Dual sources of vitronectin in the human lower urinary tract: synthesis by urothelium vs. extravasation from the bloodstream

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Zhang D, Hudson AE, Delostrinos CF, Carmean N, Eastman R Jr, Hicks B, Hurst RE, Bassuk JA. Dual sources of vitronectin in the human lower urinary tract: synthesis by urothelium vs. extravasation from the bloodstream. Am J Physiol Renal Physiol 300: F475–F487, 2011. First published November 3, 2010; doi:10.1152/ajprenal.00407.2010.—Vitronectin (VN), secreted into the bloodstream by hepatocytes, is known to anchor epithelial cells to basement membranes through interactions with cell surface integrin receptors. We report here that VN is also synthesized by urothelial cells of urothelium in vivo and in vitro. In situ hybridization, deoxy sequencing, immunohistochemistry, and ELISA of urothelial cell mRNA, CDNA, tissue, and protein extracts demonstrated that the VN gene is active in vivo and in vitro. The expression of VN by urothelium is hypothesized to constitute one of several pathways that anchor basal cells to an underlying substratum and explains why urothelial cells adhere to glass and propagate under serum-free conditions. Therefore, two sources of VN in the human urinary bladder are recognized: 1) localized synthesis by urothelial cells and 2) extravasation of liver VN through fenestrated capillaries. When human plasma was fractionated by denaturing heparin affinity chromatography, VN was isolated in a biologically active form that supported rapid spreading of urothelial cells in vitro under serum-free conditions. This activity was inhibited by the matricellular protein SPARC via direct binding of VN to SPARC through a Ca2+-dependent mechanism. A novel form of VN, isolated from the same heparin affinity chromatography column and designated as the VN(c) chromatomer, also supported cell spreading but failed to interact with SPARC. Therefore, the steady-state balance among urothelial cells, their extracellular milieu, and matricellular proteins constitutes a principal mechanism by which urothelia are anchored to an underlying substrata in the face of constant bladder cycling.

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subjects aged 0–18 yr. Specimens were obtained from normal surgical explants of renal pelves, ureters, and bladders of tissues. The results provide the first evidence for VN gene protein distribution and gene expression in lower urinary tract disease, we undertook a study of VN understanding of VN's function and its potential involvement in clinical management of urinary tract disease. To improve our understanding of VN's function and a number of diseases and disorders that lying basement membrane can be related directly to urinary tract function and a number of diseases and disorders that include interstitial cystitis. Therefore, the study of the interactions between urothelial cells and extracellular matrix is significant and would be expected to lead to a greater understanding of urothelial cell turnover and function as well as the clinical management of urinary tract disease. To improve our understanding of VN's function and its potential involvement in lower urinary tract disease, we undertook a study of VN protein distribution and gene expression in lower urinary tract tissues. The results provide the first evidence for VN gene expression in urothelial cells.

**EXPERIMENTAL PROCEDURES**

**Human tissue.** Bladder or ureter mucosa was obtained from surgical explants of renal pelves, ureters, and bladders of subjects aged 0–18 yr. Specimens were obtained from normal tissue. Normal bladder tissue was obtained from vesicoureteral reflux surgical explants, which is considered to exhibit a normal phenotype (17). All specimens were obtained with informed consent and with the approval of the Institutional Review Board of Seattle Children’s Hospital.

**In vitro propagation of urothelial cells.** Cultures were grown according to previously described procedures (4, 10, 12, 29, 66). Cells also were cultured on Matrigel and small intestine submucosa (SIS), as described previously (30, 34). Briefly, immortalized human ureteric urothelial cells were placed on Transwell supports coated with Matrigel (BD Biosciences, San Jose, CA) or SIS gel (Cook Biotech, West Lafayette, IN), and MEM culture medium that contained 10% fetal bovine serum was added outside the Transwell to just below the level of the cell layer. SIS gel is a gel-forming product prepared from porcine small intestine submucosa and supports the normal phenotype. Cells were grown for 9 days. The Transwells were removed, and the cell layer and membrane were removed with a sharp scalpel, fixed, covered in agar to prevent loss during microtoming, mounted in paraffin, and later sliced as 5-µm sections.

