their quantitative distribution pattern in the seminiferous epithelium of men with normal spermatogenesis and oligozoospermic men in order to investigate the mitotic activity of the different types of spermatogonia and the possible involvement in the low spermatogenic efficiency in testes of infertile men.

Materials and methods

Testicular tissue used in this study was obtained from men attending the Infertility Clinic of the Institute of Reproductive Medicine in Münster. Patients were subjected to a thorough clinical investigation, including at least two semen analyses according to World Health Organization (1992) guidelines and hormone analyses. Serum follicle stimulating hormone (FSH) levels were measured by fluoroimmuno-
Biopsies were performed in azoospermic patients in order to distinguish between obstructive and non-obstructive azoospermia, and in patients with oligozoospermia, when neither medical history, clinical investigation, semen analysis or hormone levels had provided an explanation for their infertility.

The testicular biopsy specimens, each about the size of a grain of rice, were routinely cut into halves and fixed by immersion in either 5.5% glutaraldehyde and embedded in Epon or in Bouin’s fixative and embedded in paraffin wax using standard techniques. For histological evaluation semithin sections (1 µm) were stained with Toluidine Blue and paraffin sections (5 µm) were stained with haematoxylin. Both were scored according to the method of Holstein and Schirren (1983), which describes the percentage of seminiferous tubules bearing elongated spermatids, i.e. score 10 means that 100% of the seminiferous tubules contain elongated spermatids.

For immunohistochemistry, paraffin sections were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma, Munich, Germany). After deparaffinization and rehydration, sections were stained with the monoclonal antibodies PC10 (Dakopatts, Hamburg, Germany) against the human proliferating cell nuclear antigen (PCNA) and MIB-1 (Dianova, Hamburg, Germany) against the human Ki-67 protein using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method (Cordell et al., 1984). In the case of the Ki-67 protein, sections were pretreated by the antigen retrieval technique using microwaves (Bremner et al., 1994).

For PCNA immunohistochemistry sections were treated with normal rabbit serum (1:5; Dakopatts) for 20 min followed by incubation with the primary antibody (1:20) for 3 h. The biotinylated secondary antibody (rabbit anti-mouse IgG, 1:25; Dakopatts, Hamburg, Germany) followed by APAAP (1:50; Dakopatts) for 30 min each (first incubation) and for 10 min each (second incubation). After each incubation sections were thoroughly washed with Tris-buffered saline (TBS; pH 7.4).

For PCNA immunohistochemistry sections were treated with normal rabbit serum (1:5; Dakopatts) for 20 min followed by incubation with the primary antibody (1:20) for 3 h. The biotinylated secondary antibody (rabbit anti-mouse, 1:400; Dakopatts) was applied for 30 min followed by incubation with alkaline phosphatase-conjugated streptavidin (1:200; Dakopatts) for 30 min. After each incubation sections were thoroughly washed with TBS (pH 7.4).

In both cases the immunoreaction was visualized by developing sections with Fast Red TR/Naphthol AS-MX (Sigma, Munich, Germany). Finally, sections were mounted in Dako Glycergel.

For each test, control incubations were performed by substituting buffer for the primary antibody. The tissue sections were completely immunonegative.

Spermatogonial proliferation is thought to be stimulated by FSH (van Alphen et al., 1988; Arslan et al., 1993). Oligozoospermic men often show elevated FSH concentrations, probably due to the occurrence of focal Sertoli cell-only syndrome (SCO) (Bergmann et al., 1994; Martin-du-Pan and Bischof, 1995). In order to evaluate a possible relationship between spermatogonial proliferative activity and FSH, we therefore analysed the percentage of labelled spermatogonia and FSH values depending on the occurrence of focal SCO. A total of 139 biopsies from 79 patients were investigated.

Results

In the normal seminiferous epithelium in biopsies from patients showing obstructive azoospermia both antibodies provided a nuclear staining of spermatogonia. In the case of the PC10 antibody, primary spermatocytes up to the pachytene stage were additionally stained (Figures 1, 2). In our material 26.6 ± 12.4%/46.3 ± 9.5% of all spermatogonia were found to be positive for Ki-67/PCNA respectively.

