Mechanical stretch regulates cell survival in human bladder smooth muscle cells in vitro

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Departments of ¹Surgery and ³Medicine and Therapeutics, Mater Misericordiae Hospital, Conway Institute of Biomolecular and Biomedical Research, University College, Dublin 7, Ireland; and ²Department of Reproductive and Surgical Sciences, Royal Victoria Infirmary, University of Newcastle, Newcastle-upon-Tyne NE2 4HH, United Kingdom

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Galvin, David J., R. William G. Watson, James I. Gillespie, Hugh Brady, and John M. Fitzpatrick. Mechanical stretch regulates cell survival in human bladder smooth muscle cells in vitro. Am J Physiol Renal Physiol 283: F1192–F1199, 2002. First published August 6, 2002; 10.1152/ajprenal.00168.2002.—Our understanding of the pathophysiology of the overactive bladder is poor. It has been proposed that localized contractions result in the abnormal stretching of bladder smooth muscle. We hypothesize that stretch regulates the cellular processes that determine tissue size. The purpose of this study was to investigate the effect of stretch on apoptosis, proliferation, cell hypertrophy, and growth factor production in human bladder smooth muscle cells in vitro. Normal human detrusor muscle was obtained from patients undergoing radical cystectomy for invasive bladder cancer, and primary cultures were established. Cells were mechanically stretched on flexible plates at a range of pressures and times. Apoptosis was assessed by propidium iodide incorporation and flow cytometry. Radiolabeled thymidine and amino acid incorporation were used to assess proliferation and cell hypertrophy. ELISA and RT-PCR were used to assess growth factor production. Mechanical stretch inhibits apoptosis in a time- and dose-dependent manner and was associated with increases in the antiapoptotic proteins heat shock protein-70 and c-IAP-1. Stretch also increases smooth muscle cell proliferation and hypertrophy, but hypertrophy is the more dominant response. These changes were associated with increases in IGF-1 and basic FGF and a decrease in transforming growth factor-β1. Mechanical stretch regulates apoptosis, proliferation, and cell hypertrophy in human bladder smooth muscle cells.

overactive bladder; apoptosis; heat shock proteins; hypertrophy

RECENTLY, THE PREVALENCE OF overactive bladder has been estimated at one in six of all adults aged >40 years (19). The effect of this is made all the more significant when the detrimental influence of bladder overactivity on all aspects of quality of life are taken into account (18). The pathological changes that occur in the overactive bladder are well characterized, consisting of detrusor smooth muscle (SM) hypertrophy, increased extracellular matrix production, and cholinergic denervation (4). These findings are considered common to most conditions leading to detrusor instability (4). Although there is a strong association among outlet obstruction (17), neurological disease (1), and bladder overactivity, little is known about the events that lead to these changes or the underlying mechanisms involved.

A theory by Coolsaet et al. (9) proposes that local bladder wall contractions, termed micromotions, which are synonymous with intrinsic activity, cause stretching of the adjacent SM (9). Not only may the sensation of bladder stretch initiate urgency, but in the presence of an overactive bladder, this spontaneous activity will spread throughout the bladder because of an increase in electromechanical coupling (12). Drake et al. (10) developed this concept further and described their model of peripheral autonomous modules. Essentially, a module is a circumscribed area of detrusor defined by its individual intramural bladder ganglia. Synchronized modular activity brings about bladder emptying, whereas unsynchronized activity causes stretching of the relaxed SM.

We hypothesize that mechanical stretch of bladder SM regulates detrusor size in bladder overactivity. We intend to establish primary human bladder SM cell (hBSMC) cultures, subject them to mechanical stretch, and assess their response in terms of apoptosis, proliferation, and cellular hypertrophy and to identify possible underlying mechanisms involved.

MATERIALS AND METHODS

Materials. RPMI-1640, penicillin and streptomycin solution, amphotericin B, t-glutamine, PBS, HEPES (1 M), and FCS were purchased from Gibco Life Technologies, Cambridge, UK). The following primary antibodies were used: α-SM actin, including fluorescently labeled antibody (Sigma); c-IAP-1 (R&D Systems, Oxford, UK); Bcl-2 (Signal Transduction Laboratories), and heat shock protein (HSP)27 and HSP70 (Stressgen Biotech). The secondary antibody used

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was mouse horseradish peroxidase conjugated (Signal Transduction Laboratories). Sigma supplied all other chemicals unless otherwise stated.

