Cadherin-11 Up-Regulation in Overactive Bladder Suburothelial Myofibroblasts

Alexander Roosen, Apostolos Apostolidis,* Sohier Elneil,† Shahid Khan, Jalesh Panicker, Sebastian Brandner, Clare J. Fowler‡ and Thomas M. Kessler†,§

From the Department of Uro-Neurology, National Hospital for Neurology and Neurosurgery, University College London Hospitals National Health Service Foundation Trust (AR, AA, SE, SK, JP, CJF, TMK) and Division of Neuropathology and Department of Neurodegenerative Disease, Institute of Neurology (SB), London, United Kingdom, Department of Urology, Ludwig-Maximilians-University (AR), München, Germany, and Second Department of Urology, Aristotle University of Thessaloniki, Papageorgiou Hospital (AA), Thessaloniki, Greece

Abbreviations and Acronyms

Cy3 = cyanine 3
DAPI = 4',6-diamidino-2-phenylindole
FITC = fluorescein isothiocyanate
LUTS = lower urinary tract symptoms
OAB = overactive bladder

Purpose: We investigated whether the adherens junction proteins cadherin-11 and β-catenin can be immunohistochemically visualized in the human bladder using commercially available antibodies and, if so, whether there are differences between patients with overactive bladder and refractory detrusor overactivity, and controls without lower urinary tract symptoms.

Materials and Methods: In a prospective, nonrandomized single center study 32 patients with overactive bladder and refractory detrusor overactivity, and 8 controls without lower urinary tract symptoms underwent cystoscopic bladder biopsy. Quantitative immunohistochemistry was performed. The primary outcome was cadherin-11 and β-catenin expression in the human bladder using commercially available antibodies. The secondary outcome was differences in cadherin-11 and β-catenin in patients with overactive bladder and refractory detrusor overactivity, and controls.

Results: Double labeling experiments showed co-localization of cadherin-11 and connexin 43 in the suburothelium. There was also strong co-localization of cadherin-11 and β-catenin in the suburothelium and detrusor. Significant 2-fold up-regulation of cadherin-11 was found in the suburothelium of patients with overactive bladder compared with that in controls (p = 0.018), whereas β-catenin was similar in the groups (p = 0.6). In the detrusor cadherin-11 and β-catenin expression was comparable in patients with overactive bladder and controls (each p = 0.5). No difference was observed in cadherin-11 and β-catenin in patients with overactive bladder with idiopathic vs neurogenic detrusor overactivity in the suburothelium and the detrusor (p >0.3 and >0.2, respectively).

Conclusions: Using commercially available antibodies cadherin-11 and β-catenin expression in human bladder suburothelial myofibroblasts and detrusor smooth muscle cells was noted. Cadherin-11 up-regulation in suburothelial myofibroblasts in patients with overactive bladder may be significant in overactive bladder pathogenesis.

Key Words: urinary bladder, overactive; osteoblast cadherin; beta catenin; fibroblasts; muscle, smooth

OVERACTIVE bladder is highly prevalent and it affects the lives of millions of people worldwide. In the United Kingdom more than a third of the population older than 40 years, ie 9 million individuals, is estimated to have OAB and a fifth, ie 5 million, requires health care for this condi-
tion. OAB is a burden for every health care system and the socioeconomic costs are equivalent to those of dementia or diabetes. Estimated yearly costs for treating OAB in Germany with a population of about 82 million individuals has been reported to be €3.98 billion and the situation may not be different proportionally in other European countries or the United States.