During normal human spermatogenesis, three different types of spermatogonia can be differentiated: A Dane, A dark and B spermatogonia. Compared to type A spermatogonia (Ki-67-positive: 22.1 ± 8.9%; PCNA-positive: 45.3 ± 9.5%) the type B spermatogonia revealed a significantly higher immunostaining for both Ki-67 (43.8 ± 15.7%) and PCNA (74.9 ± 27.6%) (Table I).

According to the different stages of spermatogenesis (Clermont, 1963), there were no significant differences between stages I–III (Ki-67: 29.9 ± 13.4%; PCNA: 45.2 ± 10.1%) and IV–VI (Ki-67: 23.5 ± 10.4%; PCNA: 47.5 ± 8.9%) (Table II).

In testes of oligozoospermic men showing mixed atrophy and a score of <8, there was a significant (P < 0.05) reduction of the percentage of labelled spermatogonia to 19.9 ± 3.0% (Ki-67) and 31.4 ± 5.7% (PCNA) at score 1 (Table III). However, significant differences between score 7 and score 1 could not be found. The score value principally represents the percentage of tubules showing elongated spermatids, but disregards the distribution pattern of defined spermatogenic defects. When summarizing the data according to defined defects such as hypospermatogenesis (only qualitatively intact spermatogenesis) or spermatogenic arrest at the level of early round spermatids, spermatocytes or spermatogonia, there was a significant (P < 0.05) reduction of PCNA-labelled spermatogonia in tubules showing spermatocytic (32.2 ± 11.8%) or spermatogonial (20.0 ± 9.5%) arrest compared to normal biopsies. For the MIB-1 antibody this significant decrease (sca: 23.7 ± 9.3%; sga: 16.2 ± 6.0%) applied in addition to spermatogenic arrest at the level of spermatids (25.6 ± 9.3%) and hypospermatogenesis (28.6 ± 8.8%) (Table IV).

FSH values of oligozoospermic patients revealed a significant increase up to 28.3 IU/l in patients showing unilateral and bilateral focal SCO. The proliferative activity of spermatogonia was significantly (P < 0.05) reduced as was found after score dependent evaluation regarding both Ki-67 and PCNA values, but was not related to FSH elevation (Table V).

Discussion

Mitotic activity of cells can be sufficiently evaluated by different methods, i.e. the counting of mitotic figures, application of labelled nucleotides such as [3H]thymidine or bromodeoxyuridine (BrdU) or by detecting specific S-phase related proteins such as PCNA (Miyachi et al., 1978) or Ki-67 antigen (Gerdies et al., 1984). Using human material, only the latter approach is possible because of ethical reasons.

PCNA is an auxiliary protein to DNA polymerase-δ, being involved in nucleotide excision repair mechanisms (Miyachi et al., 1978; Hall et al., 1993). With a biological half-life of

Statistics

Data were analysed using Student’s t-test. P < 0.05 was regarded as significant.
The proliferation of spermatogonia in the human testis

Figures 1 and 2. Immunostaining of the nuclei of spermatogonia (arrowheads) with the MIB-1 (Figure 1) and the PC10 (Figure 2) antibody. In addition, proliferating cell nuclear antigen is present in the nuclei of primary spermatocytes (arrows) up to the pachytene stage of meiosis (Figure 2). Bar = 1 µm.

Table I. Percentage of type A and type B spermatogonia immunopositive for MIB-1 and PC10 in normal human seminiferous tubules. *n = number of patients

<table>
<thead>
<tr>
<th>Type of spermatogonia</th>
<th>Spermatogonia immunopositive for the anti-Ki-67 antibody (MIB-1)</th>
<th>anti-PCNA antibody (PC10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>22.1</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>43.8</td>
</tr>
</tbody>
</table>

Table II. Percentage of MIB-1 and PC10 immunopositive spermatogonia in normal human seminiferous tubules of different stages of the seminiferous epithelial cycle. *n = number of patients

<table>
<thead>
<tr>
<th>Stages of the seminiferous epithelium</th>
<th>Spermatogonia immunopositive for the anti-Ki-67 antibody (MIB-1)</th>
<th>anti-PCNA antibody (PC10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>I–III</td>
<td>4</td>
<td>29.9</td>
</tr>
<tr>
<td>IV–VI</td>
<td>4</td>
<td>23.5</td>
</tr>
</tbody>
</table>

Table III. Percentage of MIB-1 and PC10 immunopositive spermatogonia in human testicular biopsies of different scores. *n = number of biopsies

