Ultrastructural and immunohistochemical study of basal lamina of the testis in adolescent varicocele

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Objective: To evaluate a possible involvement of the extracellular matrix (ECM) in the testes of adolescents with varicocele.

Design: Cross-sectional study.

Setting: University-based medical center.

Patient(s): Twenty-four adolescents aged between 13 and 18 years underwent surgical treatment for repair of left idiopathic varicocele.

Intervention: A testis biopsy was performed at time of surgery.

Main Outcome Measure(s): Transmission electron microscopy study of basal lamina and immunofluorescence studies of collagen type IV and laminin, two major components of basal lamina.

Result(s): Transmission electron microscopy observations showed an uneven profile of the basal lamina with a variable thickness. Immunofluorescence studies showed an irregular immunofluorescent line that appeared interrupted in some observations. Collagen type IV showed some areas of strong immunostaining with other areas with reduced immunoreactivity.

Conclusion(s): Our ultrastructural and immunohistochemical observations highlight focal damage at the level of peritubular basal lamina, but this damage is not as severe as that described in adult varicocele. Initial involvement of basal lamina could represent one of the mechanisms responsible for varicocele-induced histologic alterations of the testes. (Fertil Steril 2000;73:699–705. ©2000 by American Society for Reproductive Medicine.)

Key Words: Adolescent varicocele, basal lamina, collagen type IV, laminin, physiopathology, immunohistochemistry

Varicocele is a common pathologic condition in adolescents, with an incidence varying between 9% and 25.8% (1). Different studies have described numerous changes in testicular histology (2–5), and the mechanisms that regulate these alterations have not been completely elucidated. Little attention has been given to the role played by the extracellular matrix (ECM).

The ECM creates a network of locally secreted polysaccharides and proteins that intertwine with the cell population of different tissues. At the level of the interface between the epithelium and connective tissue, the ECM is organized in a thin but strong layer known as basal lamina, which plays a key role in regulating cell activity (6); any alteration of the basal lamina can lead to tissue dysfunction. In the testes in particular, cells cooperate in ECM component deposition (7) and interact with each other through the ECM (8). Furthermore, a well-functioning epithelium depends on cell-matrix interactions (9); interactions among Sertoli cells, peritubular myofibroblasts, Leydig cells, and germ cells are also essential for spermatogenesis (8).

To evaluate a possible role of ECM in adolescent varicocele, we performed an ultrastructural study of basal lamina, with use of a transmission electron microscope, and an immunohistochemical study of the two major components of basal lamina, laminin and collagen type IV, with use of a confocal laser-scanning microscope. Laminin is secreted by
TABLE 1