However, although OAB has a major impact on quality of life and imposes a substantial economic burden, to our knowledge the pathophysiological mechanisms involved are still incompletely understood. Suburothelial myofibroblasts and detrusor smooth muscle cells are electrically coupled via gap junctions. In addition, increased expression of the gap junction protein connexin 43 was found in suburothelial myofibroblasts and in the detrusor of patients with detrusor overactivity. How-ever, for strong physical interaction between cells intact intercellular mechanical adhesions are necessary. Adherens junctions, also referred to as zonulae adherens, are multiprotein complexes formed by cadherins and catenins. Cadherins, a superfamily of transmembrane glycoproteins, promote cell-cell adhesion and interact with cytoplasmic proteins such as β-catenin. Cadherin-11, also known as osteoblast-cadherin, belongs to the type 2 cadherin subgroup and is a marker of the connected cellular elements of the mesenchyma. Cadherin-catenin coupling is required for complete interaction between cadherins and the actin based cytoskeleton. Indeed, evidence has been provided for cadherin mediated suburothelial myofibroblast and detrusor smooth muscle cell-cell interaction. Therefore, structural changes in adherens junctions may be involved in OAB pathogenesis. Therefore, we investigated the immunohistochemical expression of the adherens junction proteins cadherin-11 and β-catenin in bladder biopsies in patients with OAB and refractory detrusor overactivity, and compared it to those in controls.

PATIENTS AND METHODS

Patients
Prospectively included in the study were 8 men and 24 women with a mean ± SEM age of 46 ± 2 years who had OAB and refractory detrusor overactivity, including 13 with idiopathic and 19 with neurogenic detrusor overactivity due to multiple sclerosis (17) and myelomeningocele (2), and 8 female controls with a mean age of 51 ± 5 years. In all patients with OAB detrusor overactivity was proved urodynamically and pharmacological treatment with more than 1 antimuscarinic drug for at least 3 months had failed. Controls were undergoing gynecological surgery under general anesthesia. They had no lower urinary tract symptoms, a macroscopically normal bladder and sterile urine on cystoscopy. Bladder biopsies were obtained from a consistent bladder area 2 cm above and lateral to the ureteral orifices. All patients provided written informed consent and the study received local ethics committee approval.

Immunohistochemistry
Biopsy specimens were snap frozen in liquid nitrogen, embedded in optimal cutting temperature medium and stored at −60°C. Three sections per specimen were cut in a cryostat at 10 μm and collected on 3-aminopropyltriethoxy-silane-coated Superfrost® slides. Sections were post-fixed in methanol at −20°C and blocked with 1% bovine serum albumin before incubation with primary antibodies for 2 hours at room temperature. For quantitative immunofluorescence 3 sections per specimen were co-labeled for cadherin-11 (rabbit polyclonal, 71-7600, 1:100) and β-catenin (mouse monoclonal, 13-8400, Invitrogen™, 1:1,000). Further sections were double labeled for cadherin-11/β-catenin and connexin 43 (mouse monoclonal, mAb 3067, Millipore®, 1:1,000 and rabbit polyclonal, 71-0700, Invitrogen, 1:500). Binding sites were visualized using Cy3 and FITC conjugated secondary antibodies (goat antimouse, AP181C, 1:500 and donkey antirabbit, AP182F, Millipore, 1:50). Nuclei were counterstained with DAPI (D1306, Invitrogen, 1:50,000) during incubation with a secondary antibody. Slides were coverslipped using Citifluor® mounting medium. Immunolabeled sections were examined using an LSM-510 laser scanning microscope (Carl Zeiss™) equipped with an argon laser (458, 488 and 514 nm), a helium-neon laser (543 and 633 nm) and a 405 nm diode laser using a 40× oil immersion objective. Fluorescence was excited at 488 m (FITC), 405 (DAPI) and 543 nm (Cy3), and recorded with separate detectors. Multitrack scanning avoided crosstalk in double labeling experiments. Three images per section (areas with highest immunoreactivity) were scanned, rendering 9 representative images of the suburothelial and detrusor adherens junction network per biopsy for analysis. In accordance with previous semiquantitative immunohistochemical studies of the suburothelium in OAB cases we first calibrated the detection system on a reference section and reused the parameter settings (pinhole, optical slice less than 1.3 μm, detector gain) for all images to ensure the comparability of fluorescence signal intensity among samples.

