Expression of Smoothelin in the Normal and the Overactive Human Bladder

From the Institute of Anatomy, University of Zürich (CM, UZ), Department of Urology, Zürich University Hospital (ML, XW, DMS, HJ) and Clinic of Urology, Hirslanden Hospital (HJ), Zürich, Switzerland

Purpose: We established the expression pattern of smoothelin, a marker protein for contractile smooth muscle cells, in the human detrusor and investigated its possible impact on bladder overactivity.

Materials and Methods: Detrusor samples of 13 overactive bladders (sensory urge and detrusor instability) were obtained before botulinum toxin injection and compared to those of 8 normally contractile, nonobstructed bladders obtained during radical cystectomy. Smoothelin mRNA expression patterns were investigated by Northern blot and variant specific reverse transcriptase-polymerase chain reaction as well as by quantitative reverse transcriptase-polymerase chain reaction on laser capture, microdissected smooth muscle. At the protein level smoothelin was investigated by standard and quantitative immunohistochemistry.

Results: The bladder muscularis expressed vascular and visceral smoothelin isoforms, and 2 of the known splice variants. In the smooth muscle of patients with detrusor instability and sensory urge a significant 2.4 and 2.2-fold increase, respectively, in smoothelin variant 1 mRNA was observed in comparison to that of normal controls. Analyses at the smoothelin protein level confirmed significant up-regulation in these bladder dysfunctions by a factor of 2.3 and 1.8, respectively. No significant difference in smoothelin expression was observed between detrusor instability and sensory urge.

Conclusions: Increased expression of smoothelin in patients with detrusor instability and sensory urge implies that the etiology of these dysfunctions includes changes in myogenic parameters. In addition, our data support the new classification of the International Continence Society for overactive bladder proposing that sensory urge and detrusor instability represent a single clinical entity.

Key Words: bladder; muscle, smooth; SMTN protein, human; muscle contraction; urination disorders

In 2002 the ICS recommended a new definition of overactive bladder.1 The term was defined as a syndrome characterized by urgency with or without urge incontinence, usually combined with frequency and nocturia.1 However, this terminology provoked a controversial discussion about its validity. Among the points that were criticized was the fact that the definition is based on symptoms only, neglecting or even excluding the pathophysiological background.2 On the other hand, regarding the underlying disease processes, we still lack the basic scientific understanding necessary not only for an improved terminology, but also for the future design of better treatment options.

With the aim of adding information about changes at the detrusor muscle level we investigated the expression of smoothelin, a recently discovered protein that in adults appears to be smooth muscle specific.3-4 Reports indicating that its expression is rapidly down-regulated in primary cultures of smooth muscle have led to the conclusion that its transcription is restricted to the phenotype of contractile smooth muscle cells.3-6 Smoothelin appears to be part of the cytoskeleton, co-localizing with α smooth muscle actin filaments3,4,6,7 through 2 actin binding domains.8,9

Due to alternative promoter use smoothelin transcription and translation give rise to 59 kDa isoform A or to N-terminally extended, 110 kDa isoform B.5,10 These distinct smoothelin isoforms appear to be differentially expressed in the smooth muscle of visceral tissues (smoothelin-A) or the vascular wall (smoothelin-B).8 In these 2 isoforms alternative splicing results in the generation of 3 variants with almost identical molecular weights.10

While the occurrence of smoothelin in the porcine bladder has been described previously,11 little is known about its expression in the human bladder. However, its proposed role for smooth muscle contractility makes smoothelin an interesting candidate for studies of bladder dysfunction, such as bladder overactivity.

PATIENTS AND METHODS

Patients. Patients underwent videourodynamic evaluation according to ICS definitions, including medium fill cystometry. A total of 8 normally contractile, nonobstructed (Schaefer grade less than 2) detrusor samples were derived from 3 female and 5 male patients with a mean age ± SD of 57.8 ± 14.3 years undergoing radical cystectomy for localized bladder cancer. Samples were dissected from tumor-free areas and showed neither gross nor microscopic signs of
malignancy. A total of 13 detrusor samples from overactive bladders were obtained by cold forceps biopsy before botulinum toxin injections in patients with a mean age of 58.9 ± 16.1 years. Based on urodynamics overactive bladders were subdivided into detrusor instability in 4 females and 3 males, and sensory urge in 1 female and 5 males. All tissue samples were snap frozen. The study was approved by the local ethics committee and all samples were taken with the written consent of patients.

