Features of impaired seminiferous tubule differentiation are associated with germ cell neoplasia in adult men surgically treated in childhood because of cryptorchidism

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Abstract: Seminiferous tubule differentiation was related to the occurrence of germ cell neoplasia in 39 men, aged 17-47, treated surgically in childhood for cryptorchidism. Tissues from 47 testes obtained from biopsies taken as a neoplastic preventive procedure or whole testes removed because of GCT were evaluated quantitatively. Paraffin sections were treated with antibodies against placental like alkaline phosphatase (PLAP), a marker of germ cell neoplasia, and cytokeratin 18 (CK-18), a marker of immature Sertoli cells. Quality of spermatogenesis and number Leydig cells were assessed with a score count. Seminiferous tubules diameter, thickness of basal membrane and size of intertubular spaces were measured with image analysis software. In 17.0% of testes spermatogenesis was normal (9.9±0.01 points) (N) and neoplasia was not found there. In the other 39 specimens (83,0%) spermatogenesis was abnormal (A). When spermatogenesis was arrested or when germ cells were absent (3.7±1.8 points), neoplastic lesions were found in 12.9% of the specimens. In A group 5.1±7.1% of tubules contained immature Sertoli cells, while in N they were not found. Tubular diameter was significantly lower in A (161.5±31.8 μm) than in N (184.6±24.3 μm) and the percentage of seminiferous tubules with the thickening of tubular basal membrane was also greater in A. Intertubular spaces were significantly larger in A (49.9±18.6%) in comparison to N group (32.6±12.5%). Mean number of Leydig cells was similar in both groups. To conclude, in most of the formerly cryptorchid testes, despite surgical treatment, impaired seminiferous tubules differentiation is predominant. Germ cell neoplasia is present in testes with retarded seminiferous tubules differentiation. Retardation of seminiferous tubule differentiation consists of inhibited spermatogenesis, presence of tubules with immature Sertoli cells, decreased tubular diameter, increased thickness of basal membrane and enlarged intertubular spaces. Examination of testicular biopsy with respect to the state of seminiferous tubule differentiation may be helpful to predict the appearance of germ cell neoplasia in adult men with cryptorchidism in anamnesis. Orchiopexy of cryptorchid testes may not prevent the occurrence of features of testicular dysgenesis and the associated germ cell neoplasia.

Key words: Testicular biopsy - Germ cell cancer - Carcinoma in situ - Spermatogenesis - Sertoli cell maturation

Introduction

Cryptorchidism refers to the absence of one or both testes from the scrotum. Testis doesn't move from an abdominal position through the inguinal canal into the ipsilateral scrotum during fetal development. The cause of cryptorchidism is unknown and the aetiology is possibly multifactorial [1].

Cryptorchidism is associated with increased risk of germ cell tumours (GCT). Dieckmann and Pichlmeier [2] in a meta-analysis evaluated the relative risk of GCT in subjects with a history of cryptorchidism as 4.8 (95% CI = 4.0-5.7). Cryptorchidism is also associated with impaired spermatogenesis. Lee *et al.* [3] revealed in their case controlled study that among a group of men treated in childhood because of bilateral cryptorchidism, infertility is about 3.5 times more frequent than in a group of men with unilateral cryptorchidism and 6 times more frequent in comparison to the control group (men without cryptorchidism). They did not find any correlation between age of orchiopexy

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and paternity for either group. Kuzanski *et al.* [4] noticed that bilateral cryptorchidism and primary abdominal testicle position appeared to have deteriorating influence on sperm quality. These authors stress that the coexistence of both these factors in one patient caused amplification of this effect.

There are suggestions that the abnormal position of the testis and about 2°C higher temperature in the inguinal canal or abdominal cavity than in the scrotum may be the cause of germ cell degeneration, especially between the 6th month and 2nd year of life [5,6]. Other hypotheses emphasize the role of testicular maldevelopment. The apparent increase in the prevalence of cryptorchidism in some geographic regions suggest that genetic and environmental factors can be involved [for review: 7].

The aim of our study was to relate seminiferous tubule differentiation to the occurrence of GCT and its preinvasive form carcinoma in situ (CIS) in adult men treated surgically in childhood for cryptorchidism.