Quantitative and Statistical Analysis
To examine the amount of cadherin-11 and β-catenin labeling a square 75 × 75 μm² section representing the minimal extension of the cadherin-11/β-catenin positive ribbon in a transverse cut section was cropped from the cadherin-11/β-catenin positive area of each image and analyzed using ImageJ software (http://rsb.info.nih.gov). Colors were split to render images for the cadherin-11, β-catenin and nuclear labels alone. Images were then converted into black and white bitmaps after equalizing the setting of threshold levels with punctate staining represented by single black particles and automatically calculated as the area fraction. The area fraction was also correlated to the number of nuclei, which was used for statistical analysis to ensure normalization for any variation in cellular components in different sections, as described previously. Data were normally distributed and
are presented as the mean ± SEM. To compare unrelated samples the 2-sided unpaired Student t test was used with p < 0.05 considered significant. Statistical analysis was done using SPSS® 16.0.

RESULTS

Immunolabeling Localization
Punctate cadherin-11 immunolabeling was widely distributed in the suburothelial layer, whereas urothelial cells showed no immunoreactivity for cadherin-11. The boundary of the cadherin-11 positive ribbon was more sharply defined on the urothelium facing side than on the submucosal side, where labeling density tended to decrease progressively with depth. Double labeling experiments showed a similar staining pattern for cadherin-11 and connexin 43 (fig. 1). A considerable part of the cadherin-11 binding sites was co-localized with connexin 43 (fig. 1, E). However, the rest of positive punctate staining was at different locations. Similar to cadherin-11 expression, prominent punctate immunoreactivity for β-catenin was found beneath the basal lamina with density progressively decreasing with depth. There was strong co-localization with cadherin-11 (fig. 2, D). Remarkably β-catenin showed strong nonpunctate diffuse expression in the urothelial layer. In the detrusor cadherin-11 and β-catenin showed the same dense and punctate expression evenly distributed between muscle bundle myocytes (fig. 3).

Patients vs Controls
There was a significant 2-fold up-regulation of cadherin-11 in the suburothelium of patients with OAB.
and refractory detrusor overactivity compared to that in controls (p = 0.018), whereas β-catenin expression was similar in the 2 groups ((p = 0.6) fig. 4, A and B). In the detrusor cadherin-11 and β-catenin expression was comparable in patients with OAB and controls (each p = 0.5, fig. 4, C and D).

When comparing patients with OAB who had idiopathic vs neurogenic detrusor overactivity, no significant differences were found in cadherin-11 and β-catenin expression in suburothelium and detrusor (p >0.3 and >0.2, respectively). In addition, cadherin-11 and β-catenin expression did not differ between the genders (p >0.1).

**DISCUSSION**

To our knowledge this is the first study of the immunohistochemical expression of the adherens junction proteins cadherin-11 and β-catenin in patients with OAB with refractory detrusor overactivity. We found cadherin-11 up-regulation in suburothelial myofibroblasts in patients with OAB compared to that in controls, whereas β-catenin expression was similar in the 2 groups. Whether this reflects dysfunction of up-regulated adherens junctions in the suburothelium of patients with OAB is unclear and warrants further investigation. In addition,
double labeling experiments showed that adherens junctions and their electrical counterparts (gap junctions) were expressed by the same cell layer of suburothelial myofibroblasts. The fact that cadherin-11 and connexin 43 were often co-localized suggests that adherens and gap junctions between suburothelial myofibroblasts might form a functional unit.

A layer of fibroblastic cells with smooth muscle-like characteristics has been identified immediately beneath the human bladder urothelium. These so-called suburothelial myofibroblasts form a functional syncytium through gap junction coupling and may modulate sensory responses from the bladder wall and spontaneous activity. It was also suggested that suburothelial myofibroblasts might act in a fashion analogous to that of interstitial cells of Cajal, which have pacemaking properties in the gastrointestinal tract, although to our knowledge no activity has been noted in the human bladder. The detrusor also has an extensive network of fibroblastic cells but they do not have a myofibroblast ultrastructure, nor is there an acquired phenotypic change in neurological cases of detrusor overactivity. Thus, suburothelial cells remain the only population of myofibroblasts identified in the human bladder to date.