**RT-PCR.** RNA extraction, RT and PCR were performed as previously described using smoothelin primers (table 1 and fig. 1). Cycling parameters were 1 cycle of 5 minutes at 95C, 33 cycles at 94C, 58C and 72C for 45 seconds each, and a final 5-minute amplification. Representative large and small PCR fragments were purified with a QIAquick® gel extraction kit and sequenced (Microsynth, Balgach, Switzerland).

**Probe preparation and Northern blotting.** To synthesize DIG labeled smoothelin antisense riboprobes 200 ng of a PCR product generated with primers SMTN1s and SMTN1as (the latter containing a T7 RNA polymerase binding site) was in vitro transcribed using a DIG RNA labeling kit (Roche Diagnostics, Rotkreuz, Switzerland). Total RNA (6 µg) from normally contractile detrusors were used for Northern blotting with 200 ng/ml DIG labeled antisense smoothelin riboprobe according to our standard protocol.

**Immunohistochemistry.** Frozen sections were fixed with 4% paraformaldehyde and subjected to standard immunohistochemical procedures using a monoclonal smoothelin antibody (Chemicon, Temecula, California) (1:30,000), biotinylated sheep anti-mouse IgG (1:100) and streptavidin/fluorescein isothiocyanate (Bioscience, Emmenbrücke, Switzerland) (1:1,000). Alternatively after application of the secondary antibody sections were incubated with streptavidin horseradish peroxidase (Bioscience) (1:100), followed by diaminobenzidine (Sigma Chemical Co., St. Louis, Missouri) for 10 minutes. Sections were analyzed with a confocal laser scanning microscope (Leica, Glattbrugg, Switzerland) or a Zeiss Axiophot (Zeiss, Zürich, Switzerland). For quantitative analyses in 3 sections per patient smooth muscle fluorescence intensity was measured under constant conditions at a fixed depth and 200× magnification using LCS Lite software (Leica). Statistical analysis was performed with the Mann-Whitney test.

**Laser capture microdissection and quantitative RT-PCR.** Frozen sections (10 µm) were mounted on polyethylene membrane slides (MMI, Glattbrugg, Switzerland), dehydrated and dried. Smooth muscle areas of 300,000 ± 500 µm² were dissected using a laser capture microdissection microscope and UVCut® system, and collected on adhesive caps (MMI). For RNA extraction an RNasy micro kit (Qiagen, Basel, Switzerland) was applied. RNA (12 µl) was divided between a RT reaction, as described except SuperScript II enzyme (Invitrogen, Basel, Switzerland) was used, and a RT-minus reaction.

For real-time RT-PCR primers and probes for the 2 smoothelin variants A/B1 and A/B2 were designed using an assay by design service (Applied Biosystems, Rotkreuz, Switzerland) (table 2). The assay on demand for β2-microglobulin (HS99999907-M1, Applied Biosystems) served as the housekeeping gene. Reactions (20 µl) were performed in triplicate, containing 1 µl cDNA in Universal PCR Master Mix (Applied Biosystems). Reactions were incubated in an ABI Prism® 7500 system for 2 minutes at 50C and 10 minutes at 95C, followed by 45 cycles with 15 seconds at 95C and 1 minute at 60C. Control reactions included efficiency curves, RT-minus reactions and no-template controls. Furthermore, samples were tested for stable expression of the housekeeping gene. To determine the relative expression levels C, values were automatically obtained by ABI 7500 System SDS software (Applied Biosystems). Thereafter the relative expression software tool for data analysis was applied.

### RESULTS

**Smoothelin is produced in the human detrusor.** The probe used for Northern blot has complete homology to the longest splice variants A2 or B2 and it detects visceral (A) and vascular (B) smoothelin isoforms. Northern analyses revealed that RNA from the bladder muscularis contained at least 4 smoothelin transcripts (fig. 2). Comparison of samples from 5 normally contractile patients showed that this complex expression pattern was repeatedly acquired. The most prominent band migrated at about 1.9 kb.

