P2x3 receptor-expression in fetal and infant bladders – implications for a new therapeutic concept for overactive bladders in children?

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KEY WORDS

P2X3 ◗ overactive bladder ◗ receptor expression

ABSTRACT

Introduction. The urothelium plays an active role in the regulation of urinary bladder function due to its sensorial properties. P2X3 receptors are critical components of this sensory pathway. They convey afferent information creating central nervous pain perception. We report the expressional time course of P2X3-receptors from fetal life to adulthood to rule out their possible implications in various bladder pathologies.

Material and methods. Fetal, neonatal, and adult bladder specimens were obtained and immunostained with anti-P2X3 antibodies. A computer assisted microscope evaluated the intensity, extension, and tissue distribution.

Results. P2X3-receptors are widely abundant in the suburothelial space containing the nerve plexuses. These suburothelial nerve fibers expressing P2X3 were demonstrated in fetal as well as infant bladder tissue. However, the overall P2X3 expression was less intense in fetuses when compared to infants. In contrast to previously described adult specimens, we found a higher density of P2X3 receptors in infants and fetuses.

Conclusions. This is the first study investigating the expressional time course of P2X3 receptors during human fetal development as well as in infancy. The disproportionately strong expression of P2X3-receptors in infants may explain the process of reflective bladder emptying in non-toilet trained children. However, P2X3 is less abundant in adults with a normal voiding pattern. Correspondingly, overexpression or deregulation of the P2X3 receptor complex may explain the lack of significant pathological findings in children with overactive bladder. Nonetheless, the inconsistent expression of P2X3 receptors in fetuses and infants may permit new therapeutic concepts.

INTRODUCTION

The P2X3 receptor is a transmembrane ion channel allowing specific electrolyte and intercellular messenger traffic. It is located in the outer cell membrane, opening subsequent to ATP binding. Hence, when the bladder is distended during filling, ATP is released by the transitional epithelium. Furthermore, it has been proposed that ATP binds to P2X3 receptors on sensory nerve terminals, regulating the degree of bladder distension [1].

ATP-regulated sensory function via the P2X3 receptor pathway comprises both, filling and emptying of the bladder, as well as the nociceptive action in pathological states [2]. Animal experiments involving P2X3 receptor knockout mice have demonstrated reduced voiding frequency, increased bladder capacity, and higher voiding volumes while bladder pressure remains normal.

Herein we analyzed fetal, neonatal, infant, and adult bladder specimens based on semiquantitative immunohistochemistry. Previous studies yielded specific P2X3 expression predominantly in the suburothelial space as well as in the urothelium itself. We characterized the time-course and tissue distribution of P2X3 expression from fetal life until adulthood. Consequently, we hypothesize that overexpression or deregulation of the P2X3 receptor complex could serve as one of the underlying mechanisms of overactive bladder in children without specific pathological findings. These observations may also have implications for future therapeutic concepts particularly due to the increasing availability of specific P2X3 antagonists.

MATERIALS AND METHODS

A total of 27 bladder specimens (14 fetuses, 8 neonates, and 5 adults) were included in this study with all human material derived from the Institute of Anatomy and Embryology. Obvious bladder disorders were ruled out, either by individual medical history or by accurate autopsy of the whole urogenital tract. Fetuses displaying syndromal features were also excluded from the study.

All investigations were done based on semiquantitative immunohistochemistry using anti-P2X3 antibodies. All samples were fixed in phosphate buffered 4% formalin and embedded in paraffin wax following standard laboratory procedures. Subsequently, sections were cut transversally to 4 µm using a Microm ERGO Star Rotations microtome (Microm, Walldorf, Germany), mounted onto Super frost Plus microscope slides (Menzel, Braunschweig, Germany), dried overnight, dewaxed with xylene, and rehydrated in a graded alcohol series (100% to 70%). Slides were stained using the automated *DiscoveryXT* staining system by Ventana Medical Systems Inc. (Ventana, Strasbourg, France).

Heat-induced epitope/antigen retrieval was performed for 20 minutes with CC1 buffer pH7.8 (Ventana, Strasbourg, France) with the *DiscoveryXT*.

For immunostaining of P2X3, a polyclonal rabbit anti-P2X3 antibody (dilution 1/750) (Chemicon International, USA, polyclonal rabbit, Nr. AB 5895, 1:750) was applied for 2 hours at room temperature.