Materials and methods

Patients. Thirty nine men, aged 17-47 (mean 31.4 years), were included in this study. All had cryptorchidism in their medical history. In 27 patients unilateral and in 12 bilateral cryptorchidism was diagnosed. All were submitted to orchidopexy in the prepubertal period of life (age 1-12 years, mean 6.6) in different centers of pediatric surgery in Poland.

In 4 patients the whole testes were removed because of the overt GCT. Testicular biopsies were taken in 35 men from an undescended testis as a neoplastic preventive procedure. In 8 men both testes were biopsied and in 27 men biopsies were performed unilaterally.

Histology and immunohistochemistry. Tissues obtained from 47 testes were fixed in Bouin's solution and embedded in paraffin. Each biopsy or the whole testis were sectioned serially in their entirety. Histological slides of each testis were stained with hematoxylin and eosin and examined histologically.

Immunohistochemistry was performed using monoclonal antibodies against placental like alkaline phosphatase (PLAP, Novocastra Laboratories, United Kingdom), a marker of GCT and CIS cells, and cytokeratin 18 (CK-18, Novocastra Laboratories, United Kingdom), a marker of immature Sertoli cells.

From each gonad 5 histological sections were treated with antibody against PLAP and 5 histological sections were treated with antibody against cytokeratin 18. Both antibodies were diluted 1:25 in 0.05 M Tris-buffered saline, pH=7.4. As the immunohistochemical visualisation system was used EnVision System-AP (DakoCytomation, Denmark). 3,3'-diaminobenzidine was used as a chromogen, giving a yellow-brownish staining of cytoplasm in the PLAP or CK-18 positive cells. Paraffin-embedded sections of testicular seminoma resected from an adult patient served as a positive control for PLAP. Histological sections of prepubertal testis served as a positive control for CK-18. For the negative control, the primary antibodies were replaced with 0.05 M Tris-buffered saline.

Assessment of spermatogenesis. Histological sections, 5 μm thick, were stained with hematoxylin and eosin 50-100 cross-sections of seminiferous tubules were evaluated in each biopsy. Spermatogenesis was assessed with a scale from (-1) to (+10) points.

Score count was based upon De Kretser's and Holstein's way [8] modified by us and is explained in the Table 1. Different lesions in the same histological section were evaluated separately and a mean number of points was assigned to the biopsy specimen.

Leydig cells assessment. Number of Leydig cells in the triangular intertubular spaces was assessed by scoring: total lack of Leydig cells - 0; 1-5 Leydig cells - 1 point; 6-10 Leydig cells - 2 points; 11-30 Leydig cells - 3 points; >30 Leydig cells (nodules of Leydig cells) - 4 points. In each biopsy 100 triangular intertubular spaces were evaluated.

Morphometry. The morphometric analyses were performed using image analysis software LxAND v3.60HM. Seminiferous tubule development was evaluated on the basis of tubular diameter and the areal fraction of intertubular space. Areal fraction of intertubular space reports the percentage participation of the intertubular compartment to the area of the histological section of the testicular biopsy [9]. The microscopic picture at 100× magnification was covered by a square lattice containing 441 intersections. The number of intersections falling on the intertubular spaces was counted by a systematic movement across the grid over the entire tissue section. The areal fraction was calculated by dividing the number of intersections, which hit the intertubular spaces, by the number of points hitting the whole vision area at the same magnification and multiplied by 100%. Thickness of tubular membrane was also measured as a parameter of tubular atrophy.

All the measurements were performed in 10 randomly selected histological sections of one gonad in each patient and showed as a mean ±standard deviation (SD), median and range of values.

Statistics. Nonparametric analysis (Mann-Whitney U test) was applied for comparison between groups after verification that values were not normally distributed. A "p" value <0.05 was considered as significant. All statistical analyses were performed using Statistica 7.1 software (Statsoft, Poland).

Results

Table 2 presents results of spermatogenesis and Leydig cells score counts, as well as morphometric measurements. In 8 testes out of 47 (17.0%) spermatogenesis was complete and intact or complete and slightly reduced (hypospermatogenesis) (group N). Mean number of points received in this group was 9.9±0.01.

