Distribution of interstitial cells of Cajal in the neurogenic urinary bladder of children with myelomeningocele

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ABSTRACT

Purpose: C-kit positive interstitial cells of Cajal (ICCs) play an important role in the regulation of the smooth muscle motility, acting as pacemakers to provide the slow wave activity in various organs. Recent studies have shown that c-kit positive ICCs are widely distributed in the urinary tract of animals and humans. The aim of our study was to examine the distribution of ICCs in the children’s neurogenic bladder.

Methods: An immunohistochemical study of specimens obtained from neurogenic urinary bladder (from the trigonum and the corpus) of children with meningomyelocele and during autopsy was performed using antibody against c-kit (CD 117). Histological morphometry of immunoexpression of c-kit positive ICCs was performed by means of an image analyzing system.

Results: Our investigation demonstrated ICCs located in the vesical muscle layers. The distribution of those cells is different in the trigonum and the corpus of the urinary bladder. No remarkable differences were observed in c-kit immunoexpression between the neurogenic and the control group.

Conclusion: There was no difference in the distribution of ICCs in the urinary bladder of healthy children as compared to children with myelomeningocele. Biopsy revealed different distribution of ICCs in particular parts of the bladder (trigonum/corpus) in both groups of children.

Key words: Interstitial cells of Cajal (ICCs), immunohistochemistry, neurogenic urinary bladder, myelomeningocele.

INTRODUCTION

Myelomeningocele is the most common cause of neurogenic bladder dysfunction of children. Decreased nerve density indicates loss of peripheral neural innervation throughout gestation. Early observations of decreased innervation and decreased contractility in the absence of morphologic abnormalities in muscle structure or extracellular matrix support a pathophysiological hypothesis that denervation is the primary insult preceding the observed alterations in bladder muscle structure and function. Literature data regarding microscopic findings in the urinary bladder of patients with a myelomeningocele are scarce. Recently a study conducted by Elbadawi et al. [1] revealed features of combined degeneration and regeneration of intrinsic axons together with activated Schwann cells. Another investigation confirmed significantly lower density of muscarinic cholinergic receptors in myelodysplastic bladders [2]. Janzen et al. [3] described leiomyomatous-like hyperplasia of the muscularis and severe fibrosis in the lamina propria and muscularis.
Staining for S-100 protein, an antigen expressed by Schwann cells of peripheral nerves, revealed marked hypertrophy and hyperplasia of the nerve fibers in the lamina propria and the muscularis [3]. These findings could be interpreted as a reaction to persistent mechanical strain on the bladder wall. They also illustrate severe morphological changes of the urinary bladder induced primarily by neurogenic deficiency and complicated by chronic inflammation [4]. The urothelium of the neurogenic bladder in young patients with myelomeningocele is also abnormal, with loss of uroplakin expression and altered urothelial proliferation [1,4,5].

In the last decade considerable evidence has shown that novel interstitial cells exist in the urinary bladder [6-9]. These cells are morphologically similar to gut interstitial cells of Cajal (ICCs), which are widely recognized as the pacemaker cells. ICCs inject depolarizing currents into neighboring smooth muscles to initiate spontaneous slow waves and corresponding phasic contractions, and play a crucial role in the neurotransmission from enteric neurons to smooth muscles cells (SMCs) [10]. There have been many debates on the correct nomenclature that should be adopted when describing these cells in organs outside the gut. There are many articles which refer to them as: ICCs, interstitial cells (ICs), ICC-like cells, myelofibroblasts. At the 5th International Symposium on ICC in Ireland, July 2007, the consensus was reached that these cells should be termed ‘ICC’. This terminology describes a group of cells found in many organs including urinary bladder, urethra, ureter and other genitourinary tissues and also blood vessels, which have morphological, ultrastructural, and physiological properties of ICCs, but do not necessarily show typical physiological function of ICCs in the gut [7].