### Table 1. Primer sequences location and specificity

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Isoform Specificity</th>
<th>Exon Location</th>
<th>Variant Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMTN1s</td>
<td>5′cgagaggctcacttggaat3′</td>
<td>Vascular/visceral</td>
<td>15</td>
<td>1–3</td>
</tr>
<tr>
<td>SMTN2s</td>
<td>5′cctgacgcaacctcttc3′</td>
<td>Vascular/visceral</td>
<td>16</td>
<td>1–3</td>
</tr>
<tr>
<td>SMTN3s</td>
<td>5′ctcttctctcttgacccagac3′</td>
<td>Vascular/visceral</td>
<td>19/20</td>
<td>2, 3</td>
</tr>
<tr>
<td>SMTN4s</td>
<td>5′ggaggcttcttcgctgctg3′</td>
<td>Vascular</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>SMTN1as</td>
<td>5′ggcttggtgcttcatgcagtc3′</td>
<td>Vascular/visceral</td>
<td>20</td>
<td>1–3</td>
</tr>
<tr>
<td>SMTN2as</td>
<td>5′cagggctgctgcttcatgcagtc3′</td>
<td>Vascular</td>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>

Sequence design was based on database information with GenBank Accession Nos. NM_006932.3 (variant 1), NM_134269.1 (variant 2) and NM_134270.1 (variant 3).

### Table 2. Primer sequences, location and specificity

<table>
<thead>
<tr>
<th>Primer</th>
<th>Real-Time PCR</th>
<th>Variant 1</th>
<th>Variant 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense</td>
<td>5′tgaggtgctgcttcatgcagtc3′</td>
<td>5′cctgacgcaacctcttc3′</td>
<td></td>
</tr>
<tr>
<td>Antisense</td>
<td>5′ggtgcttcatgcagtc3′</td>
<td>5′ggtgcttcatgcagtc3′</td>
<td></td>
</tr>
<tr>
<td>FAM labeled probe</td>
<td>5′cctgacgcaacctcttc3′</td>
<td>5′cctgacgcaacctcttc3′</td>
<td></td>
</tr>
</tbody>
</table>

Sequence design was based on database information with GenBank Accession Nos. NM_006932.3 (variant 1), NM_134269.1 (variant 2) and NM_134270.1 (variant 3).
Further investigations with immunohistochemical techniques demonstrated that smoothelin protein was present in the smooth muscle cells of the human bladder muscularis. Since the antibody recognizes the vascular and visceral isoforms, immunosignals were observed in the detrusor and in vessels (arteries and veins) (fig. 3). In detrusor smooth muscle immunoreactivity was strong and clearly defined but in some sample areas its distribution was slightly inhomogeneous in smooth muscle bundles.

Detrusor smooth muscle expresses 2 smoothelin isoforms. Since published smoothelin sequences indicate splicing in exon 20, RT-PCR primer sequences were chosen to potentially detect all 3 variants of known isoforms, as designated according to Rensen et al. Using the primers SMTN2s and SMTN1as 2 bands were amplified from the normally contractile bladder muscularis of 6 patients (fig. 4). Fragment size corresponded to the calculated size of variants 2 (364 bp) and 1 (196 bp). Sequencing these PCR fragments confirmed their complete homology to smoothelin sequences in the respective region. In none of the samples did we find a band of the expected size for variant 3 (265 bp) (fig. 4). To confirm the absence of variant 3 PCR with SMTN3s and SMTN1as was performed. In this setting the sense primer anneals to variants 2 and 3 but does not bind to variant 1. When investigating the same normally contractile bladders from

![Fig. 1. Alignment of transcripts of smoothelin vascular (B) and visceral (A) isoforms, and their variants (1 to 3) according to Rensen et al. Note location of SMTN primers for RT-PCR (top), and TaqMan® primers and probes for variants 1 (Tv1) and 2 (Tv2).](image1.png)

![Fig. 2. Nonradioactive Northern blot of 3 representative patients shows smoothelin transcript patterns in normally contractile detrusor samples. Note position of 2 ribosomal subunits.](image2.png)

![Fig. 3. Light microscopic immunohistochemistry shows normally contractile detrusor. Note dark, sometimes slightly inhomogeneous smoothelin immunosignals in smooth muscle bundles and discrete staining in vessel smooth muscle cells (arrows). Scale bar represents 50 μm.](image3.png)
the 6 patients as before, a single band corresponding to 283 bp of variant 2 was amplified, while no band of 187 bp (as calculated for variant 3) was visible (fig. 4).