**Primary hBSMC culture.** Normal detrusor tissue was obtained from patients undergoing radical cystectomy for muscle invasive bladder cancer, with both ethical approval and the patients' consent. Tissue was isolated from macroscopically normal areas of bladders from five separate patients. Whole bladder was transferred to the Pathology Department, where a suitable full thickness biopsy of normal bladder was obtained and maintained in normal buffered culture media without additional oxygenation. This tissue was assessed and deemed normal by the attending pathologist and was then processed (within 30 min). The biopsy was washed three times with sterile PBS, the urothelial and serosal layers were dissected off, and the remaining muscle layer was chopped into small pieces that could be isolated and evaluated.

Cultured cells were not pooled because experiments were performed in triplicate. The detrusor is a unitary SM with slow-wave rhythm activity. Cultured cells were subjected to continuous 30-s cycles consisting of 20 s of stretch and 10 s of relaxation.

**Assessment of apoptosis and viability.** Once the stretch protocol was complete, cells were enzymatically removed (0.05% trypsin) from their collagen substrate and were centrifuged at 300 g for 10 min. Cells were then resuspended in 200 μl of culture medium containing FITC monoclonal antibody to α-SM actin (5 μl/1 × 10⁶ cells), incubated on ice for 15 min, and then centrifuged at 300 g for 6 min. Cells were then gently resuspended in 500 μl of hypotonic fluorochrome solution [50 μg/ml propidium iodide (PI), 3.4 mM sodium citrate, 1 mM Tris, 0.1 mM EDTA, and 0.1% Triton X-100; no Triton X-100 was included when assessing cell viability] and then stored in the dark at 4°C for 15–30 min before analysis with a Coulter XL cytofluorometer (Falcon/Becton Dickinson). A minimum of 5,000 events were collected and analyzed. Apoptotic cell nuclei were distinguished from normal nuclei by their hypodiploid DNA. The rates of apoptosis and viability in SM cells were calculated on the flow cytometer by obtaining the percentage of α-SM actin-positive cells that did strictly adhere to at all times, compared with the α-SM actin-positive cells that did (see Fig. 3).

**Western blotting.** Total cellular protein was extracted and pooled by using Nonidet P-40 (NP-40) protein isolation solution (0.5% NP-40, 10 mM Tris, pH 8.0, 60 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 10 mM PMSF, and 1 μM leupeptin, pepstatin, and aprotinin). Protein content was measured by the Bradford Assay protein assay kit (Bio-Rad). Western blotting was performed as previously described by using primary antibodies directed against α-SM actin, c-IAP-1, HSP27 and HSP70, and Bel-2 (8).

**Cytokine detection.** Cell culture supernatants were collected at the corresponding times and stored at −80°C. Cytokine production from control and stretched cells released into the supernatant was assessed with ELISA kits (R&D Systems). The manufacturer guidelines were strictly adhered to at all times. The corresponding cellular protein was also collected at the corresponding time points, and the results were expressed as picograms per microgram of protein.

**RT-PCR.** Total RNA was isolated from control and treated hBSMCs by using TRizol reagent (GIBCO Life Technologies) according to the manufacturer's protocol. The extracted RNA was dissolved in diethylpyrocarbonate-treated water and then quantified by measuring the absorbance at 260 nm. RNA (5 μg) was resolved on a 1% agarose gel and the endogenous control 18S rRNA was purfied (see Western blotting) and Western blot analyses were performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems). Cycling conditions were as follows: step 1, 2 min at 50°C; step 2, 10 min at 95°C; step 3, 15 s at 95°C; step 4, 1 min at 60°C; and repeat step 3, × 40 cycles. Probes were labeled with 5′ FAM and 3′ TAMRA as a quencher, with the exception of the ribosomal probe, which was labeled with 5′ VIC to facilitate dual reporter assay. The manufacturer's guidelines were strictly adhered to at all times.

Radiolabeled thymidine and amino acid studies. Radiolabeled [H]thymidine or leucine (0.25 μCi/1 × 10⁶ cells) was added to alternative wells 24 h before mechanical stretch. In the measurement of leucine incorporation, a modified protocol was used as described. In addition, the standard culture conditions were as follows: step 1, 2 min at 50°C; step 2, 10 min at 95°C; step 3, 15 s at 95°C; step 4, 1 min at 60°C; and repeat step 3, × 40 cycles. Probes were labeled with 5′ FAM and 3′ TAMRA as a quencher, with the exception of the ribosomal probe, which was labeled with 5′ VIC to facilitate dual reporter assay. The manufacturer's guidelines were strictly adhered to at all times.