In other 39 testes (83,0%) spermatogenesis was abnormal: arrested at different levels or germ cells were absent (Sertoli-cell-only syndrome) or seminiferous tubules had features of atrophy (group A). Spermatogenesis assessed by quality received significantly lower number of points (3.7±1.8) in comparison to N group. In group A germ cell neoplasia was found in 5 testes out of 39 (12.9%). Among them in 4 testes (10.3%) overt GCT (seminoma, embryonal carcinoma) were present and in 1 testis (2.6%) CIS was found (Table 3, Fig. 1A). Germ cell neoplasia was not diagnosed in the testes from N group. In A group 5.1±7.1% of tubules contained immature Sertoli cells, revealing positive reaction with antibodies against CK-18, while in N group such tubules were not found (Fig. 1B).

Tubular diameter was significantly lower in A (161.5±31.8 μm) than in N group (184.6±24.3 μm).

Table 1. Assessment of the seminiferous epithelium status. Score count is based upon the way by De Kretser and Holstein [7], modified.

Score	Histological criteria	Diagnosis
+10	>20 mature spermatids/tubule Germinal epithelium height ≥80 μm Spermiation common	Complete, intact spermatogenesis
+9	>20 mature spermatids/tubule Germinal epithelium height <80 μm Spermiation rare	Complete, reduced spermatogenesis (hypospermatogenesis)
+8	<20 mature spermatids/tubule Germinal epithelium height <80 μm Spermiation absent	Complete, reduced spermatogenesis (hypospermatogenesis)
+7	No mature spermatids Numerous round immature spermatids	Disturbed differentiation of spermatids Spermatogenesis arrested at round spermatids level
+6	No mature spermatids Few round immature spermatids	Disturbed differentiation of spermatids Spermatogenesis arrested at round spermatids level
+5	No spermatids Numerous primary spermatocytes	Disturbed maturation of primary spermatocytes Spermatogenesis arrested at primary spermatocytes level
+4	No spermatids Few primary spermatocytes	Disturbed maturation of primary spermatocytes Spermatogenesis arrested at primary spermatocytes level
+3	No spermatids No primary spermatocytes Only spermatogonia	Disturbed differentiation of spermatogonia Spermatogenesis arrested at spermatogonia level
+2	No germ cells Only Sertoli cells	Sertoli-cell-only syndrome
+1	Degenerating Sertoli cells No germinal epithelium	Tubular atrophy
0	Testicular carcinoma in situ	Preinvasive neoplastic lesion
-1	Overt germ cell tumour	Invasive neoplastic lesion

Table 2. Spermatogenesis score count, morphometric parameters of seminiferous tubules and Leydig cells score count in testes with complete and abnormal spermatogenesis in 38 adult men with cryptorchidism in anamnesis. n - number of testes

	Complete spermatogenesis n = 8				Abnormal spermatogenesis n = 39			
	Mean	SD	Median	Range	Mean	SD	Median	Range
Spermatogenesis advance (points)	9.9	±0.01	10.0	9.0-10.0	3.7***	±1.8	3.0	1.0-6.5
Seminiferous tubules diameter (μm)	184.6	±24.3	182.0	157.9-220.7	161.5*	±31.8	170.0	83.1-228.1
Thickness of tubular basal membrane (μm)	9.3	±2.2	8.7	6.1-13.2	10.3	±3.7	10.3	4.6-23.2
Number of Leydig cells (points)	1.6	±1.2	1.0	1.0-4.0	1.7	±1.1	1.0	0.0-4.0
Areal fraction of intertubular spaces (%)	32.6	±12.5	28.5	20.8-59.0	49.9*	±18.6	47.1	17.2-83.0

* $p<0.05$; *** $p<0.001$, Mann-Whitney U test, abnormal vs. complete spermatogenesis

Mean thickness of tubular basal membrane was not significantly different in A versus N group. However, percentage of tubules with membrane thickness >10 μm was greater in A than in N group (47.4% vs. 25%). Mean areal fraction of intertubular space was significantly larger in A than in N group (32.6±12.5% vs. 49.9±18.6%, respectively, $p<0.05$). Mean number of Leydig cells did not differ significantly in both groups.

