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THE INTRAVESICAL URETER IN CHILDREN WITH VESICOURETERAL REFLUX: A MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL CHARACTERIZATION

JOSEF OSWALD, ERICH BRENNER, CHRISTIAN SCHWENTNER, MARTINA DEIBL, GEORG BARTSCH, HELGA FRITSCH AND CHRISTIAN RADMAYR*

From the Department of Pediatric Urology (JO, CS, GB, CR), Institute of Anatomy, Histology and Embryology (EB, HF) and Institute of Biostatistics (MD), University of Innsbruck, Innsbruck, Austria

ABSTRACT

Purpose: We investigated intravesical ureteral endings using immunohistochemical methods to study general morphology, smooth muscle architecture and collagen composition in children with vesicoureteral reflux.

Materials and Methods: Samples were obtained from 29 ureterorenal units in children with a mean age of 52.3 months undergoing reflux surgery. Routine histological paraffin embedded sections were stained with hematoxylin and eosin, and Masson trichrome to assess general morphology. Staining for actin, myosin and desmin was performed to evaluate the presence, allocation and architecture of the ureteral smooth muscle wrap. In addition, indirect immunohistochemical methods were used to study the collagen composition of the ureteral wall and CD68 was used for macrophage labeling as a marker of tissue remnant scavenging. All investigations were done using high power field magnification for quantification. In addition, age matched, nonrefluxing ureteral specimens served as controls.

Results: Smooth muscle α-actin, myosin and desmin expression were extensively decreased in all specimens pertaining to the ureteral ending. This distal part showed a high degree of muscle atrophy and degeneration as well as a disordered fiber arrangement associated with increased extracellular matrix collagen accumulation. In addition, CD68 positive macrophages were significantly increased. In contrast to these observations, the proximal intravesical portion of the ureter showed intact morphology and arrangement of the muscular coat.

Conclusions: Refluxing intravesical ureteral endings showed dysplasia, atrophy and architectural derangement of smooth muscle fibers. Consequently symmetrical contraction of the distal ureteral smooth muscle coat creating the active valve mechanism to protect reflux is not achievable.

KEY WORDS: ureter; muscle, smooth; vesicoureteral reflux

Congenital structural deficiency of the trigonal ureterovesical junction as well as lower urinary tract dysfunction affects approximately 1% of children and it causes the risk of long-term renal scarring and hypertension or renal failure.[1,2] The ureterovesical junction represents the area boundary between the low pressure upper urinary tract and high variations in pressure of the lower urinary tract. It protects the upper tract from reflux using active and passive antireflux mechanisms. The mechanisms of ureteral motility as well as detailed physiological knowledge of the normal valve action of the ureterovesical junction are still under investigation. The most common explanation of a competent valve mechanism is passive compression of the roof of the intravesical ureter against the underlying detrusor. The length of the intravesical ureter relative to its diameter seems to be the crucial point perpetuating the passive reflux defense mechanism.[3]

The current opinion of conservative treatment postulates that growth of the infant bladder and ureterotrigonal unit may provide the necessary difference to allow the valve to function. By creating a passive valve mechanism through construction of a long submucosal ureteral tunnel one can theorize that this high successful surgical conception leads to overestimation of naturally occurring ureteral intravesical tunnel length.[4] In addition, adequate muscular attachment on the surrounding detrusor to ensure fixation and an intact posterior muscular trigonal support is considered to be important.[5] In some children voiding dysfunction has an etiological role in vesicoureteral reflux. Abnormal bladder behavior frequently combined with constipation seems to prevent reflux resolution.[6] Furthermore, high voiding pressure with low bladder capacity caused by transient prenatal bladder outlet obstruction has been reported in male newborns with high grade reflux.[7] Minor attention has been given to a possible congenital muscular structural insufficiency of the distal ureter. Our study placed particular emphasis on changes occurring in the intravesical part of the ureteral wall in children treated with survey for reflux using histological and immunohistochemical methods with meticulous attention to general morphology, smooth muscle architecture, chronic inflammatory markers and collagen composition distribution.