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Fig. 1. Characterization of human bladder smooth muscle (SM) cells (hBSMCs). To confirm their SM cell phenotype, the cells were examined morphologically and by their protein expression. A: photomicrograph of hBSMCs in culture. Typical SM cell morphology is demonstrated. B: Western blot, as described in MATERIALS AND METHODS, demonstrating expression of α-SM actin and SM myosin and verifying that these cells are differentiated SM cells. Cultures were tested at each passage to confirm the expression of these proteins.

medium (not leucine-free) was used for these experiments. All culture plates, irrespective of their time point, were removed from stretch at the same time (t = 0) so that all cells were exposed to the radioisotope for the same period. Once stretching was complete, the culture medium was removed and each well was washed three times with PBS. Cells were lysed with 2% SDS solution, and the lysate was added to 9 ml of scintillation fluid (Ultima Gold, Sigma). Beta isotope emission was assessed by using a 2-min protocol on a beta counter (Packard, Tri-Carb).

Statistical analysis. Statistical analysis was carried out by using ANOVA-one-way ANOVA with Student’s-Newman correction. Significance was assumed for values of \( P < 0.05 \). Results are expressed as means ± SD.

RESULTS

Characterization of hBSMC cultures. Cultured cells were examined to confirm their SM cell origin and their differentiated state. Cell morphology demonstrated SM cell characteristics, namely, an ellipsoid shape with tapered ends and a single, centrally placed nucleus. Cells were tightly packed and formed a “hill and valley” appearance in culture (Fig. 1A). Western blotting confirmed expression of the SM cell markers α-SM actin and SM myosin on cell lysates derived from cells in each passage used (Fig. 1B). Flow cytometry demonstrates that ~99% of cultured cells were viable α-SM actin-expressing cells, suggesting minimal contamination (see Fig. 3).

Effect of mechanical stretch on apoptosis. hBSMCs had a high basal rate of spontaneous apoptosis, even in optimum conditions. Table 1 displays our initial rates of spontaneous apoptosis at the various time points and degrees of mechanical stretch. At the lower degree

### Table 1. Spontaneous rates of apoptosis of human bladder smooth muscle cells following mechanical stretch

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<tr>
<th>Time, h</th>
<th>Degree of Mechanical Stretch</th>
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<tr>
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<td>7.22%</td>
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<tr>
<td>0</td>
<td>27.8 ± 4.1</td>
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<td>3</td>
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<tr>
<td>6</td>
<td>28.4 ± 0.9</td>
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<tr>
<td>12</td>
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<tr>
<td>24</td>
<td>19.7 ± 1.22*</td>
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<tr>
<td>48</td>
<td>24.5 ± 1.05</td>
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Values are means ± SD. Statistical analysis was by ANOVA. This table displays the spontaneous rates of apoptosis of human bladder smooth muscle cell following mechanical stretch at 7.22 (40 cmH2O), 12.37 (80 cmH2O), and 18.47% (120 cmH2O) for the times indicated. Treated cells were enzymatically removed from their substrate and resuspended in 200 μl of culture medium containing FITC monoclonal antibody to α-smooth muscle actin (5 μl/l × 10⁶ cells) for 15 min. They were then centrifuged and gently resuspended in 500 μl of hypotonic fluorescein solution and stored in the dark at 4°C for 15–30 min before analysis with a cytometer. A minimum of 5,000 events were collected and analyzed. ND, not done. *P < 0.05.
of stretch, apoptosis is reduced at 24 h only. Mechanical stretch at 12.37% (80 cmH₂O) induced a time-dependent decrease in spontaneous rates of apoptosis (Fig. 2A). Spontaneous apoptosis was significantly decreased as early as 6 h (P = 0.026) and continued at 12, 24, and 48 h of stretch (P = 0.0036, 0.0001, and 0.015, respectively). Figure 2B confirms that there is no alteration in cell viability with mechanical stretch. Representative samples of the flow cytometry data are demonstrated in Fig. 3. At the higher degree of stretch, there was no significant change in apoptotic rates.

