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Myofibroblasts in adolescent varicocele: an ultrastructural and immunohistochemical study

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Abstract Myofibroblasts of the testes play an important role in the morphofunctional integrity of the seminiferous tubule. Previous studies in adults with varicocele have demonstrated an involvement of this cell population that tends to transform into fibroblasts. The aim of the present study was to try and verify the morphological features of myofibroblasts in the adolescent with left idiopathic varicocele. Twenty-two testicular biopsies were obtained from adolescents (aged 13–18 years, mean 15.8) and operated for left idiopathic varicocele. Biopsies were processed for electron microscopy (TEM) and immunofluorescence studies. The latter determined the level of myofibroblasts by using α -smooth muscle isoactin, a specific marker of myofibroblasts and, hence, excluding fibroblasts. TEM observations revealed a normal ultrastructure of myofibroblasts that was similar to that for the controls but an increased presence of extracellular matrix. The immunofluorescence study always demonstrated strong cell positivity to anti α -smooth muscle isoactin as also seen in the controls. This study demonstrates that adolescents with varicocele have well preserved myofibroblasts and do not show any evidence of transformation into fibroblasts, this has already been demonstrated in adult varicocele. These observations could represent an important factor for the understanding of the reversal of growth failure of the testes observed after early treatment.

Key words Adolescent varicocele · Myofibroblasts · Immunofluorescence · α -Smooth muscle isoactin

Introduction

Although varicocele is a common and well studied pathology, its management in adolescents is still controversial. Data supporting the need for early treatment in this age group are still lacking [11].

Different histologic studies have focused attention on the tubular compartment [3, 38]. Some of the changes observed have been confirmed in adolescents [15, 24]. Very little attention has been given to the extratubular compartment (lamina propria and its components).

The lamina propria of seminiferous tubules consists of three to five inner layers of myofibroblasts and one to two outer layers of fibroblasts [13]. All these cellular layers are separated by thin layers of an extracellular matrix consisting of glycosaminoglycans, proteoglycans and collagen fibers [5].

Myofibroblasts have ultrastructural features that are in between those for fibroblasts and smooth muscle cells [4, 8]. They contain actin [34], myosin microfilaments [18] and intermediate filaments [37, 39]; these last elements contain a moderate amount of vimentin [29] and abundant desmin [37]. It has also been demonstrated by Tung and Fritz [36] that myofibroblasts and smooth muscle cells represent the only cellular elements of the seminiferous tubule with immunopositivity to an actin isoform, i.e. α -smooth muscle isoactin. This protein is already expressed in the neonate [21] and represents a specific marker of myofibroblast differentiation [16, 30].

The extratubular compartment plays a key role in the maintenance of spermatogenesis. Cells of the lamina propria create an interface between tubular and interstitial cells [32]. Myofibroblasts interact particularly with Sertoli cells to maintain an epithelial polarity, and so play a pivotal role in determining the morpho-functional integrity of the seminiferous tubule [23].

In adult varicocele and in other forms of hypospermatogenesis a progressive thickening of lamina propria due to an increase in the extracellular elements has been already demonstrated [6, 12, 27]. These studies have also

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highlighted ultrastructural modifications of the myofibroblasts characterized by a progressive reduction of elements immunopositive to desmin (myofibroblasts) with an increase of those reactive to vimentin (fibroblasts) [27].

We recently demonstrated that the thickening of the lamina propria is already present in adolescent varicocele even if it is not so severe as in adult patients [26].

With the present study we wanted to investigate further the ultrastructural features of myofibroblasts in the lamina propria. An ultrastructural and immunohistochemical study of myofibroblasts in the testes from adolescents with varicocele has been carried out.

Materials and methods

Twenty-two testicular biopsies were obtained, with prior informed consent, from adolescents, aged from 13 to 18 years (mean age 15.8) and affected by idiopathic left varicocele. Five testicular biopsies from adolescents operated for hydrocele or inguinal hernia were used as controls, always after informed consent.

Patients with varicocele were diagnosed after physical examination, echocolor Doppler and phlebographic studies. A grade II or III varicocele (after Horner) was considered an indication for treatment. Surgical treatment was undertaken with a selective microsurgical ligation of ectatic intra and extrafunicular vessels, after delivery of testis, (according to Goldstein) [10] and spermatic-epigastric microsurgical derivation (according to the Belgrano technique [1]).

Biopsy specimens were processed for transmission electron microscopy (TEM) and for immunofluorescent studies with different protocols.