**Preparation of tissue and cells for immunolight microscopy and in situ hybridization.** Surgical tissues were fixed with 4% paraformaldehyde, 50% ethyl alcohol, and 5% acetic acid and prepared as described previously (66). Cultured urothelial cells on slides were fixed, washed, and dehydrated with a series of ascending concentrations of alcohol and stored at 4°C. Slides were used directly for immunostaining or in situ hybridization experiments after rehydrating.

**Immunostaining of VN by colorimetric light microscopy.** Paraffin was removed from tissue sections with three 15-min changes of xylene substitute (Sigma, St. Louis, MO). Sections were rehydrated in graded concentrations of ethyl alcohol, then in water, and subsequently blocked by incubation for 1 h with 1% (wt/vol) bovine serum albumin (BSA) (fraction V; Sigma) in TBST [10 mM Tris·HCl, pH 7.5, 250 mM NaCl, and 0.3% (vol/vol) Tween-20]. Sections were incubated for 4 h with primary antibody (polyclonal rabbit anti-human VN serum, no. 681125; Calbiochem, San Diego, CA) in the blocking solution at 1:500 dilution. Two types of controls were included: 1) omission of the primary antibody on parallel slides and 2) incubation with normal rabbit serum at the same dilution as the primary antiserum. After washing with TBST, sections were incubated for 1 h with 12.7 µg/ml of secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase, no. A3937; Sigma) in the blocking solution. Sections were washed with TBST and then developed with substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT; Roche Diagnostics, Indianapolis, IN) at pH 9.5. After counterstaining with hematoxylin and coverslipping, images were captured at image resolutions of 1,300 × 1,030 pixels with a DC200 digital camera (Leica Microsystems, Wetzlar, Germany) mounted on a Leica DMR light microscope equipped with N-plan objectives with the following magnifications: ×20 air (0.40 numerical aperture), ×40 air (0.65 numerical aperture), and ×100 oil (1.25 numerical aperture). The software used to acquire images was the Leica DC Tlain 4.1.8.0 import function of Photoshop 7.0.1 (Adobe). This software was also used to resize, crop, and label images for the figures. All postacquisition processing was performed the same way for all members of a given data set. For cultured urothelial cells,
frozen pellet of a urothelial cell culture was thawed, lysed with
were read at 450 nm on a Bio-Tek Powerwave XS plate reader.
were washed three times with the wash buffer provided. Primary
an insoluble cellular fraction. Western blot analysis of VN from cultured urothelial cells. Cultures of bladder or ureter urothelial cells and liver HepG2 cells were grown in T175-cm² flasks or in T150-cm² plates. Cultures of bladder or ureter urothelial cells and liver HepG2 cultures of bladder or ureter urothelial cells and liver HepG2 were read at 450 nm on a Bio-Tek Powerwave XS plate reader. 

Stained sections were cut and mounted on 200 mesh nickel grids for immunogold labeling, as described previously (66). After incubation with rabbit primary antiseraum, bound VN-IgG complexes were detected with goat anti-rabbit IgG tagged with 10-nm gold particles (cat. no. 25365; Electron Microscopy Sciences, Hatfield, PA). Washed and dried grids were fixed with 2% (wt/vol) osmium tetroxide (Electron Microscopy Sciences) for 6 min and sequentially stained with 2% (wt/vol) uranyl acetate (Electron Microscopy Sciences) for 5 min and with 0.25% (wt/vol) lead citrate (Electron Microscopy Sciences) for 7.5 min. After a triple wash with H₂O, grids were dried, visualized, and photographed with a Zeiss 910 transmission electron microscope (Carl Zeiss, Thornwood, NY). Acquisition of images was accomplished with a Soft Imaging System Mega View III digital camera running under analysis FIVE software (Zeiss).