The precise physiological role of ICCs in the urinary bladder wall has not been fully elucidated. Literature suggests that they play an important role in the bladder excitation regulation, mostly via mediation of the innervation [7,11,12]. Little is known about abnormalities of ICCs in the urinary tract, especially in the urinary bladder. Different distribution of these cells has been described in the urinary bladder of children with megacystis–microcolon-intestinal hypoperistalsis syndrome [13] and in the overactive bladder of patients after spinal cord injury [14]. Recognition of abnormalities in the distribution of ICCs in the urinary bladder may offer insight into a variety of pathological bladder functions, which are congenital and/or acquired conditions, thought to be a consequence of a deficiency in bladder musculature innervation.

The aim of our study was to examine the distribution of c-kit positive interstitial cells of Cajal in different parts of the neurogenic urinary bladder wall of children with myelomeningocele. We would like to know if ICCs play a role in the etiopathology of neurogenic urinary bladder of children with myelomeningocele.

### MATERIALS AND METHODS

The study comprised 13 patients with myelomeningocele. All of them had well documented neurogenic bladder dysfunction with overactivity. Most of them received anticholinergic medications or were on intermittent catheterization. All children were scheduled for urological surgery. Parents were informed about the procedure and signed consent for bladder biopsy. Urinary bladder muscles samples were obtained with a cold cup biopsy forceps from the trigonum of the bladder (Group A) and from the anterior wall of the bladder corpus (Group B). Tab. 1 shows the characteristics of those patients. In the control group urinary bladder specimens were obtained from the trigonum of the bladder (Group C) and anterior wall of the bladder corpus (Group D) during autopsy. The characteristic of this group of children is presented in Tab. 2. The study was approved by the Ethical Committee of Polish Mother’s Health Centre Institute in Lodz.

Immunohistochemistry. Samples of bladder tissue were fixed in 4% neutral phosphate-buffered formalin for 24 hours. Immunohistochemistry was performed on paraffin-embedded sections (five micron-thick sections). Immunohistochemical localization of the protein was performed using polyclonal rabbit anti-human antibody for CD117/c-kit (DakoCytomation, 1:600 at antibody dilution). Immunohistochemical section was deparaffinized and hydrated by standard methods. Slides were treated with 3% H2O2 in methanol for 20 minutes to block the endogenous peroxidase activity. For antigen retrieval, the slides were subjected to a 20-min steamer treatment (96 °C) in citric acid buffer, pH 6.0.

<table>
<thead>
<tr>
<th>Children with myelomeningocele and neurogenic urinary bladder</th>
<th>Age of children</th>
<th>Group A Mean number of ICCs p&lt;0.05</th>
<th>Group B Mean number of ICCs p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5 years</td>
<td>11.33</td>
<td>6.00</td>
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<tr>
<td>2.</td>
<td>8 years</td>
<td>23.80</td>
<td>11.00</td>
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<tr>
<td>3.</td>
<td>4 years</td>
<td>24.00</td>
<td>18.75</td>
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<tr>
<td>4.</td>
<td>4 years</td>
<td>9.00</td>
<td>9.00</td>
</tr>
<tr>
<td>5.</td>
<td>2 years</td>
<td>31.75</td>
<td>18.25</td>
</tr>
<tr>
<td>6.</td>
<td>5 years</td>
<td>35.00</td>
<td>30.40</td>
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<tr>
<td>7.</td>
<td>11 years</td>
<td>20.00</td>
<td>11.00</td>
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<tr>
<td>8.</td>
<td>3 years</td>
<td>7.00</td>
<td>1.00</td>
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<tr>
<td>9.</td>
<td>2 years</td>
<td>18.00</td>
<td>15.00</td>
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<tr>
<td>10.</td>
<td>5 years</td>
<td>17.35</td>
<td>13.20</td>
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<tr>
<td>11.</td>
<td>3 years</td>
<td>14.00</td>
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<tr>
<td>12.</td>
<td>2 years</td>
<td>12.00</td>
<td>6.00</td>
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<tr>
<td>13.</td>
<td>5 years</td>
<td>21.25</td>
<td>15.50</td>
</tr>
<tr>
<td>Mean value</td>
<td>18.81</td>
<td>8.42</td>
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</tbody>
</table>
After cooling down to room temperature and rinsing with phosphate buffered saline (PBS, DakoCytomation), the slides were incubated with a primary antibody for 30 min at room temperature. Slides were rinsed twice with PBS. For visualization EnVision+HRP Kits (DakoCytomation) was used. Slides were then rinsed twice with PBS. Peroxydase was detected by 3,3’-diaminobenzidine tetrahydrochloride, DAB (DakoCytomation). Finally, slides were rinsed with running tap water and counterstained with hematoxylin, dehydrated by standard methods and mounted. Negative controls were performed by omission of the primary antibody. All immunohistochemical staining was performed under equal circumstances. Only cytoplasmic cellular staining pattern was analyzed as positive.

