Expression of P2X3 purinoceptors in suburothelial myofibroblasts of the normal human urinary bladder

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Objectives: To investigate the possible localization of P2X3 receptors on suburothelial myofibroblasts and the structural relationship between these cells and sensory nerves in the human bladder.

Methods: Bladder specimens were obtained from 17 patients. Cryosections were prepared for immunofluorescent investigations using various antibodies, including cytoskeletal marker vimentin, α-smooth muscle actin (α-SM actin), desmin, P2X3 purinoceptors, afferent nerve fibers marker calcitonin gene related peptide, substance P and Griffonia simplicifolia isoelectin B4. Double-labeling was employed to determine the spatial relationship of myofibroblasts with P2X3 purinoceptors and afferent fibers.

Results: In the bladder suburothelium, there was a network of fusiform vimentin-positive cells with branching processes. Almost all of these vimentin-positive myofibroblasts showed immunoreactivity for α-SM actin. P2X3 receptors’ immunoreactivity was not distributed on any of the suburothelial afferent nerve fibers including calcitonin gene related peptide, substance P and isoelectin B4-containing nerves in the bladder. However, abundant P2X3 receptors localized on the small soma and branching processes of suburothelial myofibroblasts. Furthermore, a large number of suburothelial afferent fibers were found to contact closely with myofibroblasts, or intermingle with each other.

Conclusions: In the suburothelium of the human bladder, there was a layer of vimentin-positive myofibroblasts. Almost all vimentin-positive myofibroblasts showed double labeling for α-SM actin. These cells expressed P2X3 receptors. Suburothelial myofibroblasts may be intermediate in processing adenosine triphosphate-mediated sensory activation.

Key words: bladder, myofibroblasts, P2X3 receptors, sensory nerves, vimentin.

Introduction

The afferent input from the bladder triggers the perception of bladder fullness and initiates the micturition reflex. The sensory afferents that transmit the sensation of bladder fullness are thought to be small myelinated (Aδ) and unmyelinated (C) fibers. The bladder sensory afferents generally have endings in the suburothelial layer of the bladder wall, but in some cases, they also penetrate the urothelium.1 It has been demonstrated that there are generally two types of C-fiber afferents distinguished by central projects as well as by the presence of neuropeptides.2,3 The first type of afferent projects primarily to the spinal lamina I and outer lamina II, being neuropeptidergic, and positive for calcitonin gene-related peptide (CGRP) and substance P (SP). The second type projects to the inner lamina II of the spinal dorsal horn, being non-peptidergic, Griffonia simplicifolia isoelectin B4 (IB4)-binding nerves.3,4

The mechanisms of transduction between bladder filling (mucosal stretch) and unmyelinated afferents have been studied, demonstrating that stretching of the urothelium releases adenosine triphosphate (ATP).5,6 In addition, ATP receptors (P2X3) were shown to be expressed on suburothelial afferents and sensory afferent neurons.5,7 Thus, these studies suggest that during bladder filling, ATP released from the urothelium activates afferent nerves via P2X3 receptors. Supporting this view, P2X3-null mice showed a marked urinary bladder hyporeflexia, with decreased voiding frequency and increased bladder volume.9,10 However, in studies of the rat and the human bladder, no clear evidence was found for the presence of P2X3 receptors on sensory nerve fibers containing CGRP.11 This observation shows that the sensory process is incompletely understood.

Recently, the potential role of suburothelial myofibroblasts in the sensation of bladder fullness has become the focus of intensive interest. These special mesenchymal cells, named myofibroblasts, interstitial cells of Cajal (ICC), or interstitial cells were first recognized in the gut.12 Myofibroblasts are a unique group of smooth-muscle-like fibroblasts that have similar morphological characteristics and function regardless of their tissue location.13 They are ubiquitous cells with similar properties and functions that play important roles in growth and development, wound repair, and disease.14 A comprehensive survey of the suburothelial region of the human bladder has suggested that there is a network of functionally connected myofibroblasts immediately below the urothelium.15,16 Electron microscope studies have also shown that myofibroblasts interconnect through gap junctions, and these cells also form close contacts with unmyelinated axonal varicosities in bladder suburothelium.14,16 Moreover, Sui et al. also demonstrated that vimentin-labeling myofibroblasts were confined to the suburothelial layer of the guinea pig bladder.15,17 Isolated myofibroblast cells were identified, labeled with vimentin and were stronger for the P2Y6 antibody, but weaker for P2X3, P2Y2 and P2Y4, with none for P2Y1.17 In the human bladder, increased muscarinic receptor subtypes 2 and 3 immunostaining were observed in suburothelial myofibroblasts in patients with painful bladder syndrome.18