<table>
<thead>
<tr>
<th>Score</th>
<th>Spermatogonia immunopositive for the anti-Ki-67 antibody (MIB-1)</th>
<th>anti-PCNA antibody (PC10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>10, 9 (Normal)</td>
<td>15</td>
<td>30.2</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>28.6</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>20.9*</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>22.6</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>26.0</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>25.3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>23.5*</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>23.3*</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>19.9*</td>
</tr>
</tbody>
</table>

*Indicates values which are significantly different ($P < 0.05$) from normal values (score 9, 10).
Table IV. Percentage of MIB-1 and PC10 immunopositive spermatogonia in human seminiferous tubules with hypospermatogenesis (hyp) and spermatogenic defects such as arrest of spermatogenesis at the level of round spermatids (sda), spermatocytes (sca) and spermatogonia (sga). 

<table>
<thead>
<tr>
<th>Spermatogenic defect</th>
<th>Spermatogonia immunopositive for the anti-Ki-67 antibody (MIB-1)</th>
<th>anti-PCNA antibody (PC10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean (%)</td>
</tr>
<tr>
<td>Normal</td>
<td>32</td>
<td>34.6</td>
</tr>
<tr>
<td>hyp</td>
<td>131</td>
<td>28.6*</td>
</tr>
<tr>
<td>sda</td>
<td>56</td>
<td>25.6*</td>
</tr>
<tr>
<td>sca</td>
<td>135</td>
<td>23.7*</td>
</tr>
<tr>
<td>sga</td>
<td>34</td>
<td>16.2*</td>
</tr>
</tbody>
</table>

*Indicates values which are significantly different ($P < 0.05$) from normal values.

Table V. Percentage of MIB-1 and PC10 immunopositive spermatogonia depending on the serum follicle stimulating hormone (FSH) from men with obstructive azoospermia and oligozoospermic men without Sertoli cell-only syndrome (SCO), with unilateral SCO and with bilateral SCO.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Without SCO</th>
<th>Unilateral SCO</th>
<th>Bilateral SCO</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (IU/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>20</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Mean (IU/l)</td>
<td>3.7</td>
<td>5.1</td>
<td>8.7*</td>
<td>16.1*</td>
</tr>
<tr>
<td>SD (IU/l)</td>
<td>1.9</td>
<td>3.8</td>
<td>5.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Ki-67</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Mean (%)</td>
<td>35.5</td>
<td>23.4*</td>
<td>24.3*</td>
<td>26.1*</td>
</tr>
<tr>
<td>SD (%)</td>
<td>2.1</td>
<td>5.1</td>
<td>4.8</td>
<td>3.9</td>
</tr>
<tr>
<td>PCNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>16</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Mean (%)</td>
<td>44.5</td>
<td>36.9*</td>
<td>36.2*</td>
<td>33.6*</td>
</tr>
<tr>
<td>SD (%)</td>
<td>5.0</td>
<td>9.4</td>
<td>6.6</td>
<td>10.5</td>
</tr>
</tbody>
</table>

*Indicates values which are significantly different ($P < 0.05$) from normal values.

The proliferation-associated antigen Ki-67 is expressed in the nuclear matrix of cells during all phases of the cell cycle with a maximum in S- and G2-phases (Hall et al., 1990; Casasco et al., 1993). The proliferation-associated antigen Ki-67 is expressed in the nuclear matrix of cells during late G1-, S-, G2- and M-phases of the cell cycle with a maximum in G2- and early M-phases (Gerdes et al., 1984; Sasaki et al., 1987). Its biological half-life is ~1 h (Duchrow et al., 1994). The Ki-67 protein is absent in resting cells (G0-phase of the cell cycle) and in cells during early G1-phase, nor is it detectable during DNA repair processes (Hall et al., 1993; Kubbhat et al., 1994). Its biological half-life is ~1 h (Duchrow et al., 1994). The Ki-67 protein is absent in resting cells (G0-phase of the cell cycle) and in cells during early G1-phase, nor is it detectable during DNA repair processes (Hall et al., 1993; Kubbhat et al., 1994). Becker et al. (1992) developed and characterized the monoclonal antibody MIB-1, which after microwave treatment of the tissue sections recognizes the Ki-67 antigen even in formalin-fixed and paraffin-embedded tissues (Cattoretti et al., 1992). The specific characteristics of both proteins are summarized in Table VI.