Transmission electron microscopy

The specimens were immediately fixed for 3 h in 4% glutaraldehyde in phosphate buffer 0.2 M pH 7.4 at 4°C. After rinsing in the same buffer, the specimens were postfixed in 1% OsO₄ in 0.2 M phosphate buffer pH 7.2–7.4. They were then dehydrated in graded alcohol and acetone and flat embedded in Durcupan (Fluka, Milan, Italy). The ultrathin sections were cut on a LKB Ultratome V ultramicrotome (Turku, Finland) stained with uranyl acetate and lead citrate and photographed using a TEM Philips CM10 (Eindhoven, The Netherlands).

Immunofluorescence

The biopsies were fixed for 4 h in 4% paraformaldehyde in 0.2 M phosphate buffer pH 7.4 at 4°C. After repeated rinsing in 0.2 M phosphate buffer and in phosphate-buffered saline (PBS), the biopsies were infused in 12% and 18% saccharose and then frozen in liquid nitrogen and sectioned using the Bright cryostat (Bright, Huntingdon, UK). The 20- μ m seriate sections (40–50 sections for each sample), were placed on gelatin-coated glass slides and pre-incubated with a solution containing 0.6 ml sheep serum, 1 ml 45 mM NaCl and 1 ml 20 mM phosphate buffer for 15 min in to avoid unspecific background staining. The sections were incubated for 15 min with a solution containing PBS, bovine serum albumin 1% and Triton X-100 0.3%. This solution was also used as dilution and rising buffer. The primary antibody employed was anti α -smooth muscle iostatin (Mouse ascites fluid, Clone 1A4; Sigma Chemicals ST. Louis, Mo.) at a dilution of 1:400. As a secondary antibody we used an Ig-biotinilate antimouse in sheep (Amersham International, Amersham, UK) at a dilution of 1:500 and as fluorochrome Texas-red streptavidin (Amersham International,

Amersham, UK) at a dilution of 1:100. Controls were performed either by omitting the primary antibody or by replacing the primary antibody with a non immune rabbit or mouse serum.

The sections were observed and photographed using a Leica TCS 4D (Heidelberg, Germany) upright confocal microscope with immersion objectives. It is equipped with an argon krypton laser (λ 568 nm, 100 mW; λ 488 nm, 100 mW and λ 647 nm, 100 mW). Collected images were digitized at a resolution of 8 bits into an array of 512 \times 512 pixels. The software controlling the microscope and the processing of the images was provided by the manufacturer. Optical sections of fluorescence specimens were obtained using a laser line 568 nm and a TRIC set of filters at 1" scanning speed with an average of up to 8.

Results

Transmission electron microscopy

In the control testes the lamina propria of the seminiferous tubules was composed of three to five inner cellular layers of myofibroblasts and one to two more outer cellular layers of fibroblasts; these cellular layers were separated by laminae of extracellular connective-tissue components. Myofibroblasts were individual flat cells, not forming continuous cellular layers. Their cytoplasm split up into two or more layers and they were not completely covered by a basal lamina (Fig. 1a). Normal organelles, myofilament bundles and electron-dense bodies were observed in the cytoplasm.