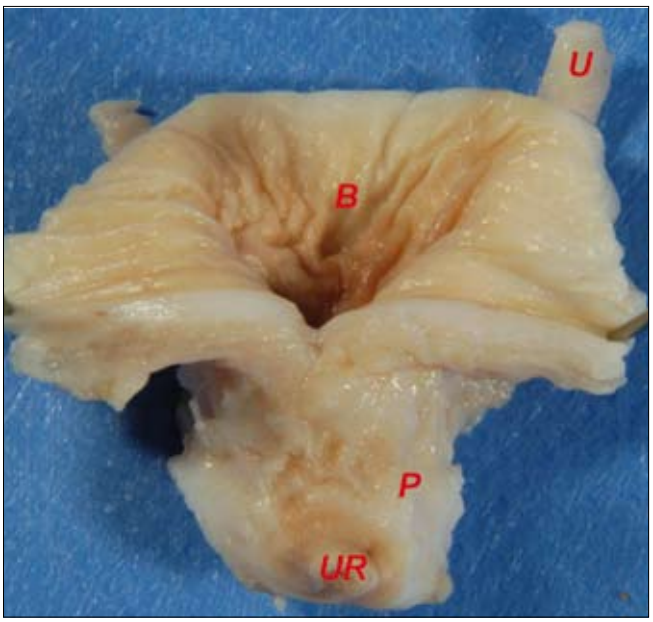

Fig. 1. Macroscopic specimen of a 12 month old male fetal bladder-prostate complex. (P - Prostate, B - Bladder, U - Ureter, UR - Urethra)

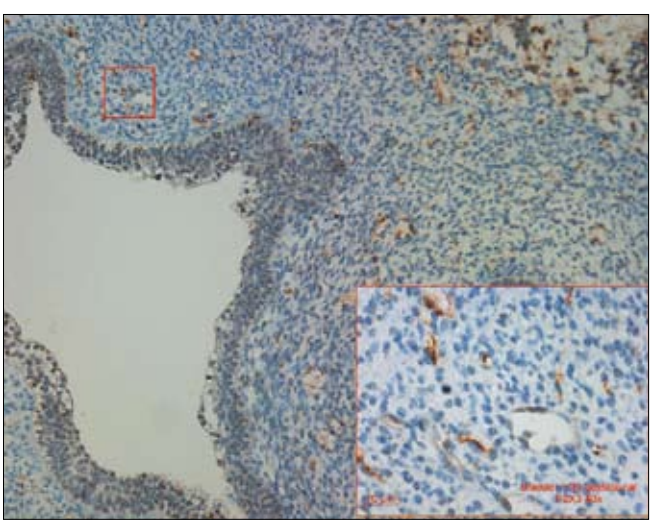

Fig. 2. Representative positive immunohistochemical staining of an 11 week old male bladder specimen (magnification 100x). Strong urothelial staining while the suburothelial compartment (display window, magnification 400x) contains fewer positive cells.

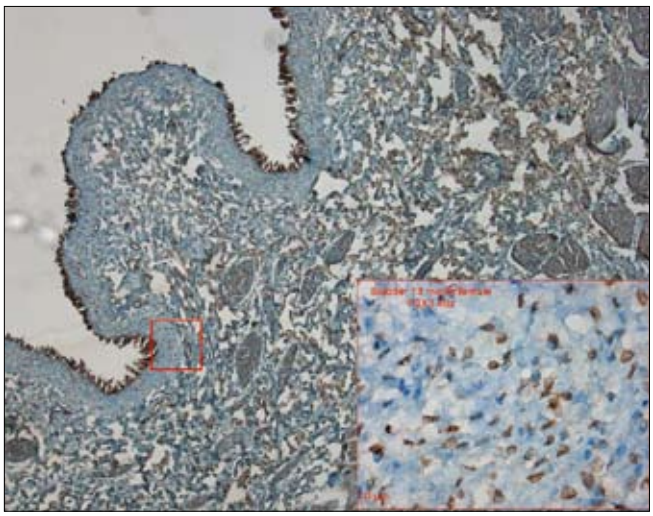

Fig. 3. Thirteen month old infant specimen (magnification 100x). P2X3 positive cells (display window, magnification 400x) are significantly more abundant in the suburothelium.