Study patients summarized by: age, pubertal stage (Tanner), varicocele degree (Horner), testicular volume (sonography), hormonal parameters, and main histological features (pathology reports). Dx = right. Sn = left.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (y)</th>
<th>Pubertal stage</th>
<th>Grade</th>
<th>Testicular volume</th>
<th>Hormones (FSH, LH, SHBG)</th>
<th>Histologic findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18 V</td>
<td>II</td>
<td>Dx = 52 × 33; Sn = 50 × 32.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17 V</td>
<td>II</td>
<td>Dx = 50 × 32.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>16 II</td>
<td>Dx = 48 × 19 × 34; Sn = 47 × 18 × 35.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18 IV</td>
<td>II</td>
<td>Dx = 54 × 33 × 42; Sn 53 × 30 × 41.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>16 IV</td>
<td>III</td>
<td>Dx = 42 × 28; Sn = 41 × 28.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; incomplete spermagenesis</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>15 IV</td>
<td>III</td>
<td>Dx = 39 × 20 × 27; Sn = 38 × 21 × 27.</td>
<td>Within normal ranges</td>
<td>Peritubular fibrosis; incomplete germinal epithelium</td>
<td></td>
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<tr>
<td>7</td>
<td>17 IV</td>
<td>II</td>
<td>Within normal ranges</td>
<td>Interstitial edema; fibrosis and metaarteriovenular stasis</td>
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<tr>
<td>8</td>
<td>13 I</td>
<td>I</td>
<td>Atrophy Dx Consistency Sn ↑</td>
<td>Within normal ranges</td>
<td>Interstitial edema; fibrosis</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>17 IV</td>
<td>II</td>
<td>Dx = 43 × 16 × 28; Sn = 41 × 17 × 28.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis; blocked spermatogenesis in many tubules</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16 IV</td>
<td>II</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis; blocked acromosomogenesis</td>
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<td></td>
</tr>
<tr>
<td>11</td>
<td>17 IV</td>
<td>II</td>
<td>Consistency Sn ↓</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>17 IV</td>
<td>III</td>
<td>Dx = 45 × 18 × 29; Sn = 44 × 20 × 29.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis; blocked spermatogenesis in many tubules</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>13 I</td>
<td>I</td>
<td>Consistency Sn ↓</td>
<td>FSH ↑; LH ↑; SHBG ↑</td>
<td>Peritubular fibrosis; interstitial edema</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>16 IV</td>
<td>II</td>
<td>Dx = 55 × 34; Sn = 23 × 09.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; fibrosis; germinal line disarranged</td>
<td></td>
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<tr>
<td>15</td>
<td>16 IV</td>
<td>II</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>17 IV</td>
<td>III</td>
<td>Dx = 45 × 30 × 22; Sn = 43 × 29 × 23.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
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<tr>
<td>17</td>
<td>17 IV</td>
<td>III</td>
<td>Within normal ranges</td>
<td>Interstitial edema; interstitial fibrosis</td>
<td></td>
<td></td>
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<tr>
<td>18</td>
<td>15 III</td>
<td>II</td>
<td>Dx = 43 × 24 × 21; Sn = 43 × 21 × 24.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; peritubular fibrosis</td>
<td></td>
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<tr>
<td>19</td>
<td>15 IV</td>
<td>II</td>
<td>Within normal ranges</td>
<td>Interstitial edema; interstitial fibrosis</td>
<td></td>
<td></td>
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<tr>
<td>20</td>
<td>16 IV</td>
<td>II</td>
<td>Dx = 20 × 34 × 26; Sn = 17 × 32 × 25.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; interstitial fibrosis; blocked spermatogenesis in many tubules</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>15 II</td>
<td>II</td>
<td>Within normal ranges</td>
<td>Interstitial edema; interstitial fibrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>18 V</td>
<td>II</td>
<td>Dx = 41 × 29 × 20; Sn = 40 × 27 × 19.</td>
<td>Within normal ranges</td>
<td>Peritubular fibrosis; interstitial edema</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>17 IV</td>
<td>III</td>
<td>Dx = 44 × 28 × 19; Sn = 37 × 19 × 22.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; fibrosis</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>16 III</td>
<td>II</td>
<td>Dx = 38 × 17 × 20; Sn = 30 × 13 × 19.</td>
<td>Within normal ranges</td>
<td>Interstitial edema; fibrosis</td>
<td></td>
</tr>
</tbody>
</table>


Sertoli cells (10) and to a lesser degree by peritubular cells (11, 12) and localized at the level of the lamina rara of the basal lamina. Its main function is regulating the processes of adhesion, proliferation, and cell differentiation (6). Collagen type IV, secreted by myofibroblasts and Sertoli cells (7, 13), is mainly localized at the level of the lamina densa and is a ubiquitous component of basal lamina in different tissues (6). The results are discussed in relation to those obtained by other investigators in normal testes (14).

We hypothesize that modifications of ECM components could be already present in adolescent varicocele and play a role in varicocele-induced morphological changes.

**MATERIALS AND METHODS**

Twenty-four testicular biopsy specimens were obtained, with prior informed consent, from adolescents aged from 13 to 18 years (mean age, 16.2), affected by left idiopathic varicocele. Patients with varicocele were diagnosed after physical examination, echocolor Doppler, and phlebographic studies. A grade II or III varicocele (after Horner) was an indication for treatment. Surgical treatment was undertaken with selective microsurgical ligation of ectatic intrafunicular and extrafunicular vessels, after delivery of testis (according to Goldstein) (15), and spermaticoepigastric microsurgical derivation (a microsurgical vascular anastomosis between the spermatic vein and the inferior epigastric vein) (according to the Belgrano technique) (16). Further information on the patients they treated as well as the main histologic observations are reported in Table 1.

Biopsy specimens were processed for transmission electron microscopy and for immunofluorescent studies using different protocols.

In transmission electron microscopy the specimens were immediately fixed for 3 hours in 4% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, at 4°C. After rinsing in the same buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M buffer, the specimens were postfixed in 1% OsO4 in 0.2 M 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 (Wallac Oy, Turku, Finland). The ultrathin sections were cut on an LKB Ultratome V ultramicrotome, stained with uranyl acetate and lead citrate, and photographed with a transmission electron microscope Philips CM10.