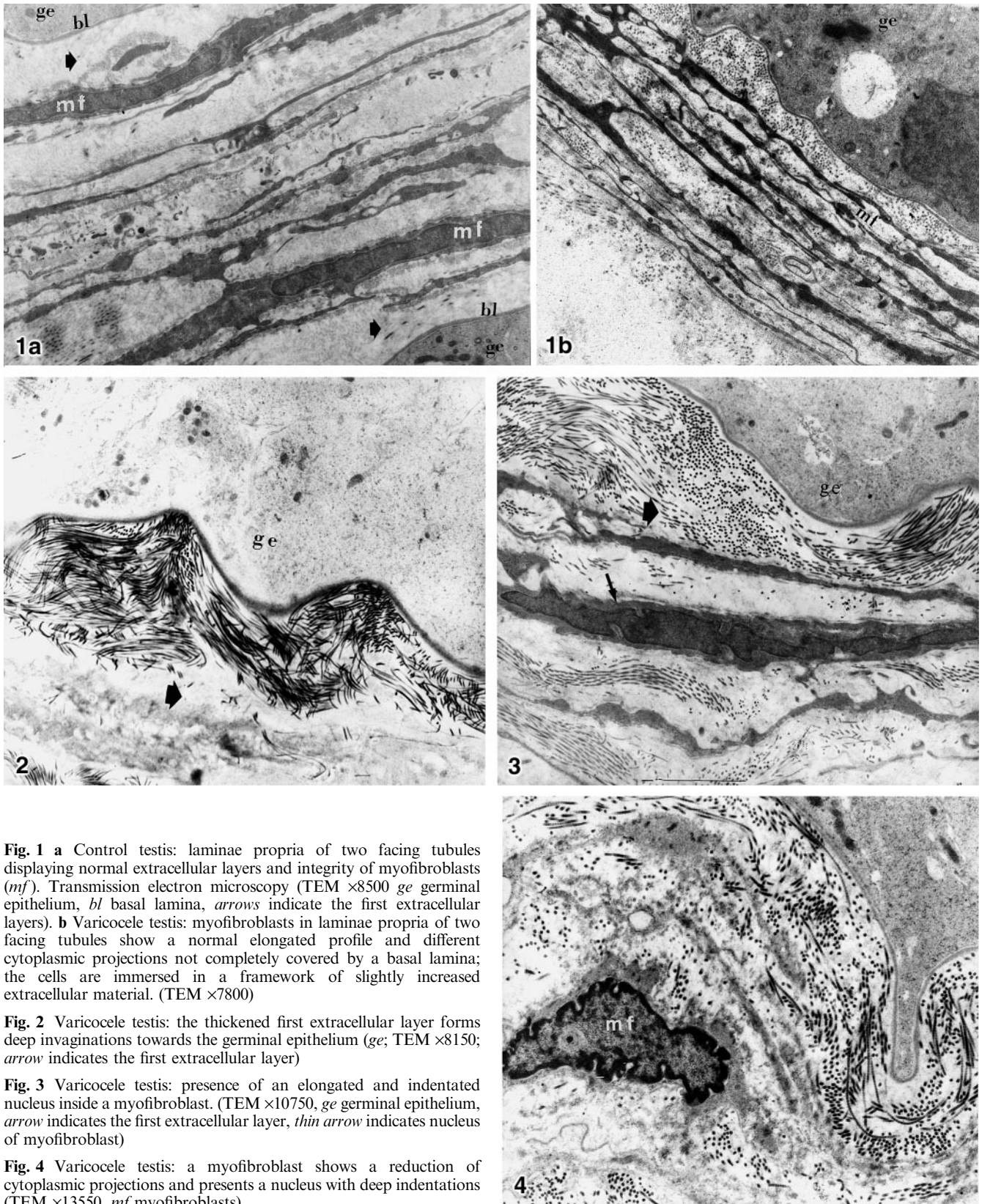
Myofibroblasts of the seminiferous tubules from adolescents with varicocele were characterized mainly by a normal ultrastructure but they were immersed in an increased extracellular matrix (Fig. 1b); this was particularly evident in the first extracellular layer where deep invaginations toward the germinal epithelium were observed (Fig. 2). In some myofibroblasts, a nucleus irregularly marked with indentations was observed (Fig. 3). In rare cases myofibroblasts with a cell membrane of an uneven profile, reduction of the cytoplasmic projections, microfilaments and electron-dense bodies and a nucleus with deep indentations were observed (Fig. 4).

Immunofluorescence

The main results are summarized in Table 1. α -smooth muscle immunoreaction, specific for myofibroblasts, did not show any major differences between the control and the pathology samples. A positive immunoreaction was always observed, with a regular distribution around the tubular lumen delineating myofibroblasts morphology (Fig. 5).

Discussion

In 1951 Roosen-Runge first showed a peristaltic activity in seminiferous tubules mediated by cells of the lamina propria [25].



In 1958 Clermont using electron microscopy, identified peritubular cells of the seminiferous tubules similar to smooth muscle cells with contraction activity that

were defined as “interlamellar cells” [4]. Subsequently these cells were known as “peritubular contractile cells” [26].

Table 1 Grade of positivity to anti- α -smooth isoactin of myofibroblasts in the lamina propria of seminiferous tubules in adolescent varicocele and control patients. (– absent, + weak, ++ moderate, +++ strong)

Myofibroblasts	+++	++	+	–
Control (<i>n</i> = 5)				
Anti- α -smooth isoactin	5	0	0	0
Varicocele (<i>n</i> = 22)				
Anti- α -smooth isoactin	18	4	0	0

In 1969 Fawcett et al. introduced the term “myoid cells” due to their cytological resemblance to smooth muscle cells in electron micrographs [9].

In 1972 Böck et al. demonstrated that these cells have also characteristics similar to fibroblasts identifying them as “myofibroblasts” [2].