However, in most detrusor samples 3 bands appeared when using primer pair SMTN1s and SMTN1as (fig. 5, E). The amplicons migrate according to the calculated size of not only variants 2 (580 bp) and 1 (415 bp), but also apparently variant 3 (484 bp). Nonetheless, the identity of the latter amplicon remained unclear since it failed to be re-amplified and sequenced. In addition, the same cDNA samples lacked amplification of variant 3 with SMTN2s and SMTN1as, although this primer combination has the potency to generate a variant 3 amplicon of 265 bp (fig. 5, E). Thus, variant 3 was excluded from real-time PCR studies.

Expression of smoothelin is up-regulated in the detrusor smooth muscle of patients with overactive bladder. Primers/probes used for quantitative analysis of smoothelin variant expression were designed according to sequence differences at their 3′ end. Thus, isoforms A and B could not be distinguished simultaneously due to sequence differences at their 5′ end. However, smoothelin A has been reported to be expressed in visceral smooth muscle cells only, whereas smoothelin B appears to be exclusively vascular.8 To confirm this observation in the human bladder we compared PCR patterns from whole detrusor with those from microdissected smooth muscle (fig. 4, A to D). From each sample amplicons for variants 1 and 2 could be amplified (fig. 5, E). In contrast, the use of primers specific for the vascular isoform resulted in amplification of a band only in the whole detrusor sample, while almost no such band was visible in dissected smooth muscle.

As a prerequisite for real-time PCR, we performed a series of controls (data not shown). RT-minus controls showed no amplification with TaqMan® chemistry, excluding reactions with genomic DNA. Efficiency curves in a range of 10,000-fold dilution demonstrated 99% efficiency for the housekeeping gene and smoothelin variant 1 or 2. The validity of β2-microglobulin as a housekeeping gene was confirmed since no statistically significant difference in its expression was observed among the sample groups.

Thereafter we investigated by quantitative PCR if the expression of smoothelin variants 1 and 2 was altered in laser microdissected smooth muscle of overactive vs normally contractile bladders. Some of the initial samples had to be excluded due to insufficient RNA quality, so that 5 samples per group were incorporated in this part of the study. In general smoothelin variant 1 appeared to be more highly expressed than variant 2 in detrusor with a mean C_{t} for variants 2 and 1 of 33.77 ± 0.44 and 33.02 ± 0.14 in controls, 32.9 ± 0.57 and 31.92 ± 0.78 for sensory urge, and 33.02 ± 0.15 and 31.77 ± 0.13 for detrusor instability, respectively. Comparison of normally contractile controls with sensory urge or detrusor instability samples showed that smoothelin expression was up-regulated in the patient groups (table 3). However, randomization tests of the relative expression software tool indicated that only the change in variant 1 achieved statistical significance. Between the sensory urge and detrusor instability groups no statistically significant difference in smoothelin variant expression was observed.

At the protein level the up-regulation of smoothelin in overactive bladders could be confirmed, although the antibody did not distinguish variants. By measuring the inten-
sity of smoothelin immunoreactivity in smooth muscle bundles we found a significant increase in smoothelin in the sensory urge and detrusor instability groups in comparison to that in individuals with a normally contractile bladder (mean 0.98 ± 0.24 and 1.27 ± 0.31 AU, respectively, vs 0.54 ± 0.089, fig. 6).

DISCUSSION

The high socioeconomic relevance of overactive bladder syndrome is widely appreciated. However, little is known about the patho-etiology of this condition. We focused on smoothelin, a protein thought to be involved in smooth muscle contractility. To our knowledge we present the first evidence of its change in bladder overactivity.

Using molecular biological and morphological methods we observed for the first time that the human bladder contains smoothelin. This extends a recent study in human bladder stromal cells, in which serum starvation resulted in smoothelin expression. In pigs the occurrence of smoothelin in the bladder had been shown by Western blot. Interestingly as found by Northern blotting in our study, smoothelin transcript patterns differ slightly from those published for other organs. The prominent band of about 1.9 kb in our study is comparable to previously published data that have been interpreted as being visceral smooth muscle derived, while the weaker, high molecular weight band in our blots represents the vascular smoothelin transcript(s). To our knowledge the presence of at least 2 further bands of lower molecular weight, as in our study, has not been shown before and their identities are undefined.

Using the smoothelin antibody smo2 Kramer et al observed the presence of low molecular weight bands on Western blot of porcine bladder extracts, which had been considered degradation products. However, in the context of our Northern data results this may indicate the occurrence of further and maybe bladder specific smoothelin (iso)forms.