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* Corresponding author: Department of Pediatric Urology, University of Innsbruck, Anichstr. 35, 6020 Innsbruck, Austria (telephone: 43-512-504-4810; FAX: 43-512-504-4873; e-mail: Christian.Radmayr@uibk.ac.at).

MATERIALS AND METHODS

Specimens. After informed consent was obtained 29 specimens of the distal intravesical part of ureters in children with vesicoureteral reflux were obtained from 6 males and 9 females with a mean age of 52.3 months (range 18 to 108) undergoing reimplantation. Neither neurogenic bladder dysfunction nor voiding dysfunction was found in any of these

children. Reflux was grades I to V in 1, 9, 8, 9 and 2 ureters, respectively (table 1). Grading was done according to the International Reflux Study.[8]

Ureteral tissues of the ureterovesical junction from 7 age matched autopsies without a history and with no evidence of any urological disease, as demonstrated by accurate autopsy of the whole urogenital tract, served as controls. To identify the distal part of the ureter colored tracer threads were used. Paraffin blocks were labeled with a numerical code, stained and evaluated while blinded to clinical data.

Immunohistochemistry. Paraffin sections were cut transversely at 5 μm using an ERGO-Star Rotations (Microm, Walldorf, Germany) microtome. They were then dewaxed and rehydrated. Microwave application for antigen retrieval was used. Samples were heated to recover antigenicity. After cooling the staining procedure was initiated using the automated Nexes IHC (Ventana, Strasbourg, France) staining system. Sections were incubated with monoclonal antibodies. Primary antibodies to smooth muscle actin, muscle specific myosin, desmin, collagen types I, III and IV, and CD68 were applied.

Smooth muscle cell staining. Antibodies included SM1 antismooth muscle α-actin (1:200), SMMS-1 antismooth muscle myosin and DE-R-11 antismooth muscle desmin. Chromogenic detection using a ChemMate (Ventana, Strasbourg, France) peroxidase/diaminobenzidine detection kit was applied according to manufacturer guidelines. Specimens were dehydrated and subsequently mounted permanently in entellan (Merck, Darmstadt, Germany) embedding medium.

Collagen staining. Antibodies to type I clone COL-1 (1:1,500 dilution), type III clone FH-7A (Sigma Chemical Co., St. Louis, Missouri) (1:10 dilution) and type IV clone CIV 22 collagens (Ventana) (prediluted) were used. The Nexes IHC (Ventana) detection protocol comprised proteolytic digestion step using protease 1, followed by primary antibody incubation, an amplification step and fixation with 0.05% glutaraldehyde. The chromogen reaction, counterstaining and mounting were performed as described.

Macrophage staining. CD68 staining was performed with clone KP-1 (Ventana) antihuman CD68. Digestion, incubation, detection and counterstaining were performed according to manufacturer instructions.

Morphological analysis and interpretation. Morphological analysis was done using an Axiovision (Zeiss, Jena, Germany) computer assisted light microscope and an Axioplan (Zeiss, Oberkochen, Germany) microscope. The software package was used for modular image acquisition. Slices were examined under light microscopy by 2 independent observers. Criteria for pathological arrangement were scored on a scale of 0—absent, 1—mild (25% or less of each microscopic field), 2—moderate (26% to 50%), 3—severe (51% to 75%) and 4—extremely severe (more than 75%) based on a partially absent muscular coat, replacement of muscle fibers with fibrotic tissue, enhancement of interstitial collagen and change in collagen subtype composition. The mean number of CD68 positive macrophages was counted in 2 random high power fields per slice.

Statistical analysis. Descriptive statistics were used analyzing the results of semiquantitative evaluation of smooth muscle and collagen composition. Contingency tables were used for clinical correlations. Kendall's τ correlation coefficient was calculated to show the association between reflux grade and CD68. Differences in CD68 between the 2 groups were tested by the Mann-Whitney U test. Data are expressed as the mean $\pm$ SD or mean and range with statistical significance considered at $p < 0.05$. SPSS for Windows 11.0 software (SPSS, Chicago, Illinois) was used for all analyses.