For immunofluorescence studies, the biopsy specimens were fixed for 4 hours 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 biopsy specimens were infused in 12% and 18% saccharose and then frozen in liquid nitrogen and sectioned with use of the Bright cryostat. The 20-μm serial sections (40–50 sections per sample) were placed on gelatin-coated glass slides and preincubated with a solution containing 0.6 mL of sheep serum, 1 mL of 45 mM NaCl, and 1 mL of 20 mM phosphate buffer for 15 minutes to avoid unspecific background staining. The sections were incubated for 15 minutes with a solution containing PBS, bovine serum albumin (BSA) 1%, and Triton X-100 0.3%. This solution was also used as dilution and rinsing buffer.

The primary antibodies used were as follows: anticollagen type IV (mouse ascites fluid, Clone COL-94; Sigma Chemical Co., St. Louis, MO) at a dilution of 1:500, and antilaminin (mouse ascites fluid, Clone LAM-89; Sigma Chemical Co.) at a dilution of 1:2,000. As a secondary antibody we used a sheep anti-mouse IgG biotinylated (Amer sham International, Little Chalfont, Buckinghamshire, United Kingdom) at a dilution of 1:500 and as fluorochrome Texas red streptavidin (Amersham International) at a dilution of 1:100. Negative controls were performed either by omitting the primary antibody or by replacing the primary antibody with nonimmune rabbit or mouse serum. Positive controls were performed with use of antisera on normal human skin, for which data for laminin and collagen type IV are already available (17).

The sections were observed and photographed with a Leica TCS 4D upright confocal microscope (Leica, Heidelberg, Germany) with immersion objectives. It is equipped with an argon krypton laser (lambda 568 nm, 100 mW; lambda 488 nm, 100 mW; and lambda 647 nm, 100 mW). Collected images were digitized at a resolution of 8 bits into an array of 512 × 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 with a laser line (568 nm) and a tetramethylrhodamine-isothiocyanate (TRIC) set of filters at 1-second scanning speed with an average of up to 8. The stacks of images obtained were processed with different software functions: [1] single section, [2] 3D rotate 0° single view (overlay): overlapping the images obtained from the single optical sections (either the whole stack or only a part) to obtain a reconstruction of the sample throughout its thickness, [3] rotation: rotation at different angles of the images of the whole sample or of its parts, [4] shadow forming process: it enhances cell profile through the creation of different shadow effects.

The ultrastructural aspect of a normal basal lamina and the immunohistochemical distribution of laminin and collagen type IV in a normal testis, reproduced with the permission of Archives of Histology and Cytology (14), were used as a control testis.

**RESULTS**

**Transmission Electron Microscopy**

Lamina propria always appeared thickened because of an increased deposition of collagen fibrils, which was principally localized in the first extracellular layer. The increased thickness was responsible for the formation of the observed deep invaginations facing the germinal epithelium. Basal lamina appeared around the seminiferous tubule; it was formed by two different layers, one electron-dense and the other electron-transparent, displaying an uneven profile with a variable thickness; myofibroblasts were partly surrounded by basal lamina (Fig. 1).
Immunofluorescence

Laminin immunostaining displayed an irregular immunofluorescent line with a wavy profile that in some places appeared to be interrupted at the peritubular level. Outside this line of fluorescence, other areas of positivity were seen but were less intense and more irregular; this finding was probably due to the interrupted presence of the basal lamina of myofibroblasts (Fig. 2). The 3D reconstruction and the 20° rotation of the entire thickness of the section displayed a wider surface of the peritubular basal lamina. This projection highlighted areas of irregular immunostaining that create wide empty spaces in the thickness of the peritubular basal lamina (Fig. 3).

Collagen type IV immunostaining was mainly localized at the level of the basal lamina facing the germinal epithelium, surrounding blood vessels, and to a lesser degree inside the lamina propria (Fig. 4). The 3D reconstruction and the 20° rotation of the sample displayed a peculiar deposition of collagen at the peritubular level, which seemed to be organized in areas of annular thickness with a wavy profile, alternating between areas of strong immunopositivity and areas of reduced immunoreactivity (Fig. 5). Use of the shadow-forming process highlighted the peculiar aspect of the wavy distribution of the peritubular basal lamina and a different immunopositivity of collagen type IV (Fig. 6).

DISCUSSION

The presence of a varicocele influences spermatogenesis, and early treatment during childhood or adolescence could have a beneficial effect (18). This recommendation has been derived from clinical and histologic observations. Clinically, varicocele in adolescents have been associated with a smaller, softer testis similar to that observed in adult varicocele. Histologically, morphological alterations observed in adolescents are similar to those observed in adult varicocele. Previous studies on testicular damage have focused attention on the intratubular and interstitial components (2, 4, 5, 19, 20). Moreover, these alterations are progressive (19, 21). Little attention has been focused on ECM, in particular in the basal lamina.