Morphometric analysis was performed without previous knowledge of the patient’s clinical findings. Morphometry was performed on the level of light microscope (Olympus AX60) using ImageJ software (Java –based image processing program developed at the National Institutes of Health). Slides were evaluated under a microscope with camera (Olympus C5050 200M). Minimum 2 pictures (only from the muscular layer of the bladder) were taken from each slide and were separated by one visual field (magnification 400x). Scanned color images (magnification 400x) were converted to 8-bit gray scale. Then, obtained images were converted to binary pictures (black and white) using the automatic thresholding method based on Renyi’s entropy. White spots on converted black and white pictures represented Cajal’s cells. Selected areas were calculated using Image J option “Analyze Particles”. The minimum particle size was set to 600 pixels. The procedure was performed using macroinstruction. A summary of the particle count was shown in a data window. The technique was manually validated before collecting experimental data.

**Statistical methods**

Differences between groups were tested using Student’s t-test preceded by evaluation of normality and Fisher’s test. Results were considered statistically significant if p<0.05.

**RESULTS**

Our investigation revealed c-kit positive cells (fusiform cells with a thin cytoplasm, two dendritic processes and with large oval nuclei) located in the vesical muscle layers. These cells displayed morphological characteristics of cells that had previously been defined as the ICCs, or pacemaker cells in the gut. Because the anti-c-kit is a specific marker for ICCs, we referred to the labeled cells as ICCs. C-kit positive mast cells were sporadically present also in the muscle layer of the urinary bladder. These cells were round and had round nuclei, but no dendritic processes as ICCs.

In Group A (neurogenic urinary bladder specimens obtained from the trigonum) the mean value of ICCs was statistically significantly higher (t A:B, p=0.0474) than in
Group B (neurogenic bladder specimens obtained from the anterior wall of the corpus) (Fig. 1). Also in the control group (C and D) our study showed significantly higher c-kit immunoeexpression in the trigonum (group C) of the urinary bladder then in the corpus (group D) (tC:D, p=0.0003) (Fig. 2).

There was no remarkable differences in c-kit immunoeexpression in the muscularis layer of the urinary bladder between children with myelomeningocele (neurogenic urinary bladder with overactivity) and control group (t A:C, p=0.4279; t B:D, p=0.4326). The results of morphometric evaluation of the c-kit positive cells are shown in Tab. 1 and Tab. 2.

**DISCUSSION**

The kit receptor, which is encoded by proto-oncogene c-kit, is a specific marker for ICCs and kit antibodies have been used to identify those cells in the muscles of gastrointestinal wall and other organs [15,16]. The literature contains substantial data
that several subpopulations of ICCs are present in the wall of urinary bladder of mammalians [11,17] and among them humans [6-9]. They are located throughout the bladder wall and can be divided into at least two subpopulations by their morphology and orientation, that is ICCs in detrusor smooth muscles layers (detrusor ICCs) and ICCs in the suburothelial layers (suburothelial ICCs) [7,18,19]. Investigations conducted by Johnston et al. [9] showed the presence of ICCs in the lamina propria and detrusor region, but also unknown ICC subtype associated with microvessels in the human urinary bladder.