Discussion

Our results indicate that the retardation of seminiferous tubule differentiation exists in 83% of histological specimens of testes obtained from adult men operated in childhood because of cryptorchidism. The retardation consists of inhibited spermatogenesis (from the total lack of germ cells to the inhibition of spermatoge-

Table 3. Number of testes with overt germ cell tumors (GCT) or testicular carcinoma in situ (CIS) and mean ±SD percentage of seminiferous tubules with immature Sertoli cells in testes with complete and abnormal spermatogenesis in 38 adult men with cryptorchidism in anamnesis.

Groups	GCT no. of cases (%)	CIS no. of cases (%)	Seminiferous tubules with immature Sertoli cells Mean±SD (%) Median Range
Complete spermatogenesis n = 8	0	0	0
Abnormal spermatogenesis n = 38	4 (10.5)	1 (2.6)	5.1±7.1*** 3.4 0.0-32.2

*** p<0.001, Mann-Whitney U test, abnormal vs. complete spermatogenesis; n - number of testes

nesis at different levels), the presence of tubules with immature Sertoli cells, the decreased diameter of seminiferous tubules, the increased thickness of basal membrane and enlarged intertubular spaces. Moreover, developmental retardation of seminiferous tubules was associated with high incidence (12.9%) of germ cell neoplasia. Neoplastic lesions were found in testes with abnormal spermatogenesis, but were absent, when complete spermatogenesis was diagnosed. More dramatic findings were described by us in dysgenetic gonads of intersex children, where germ cell neoplasia was found in almost 65% of cases [10].

Skakkebaek *et al.* [10] suggested that male reproductive problems may be one entity with the same etiology. They proposed the existence of a new clinical syndrome - a testicular dysgenesis syndrome (TDS), which comprises the wide range of developmental retardations of the testes, including disturbed organogenesis and cryptorchidism. The authors proposed that male infertility (oligo- and azoospermia) and testicular GCT resulted also from TDS. Our data may support these suggestions. The important finding of our study is that the presence of immature Sertoli cells in cryptorchid testes of adult men was evidenced by specific immunostaining against CK-18 which was not reported before.

All the disorders included in TDS are the risk factors for GCT [for review: 12]. Dieckmann and Pichlmeier [2] performed epidemiological analysis of GCT and established the suspected risk factors. They attributed levels of evidence to each of the putative risk factors in analogy to the methods of evidence based medicine and adapted to clinical epidemiology. The authors established that cryptorchidism is GCT risk factor with the highest level of evidence. In our previous study we reported that CIS was detected in 33% of patients with undescended testes, but the num-
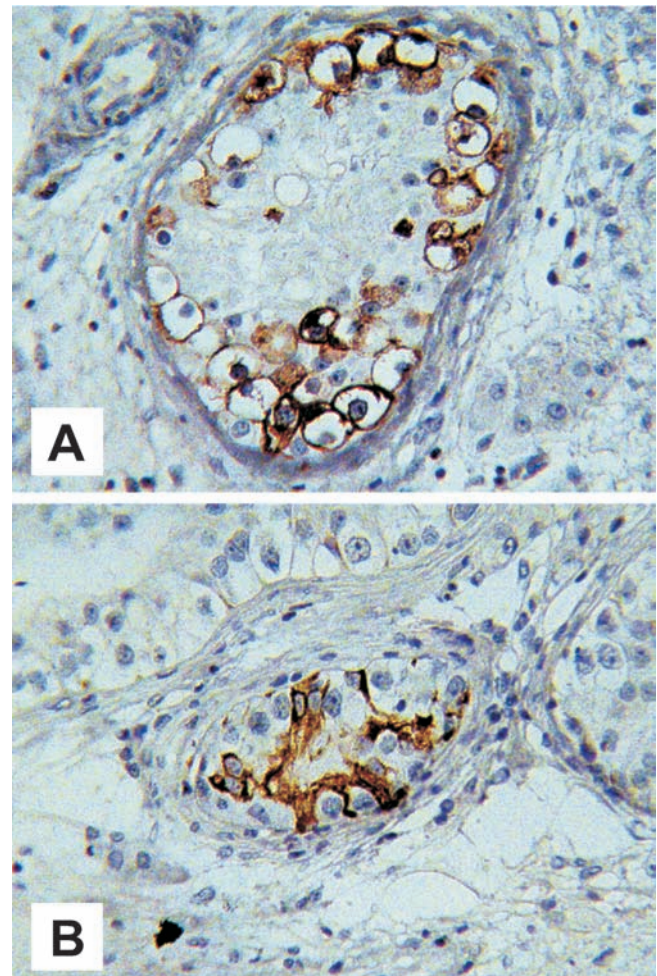
Fig. 1. Positive immunohistochemical reactions for: A) placental like alkaline phosphatase (PLAP), a marker of germ cell neoplasia, in a seminiferous tubule with the *carcinoma in situ* in a cryptorchid testis of a 36-year old man submitted to orchiopexy in childhood; B) cytokeratin 18 (CK-18), a marker of immature Sertoli cells, in a formerly cryptorchid testis of 24-year-old man (magnification ×200).