ELISA analysis of VN in human cell culture fractions. Cultures of bladder or ureter urothelial cells and liver HepG2 cells were grown in T175-cm² flasks or in T150-cm² plates. Conditioned medium was collected and concentrated via Centricon-10 (Millipore, Bedford, MA) centrifugal devices at 3,000 g for 40 min. Scraped cells were treated with either M-PER reagent according to the manufacturer’s instructions (Pierce, Rockford, IL) or lysis buffer [150 mM NaCl, 1.5 mM MgCl₂, 0.65% (vol/vol) NP-40, 10 mM Tris·HCl (pH 8.0), 25 mM sodium vanadate] that contained 1 × protease inhibitors (Complete; Roche). After 30 min on ice, cells in lysis buffer were centrifuged at 1,000 g for 50 min to yield a soluble cellular fraction and an insoluble cellular fraction.

An ELISA assay kit (Molecular Innovations, Southfield, MI) was used for detection of human VN. Stock human VN, rabbit anti-human VN IgG polyclonal antibody (primary), and goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody (secondary) were all provided, as was the blocked Immulon-2 96-well plate that contained dried anti-human VN capture antibody. The primary and secondary antibodies were diluted according to the manufacturer’s instructions in blocking buffer [3.0% BSA in 10 mM Tris·HCl (pH 7.5), 150 mM NaCl]. Standards and samples were added to the plate at a volume of 0.1 ml and incubated at ambient temperature for 30 min with gentle shaking. The fluid was aspirated, and the wells were washed three times with the wash buffer provided. Primary antibody (0.1 ml) was added to each well and incubated at ambient temperature for 30 min with gentle shaking. After three washes, 0.1 ml of secondary antibody was added to each well and incubated at ambient temperature for 30 min. The wells were washed three times again, and 0.1 ml of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and incubated at ambient temperature for 5 min. The absorbance values were read at 450 nm on a Bio-Tek Powerwave XS plate reader. Western blot analysis of VN from cultured urothelial cells. A frozen pellet of a urothelial cell culture was thawed, lysed with 2× sample buffer [0.125 M Tris·HCl (pH 6.8), 4% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.1 M DTT] that contained protease inhibitors (Complete; Roche), fractionated by a 12% polyacrylamide electrophoretic gel that contained 0.1% SDS, electrotransferred to a nitrocellulose membrane, and detected by Western blot procedures. The membrane was wet with TBST [10 mM Tris·HCl (pH 7.5), 500 mM NaCl, 0.3% (vol/vol) Tween-20], blocked by incubation for 2 h with TBST that contained 1% (wt/vol) bovine serum albumin (fraction V; Sigma), and then incubated for 4 h with the primary antibody rabbit anti-human VN serum (Calbiochem) at 1:1,000 dilution in the blocking solution. The membrane was washed with TBST for four changes, 10 min for each change. Following these washes, the membrane was incubated for 1 h with the secondary antibody, a goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma), at a 1:5,000 dilution in blocking solution. The membrane was washed four times with TBST and developed with substrates NBT and BCIP at pH 9.5.

For urothelial cells cultured in SIS, the cells and SIS piece were mashed at frozen temperature (liquid nitrogen), extracted with the SDS sample buffer containing protease inhibitors (Complete; Roche), and separated by running a 12% PAGE gel. Two control lanes (SIS only) were loaded at the same volume and the same amount of protein as SIS plus cell sample (25 µg/lane). The above Western blot procedures were followed, except that the secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Resultant signals were visualized by enhanced chemiluminescence.

In situ hybridization. A human VN clone (pOTB7-Vn, clone no. 4040317) was purchased from Invitrogen (Carlsbad, CA). Plasmid DNA was isolated, and the insert identity was confirmed byideoxy sequencing. The plasmid was linearized with EcoRI and XhoI (Fermentas, Hanover, MD). After digestion was confirmed by electrophoresis through a 1% (wt/vol) agarose gel, contaminants were removed from linearized plasmids by extraction with phenol-chloroform, and DNA was collected by precipitation with ethyl alcohol. Sense and antisense single-stranded RNA probes were synthesized from SP6 and T7 promoters, respectively, with a ribonucleotide mixture containing digoxigenin-11-UTP (DIG-RNA Labeling SP6/T7 Kit; Roche). Concentrations of the synthesized probes were analyzed by following the manufacturer’s procedures.