TABLE 1. *Sample distribution by reflux grade*

Reflux Grade	No. Ureteral Units
I	1
II	9
III	8
IV	9
V	2
Total	29

RESULTS

A control ureteral ending with a typically stellate lumen showed a lining of transitional epithelium with underlying lamina propria of loose connective tissue, surrounded by a solid predominantly inner longitudinal and slight outer circular layer of smooth muscle (fig. 1). In contrast, the average results of refluxing specimens using antiα-actin SM1, antimyosin SMMS-1, antidesmin DE-R-11 and anticollagen types I COL-1 and III showed a completely different appearance (table 2).

Semiquantitative evaluation in refluxing ureteral specimens with α-actin, desmin and myosin showed a score of 4, 3 and 2 smooth muscle architecture decomposition in 24.1%, 31.0% and 27.6%, respectively, and score 1 disintegration in 13.8%, while in 1 specimen a normal muscular arrangement (score 0) could be determined (fig. 2). The degree of smooth muscle deterioration was not uniform. In particular, there was no significant correlation between reflux grade and the degree of smooth muscle damage. In most samples the whole muscle bundles appeared to be affected, in particular the diameter, amount, distribution and configuration of smooth muscle fascicles. Perimysial and endomysial connective tissues were increased irregularly (fig. 2, *D*). In all specimens except 1 smooth musculature was decreased and replaced by connective tissue. In contrast to these observations, the proximal intravesical proportion of the ureter, representing about 80% to 85% of the whole intramural length, showed intact morphology and arrangement of the muscular coat. Only the distal 15% to 20% of the ureteral ending had pathological changes.

Collagen type IV was constantly found as a part of all basement membranes, including urothelium, vessels and smooth muscle cells. No thickening, hydrolysis or degradation was observed in any specimen. Corresponding to the

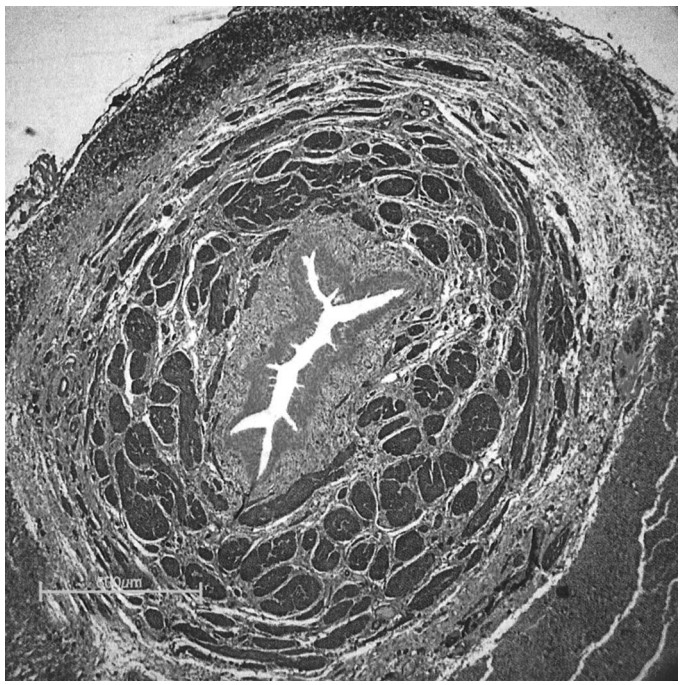

FIG. 1. Normal ostial ureter with predominantly inner longitudinal and slight outer circular layers of smooth muscle. α-Actin staining.