In the present study, we determined the presence and distribution of myofibroblasts in the suburothelial region of the human bladder, and investigated their structural interactions with sensory nerves. In addition, we also investigated the possible co-localization of P2X3 receptors with myofibroblasts in the urinary bladder.
**Methods**

**Patients**

The patients who participated in this study provided written informed consent approved by the ethics committee at our university and their anonymity was kept preserved. The study conformed to the standards set by the Declaration of Helsinki, and the procedures were approved by the local ethics committee. The bladder samples were taken from macroscopically normal areas. Hematological or biochemical blood tests were normal in the 17 patients and all had negative microbiological urinary investigations. Clinical history, physical examination including neurological assessment and ultrasound of the whole urinary tract were done in all patients before entering the study to diagnose concomitant neurological disease, bladder outlet obstruction or bladder disease that could influence bladder innervation. The patients who suffered from such disorders that could influence bladder innervation or bladder function were excluded from the study. In addition, patients with cardiovascular, renal, hepatic, psychiatric or malignant disorders and pregnant patients were excluded from analysis. No patient underwent chemotherapy before surgery and none were diabetic.

**Tissue processing**

A total of 12 full-thickness specimens approximately 1 × 1 cm were obtained at the time of cystectomy from eight male and four female patients (mean age 54.1 years, range 30 to 72). Another five biopsies measured approximately 4 × 4 mm, including epithelium, lamina propria, and some detrusor muscle were obtained during cystoscopy using cold-cup biopsy forceps from the region adjacent to the trigone of the bladder from three male and two female patients with hematuria (mean age 53.4 years, range 40 to 60). The urinary bladder samples were removed and immediately immersion-fixed in fresh 4% paraformaldehyde, pH 7.4, for 2.5 h at 4°C. The specimens were transferred into 25% sucrose in phosphate buffer at 4°C until the blocks sank. The bladders were then encapsulated in O.C.T. compound 4583 (Sakura Finetechnical Co., Tokyo, Japan). The tissue blocks were oriented to allow complete cross-section of the bladder tissue perpendicular to the surface permitting all ‘layers’ to be included in sections in the coronal plane. The blocks were cut on a Cryostat and sections (14 μm) were mounted on Poly-L-lysine (Muto Pure Chemicals Co., Japan) -coated slides.

**Double-label immunofluorescent methods**

For staining myofibroblasts, we used anti-vimentin-antibody raised in mouse (Novocastra, Newcastle, UK; 1:200). For staining afferent nerve fibers, we used guinea pig anti-CGRP antibody (Progen Biotechnik, Heidelberg, Ger; 1:800), guinea pig anti-SP antibody (Peninsular Lab Inc, San Carlos, CA; 1:400), or fluorescein isothiocyanate (FITC)-conjugated IB4 (Sigma-Aldrich, Dorset, UK; 10 μg/mL). Rabbit anti-P2X3 antibody was obtained from Oncogen Research products (San Diego, CA; 1:20). Rabbit anti-α-smooth muscle actin (anti-α-SM actin) antibody was obtained from Abcam Ltd. (Cambridge, UK; 1:400). Rabbit anti-desmin antibody was obtained from Progen Biotechnik (Heidelberg, Ger; 1:100). Fluorescent (Alexa 488 or Alexa 594) conjugated secondary antibodies were obtained from Molecular Probes (Eugene, USA; 1:200–400). To avoid cross reaction we used fluorescent secondary antibodies raised in the animal species different from the animals used for raising primary antibodies. The sections were permeabilized with 0.3% TritonX-100 in phosphate buffer saline for 30 min, and incubated with pairs of the above primary antibodies, or P2X3 antibody and FITC-conjugated IB4 at 4°C for 16–18 h. After washing, sections were incubated with a mixture of species-specific secondary antibodies for 1.5 h. Subsequently, the sections were mounted in Fluorescent Mounting Medium (DakoCytomation, Denmark), and examined with an Olympus BX51 fluorescent microscope (Olympus, Japan). Images of immunofluorescence labeling were taken with an Olympus DP70 digital camera (Olympus, Japan) attached to Olympus BX51 microscope. Images were imported into a graphics package and the two-channel readings for green and red fluorescence were merged (DP70-BSW Basic software).