ber of patients was relatively low [13]. We showed also that the presence of neoplastic germ cells appeared to be independent of the structural and numerical aberrations of sex chromosomes, which were generally considered before to be a main cause of gonadal dysgenesis and development of GCT [10,14]. Those and the presented here results suggest that disturbances of testicular organogenesis may by itself predispose to the initiation of germ cells neoplastic changes. CIS cells located inside underdeveloped seminiferous tubules may have an environment favourable for their survival [15]. We have found previously that CIS cells are already neoplastic in the early childhood. Namely, in dysgenetic testes of intersex children, aged 8 months to 3 years, DNA pattern of CIS cells was predominantly aneuploid (tri- and tetraploid), characteristic for GCT [16]. The data presented here indicate that examination of testicular

biopsy with respect to the state of seminiferous tubule differentiation may predict the risk of germ cell neoplasia in men with cryptorchidism in their past medical history.

The thickening of seminiferous tubules basal membrane in cryptorchid testes in adults was described previously [17]. Pronounced fibrosis of the tubular lamina propria in cryptorchid boys was observed already in the 1st year of life [18] or the 3rd year of age [19]. There are suggestions that lesions of tubular membrane in cryptorchid testes might be related to the inhibited maturation/differentiation of Sertoli, Leydig and peritubular cells [20]. The thickening of basal membrane increases with age in dysgenetic testes [21], indicating that age by itself may have a detrimental effect. In our present study the percentage of seminiferous tubules with the thickening of tubular wall was greater in the testes bearing germ cell neoplastic lesions. This indicates that the thickness of tubular membrane, considered up to now as nonspecific testicular pathology, may be an important parameter of TDS, indicative for the appearance of germ cell neoplasia.

Cryptorchidism is associated with infertility [3]. Results from several studies indicated that only 13-62% of men with bilateral cryptorchidism (after orchiopexy) were able to father at least one child [22-24]. For many years it has been stressed that cryptorchidism if not operated, decreases dramatically the number of germ cells within the first two years of life [25-27]. The presented here data on structural lesions of testes may suggest that the disturbed function of Sertoli cells may be a primary cause that is followed by disturbed testicular descent and spermatogenic failure. It seems unlikely that impaired maturation of Sertoli cells results predominantly from the increased temperature of testis environment in childhood. Regadera *et al.* [28] have found that the absence of androgen receptor in immature Sertoli cells of cryptorchid testes correlated with hypoplasia of the seminiferous tubules and the absence of germ cell differentiation. The authors suggested that congenital focal lesions might be present at the level of somatic cells in cryptorchid testes. The presence of such lesions in seminiferous tubules may help to explain why surgical intervention to descend the testis into the scrotum, even when performed early in life, fails to restore the normal onset of spermatogenesis and fertility. Our data indicate in addition that orchiopexy of cryptorchid testes may not prevent the occurrence of TDS and the associated germ cell neoplasia.

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