TABLE 2. *Pathological derangement score of ostial smooth muscle wall and extracellular matrix*

Score	No. Ureteral Smooth Muscle (%)	No. Anticollagen (%)	
		Type I	Type III
0—Absent	1 (3.5)	1 (3.5)	1 (3.5)
1—Mild	4 (13.8)	3 (10.3)	6 (20.7)
2—Moderate	8 (27.6)	7 (24.1)	9 (31.0)
3—Severe	9 (31.0)	10 (34.5)	7 (24.1)
4—Extremely severe	7 (24.1)	8 (27.6)	6 (20.7)

There were 29 samples per group.

severity code of smooth muscle deprivation, a 2 to 3-fold increase in connective tissue or extracellular matrix protein was observed (fig. 3, *A*). In these regions lacking smooth muscle tissue interstitial collagen was substituted. In addition, the increase in the deposition of collagen types I and III was caused by collagen proliferation (fig. 3, *B*). Collagen type I was increased to scores 4 to 1 in 27.6%, 34.5%, 24.1% and 10.3% of preparations, respectively, while no pathological proliferation was seen in 1 ureter. Collagen type III was increased to scores 4 to 1 in 20.7%, 24.1%, 31.0% and 20.7% of preparations, respectively, while no proliferation was seen in 1 specimen. In particular, collagen type III was localized to the lamina propria connective tissue matrix within muscle bundles and perimysial tissue. No significant correlation between reflux grade and the degree of increased collagen type I or III could be assessed.

Despite remodeling and sclerotic changes in the ureteral wall no deformation of the ureteral lumen could be noticed. The mean number of CD68 immunoreactive macrophages was significantly increased in refluxing specimens compared with healthy controls (28.8 ± 6.76 vs 16.9 ± 2.38, $p < 0.001$, fig. 4). Correlation analysis of reflux grades vs CD68 labeled macrophages revealed no significant coherence ($p = 0.860$). Except for macrophages neither specific immunoresponsive cells nor inflammatory cells were noted.

DISCUSSION

The nature of the abnormality at the ureterovesical junction in children with vesicoureteral reflux remains controversial. Although various functional and histological abnormalities have been described, many investigators believe that a lateral ostium with a short transmural course and short submucosal tunnel is the main cause generating vesicoureteral reflux.[9] Consequently spontaneous reflux resolution in children is explained by bladder growth, during which submucosal tunnel elongation occurs.[10] On the other hand, Cussen reported that growth of the intravesical ureter occurs simultaneously in relation to the surrounding tissue components.[11] By correlating length, muscle mass and muscle population of the intravesical ureter with height, weight, body surface area and patient age he found definitive growth in all of these elements with time. The morphological and functional integrity of the ureterotrigonal unit providing the active muscular control of the ostial lock mechanism seems as important as the so-called passive antireflux mechanism, most notably since it was demonstrated that the ratio of the intravesical ureteral length to ureteral diameter is obviously lower than assumed to date.[12,13] Tokunaka et al established the term ureteral muscle dysplasia for alterations in the distal intravesical ureter of children with reflux.[14]

In contrast to other studies of the ureter just outside of the bladder wall, our region of interest focused on the ostial part of the ureterovesical junction.[15] Our results clearly demonstrate a decrease in smooth muscle α-actin, myosin and desmin expression in all except 1 of our investigated specimens. This distal part showed a high degree of muscle atrophy and degeneration as well as a disordered, disrupted and scattered arrangement of fibers associated with changes in extracellular matrix collagen composition. The proportion of muscle to collagen decreased consequently in scores 3 and 4 pathological derangements from a normal 1:0.3 to 1:3. Gearhart et al described higher amounts of collagen deposition compared

