Initial Characterization of Exstrophy Bladder Smooth Muscle Cells in Culture

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Purpose: Previous studies have suggested that exstrophic bladder smooth muscle cells grown in culture show contractility similar to that of normal bladder smooth muscle cells. Despite this similar contractility, other cellular characteristics may vary between exstrophic and normal bladder smooth muscle cells.

Materials and Methods: Primary cultures of bladder smooth muscle cells were established from patients with bladder exstrophy (14) and vesicoureteral reflux as a control (10). Expression of smooth muscle specific α-actin and heavy chain myosin was determined with immunohistochemistry. Response of smooth muscle cells to high potassium Krebs solution or acetylcholine (0.1 mM) was assessed using a calcium sensitive fluorescent dye. Intracellular calcium concentration was measured after 48 hours in basal media. Cell migration in basal media during 24 hours was determined using transwell assays. Baseline proliferation and response to 10% fetal bovine serum were assessed with bromodeoxyuridine incorporation assays.

Results: More than 95% of exstrophy and control smooth muscle cells stained positive for actin and myosin. Functional integrity was verified in each exstrophy and control cell line by response to high potassium Krebs solution or acetylcholine. The intracellular calcium concentration was lower in exstrophy smooth muscle cells than in control smooth muscle cells (71 vs 136 nM, $p < 0.001$). More exstrophy cells migrated than control cells (37% vs 18%, $p = 0.004$). There was no statistically significant difference in proliferation between exstrophy and control smooth muscle cells in basal or growth media.

Conclusions: Cultured exstrophy smooth muscle cells demonstrate some differences in baseline characteristics compared to control cells. Differences in migration and intracellular calcium may have implications for in vivo detrusor function and tissue engineering.

Key Words: urinary bladder; bladder exstrophy; muscle, smooth; translational medical research

Abbreviations and Acronyms

ACh = acetylcholine

$[Ca^{2+}]_i$ = intracellular calcium concentration

CBE = classic bladder exstrophy

FBS = fetal bovine serum

HBSS = HEPES buffered saline solution

HCM = heavy chain myosin

KCl = high potassium Krebs solution

PBS = phosphate buffered saline

SMA = smooth muscle specific α-actin

SMC = smooth muscle cell

VUR = vesicoureteral reflux

Study received institutional review board approval.

Supported by award Number T32DK007552 from the National Institute of Diabetes and Digestive and Kidney Diseases. The content is solely the responsibility of the authors, and does not necessarily represent the official views of the National Institute of Diabetes and Digestive and Kidney Diseases or the National Institutes of Health.

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CLASSIC bladder exstrophy is a rare congenital birth defect occurring in the United States with a weighted incidence of approximately 1 in 50,000. The major clinical manifestations involve anomalous development of the bladder and bony pelvis, although other comorbidities are sometimes reported.[1] The function of the bladder as a continent reservoir is of major concern to parents and patients, with bladder capacity an important determinant in achieving eventual continence.[2] Although early successful primary closure is likely the most important modifiable factor contributing to improved

0022-5347/12/1884-1521/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2012.02.028
Vol. 188, 1521-1527, October 2012
Printed in U.S.A.

bladder capacity and compliance,[3] little is known about inherent tissue characteristics that differ between exstrophic and normal bladder smooth muscle.

Previous studies of cultured neuropathic and exstrophic SMCs used bladder biopsies of patients with VUR as a control.[4–6] Although initial studies found no significant phenotypic or functional differences between exstrophy and control SMCs,[5] later reports indicated sustained differences in cultured neuropathic SMCs.[7] Moreover, altered gene expression in cultured exstrophy SMCs suggested a distinct phenotypic difference inherent to the cells that remained evident after removal from the in vivo environment.[6]

Given the limited data available regarding the functional changes that occur in SMCs in the exstrophy complex, we performed an initial characterization of cultured exstrophy SMCs as a foundation for future research. We hypothesized that there would be significant differences in baseline cellular characteristics, including intracellular calcium concentration, migration and proliferation, of cultured exstrophy and control SMCs.