**Control**

Pre-absorption of each antibody with an excess of the synthetic peptides used for its generation was tested to prove the specificity of the primary antibodies. Potential inappropriate cross-reaction between primary and secondary antisera was also assessed. Furthermore, no immunoreactivity was observed when the primary antibodies were omitted from the incubation and/or replaced with non-immune serum or when the secondary antibody was omitted. Routine hematoxylin and eosin staining was performed on all human specimens.

**Results**

**Identification of myofibroblasts**

Abundant vimentin-immunoreactive (-ir) myofibroblasts were identified in the suburothelial region in the bladder. These cells had a fusiform appearance and dendritic processes, and they formed a roughly continuous network beneath the urothelium (Figs 1a,d,2b,d). Thus, vimentin-ir cells in the suburothelium were characteristic of the description of myofibroblasts. Double-labeling fluorescence studies showed α-SM actin was present in almost all suburothelial vimentin-ir cells (Fig. 1a–c). In addition, there were few vimentin-positive myofibroblasts containing desmin immunoreactivity in the suburothelial region (data not shown). The myofibroblasts distribution was similar in all parts of the bladder.

**Immunofluorescence localization of afferent nerve fibers**

In the bladder wall, CGRP and SP were reliable markers to show afferent nerve fibers (Fig. 2a,b,d), although the number of CGRP-ir fibers slightly exceeded that of SP-ir fibers. In addition, the immunoreactivity for CGRP and SP was very rich in the bladder neck but relatively sparse in the bladder dome. The peritrigonal areas were relatively rich of CGRP/SP-ir nerve fibers compared to other parts of the bladder. Numerous CGRP-ir and SP-ir nerve fibers were observed beneath the urothelium (Fig. 2a,b,d), around vessels in the suburothelium, and parallel to the smooth muscle bundles. These afferent fibers often exhibited the bead-like appearance of their varicosities and thick nerve bundles in the suburothelial region (Fig. 2a,b,d). Furthermore, no obvious IB4-positive nerve fibers were found in the bladder wall, either in varicosities or in nerve bundles (Fig. 2c). Non-neuronal IB4-positive staining was observed in the plasma membranes of urothelium (Fig. 2c), smooth muscle cells and endothelial cells of blood vessels.
Immunofluorescence localization of P2X3

P2X3 receptors immunoreactivity was detected on the urothelium and suburothelium of the human bladder (Fig. 1e,f,2a,c). The endothelium of suburothelial capillary also showed weak staining for P2X3 subunits. The expression of P2X3 receptor was similar in all parts of the bladder. The double-labeling studies in the human bladder showed that no clear P2X3 receptor immunoreactivity was observed on CGRP-ir (Fig. 2a) or SP-ir afferent fibers in the suburothelium.

To determine whether suburothelial myofibroblasts express P2X3 receptors, double-label for P2X3 and vimentin was carried out. P2X3 receptor antibody was present strongly in vimentin-containing myofibroblasts in the bladder suburothelium (Fig. 1d–f). The suburothelial P2X3 immuno-labeling concentrated in a reticular meshwork of spindle-like vimentin-positive cells, which extended the branching processes within the matrix and along the basal margins of urothelium (Fig. 1e,f). The double-labeling studies showed that the vimentin-ir signals were usually present in the elongated and filamentous processes while the P2X3 positive signals tended to be confined to the cellular body or main part of the processes (Fig. 1d–f).

Immunofluorescent localization of myofibroblasts and afferent nerve fibers

In the suburothelium of the urinary bladder, close appositions were found between CGRP/SP-ir varicose terminals and vimentin-ir myofibroblasts (Fig. 2b,d).

Discussion

A current view of the mechanisms of micturition reflex in normal and pathological conditions is that ATP released from the urothelium may play an important role in afferent activation. Several investigators showed that the afferent nerve fibers in the suburothelial plexus in the mouse and human bladder are immunoreactive to P2X3 antibody. This P2X3-positive staining was completely absent in the suburothelial plexus of P2X3 receptor-deficient mice. These mice were found to have bladder function abnormalities, suggesting that P2X3 receptors on a subpopulation of afferent nerves have a role in bladder activation.

On the other hand, a previous study showed that in human and rat bladders, P2X3 receptors were found in the urothelium, but not on CGRP-containing nerves, suggesting that P2X3 receptors may not have...
a direct role in the mediation of sensory response to ATP in the bladder. In agreement with the above findings, the present study also shows that P2X3 receptors and CGRP or SP immunoreactivity are not co-localized in the suburothelial region of human bladders. In addition, the present study evaluated whether IB4-binding nerves express P2X3 receptors. However, the results show that no IB4-positive nerve fibers were observed in the human bladder. Therefore, it is not likely that in human bladders, P2X3 receptors are expressed on CGRP- or SP-containing nerves and IB4-positive nerves. However, we can not rule out the possibility that neuronal P2X3 receptors may express on a subpopulation of CGRP-, SP- and IB4-negative sensory nerves.