Although the immunohistochemical detectability of Ki-67 protein and PCNA is highly influenced by the fixation procedure (Bruno et al., 1992; Kujat et al., 1996), it has been demonstrated that these techniques yield labelling indices comparable to nucleotide incorporation (Böswald et al., 1990), giving results which are generally in accordance with the proliferative pattern obtained by counting of mitotic figures (Mieting, 1993). However, the more widely spreading the Ki-67 protein and PCNA within the cell cycle, the greater will be the increase in the proliferative pattern obtained by MIB-1 and PC10 immunohistochemistry compared to nucleotide incorporation calculations of S-phase cells. In the mouse an average of ~25% of the spermatogonia show radiolabelling with [3H]thymidine (Oakberg, 1970). In the normal human seminiferous epithelium, Lamont et al. (1981) found an average of ~10% of the spermatogonia at the metaphase of mitosis by counting mitotic figures. Our material revealed 26.6 ± 12.4%/46.3 ± 9.5% positive spermatogonia for Ki-67/PCNA respectively.

The higher percentage of PCNA staining as well as the labelling of primary spermatocytes corresponds with results from bull (Wrobel et al., 1996), rodent and primate testes (Schlatt and Weinbauer 1994), but it cannot be explained simply by the long half-life of the PCNA. It is probably due to the involvement of PCNA in nucleotide excision repair mechanisms (Hall et al., 1993; Kubbhat et al., 1994). With the MIB-1 antibody there is no immunostaining of post-spermatogonial germ cells in the human testis. This is in accordance with the fact that the amount of the Ki-67 protein is at its lowest level immediately after mitosis (Du Manoir et al., 1991) and that cell nuclei are generally MIB-1 immunonegative during early G1- and G0-phase of the cell cycle (Gerdes et al., 1984).

Thus, the change of spermatogonial immunoreactive pattern obtained with both antibodies reflects the differences associated with...
with spermatogenic impairment, but these data have to be considered in respect of the specific methodological conditions.

Comparing the different types of spermatogonia occurring during normal human spermatogenesis, a significantly higher percentage of the B spermatogonia were immunoreactive for both Ki-67 and PCNA. In men the cycle of the seminiferous tubules can be divided into six stages (Clermont, 1963). Both the Ki-67 protein and the PCNA immunoreactive spermatogonia reveal no significant stage-dependent differences. As shown by Chaturvedi and Johnson (1993) and Johnson et al. (1996) the architectural make-up of stages within seminiferous tubules and the presence of atypical cell types within stages depend on the efficiency of spermatogenesis. These variations cause difficulties concerning the exact classification of the stages and stage specific quantification.

Testis biopsies with score 7 to score 1 obtained from oligozoospermic men reveal a significant decrease of immunopositive spermatogonia compared to normal control testes. However, there is no clear score-related decrease of spermatogonial labelling. This was also found by Lamont et al. (1981) who counted metaphase spermatogonia in testes with normal spermatogenesis and in subfertile testes with mixed atrophy using the method of Johnsen (1970). In both cases immunopositive spermatogonia were counted in whole biopsies comprising a variety of spermatogenic defects in adjacent seminiferous tubules. Considering the phenomenon of mixed atrophy we also looked at single seminiferous tubules instead of whole biopsies comprising immunopositive spermatogonia in tubules with the same spermatogenic defect; there was a decrease of the spermatogonial proliferation dependent on the defined spermatogenic defect including an arrest at the levels of spermatogonia, primary spermatocytes, round spermatids or even spermatogonial proliferation only occurs in contact with Sertoli cells assuming that mediator molecules stimulate the proliferative activity of spermatogonia (Maekawa et al., 1995). Using fetal and prepubertal markers such as nuclear structure (Bruning et al., 1993), intermediate filament expression (Bergmann and Kliesch, 1994), or the persistence of anti-Muellerian hormone protein (Steget et al., 1996), it was recently shown that spermatogenic arrest and hypospermatogenesis are also associated with a population of Sertoli cells lacking full differentiation.

Therefore, we conclude that the observed high levels of FSH cannot trigger spermatogonial proliferation because of the functionally impaired population of Sertoli cells in these seminiferous tubules.

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