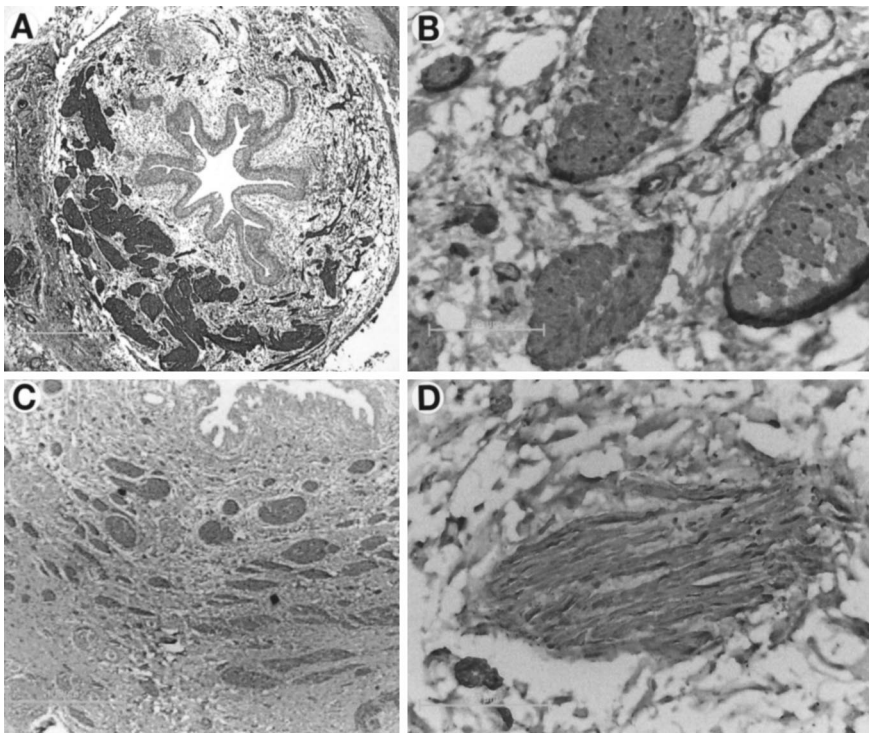

FIG. 2. *A*, score 4 derangement of refluxing intravesical ureter with severe absent smooth muscular wall. Smooth muscle α-actin staining. *B*, smooth muscle fascicle with deteriorated smooth muscle replaced by connective tissue in perimysial and endomysial regions. Myosin SM staining. *C*, immunohistochemical staining for desmin in ureteral ostia with reflux demonstrates score 3 derangement of smooth muscle wall with disintegrating and dissolving muscle fascicles. *D*, perimysial and endomysial increased extracellular matrix in smooth muscle fascicle of refluxing ureteral ostium. Myosin staining.

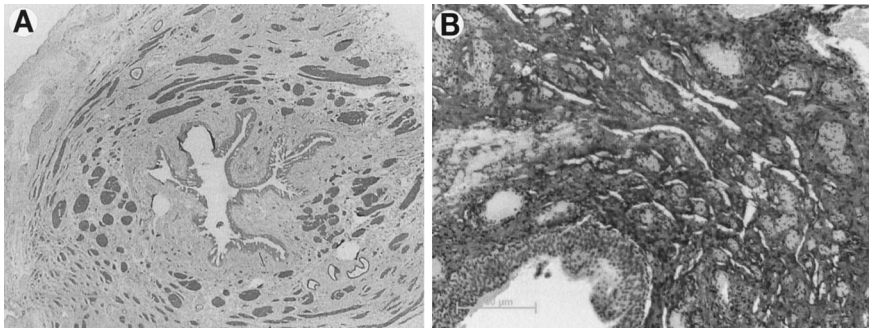

FIG. 3. *A*, disrupted muscle bundles of refluxing ureter with increased extracellular matrix protein surrounding individual muscle cells. *B*, immunolocalization of increased type III collagen shows mainly perimysial staining in deteriorated intravesical ureteral smooth muscle.

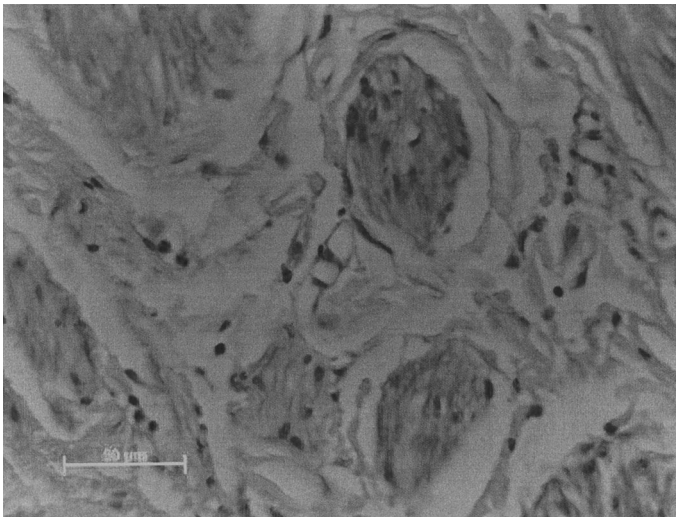

FIG. 4. Immunohistochemical staining for CD68 in ureteral ostia with vesicoureteral reflux.