In normal human testes, as reported by Holstein et al. (14), it is possible to observe a basal lamina (Fig. 7) localized around the seminiferous tubules, that divides tubules from lamina propria, and a basal lamina that surrounds the myofibroblasts in part. The peritubular basal lamina has a regular profile and constitutes two different thin layers: one electron-transparent, known as lamina lucida or rara, close to the plasma membrane of germinal epithelium, and the other electron-dense, known as lamina densa, in close relationship to the first extracellular layer of lamina propria (6).

Basal lamina is synthesized by surrounding cells (7, 10–
13), and its major components are collagen type IV and laminin; it is formed by a strong layer of collagen type IV connected to other specific molecules, such as laminin, which are necessary for connection to adjacent cells.

Collagen type IV has been identified as a continuous and uniform line in normal human testes (14), with immunohistochemical reactions in both layers of the peritubular basal lamina and in all the peritubular layers but with a reduced intensity. It is also present in the connective tissue surrounding blood vessels with a strong immunoreaction (Fig. 8).

Moreover, laminin immunolocalization was identified, by the same investigators, at the level of lamina lucida, as a continuous and uniform line, and in the extracellular matrix of some external layers (14) (Fig. 9). Laminin plays an important role in binding epithelial cells, via specific members of the integrin family (22), and connecting other ECM components such as collagen type IV (23).

These two molecules play a fundamental role in the regulation of major biological cellular functions (24–26). Through the relationship with integrins, messages are transmitted from the ECM to the nuclear compartment (through the cytoskeleton), thus controlling the processes of differentiation, proliferation, adhesion, migration, and gene expression (9, 27). It seems clear that quantitative and qualitative alterations in the basal lamina components and/or in their genetic regulation could be the basis of an acquired or inherited pathology.

In the present study, adolescents with varicocele displayed an irregular and sometimes interrupted immunolocalization of laminin at the level of basal lamina facing germinal epithelium. This finding could be interpreted as a sign of distress of the basal lamina itself. Because an intact basal lamina regulates cell polarity and cellular metabolism as well as induces cellular migration and differentiation (6), and because ECM is fundamental for spermatogenesis (8), it is possible to hypothesize that the morphological alterations reported in varicocele could also depend, at least in part, on modifications of the relationship among cells of the lamina propria, ECM, and germinal epithelium.

Collagen type IV in adolescents with varicocele displayed areas of annular thickening of the immunoreaction alternating with areas of interrupted and reduced immunopositivity at the peritubular level. Moreover, distribution of the immunoreaction appeared as irregular and wavy, following the morphology of the peritubular basal lamina observed with a transmission electron microscope along the deep invaginations; a similar ultrastructural morphology has been observed in adult varicocele and in other testicular pathologies (28, 29). The deep invaginations can be a consequence of an
increasing deposition of ECM components (30). The increased deposition of collagen fibrils starts from the first extracellular layer and successively involves the other extracellular layers. Because of this finding, the overall thickness of the lamina propria is increased but without signs of sclerosis, as is observed in adult varicocele (31).

In the present study, there was a high distribution of collagen type IV around blood vessels; its deposition in the lamina propria could depend on both Sertoli cells and myofibroblasts secreting activity. In particular, cultured Sertoli cells have a higher proportional synthesis than myofibroblasts. In vitro cocultures of Sertoli cells and myofibroblasts display a higher synthesis rate, relative to total synthesis, than monoculture alone, indicating an in vitro biosynthetic cooperation between these two cell types (13). The altered distribution of collagen type IV, observed in our study in the peritubular basal lamina, could result in unfavorable conditions for cooperation between myofibroblasts and Sertoli cells.

In conclusion, our ultrastructural and immunohistochemical observations highlight focal damage principally localized at the level of the peritubular basal lamina, but this...
damage is not as severe as that already described in adult varicocele (31). Although the clinical implications of this study are difficult to interpret because of the young age of the patients, we believe that these observations could contribute to an understanding of the natural history of adolescent varicocele. Because varicocele should be regarded as a dynamic lesion that does not disappear spontaneously in adulthood (32) and histologic changes reported seem to be progressive (19, 21), it is possible to speculate that the initial damage to the basal lamina observed in this study could only worsen if the pathophysiological mechanisms responsible for varicocele persist.

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References