To identify a possible underlying mechanism for the changes seen in spontaneous apoptosis rates, we examined a range of antiapoptotic proteins. Figure 4A demonstrates alterations in the expression of cIAP-1, a member of the inhibitors of apoptosis family, and HSP70 but not HSP27. The expression of both cIAP-1 and HSP70 increase in a time-dependent manner in response to mechanical stretch. The regulation of HSP70 by stretch is not surprising, because mechanical deformation of cells may be perceived as a cellular...
significant increase at 3 h of stretch ($P = 0.002$), representing a 13.6% increase in proliferation compared with control. Maximal increases in proliferation were seen at 6 h of stretch ($P = 0.0001$), representing a 20.5% increase in proliferation. Thymidine incorporation remained significantly increased at 12 h ($P = 0.032$) compared with control.

Western blotting demonstrated a time-dependent increase in α-SM actin expression in hBSMCs in response to mechanical stretch (Fig. 6A). Actin is an important cytoskeletal and contractile protein, and alterations in its expression suggest a change in cell size and contractile function (14). Further evidence to suggest that mechanical stretch may increase cell size was provided by quantifying the relative incorporation of radiolabeled thymidine compared with leucine. Figure 6B demonstrates that mechanical stretch increases both thymidine and leucine incorporation into hBSMCs but that leucine is incorporated to a greater degree than thymidine and over a more sustained period. Leucine incorporation was increased by 25% at 6 h and by 75% at 24 h. This suggests that bladder SM cell hypertrophy is the dominant physiological process occurring in response to mechanical stretch at these parameters.

**Effect of mechanical stretch on growth factor production.** To study possible mediators for the alterations in apoptosis, proliferation, and hypertrophy, the effect of mechanical stretch on growth factor production was evaluated. Although hBSMCs are known to produce a number of cytokines, we focused on three growth factors known to mediate the effects of stretch in animal models (6, 24). The initial response of the SM cells in terms of growth factor production is to increase both IGF-1 ($P = 0.006$ at 6 h) and basic FGF (bFGF) levels ($P = 0.006$ at 3 h) in response to cellular stretch. IGF-1 (Fig. 7A) and bFGF levels (Fig. 7B) then become significantly reduced at 48 h. This reduction may be related to the limitations of the in vitro model established, wherein cells may adapt to long-term stretch. In contrast, TGF-β1 levels are seen to decrease significantly at 6 and 24 h ($P = 0.005$ and 0.02, respectively) in response to mechanical stretch (Fig 7C). There was a trend toward a recovery of the TGF-β1 levels as mechanical stretch is continued to 48 h, which again may reflect the ability of the cells to adapt to stretch.
Between a vascular and a bladder SM cell culture, the comparison of apoptotic rates and underlying mechanisms is well described (11). A direct comparison of SM cell apoptosis is demonstrated, because its role in vascular hBSMCs was not expected, because its role in vascular SM cells undergoing mechanical stretch. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vivo.

By using RT-PCR techniques, the alteration in TGF-β1 protein was demonstrated to be as a direct result of a decrease in TGF-β1 mRNA production in response to mechanical stretch (Fig. 7C2). VEGF is a potent angiogenic agent, and its production is increased in vascular SM cells undergoing mechanical stretch (22). The initial response of hBSMCs to mechanical stretch is to increase VEGF production (data not shown) at 3 and 6 h compared with control (350 ± 5.9, 531 ± 11.5, and 639 ± 0.2 pg/μg protein at 0, 3, and 6 h, respectively). This suggests that mechanical stretch may stimulate angiogenesis in SM cells.

**DISCUSSION**

Apoptosis, proliferation, and cell hypertrophy are the three processes that regulate tissue size. We have demonstrated, by using human bladder tissue, that mechanical deformation regulates all three processes in vitro. The accumulative effect in vivo would be increased tissue size. The inhibition of apoptosis in detrusor muscle by mechanical stretch has not been documented previously, although apoptosis has been shown to have a role in the progression of the hypertrophied rabbit bladder (23). The potential role of apoptosis in bladder overactivity may provide us with new therapeutic targets. The high basal rate of spontaneous apoptosis in our cells is in contrast to the low rates of apoptosis seen in vivo. This may be explained by the dynamic environment that the cells are subjected to in vivo. It is possible that by removing these cells from their dynamic environment in the bladder, we induce apoptosis, which is then reversed in our model of in vitro stretch.