The observed inhomogeneous distribution of smoothelin immunosignals in smooth muscle bundles may be related to uneven tissue fixation processes, although it may also reflect the functional status of smooth muscle cells with different contractile properties. As found in vessels in our study, smoothelin immunoreactions are in accordance with those in previous reports. They correspond to the vascular smoothelin mRNA found in our Northern blot and PCR studies.

Since little is known about the role of smoothelin splice variants, we included this topic in our investigation. In a previous report in mouse organs not including the bladder all 3 smoothelin splice products were detected by RT-PCR, except in the prostate, where only variants 1 and 2 were expressed. Thus, together with our data on the human bladder and corpus cavernosum (Maake et al, unpublished data) it may be speculated that in the human urogenital tract splice variants 1 and 2 are the major forms.

For quantitative studies we decided to use a microdissection based approach because samples inevitably contain a highly variant ratio of smooth muscle to connective tissue, which may lead to observations of changes in gene expression that are in fact attributable to different portions of smooth muscle in the sample. Furthermore, it was necessary to exclude the vascularized connective tissue because our chemistry techniques could not discriminate the vascular from the visceral smoothelin isoform simultaneously with its variants. By applying this methods to our knowledge we report for the first time that smoothelin transcription and translation increased in the detrusor smooth muscle of patients with sensory urge or detrusor instability compared to controls. However, the functional consequence of this expressional alterations is speculative since only limited information is available about the functions of smoothelin in vivo. A previous report showing up-regulation of uterine smoothelin during pregnancy may indicate that smoothelin levels directly mirror the potential of smooth muscle to contract. Our study provides evidence that approximately doubled smoothelin expression under pathological conditions may be associated with smooth muscle dysfunction.

Our data support the hypothesis that sensory urge and detrusor instability are related entities regarding their etiology. In this context increasing smoothelin levels may reflect an ongoing process of myogenic dysregulation. Although

**Fig. 6.** Fluorescence immunohistochemistry and confocal microscopy of smoothelin. Primary antibody was applied at low concentration to see gradual differences among sample groups. A, individuals with normally contractile bladder. B, patients with sensory urge. C, patients with detrusor instability. B and C, inhomogeneous distribution of smoothelin immunosignals are especially visible. Scale bar represents 25 μm.
to our knowledge the pathways are unknown, it may be hypothesized that the increase in smoothelin expression is related to symptoms of urgency and frequency in the 2 patient groups. However, it may well be that these symptoms are caused by factors other than smoothelin and its expression must pass a certain threshold until it results in functional consequences. Although the relatively lower smoothelin protein content in sensory urge compared to detrusor instability was statistically not significant, it may characterize an earlier stage of bladder overactivity, from which a trend of further smoothelin protein up-regulation in detrusor instability eventually leads to involuntary detrusor contractions.

To our knowledge our study is the first to separately investigate the quantitative expression of smoothelin variants. The higher mRNA expression of variant 1 with its significant increase in bladder overactivity may point to the prominent role of this splicing form, making it likely that the increase in smoothelin (total) protein is mainly due to the up-regulation of variant 1. This is of interest since alternative splicing in smoothelin occurs in a region where actin binding sites are coded, thus, probably resulting in proteins with distinguished contractile properties. The apparently altered translation ratio of mRNA transcripts may be a further component of disease development.

CONCLUSIONS

We provide further evidence that bladder overactivity is associated with changes in myogenic parameters. Among the factors that contribute to pathological contractility of detrusor smooth muscle cells smoothelin may be an interesting novel candidate. However, the changed ratio in expression of its variants in overactive bladders points to the complexity of its action. In support of the ICS classification of overactive bladder we propose that sensory urge and detrusor instability share common pathways and, thus, may represent different stages of a single etiological entity.

ACKNOWLEDGMENTS

T. Lehmann provided technical assistance.

Abbreviations and Acronyms

\[\begin{align*}
C_t & = \text{threshold cycle} \\
DIG & = \text{digoxigenin} \\
ICS & = \text{International Continence Society} \\
PCR & = \text{polymerase chain reaction} \\
RT & = \text{reverse transcriptase} \\
RT-minus & = \text{control RT reaction without enzyme}
\end{align*}\]

REFERENCES