Platelet Derived Growth Factor Has a Role in Pressure Induced Bladder Smooth Muscle Cell Hyperplasia and Acts in a Paracrine Way

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Purpose: Bladder outlet obstruction is a finding in many urological disorders, leading to bladder wall hyperplasia. We investigated platelet derived growth factor and its receptor in human bladder smooth muscle cells and urothelial cells exposed to hydrostatic pressure or PDGF in vitro.

Materials and Methods: Bladder smooth muscle cells and urothelial cells were exposed to elevated hydrostatic pressure for 1 hour. The expression of PDGF and PDGFR was evaluated using reverse transcriptase-polymerase chain reaction and Western blot analysis. Pressure or PDGF induced proliferation of bladder smooth muscle cells with or without pretreatment with lovastatin or imatinib was measured by enzyme-linked immunosorbent assay. PDGFRα was knocked down with siRNA.

Results: After hydrostatic pressure bladder smooth muscle cells showed increased PDGFRα and β expression. PDGF was not expressed in bladder smooth muscle cells. Urothelial cells showed no expression of PDGFR but PDGF expression was noted. Western blot analysis of bladder smooth muscle cells revealed a pressure induced increase in PDGFR in the membrane fraction. Phosphorylation of PDGFR occurred with pressure induction. Bladder smooth muscle cell proliferation was increased in pressure and PDGF mediated fashion. Pretreatment with lovastatin or imatinib prevented proliferation. There was no cell proliferation after PDGFRα knockdown.

Conclusions: Increased expression and phosphorylation of PDGFR in bladder smooth muscle cells after hydrostatic pressure suggests a pivotal role of the PDGF pathway in pressure induced hyperplasia of bladder smooth muscle cells. PDGF expressed in urothelial cells may act in a paracrine way. Cholesterol depletion, inhibition of receptor tyrosine kinase activity and knockdown of PDGFRα in bladder smooth muscle cells prevent pressure and PDGF mediated cell proliferation. Targeting PDGFR seems a promising way to influence pressure induced bladder wall hyperplasia.

Key Words: urinary bladder; muscle, smooth; receptor, platelet-derived growth factor alpha; hyperplasia; gene knockdown techniques

Abbreviations and Acronyms

BOO = bladder outlet obstruction
BrdU = 5-bromo-2-deoxyuridine
BSMC = human bladder smooth muscle cell
DMEM = Dulbecco's modified Eagle's medium
FCS = fetal calf serum
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PDGF = platelet derived growth factor
PDGFR = PDGF receptor
pPDGFR = phosphorylated PDGFR
RT-PCR = reverse transcriptase PCR
TBP = TATA box-binding protein
UC = human urothelial cell
VSMC = vascular smooth muscle cell

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ELEVATED hydrostatic pressure secondary to BOO develops in many urological disorders. BOO often results in structural and functional changes in the detrusor muscle, which is considered partly a compensatory response to overcome resistance to bladder emptying.[1] Experimental infravesical

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obstruction shows a rapid, significant increase in the thickness of bladder smooth muscle and connective tissue.[2]

Several soluble mitogens have been implicated in smooth muscle cell proliferation, including PDGF. Ligand binding leads to activation of the PDGF receptors tyrosine kinase α and β via autophosphorylation.[3] It also triggers sequential activation of Ras/MAPK and PI3K/Akt pathways, which have a significant role in cell proliferation and differentiation.[4] Thus, PDGFR signaling can be inhibited by blocking PDGF ligand binding, eg using IMC-3G3,[5] or by impairing the cytosolic tyrosine kinase domain via administration of, eg AG1296,[6] or imatinib, one of the receptor tyrosine kinase inhibitors. Imatinib is an effective drug in the treatment of malignancies that was shown to have antiproliferative effects on vascular and pulmonary smooth muscle cells.[7,8]

Previous studies revealed that cholesterol enriched membrane microdomains such as caveolae and lipid rafts contain various growth factor receptors, eg PDGFR.[9,10] Disruption of caveolae and lipid rafts using 2-methyl-β-cyclodextrin inhibited PDGF-BB mediated proliferation of BSMCs, suggesting that these cellular domains are an essential locus of signaling processes relevant to urinary tract function.[11]

Additionally it was demonstrated that there is a direct link between increased expression of PDGFR and proliferation in response to hydrostatic pressure in VSMCs.[12] Furthermore, physical forces rapidly induce phosphorylation of PDGFR in VSMCs and BSMCs.[13,14] In BSMCs it was reported that mechanical forces[15] are also implicated in bladder wall hyperplasia and the serum growth factor PDGF-BB is a particularly relevant stimulator of mitogenic signaling.[11]

We focused on the proliferative effect of PDGF and elevated hydrostatic pressure on BSMCs and on its possible role in pressure induced bladder wall hyperplasia on the molecular level. Also, we examined the site of synthesis of PDGF in the human bladder. To attenuate PDGF or pressure induced BSMC proliferation we used statins and tyrosine kinase inhibitors.