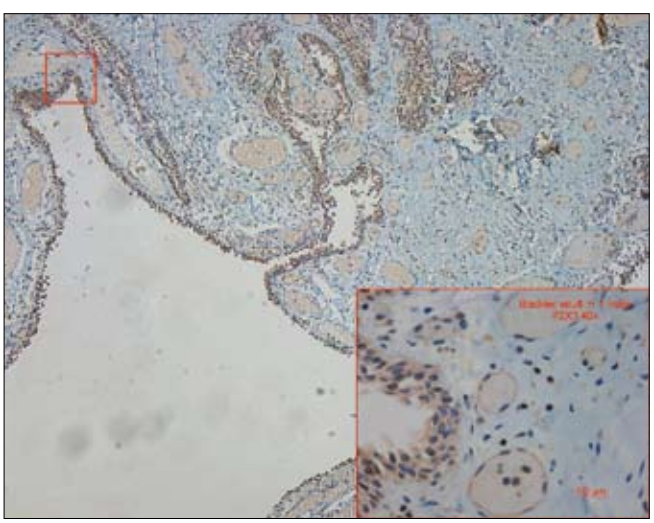

Fig. 4. Adults exhibited a similar staining pattern as that observed in fetuses (magnification 100x). Fewer cells expressing P2X3 in the urothelium, but conspicuous staining of the suburothelial space (display window, magnification 400x).

Subsequently, the antigen-antibody complex was fixed with 0.05% glutaraldehyde in 0.9% sodium chloride. Chromogenic detection was performed using a newly developed non-polymer, biotin free universal detection system (OmniMap®, Ventana Medical Systems, Inc.), which is able to detect both, rabbit and mouse primary antibodies.

Sections were counterstained with Hematoxylin and Blueing Reagent (Ventana, Strasbourg, France) for 4 minutes. After completing the staining procedure, specimens were dehydrated in an ethanol and xylene series. They were mounted permanently in Cytoseal embedding media (Microm, Walldorf, Germany).

Negative controls were achieved by simply omitting the primary antibody. Normal adult bladder tissue served as the internal positive control.

Interpretation: All specimens were investigated using a computer assisted light microscope (Zeiss®,Germany) under high power magnification (400-fold).

AxioVision® (Zeiss, Germany) software was used for counting and interpretation. Two independent observers graded all specimens. Serial sections included a 12 to 20 µm slice guaranteeing comparability in layers. A nonparametric quantification procedure was applied in all cases. The relative amount of positively stained cells in one microscopic high power field was graded at 4 levels, which included 0 to 25%, 26 to 50%, 51 to 75%, and 76 to 100% of all cellular components. Analogous to commercial detection kits, cellular staining intensity was arbitrarily considered absent (no staining or -), weak (faint and punctual or +), medium (moderately homogenous or ++), or strong (completely homogenous or +++). To assess the staining characteristics of the epithelial and stromal compartments, all positively stained cells were digitally labeled and quantified. A simple algorithm comprising nonparametric quantification and cellular staining intensity was established for final quantification.

RESULTS

In contrast to previous papers, P2X3 expression was found in all specimens including infants and fetuses [3].

Both, the urothelium as well as the suburothelial space exhibited P2X3 receptor expression. The aforementioned staining pat-

Table 1. Fetal specimens suburothelium (Intensity).

Gestational week	Nuclear staining	Cytoplasmic staining
11	+	+
14	+	+
16	+	+
16	+	+
21	-	-
23	+	+
28	++	+
29	+/-	-
32	+	++
36	+	+
36	++	+
38	++	++
39	+	+
40	++	+

Fetal specimens urothelium (Intensity)

Gestational week	Nuclear staining	Cytoplasmic staining
11	+	+
14	++	+
16	+	+
16	+	+
21	+	+/-
23	+	+
28	+	+
29	+	+
32	++	++
36	++	++
36	+	++
38	++	++
39	++	+
40	++	+++

Immunohistochemical staining characteristics of all examined fetuses. The staining intensities are given for the urothelium and the suburothelium. Additionally, the nuclear and cytoplasmic compartments were examined separately.

tern was consistent in all but two fetal specimens (21st gestational week and 29th gestational week), showing no suburothelial P2X3 expression. Interestingly, immunostaining was not only noted in the cytoplasm, but also in the nuclear compartment.

Generally, staining intensity was more distinct in the urothelium as compared to the adjacent connective tissue. P2X3 receptors are homogenously found throughout all urothelial layers comprising basal as well as umbrella cells. Infants, however, displayed a characteristic staining pattern: P2X3 was significantly more abundant in the suburothelial tissue as compared to the urothelium itself being therefore different from adults and fetuses (Figs. 1-4).