Slides with tissue sections were dewaxed with xylene substitute. Dewaxed tissue sections and slides with cultured cells were rehydrated with a graded series of ethyl alcohol made with water treated with diethylpyrocarbonate and treated for 20 min with 100 µg/ml proteinase K (Roche) at 55–60°C. Prehybridization was performed for 4 h with a solution that contained 50% (vol/vol) deionized formamide, 4 × standard saline citrate (SSC; 600 mM NaCl, 60 mM sodium citrate, pH 7.0), 5% dextran sulfate, 1 × Denhart’s solution (Sigma), 0.2 mg/ml salmon sperm DNA (Invitrogen), and 100 U/ml RNase inhibitor (Roche) at 58–60°C. Specimens were incubated for 18 h with 1 µg/ml probe in prehybridization solution at 58–60°C. Unbound probes were removed by a series of extensive washes with 2 × SSC at room temperature and with 2 × SSC that contained 50% (vol/vol) formamide at 60°C. A final series of washes was performed with 1 ×, 0.25 ×, 0.1 ×, and 0.05 × SSC at room temperature. The slides were blocked
with Blocking Solution (Roche) for 30 min and incubated for 1 h with 3.75 U/ml anti-digoxigenin IgG conjugated to alkaline phosphatase (Roche). Signals were visualized by color development in the same manner as the immunostaining above.

**Analysis of VN mRNA by RT-PCR.** Reverse transcription of mRNA and amplification of VN cDNA by PCR was used to further determine the extent of VN expression by urothelium and cultured urothelial cells. Total RNA was recovered from both tissue and cultured cells by using the Micro-to-Midi Total RNA Purification System (Invitrogen). After dissection away from the lamina propria, bladder urothelium was incubated in 0.6 ml of cell lysis buffer (provided in the kit) plus 250 U/ml RNase inhibitor (Roche) and 1% β-mercaptoethanol. After mixing with one volume of 70% ethanol, the extract was loaded into a cartridge and centrifuged at 12,000 g for 15 s. Flow-through was discarded, and the cartridge was washed once with wash buffer 1 and twice with wash buffer 2. Total RNA was eluted using RNase-free water and diluted to 50 ng/μl for RT-PCR use. Cultured cells were lysed directly via the same RNA isolation procedures. Total RNA was treated with RNase-free DNase (Roche); the enzyme was inactivated by heat and removed by phenol-chloroform extraction and ethanol precipitation. Specific oligonucleotide primer pairs were designed across introns according to the published sequences for human VN (GenBank accession no. NM_000638). The oligo primers used for RT-PCR and subsequent sequencing (“primer walking”) are listed in Table 1. The RT-PCR reaction solution was prepared with 2× buffer that contained deoxyribonucleotides and an enzyme mixture that contained reverse transcriptase and Taq polymerase (SuperScript III One-Step RT-PCR System; Invitrogen). The reaction conditions were 30 min at 55°C for cDNA synthesis and 2 min at 94°C for denaturation followed by 36 cycles of 94 (15 s), 60 (30 s), and 68°C (1.75 min). RT-PCR products were fractionated by electrophoresis on 12% agarose gel and stained with ethidium bromide staining with UV light. To increase the product signal and amount for sequencing, the products were further amplified by PCR. Products were verified by dieoxy sequencing on a 377 DNA Analyzer (Applied Biosystems, Foster City, CA).