MATERIALS AND METHODS

Primary BSMC and UC Isolation, Culture and Characterization

Biopsies were obtained from the bladder dome of pediatric patients undergoing ureteral reimplantation surgery. BSMCs were isolated and propagated as described previously.[16] Cells were maintained in RPMI Medium 1640 (Invitrogen™) supplemented with 10% FCS and 1% penicillin/streptomycin. UCs were collected by bladder washing from pediatric patients during cystoscopy.[17] After centrifugation the remaining cells were pooled and resuspended in complete keratinocyte serum-free medium (Invitrogen) containing 50 μg/ml bovine pituitary extract, 5 ng/ml human recombinant epidermal growth factor and 30 ng/ml cholera toxin (Sigma®). UCs were seeded in 25 cm^2 cell culture flasks coated with 500 μl collagen IV (Sigma). Cells were characterized via immunocytochemistry using antibodies, including mouse anti-human α-smooth muscle actin (1:50), mouse anti-human vimentin (1:100), mouse anti-human CD31 (1:50), mouse anti-human pan cytokeratin AE1/3 (1:50) and mouse anti-human cytokeratin 19 (1:50) (DakoCytomation, Hamburg, Germany). To detect PDGFR in BSMCs/UCs we used PDGFRα (D1E1E) (1:1,000) and PDGFRβ (28E1) (1:100) (Cell Signaling Technology®). For immunofluorescence we used fluorescein isothiocyanate conjugated IgG (1:320) as a secondary antibody.

Hydrostatic Pressure Experiments

A pressurized chamber based on the computer controlled Flexcell® Strain Unit enables the exertion of hydrostatic pressure in cells in vitro with dynamic airflow and a defined membrane extension regulated by spacers.[18] BSMCs and UCs cultured on collagen coated 6-well BioFlex® Culture Plates were placed in this pressurized chamber and subjected to a sustained hydrostatic pressure of 100 mm Hg (136 cm H_2O) for 1 hour at 37C. Membrane extension was fixed at 0 using a spacer inserted beneath the flexible membrane to restrict the effect to pure hydrostatic pressure. Controls were handled identically except for the exertion of pressure. All experiments were run in triplicate.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from BSMC/UC using TRIzol® Reagent according to the manufacturer protocol. Total RNA (2 μg) was reverse transcribed into complementary DNA using random hexamers (Roche Diagnostics, Penzberg, Germany) and BioFlex Culture Plates. The respective genes were PCR amplified with 40 ng cDNA, 500 nm forward and reverse primer, and iTaq™ SYBR® Green Supermix in a final volume of 20 μl. PCR reactions were run for 40 cycles consisting of 15-second denaturation at 95C, primer annealing for 15 seconds at 55C and extension for 20 seconds at 68C. The primer pairs (5′→3′ orientation) used were *PDGF-B* TGCTGCAACAACC GCAAC and GCTTCTTCCGCACAATCTCG, *PDGFRα* TGGATTGAACCCTGCTGATG and ATCAGCCTGCTT CATGTCCAT, *PDGFRβ* CACAATGACTCCCGTGGACTG and CATCATTAGGGAGGAAGCCCA, and *TBP* GCCCG AAACGCCGAATAT and CCGTGGTTCGTGGCTCTCT. RT-PCR amplifications were performed on a Mastercycler® Realplex^2 cycler. All experiments were done in duplicate. Expression of the housekeeping gene TBP was analyzed for normalization purposes. Relative quantification was performed using the ΔΔct method.[19]

Membrane Protein Extraction

Confluent monolayers were washed twice with PBS and covered with 1 ml ice-cold homogenization buffer composed of 1 mM ethylenediaminetetraacetic acid, Complete Protease Inhibitor Cocktail (Roche Diagnostics)