The myofibroblasts of the lamina propria of the seminiferous tubules of human testis are large flat cells with thin cytoplasm projections and are arranged in non-continuous cell layers; they stain intensively with anti-desmin antibodies [37] and weakly with anti-vimentin antibodies [39]. In contrast, the fibroblasts present intense immunostaining with anti-vimentin antibodies [22] and negative immunostaining with anti-desmin antibodies [35]. Konhen et al. reported that, in human myofibroblasts, in addition to desmin and vimentin, α -smooth muscle isoactin and GB-42 immunoreactivity could be established [16].

Myofibroblasts are responsible for seminiferous tubule contraction for the transport of testicular fluid and spermatozoa. They also are capable of secreting fibronectin, collagen type I and IV and proteoglycans [31, 32]. Thus, myofibroblasts, play a key role in the morpho-functional integrity of the seminiferous tubule regulating spermatogenesis. They also contribute to the creation of

the blood testis barrier, although Sertoli cell junctions have a main role in this barrier [7, 19].

Davidoff demonstrated in adult patients with disturbed spermatogenesis a progressive modification of the lamina propria both in the extracellular and cellular components [5]. He described an increase in the thickness of the lamina propria together with a decrease of desmine-like immunoreactive cells. Similarly, other studies have demonstrated in adults with varicocele an increase in lamina propria thickness, a reduction of cell positivity to anti-desmine antibodies with an increase to cell positivity to vimentin. This suggests that in the lamina propria, myofibroblasts tend to transform into fibroblasts [27].

In the present study we used as the specific marker α -smooth muscle isoactin for the differentiation of myofibroblasts, this actin isoform is specifically expressed by vascular smooth muscle cells and by myofibroblasts in situ. Tung and Fritz confirmed that α -smooth muscle isoactin is shown to be readily detectable in myofibroblasts in situ and in vitro, but not in other types of cells within the seminiferous tubules, including fibroblasts, Sertoli cells, and all classes of germinal cells [36]. On the other hand, Holstein, on the basis of α -smooth muscle isoactin immunopositivity, considered the inner cell layers situated beneath the basal lamina of the human seminiferous tubules to be true myofibroblasts [13].

Our observations have always demonstrated a cell population in the lamina propria strongly positive to α -smooth muscle isoactin in both controls and pathology samples suggesting that myofibroblasts have not transformed into fibroblasts as already demonstrated in adult varicocele [27].

The ultrastructural study has also shown in that the morphology of myofibroblasts appears well preserved without evidence of major modifications when compared with controls. On the other hand, the observed thickening of the lamina propria with the presence of abundant collagen fibrils and deep invaginations of the basal membrane, ascribed to an increased accumulation of ECM components, confirms our previous study on the lamina propria of adolescent affected by idiopathic varicocele [28].

Our results suggest that myofibroblasts, in the testes of adolescents, still maintain their morphological features with only mild modifications. Their integrity may be an important feature of adolescent varicocele since their transformation into fibroblasts, as described in adults, corresponds to a progressive sclerosis of the lamina propria [27].

Natural history and surgical treatment of adolescent varicocele are still controversial. Previous clinical studies have considered the lesions observed in adolescent varicocele to be progressive and responsible for a growth arrest [14, 29]. In this regard, short-term prospective randomized trials in adolescents by Laven et al. and Paduch and Niedzielsky have cautiously suggested a positive effect of surgery in this age group to reverse this phenomenon [17, 20].

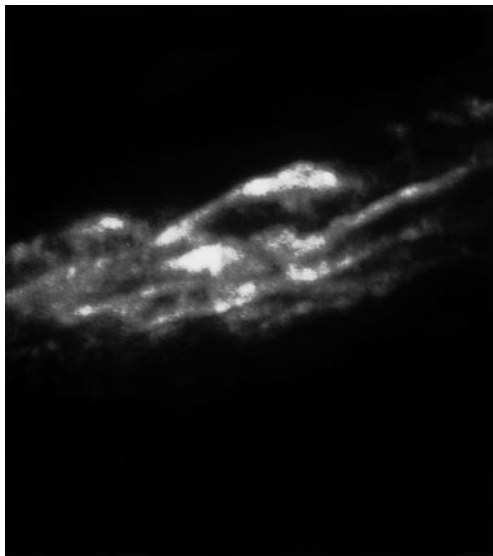


Fig. 5 Varicocele testis: confocal micrograph showing immunofluorescent α -smooth muscle isoactin along the entire cell-profile of layered myofibroblasts (x2200)

More long-term prospective studies that can confirm the above results are still necessary to better define criteria for treatment of adolescent varicocele.

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