MATERIALS AND METHODS

Patient Selection and Establishment of SMC Cultures

Institutional review board approval was obtained for the acquisition of bladder biopsies from patients with CBE undergoing primary or secondary bladder closure, bladder neck reconstruction or augmentation cystoplasty, and from controls with VUR undergoing open ureteral reimplantation. Bladder biopsies were obtained from 19 children with CBE and 12 with VUR. Patient characteristics are presented in the table.

Bladder biopsies were obtained from the anterior wall or dome, and placed immediately in cold HBSS containing (in mM) 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose and 1.5 $CaCl_2$. The pH was adjusted to 7.2 with NaOH. Under microscopy, feather cuts were made to the detrusor to increase the surface area. The tissue was digested in low calcium (20 μM) HBSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml) and dithiothreitol (1 mM) at 37C for 20 minutes, cells dispersed in calcium-free HBSS, and plated on tissue culture plates in Dulbecco's Modification of Eagle's Medium (Cellgro®) with 1% penicillin-streptomycin and 10% FBS (HyClone®). Cells were used at passages 2 to 6 and placed into basal media (0.5% FBS) 24 hours before experiments.

Patient characteristics

	Control	Exstrophy	p Value
Mean ± SD age (mos) at biopsy	68.0 ± 10.0	51.0 ± 10.0	0.295
No. gender:			0.13
M	5	14	
F	7	5	
No. procedure:			
Ureteral reimplantation	12		
Newborn closure		4	
Secondary closure		6	
Bladder neck reconstruction		5	
Bladder augmentation		4	

Immunofluorescence

Cells were grown on glass coverslips, fixed with 10% formalin for 10 minutes at room temperature, washed with PBS, permeabilized with 0.5% Triton™-X and blocked with 20% goat serum. SMA and HCM expression was visualized using monoclonal antibodies against SMA (Sigma-Aldrich, St. Louis, Missouri) and HCM (Abcam, Cambridge, Massachusetts), and fluorescent secondary antibody (Cy3®). Cells were counterstained with the nuclear dye YO-PRO®. Random fields were examined to achieve a minimum total cell count of 100, and a ratio of SMA/HCM positivity-to-total cell count was obtained.

Intracellular Calcium Concentration and Functional Integrity

Cells were incubated with 7.5 μM Fura-2 AM (Molecular Probes®) for 60 minutes at 37C. Coverslips containing SMCs were mounted in a closed, heated chamber on the stage of an inverted microscope, and superfused with modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 0.57 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 10 glucose gassed with 16% O_2-5% CO_2 at 38C for 15 minutes before beginning experiments to remove extracellular dye. Ratiometric measurement of Fura-2 fluorescence was performed using light from a xenon arc lamp filtered at 340 and 380 nm and focused onto SMCs via a 20× fluorescence objective. Emitted light was returned through the objective and detected at 510 nm by an imaging camera. Protocols were executed and data collected online with InCyte software (Intracellular Imaging Inc., Cincinnati, Ohio). Intracellular calcium concentration was calculated from Fura-2 fluorescence ratios using an in vitro calibration curve. Measurements of basal $[Ca^{2+}]_i$ were obtained during 5 minutes, after which cells were stimulated with 0.1 mM ACh or KCl containing (in mM) 59.2 NaCl, 58.4 KCl, 0.57 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 10 glucose. Maximum response and average during 10 minutes of exposure were obtained.

Cell Migration

SMC migration was determined by the 2 methods of scratch (wound) motility assay and modified Boyden chamber (transwell). For the wound assay a linear scratch was made with a cell scraper across the diameter of the well and nonadherent cells removed by rinsing with PBS. Cells were incubated with 5 μl calcein and visualized via fluorescent microscopy. After images were obtained, cells were placed in low serum medium for 24 hours, reincubated with 2.5 μl calcein and images captured on an inverted microscope at 40× magnification. The transwell assay was performed using polycarbonate membrane chambers (8 μm pores) inserted into 12-well culture plates. Basal medium was added to both chambers, and 5,000 cells were added to the upper chamber and incubated for 24 hours at 37C. Adherent cells were washed