Our understanding of the physiological functions of ICCs in the urinary bladder is relatively limited. Suburothelial ICCs form a loose network connected via Cx43 gap junctions and are associated with mucosal nerves. Detrusor ICCs track the smooth muscle bundles and make frequent contacts with intramural nerves [7,9]. Both types of ICCs exhibit spontaneous electrical and Ca\(^{2+}\) signaling and also respond to neurotransmitter substances including adenosine triphosphate (ATP) and acetylcholine (Ach) [7]. These findings suggest that ICCs may be crucial in generating spontaneous excitation and also in the mediation of the propagation of acting potentials along the muscles bundles [7,17,20]. Rahnama’i et al. [21] showed the expression of the purinergic (P2Y\(_{1}\)) and cholinergic (M\(_{3}\)) and prostaglandin types 1and 2 (EP1, EP2) receptors in the ICCs. All these findings may support suggestion that ICCs may also be involved in the sensory processes of the bladder by responding to ATP, Ach, and prostaglandin, and might play an important role in the control of bladder function. Recent reports have also suggested that kit is not only a detection marker of these cells, but also may play a crucial role in the control of bladder function, especially under pathological conditions, and certain voiding disturbances may be associated with impaired KIT signaling in ICCs [17,20].

There is little information in the literature on the distribution of ICCs in the urinary bladder of children, especially in the neurogenic urinary bladder of children with myelomeningocele. The current study identified ICCs within the junctional area between smooth muscles fibers in the wall of the urinary bladder of children with myelomeningocele, as well as in the normal bladder. Because our specimens contained only muscularis layer of the bladder we have not investigated other types of ICCs, such as suburothelial ICCs and perivascular ICCs.

There are several reports in the literature that show an increase of c-kit expression of ICCs in the urothelium and muscle layers in overactive urinary bladder detrusor of humans and mammalians in general [14,22]. These findings suggest that suburothelial and detrusor ICCs may contribute to the pathophysiology of overactive bladder. The overactive neurogenic urinary bladder is very often observed in children with myelomeningocele. In our study all patients with myelomeningocele had well documented detrusor overactivity. Our study did not confirm any significant difference in the distribution of ICCs between normal and overactive urinary bladders of patients with myelomeningoce. All children in our group with neurogenic bladder and detrusor overactivity required treatment with anticholinergics and were on intermittent catherization. In the literature, the effects of experiments with c-kit receptor inhibitor, imatinib mesylate, on bladder function seem encouraging. This tyrosine kinase blocker decreased spontaneous electrical and mechanical activity in human and guinea pig bladder strips, and improved the capacity and compliance in animal cystometry experiments [14,20,23]. Imatinib mesylate was more effective in overactive bladders from spinal cord transected animals than from controls [24]. The results indicate that kit-positive ICCs may be the therapeutic target cells to reduced bladder overactivity and that a blockage of c-kit receptor may offer a new therapeutic strategy for overactive bladder treatment, although further study will be needed.

Additionally, our investigation shows that in both groups of children (with myelomeningocele and with normal bladder) mean value of ICCs was statistically significantly higher in the specimens obtained from the trigonum of urinary bladder than in those from the anterior wall of the urinary bladder corpus. Investigations conducted by Davidson et al. [18] also showed different distribution of ICCs in particular parts of guinea pig urinary bladder. They found a big mass of ICCs in the superior wall of urinary bladder. In the other parts of the bladder there were dispersed cells or small groups containing a few ICCs. The authors of that study suggested that a large collection of ICCs, detected in the dome, appeared to constitute the “primary” vesical pacemaker that initiates the slow waves that spread to the other parts of urinary bladder wall [18]. They also suggested that an absence or deficiency of ICCs may be responsible for urinary bladder motility disorders.

**CONCLUSIONS**

We have identified ICCs in the neurogenic urinary bladder of children with myelomeningocele. Our study did not confirm, as suggested in other investigations, different distribution of ICCs in the overactive neurogenic bladder of children with myelomeningocele, as compared to normal bladder of children. But the results of this study clearly showed different distribution of ICCs in particular parts of the urinary bladder, which may be correlated with the discrepant roles of the trigonum and the corpus of the urinary bladder. However, additional studies are needed to delineate the role of these cells in the human urinary bladder physiology and also in the pathologic conditions such as neurogenic urinary bladder, with a special attention for detrusor overactivity.
ACKNOWLEDGEMENTS

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REFERENCES