The absence of the antiapoptotic protein Bcl-2 in hBSMCs was unexpected, because its role in vascular SM cell apoptosis is well described (11). A direct comparison of apoptotic rates and underlying mechanisms between a vascular and a bladder SM cell culture would help to clarify this situation. Other antiapoptotic mechanisms were examined. Mechanical stretch is a cellular stress, so it is not surprising that the expression of HSP70 is regulated by stretch. Similar results were obtained by Chen et al. (5) in their rabbit model of acute overdistension, suggesting that stretch may be the common mechanism in stimulating HSP70. We have also demonstrated the regulation of cIAP-1 by mechanical stretch. Although HSP70 inhibits the formation of the apoptosome (procaspase-9, Apaf-1, and cytochrome c), cIAP-1 is known to bind to and inhibit procaspase-3, a terminal effector cell death protease, and thus prevent cell death (25).

This study demonstrates that hBSMCs increase both proliferation and cell size in response to mechanical stretch. This is in agreement with a recent study by Orsola et al. (20), who also demonstrated that over a 24-h period, the increase in hBSMC size is greater than the increase in proliferation, at 12% stretch. They also examined the effect of 6 and 20% stretch and concluded that the decision to undergo proliferation or hypertrophy is dependent on the degree of stretch. Increased detrusor thickness is well described in the overactive bladder, but neither the stimulus nor the relative contributions of hyperplasia or hypertrophy are known in vivo (16). Karim et al. (15) have previously demonstrated both detrusor hyperplasia and hypertrophy in the obstructed guinea pig bladder and suggested a biphasic response, whereby hyperplasia precedes hypertrophy. Park et al. (21) have also demonstrated an increase in rat bladder SM cells in response to mechanical stretch. We also noted an early increase in proliferation and a more sustained hypertrophic response in our in vitro model of detrusor stretch. It was not possible to quantify a change in cell numbers in response to stretch because of the opaque culture well, but proliferation in the otherwise slow-growing...
hBSMCs was significantly enhanced by mechanical stretch. This study also demonstrates the regulation of growth factors in hBSMCs by mechanical stretch. bFGF is an SM mitogen with antiapoptotic effects and is upregulated early in the bladder’s response to distension (6). IGF-1 is also a SM cell mitogen with antiapoptotic effects, and its expression in obstructed rat bladder SM is increased (7). The increased production of both growth factors in response to mechanical stretch in human tissue may be mediating the alterations in apoptosis, proliferation, and hypertrophy in a paracrine or autocrine fashion, although inhibition studies would be needed to clarify this.

TGF-β1 is a multifunctional cytokine that stimulates fibroblast proliferation, regulates the synthesis of matrix components, stimulates SM cell apoptosis, and is regulated by bladder distension (6). We have demonstrated decreases in TGF-β1 at both the mRNA and the protein levels in response to stretch, and this may reduce its proapoptotic effects. The effect of mechanical stretch on VEGF production in vascular SM has been noted previously (22). We have demonstrated that mechanical stretch significantly increases VEGF production by hBSMCs. The regulation of VEGF by mechanical stretch in bladder SM does provide a possible mechanism for the increased microvasculature seen in hypertrophied SM (e.g., overactive bladder) (3, 13). It also suggests that mechanical stretch may have effects on other tissues in the bladder and that SM may mediate these secondary effects. In addition, VEGF has recently been demonstrated to be a survival factor in malignant cells (Bouchier-Hayes D., personal communication) and may even mediate the antiapoptotic effects of mechanical stretch. The accumulative effect of altered cytokine expression is increased cell survival and stimulation of cell number and size.

We have utilized a previously validated model for examining the effect of mechanical stretch on bladder SM cells (21). hBSMCs were maintained in culture up to their fifth passage, wherein they maintained their morphology, their proliferative capacity, and their SM marker expression. To provide a deformable matrix for the cells to adhere to, the cells were subcultured onto collagen (type 1)-coated elastomer membranes, similar to their in vivo situation. The negative pressure applied to the base of the well subjects the adherent cells to a reproducible degree of mechanical deformation. It is not known to what degree bladder SM is stretched in vivo, but the pressures applied to the base of the plates does correspond to clinical urodynamic parameters of intravesical pressure, and as mentioned previously, the detrusor possesses slow-wave activity, justifying the pressure and time variables selected.

In conclusion, mechanical stretch of bladder SM adjacent to contracting modules has been proposed as a mechanism for the overactive bladder. We have demonstrated, by using hBSMCs, that mechanical stretch does regulate processes central to tissue size, such as apoptosis, proliferation, and cell hypertrophy.

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