Interestingly, adult and fetal bladders exhibited a virtually identical P2X3 receptor pattern. During fetal development, staining intensity and tissue availability increased until the end of gestation. Subsequently, P2X3 expression achieved a peak value in early infancy. The relative expression in infancy seemed to correlate with the ability to control voiding voluntarily (Table 1-6).

Table 2. Infant specimens suburothelium (Intensity).

Age	Nuclear staining	Cytoplasmic staining
3 days	+++	++
15 days	++	+
1 month	+++	++
3 month	+++	++
3 month	++	+++
4 month	+++	++
5 month	+++	+++
13 month	+++	++

Infant specimens urothelium (Intensity)

Age	Nuclear staining	Cytoplasmic staining
3 days	++	++
15 days	++	+++
1 month	+++	+++
3 month	++	+++
3 month	+++	++
4 month	++	+++
5 month	++	+++
13 month	+++	+++

Immunohistochemical staining characteristics of all examined infant specimens. Conspicuously stronger staining reactions are noted in all infant bladders.

Table 3. Adult specimens suburothelium (Intensity).

Age	Nuclear staining	Cytoplasmic staining
53 years ♀	++	+
58 years ♀	+	+
62 years ♂	+	+/-
64 years ♀	+	+
71 years ♂	+	+

Adult specimens urothelium (Intensity)

Age	Nuclear staining	Cytoplasmic staining
53 years ♀	++	++
58 years ♀	+	+
62 years ♂	+	+
64 years ♀	+	+
71 years ♂	+	+

Adult bladder specimens showing similar staining characteristics when compared to Table 1 (except the late gestational weeks).

DISCUSSION

Urgency and frequency are typical features of overactive bladder (OAB) leading to a significant decrease in the quality of life in affected patients. Children frequently present with additional symptoms such as incontinence, enuresis, or recurrent UTI`s. Despite being a very common disorder its causes remain largely unknown. Since the availability of effective anticholinergic treatment options, such as oxybutinin and others, a possible role for deregulated neural signal transmission in the pathophysiology of OAB is discussed.

Table 4. Fetal specimens (grading).

Gestational week	Urothelium	Suburothelium
11	3	1
14	3	1
16	2	1
16	2	2
21	1	1
23	2	2
28	3	2
29	1	1
32	3	2
36	4	1
36	4	1
38	4	2
39	3	1
40	4	1

Non-parametric quantification of all specimens. Grading is presented in four levels. Level 1 – 0-25%; level 2 – 26-50%; level 3 – 51-75%; level 4 – 76-100%.

Changes in bladder innervation have been observed in OAB, resulting from increased neutrophin production by the detrusor muscle. ATP released from nerve endings stimulates nerve growth factor production in the bladder muscle cells. Hence, ATP may be involved in the initiation of the vicious circle of bladder overactivity [4]. Furthermore, ATP is supposed to act as an excitatory co-transmitter with acetylcholine (Ach) in parasympathetic nerves. ATP is released together with Ach in response to nerve stimulation, while the ATP response is mediated via P2X3 receptor signaling [5, 6].

Urinary frequency, urgency, and nocturia are key symptoms of OAB in infants. Even though the precise pathophysiology of OAB remains to be elucidated, it is supposed that the urothelium and the suburothelial network of interstitial cells play a key role in this process. Urothelial cells release ATP in response to distension in order to translate mechanical information into electrical, neural signals. ATP subsequently binds to the purinergic P2X3 receptor located in suburothelial neuronal sensory fibers. Depolarization of these neurons initiates the afferent pathway of the micturition reflex [7].

P2X3 is a member of the family of ATP-activated ion channels. These receptors consist of two transmembrane domains with intracellular N- and C-termini. They can form homodimeric and heterodimeric channels conjoining with other P2X subunits. These 66 kDa proteins are also expressed in the suburothelium of the urinary bladder [8]. P2X3 is known to be largely restricted to a subset of sensory neurons and in our study we present, in detail, the expressional changes of the P2X3 receptor at different stages of embryonic and postnatal development [9].

The relative importance of these receptors in bladder physiology was further characterized by studies investigating P2X3 knockout mice. Bladders lacking the P2X3 receptor gene exhibited hyporeflexia with reduced voiding frequency and increased voiding volume, concluding that these receptors are involved in mechanosensory transduction. Further animal trials at different fetal stages, as well as postnatally, showed that sensory neurons in dorsal root ganglia, trigeminal ganglia, and nodose ganglia expressed P2X3 receptors [9]. Hence, P2X3 may be essential for the activation of afferent nerve fibers during bladder filling [10].