with normal ureters only in dilated ureters in association with primary reflux.[16] We also noted in lower reflux grades disintegrated or sparse smooth muscle filaments at the periphery or interior part of the muscular wall. In contrast to Lee et al,[15] when analyzing the collagen subtypes in prevesical obstructed and refluxing megaureters, we found a rather higher amount of collagen type I than III in refluxing intravesical ureters. The findings of an increase in type I collagen and a lower amount of type III collagen could point to higher collagen synthesis by interstitial fibroblasts typically at later stages of fibrotic lesions.[17] Further studies analyzing more ostial specimens would be needed to corroborate these findings.

Fibrosis with tissue destruction and granulation tissue formation could also be the result of chronic inflammation. No specific immunoresponsive cells, such as lymphocytes or plasma cells, could be observed in our specimens. In detail the histological hallmarks of chronic inflammation, including altered microvascular endothelium, an influx of perivascular mononuclear infiltrates, activated spindle-shaped fibroblasts and lymphoid follicles, were not noted. In adjacent bladder mucosa chronic inflammatory alterations such as pathological impairment, and the composition of the bladder musculature and collagen could also not be proved. Consequently, one can emphasize that these specific pathological changes that primarily appear to be a congenital intrinsic abnormality at the ureteral ostial valve could be linked to the mechanisms of reflux maturation, that is the abnormal accumulation of fibrillar collagen and successive extracellular matrix remodeling could explain via cell surface integrins the tissue contraction and, hence, decrease in ureteral diameter, modifying the ratio of ureteral length to diameter.[18] Further studies investigating matrix turnover in the muscular wall of refluxive ostial specimens and estimating metalloproteinases are required to characterize these changes.

CD68 positive macrophages, which are responsible for scavenging cellular remnants, were significantly increased. Although the function of these macrophages has not been fully investigated, the biology of human macrophages seems critically important for scavenging cellular remnants of damaged muscle cells after muscle dysplasia or injury.[19] Furthermore, CD68+ macrophages generate cytokines, inducing increasing collagen types III and I production in atherosclerotic plaques.[20] CD68+ cells have a particular affinity for collagen rich regions, most notably types I and III collagen, facilitating their accumulation.[21] Further extracellular matrix remodeling is perpetuated by macrophages by secreting collagenases. Interestingly no significant correlation between the degree of ureteral muscle wall damage and clinical reflux grade was observed, whereas some degree of damage can be found in all cases of reflux. One can guess that most of the children in our series presenting for the first time with low grade reflux may have had a higher grade of reflux in infancy and consequently a limited correlation of pathological findings is achievable. Further research is needed to determine whether the degree of muscle wall impairment can alter clinical outcomes concerning so-called spontaneous maturation of the ureterovesical junction in childhood. Irrespective of these pathological findings described the surgical creation of a long submucosal ureteral tunnel acting as a passive valve mechanism is independent of resecting the abnormal distal intravesical ureter.

CONCLUSIONS

Primary vesicoureteral reflux is the consequence of a congenital abnormality of the ureterovesical junction, while deficiency of the longitudinal muscle coat leading to dysfunction of the ostial valve mechanism may have some role in the active antireflux mechanism. We found that the muscular wall of the distal ureter in children with reflux is decreased. Disorganized muscle fiber architecture leads to dysfunction of the active valve mechanism. Atrophic and dysplastic smooth muscle cells packed in a thick basal lamina are increasingly separated by expanded extracellular matrix. These morphological changes in the ureteral wall frequently result in deformation of the smooth muscle wall structure. Refluxing ureters are characterized by decreased and degraded muscle fascicles of the distal ureteral wall, leading to an insufficiently active valve mechanism. Hence, sufficient contraction of the muscular conduit to close the ostium and prevent vesicoureteral reflux is unlikely.

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