Apart from the neuronal P2X3 receptors, this study demonstrated that there was a strong immunoreactivity for P2X3 receptors in the urothelium. This result is consistent with former findings showing positive staining of P2X3 in the bladder urothelium. The urothelium has been suggested to be a source of the ATP released by distension of the urinary bladder. P2X3 receptors found on these cells may modulate the release of ATP, but the role for P2X3 receptors in the urothelium has yet to be investigated.

In the present study, P2X3 receptors immunoreactivity was also present in the suburothelium, which was separated from CGRP or SP immunoreactive afferent nerve fibers. The double labeling studies clearly showed that these P2X3 receptors were located on myofibroblasts in the suburothelial region of the human bladder. In addition, the distribution of myofibroblasts and expression of P2X3 receptors showed no difference in all parts of the bladder. To our knowledge, this is the first morphological finding to show non-neuronal P2X3 receptors that are expressed in suburothelial myofibroblasts in human bladder tissue.

Myofibroblasts are thought to possess a special cell phenotype identified by the dual expression of structural and functional properties pertaining to the fibroblast and the smooth muscle cell. It has been suggested that myofibroblasts have a role in pacemaker activity and transmission of excitation in the gut and possibly in the urinary...
tract. Based on electron microscopic findings, myofibroblasts have been defined. The ultrastructural characteristics required for a cell to be identified as a myofibroblast have been clearly defined. In human bladder, the cells of a suburothelial layer were shown to fulfill the above defining criteria. These cells were also shown to stain intensively for vimentin. In addition, Kuijpers et al. recently showed in the human bladder the presence of vimentin-ir and actin-ir suburothelial myofibroblasts. Thus, in the present study, vimentin-positive cells identified in the suburothelium may be referred to as myofibroblasts.

Vimentin, desmin and alpha-SM actin are the three filaments most often used to classify myofibroblasts. Myofibroblasts that express only vimentin are termed V-type myofibroblasts; those that express vimentin and desmin are called VD-type; and those that express vimentin and alpha-SM actin are called VA-type. As our study showed the presence of alpha-SM actin immunoreactivity in almost all vimentin positive cells, suburothelial myofibroblasts in the human bladder may be classified as VA-type.

As shown from this study, myofibroblasts interpose between urothelium and afferent nerve endings, which seems to be an ideal position to pass the sensory message to afferent nerves. Moreover, this study indicated a close contact of myofibroblasts with CGRP and SP containing afferent nerves. A similar finding was obtained from an electron microscopic study showing that these cells make close appositions with unmyelinated axonal varicosities.

As P2X3 purinoceptor immunoreactivity was demonstrated to be located on myofibroblasts, a possible role for suburothelial myofibroblasts might be to act as an intermediary stage in the ATP-induced sensory transduction process of bladder filling. Because of their contractile phenotype and intimate association with afferent nerves, myofibroblasts may respond to ATP released from the urothelium and mechanically alter afferent nerve function. Recently, isolated myofibroblasts with positive staining for vimentin were shown to respond to the application of ATP through P2Y purinoreceptors. These isolated vimentin-ir myofibroblasts from the guinea-pig bladder were strongly labeled for P2Y6, but weaker for P2X3, P2Y2 and P2Y4, and with no staining for P2Y1. As our data showed the intense staining of P2X3 receptors in the suburothelial myofibroblasts in the human bladder, this difference in the expression of purinoceptor subtypes may reflect the species difference.

Conclusions

A network of vimentin-positive myofibroblasts was found to localize in the suburothelium of the human bladder. These cells have close associations with afferent nerve fibers. The P2X3 receptors are commonly located on myofibroblasts, a possible role for suburothelial myofibroblasts in the bladder. Myofibroblasts might play a key role in ATP-mediated afferent activation in the urinary bladder.

Acknowledgments

We acknowledge with gratitude the valuable suggestions from Professor Hiroyuki Yaginuma, Department of Anatomy, Fukushima Medical University School of Medicine, Fukushima, Japan; Professor Christopher H. Fry, Institute of Urology, University College London, London, United Kingdom; and Professor Bai-ren Wang, Institute of Neurosciences, The Fourth Military Medical University, Xi’an, China. This work was supported by research grants from the National Natural Science Foundation of China (grant 30801142), Programs for Science and Technology Development of Shaanxi Province (grant 2008K15-06).

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