The detrusor is thought to comprise a set of functional units of smooth muscle cells that act in coordinated fashion and involve electrical coupling of suburothelial myofibroblasts and detrusor smooth muscle cells via gap junctions. In animal experiments suburothelial myofibroblasts generated intracellular Ca\(^{2+}\) and electrical responses to stimuli known to influence bladder activity. These responses were significantly increased by physical connection between adjacent cells. Such augmentation was suggested to depend on adherens junction formation since no evidence was found of gap junction coupling in isolated pairs of myofibroblasts. This is in accordance with our findings that cadherin-11 and β-catenin were co-localized in the suburothelium and detrusor, indicating that the 2 proteins are a prerequisite to form the functional unit necessary for mechanical cell coupling. Wagener et al found no cadherin expression using pan-cadherin antibody and concluded that cadherins are not involved in human detrusor mechanical coupling. Nevertheless, Kuijpers et al recently visualized the expression of cadherin-11 and β-catenin in suburothelial myofibroblasts and detrusor smooth muscle cells in the human bladder. They used noncommercially available antibodies and the bladder tissue was obtained from patients undergoing radical cystectomy for localized bladder cancer. Thus, it may be argued that antibody and tumor related factors might have influenced their results. However, using commercially available antibodies our findings in patients with OAB and controls are supportive of those of Kuijpers et al.

In addition, to our knowledge we report for the first time 2-fold up-regulation of cadherin-11 in suburothelial myofibroblasts in patients with OAB compared with that in controls, whereas β-catenin expression was similar in the 2 groups. This is of particular interest since we also found co-localization of adherens and gap junctions in the suburothelial space, and 2-fold up-regulation of gap junctions in the suburothelium of patients with OAB was previously noted. These findings suggest that adherens and gap junctions between suburothelial myofibroblasts form a functional unit and might have a role in the pathogenesis of OAB. Indeed, it was observed in myocardium that gap junctions require the mechanical support of adherens junctions for normal functioning. N-cadherin gene deletion in the heart resulted in the dissolution of adherens junctions and a decrease in gap junctions. In addition, cardiac specific loss of N-cadherin led to an alteration in connexins with the clinical consequence of slowed conduction and arrhythmia. Although we aimed to minimize confounding bias by using standardized procedures for tissue processing, staining and analysis, our study has limitations, mainly due to the methodological restrictions of semiquantitative immunohistochemistry. Corroboration of our findings by additional methods such as Western blotting or polymerase chain reaction would be desirable. Microdissection of urothelium, suburothelium and detrusor would be necessary for layer specific assessment. However, this is a technically demanding task in small frozen biopsies and laser dissection microscopy is not yet available at our department. Thus, immunohistochemistry is the only widely available method to semiquantitatively examine urothelium, suburothelium and detrusor selectively. Moreover, immunohistochemistry as a tool for quantifying bladder structures has been successfully used previously by us and by many other groups. Our observation of up-regulated cadherin-11 and unchanged β-catenin in suburothelium, in contrast to unchanged cadherin-11 and β-catenin in detrusor, serves as an internal control and supports the validity of our semiquantitative analysis. Because gap junctions are represented by distinct punctate staining, our study did not have the methodological problem of the quantification of fiber-like staining, e.g., nerves, since the orientation of elongated structures in the section determines their representation as punctate or fiber-like.
CONCLUSIONS

Using commercially available antibodies we reproduced the results of Kuijpers et al\(^9\) and noted cadherin-11 and β-catenin in human bladder suburothelial myofibroblasts and detrusor smooth muscle cells. In addition, we found 2-fold up-regulation of cadherin-11 in suburothelial myofibroblasts in patients with OAB and refractory detrusor overactivity compared with that in controls, whereas β-catenin expression was similar in the 2 groups. These findings may be significant in OAB pathogenesis.

REFERENCES