The principal findings are that purinoceptor expression and function are altered in patients with detrusor instability. Therefore, abnormal purinergic transmission may explain the altered bladder motility in patients with detrusor instability [4].

Table 5. Infant specimens (grading).

Age	Urothelium	Suburothelium
3 days	4	3
15 days	3	2
1 month	4	3
3 months	4	2
3 months	3	3
4 months	4	3
5 months	4	4
13 months	4	3

Non-parametric quantification of all specimens. Grading is presented in four levels. Level 1 – 0-25%; level 2 – 26-50%; level 3 – 51-75%; level 4 – 76-100%.

Table 6. Adult specimens (grading).

Age	Urothelium	Suburothelium
53 years ♀	3	2
58 years ♀	3	2
62 years ♂	3	1
64 years ♀	3	2
71 years ♂	3	1

Non-parametric quantification of all specimens. Grading is presented in four levels. Level 1 – 0-25%; level 2 – 26-50%; level 3 – 51-75%; level 4 – 76-100%.

Traditional treatment strategies for OAB are still used in clinical routine. Adult OAB patients were treated with injections of both, intramuscular Botulism neurotoxin type A (BoNTA/A) and the C-fiber toxin, resiniferatoxin (RTX) [11]. Both treatment modalities lead to the reduction of P2X3 expression in the suburothelial space in patients with neuropathic OAB. Furthermore, urgency and frequency were concomitantly improved. Thus, drugs targeting P2X receptors may present a novel and effective way of treating these conditions [4].

Additionally, similar findings were reported in non-obstructive OAB suggesting that C-fibers may also be pivotal in the pathophysiology of OAB.

The reduced level of P2X3 receptors correlated with improvement in urgency sensations. Additionally, improvements in frequency and incontinence episodes were substantial. However, no significant changes were noted in the maximum cystometric capacity and the maximum detrusor pressure during filling [12, 13].

These findings underline that the urothelium as well as the suburothelial tissue play an active role in sensory functions. The connection between the urothelium and the nervous system involves multiple interactions. Substances released from the urothelium can alter bladder neurons. These interactions appear to be important for normal bladder physiology and could therefore serve to explain the pathophysiology of OAB and other related disorders [14]. In the case of negative receptor expression or selective blocking hyporeflexia, reduced frequency and increased voiding volume suggest that P2X3 receptors are essential for mechanosensory transduction, being involved in inflammation as well as physiological reflexes [14]. Currently, a wide variety of therapeutic strategies for OAB, incontinence, and interstitial cystitis are under investigation [15]. ATP release from urothelial cells with hypo-osmotic stimulation is increased several times in inflamed bladders. Botulism toxin inhibits the release of ATP [16] and is effective in blocking simple OAB induced by ATP [17, 18].

Further insights into these mechanisms will likely contribute to the understanding of bladder function and dysfunction. These aforementioned concepts and findings combined with the knowledge of P2X3 expression in fetuses and infants, led to the hypothesis of a receptor shift during bladder development. Despite being less abundant, P2X3 receptors are homogenously expressed in fetuses and significantly increased at the end of gestation. After birth, P2X3 receptor expression markedly increases, yielding its' highest values during early infancy. After acquiring voluntary control of voiding, P2X3 receptor expression gradually decreases with age. This hypothesis could add to the understanding of reflective micturition after birth and intravesical alterations in pathologies like OAB.

CONCLUSIONS

This represents the first study investigating P2X3 receptors in human fetal specimens. The differential expression in the suburothelial and urothelial tissue in fetuses, infants, and adults leads to the hypothesis that non-toilet-trained children have higher expressions of P2X3 receptors This overexpression in infants might be of physiological nature in non-toilet-trained children and a possible cause of OAB. Furthermore, these findings may contribute to the development of new therapeutic concepts. Additionally, the tissue distribution of P2X3 in the urothelium, as well as in the suburothelial space, may possibly permit local therapy in order to avoid systemic side effects.

Abbrevations and acronyms

ATP – adenosin triphosphate
Ach – acetylcholine
kDA – kilo Daltons
DO – detrusor overactivity
BoNTA/A – Botulism neurotoxin type A
RTX – resiniferatoxin

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