**Isolation of VN from human blood plasma.** VN was isolated from outdated human blood plasma (Puget Sound Blood Center, Kent, WA) by heparin affinity chromatography (21, 64) with the following modifications. Plasma proteins were applied to a chromatography column that contained heparin affinity resin, and the flow-through fraction was collected. To this flow-through fraction, urea was added to a final concentration of 8 M and mixed at room temperature for 1 h. Denatured proteins were applied to a fresh heparin column, which was preequilibrated with 0.15 M NaCl, 8 M urea, 0.1 M sodium phosphate (pH 7.7), and 0.005 M EDTA. The column was then washed with 10 column volumes of 0.15 M NaCl, 8 M urea, 0.1 M sodium phosphate (pH 7.7), and 0.005 M EDTA; the VN chromatomer was sequentially eluted with 0.5 M NaCl, 8 M urea, 0.01 M sodium phosphate (pH 7.7), and 0.005 M EDTA. Samples were dialyzed against 1× Hanks’ Balanced Salt Solution that contained 0.01 M HEPES (pH 7.4) prior to storage at −20°C. The isolation of VN and VN(c) was successfully repeated with a separate lot of outdated plasma. The apparent molecular weights (Mr) of VN and VN(c) were calculated via Rf analysis of electrophoretic mobilities relative to unstained or prestained molecular weight protein standards in 10% polyacrylamide electrophoretic gels that included 0.1% (wt/vol) SDS. Proteins were stained with Coomassie Brilliant Blue R250 after electrophoresis.

**Mass spectrometry of VN isoforms isolated from human plasma.** Plasma VN and its VN(c) chromatomer were fractionated by SDS-PAGE (12%) and stained with CuCl2. Stained bands were excised, treated with trypsin, and subjected to tandem mass spectrometry. The masses of resultant peptides were searched by the Mascot search engine (Matrix Science, Boston, MA) against entries of the SwissProt version 46.4 database (UniProtKB/Swiss-Prot, Cambridge, UK).

**Recombinant proteins.** Recombinant (r), wild-type SPARC and its third extracellular Ca\(^{2+}\)-binding domain (“rEC”) were expressed in Erichisia coli, isolated by nickel-chelate affinity chromatography, and renatured into biological active forms, as described previously (6, 12).

**SPARC-VN binding assay.** Maxi-sorp 96-well plates (Nalgene/Nunc, Rochester, NY) were coated with 5 μg/ml rsPARC (wild-type or rEC) in coating buffer [15 mM NaCO3-35 mM NaHCO3 (pH 9.6)] for 2 h at 37°C. Wells were then blocked with 3% BSA (wt/vol) in TBS [10 mM Tris-HCl (pH 7.4), 150 mM NaCl] for 1 h. Selected wells were washed twice for 15 min each with one of the following concentrations of EDTA in water: 0, 0.1, 0.25, 0.5, 2.5, or 10 mM. VN was then added at 5 μg/ml in binding buffer [0.2% (wt/vol) BSA in TBS with 0.1% (vol/vol) Tween-20] and incubated for 2 h. Included in the binding buffer of each condition was one of the following concentrations of CaCl2: 0, 0.25, 0.5, 1.0, 2.0, or 5 mM. Wells were briefly washed with TBS containing 0.1% Tween-20 prior to incubation of a mouse monoclonal antibody specific for VN (0.1 μg/ml; Abcam, Cambridge, MA) for 16 h at 4°C. After washing with TBS containing Tween-20, a goat anti-mouse secondary antibody conjugated to hors eradish peroxidase (dase: 1:10,000 dilution; Jackson ImmunoResearch) was added. Wells were incubated with the secondary antibody for 1 h and washed, and 100 μl of TMB substrate solution (Vector Laboratories, Burlingame, CA) was added for colorimetric development. The reaction was stopped with 50 μl of 1 N sulfuric acid, and the plate was read at 450 nm with a Bio-Tek

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**Table 1. Oligonucleotide PCR primers for sequencing of urothelial cell VN**

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<td>Forward</td>
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<tr>
<td>Exon 1</td>
<td>5'-GGCTGTCTCGACTTGCTGGG-3'</td>
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<tr>
<td>Exon 1</td>
<td>5'-CTAGGGAGGGATCAGG-3'</td>
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<td>Exon 2</td>
<td>5'-ATGCTGAGGAGCC-3'</td>
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<td>Exon 2</td>
<td>5'-CTAGGGAGGGATCAGG-3'</td>
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<td>Exon 3</td>
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<td>Exon 5</td>
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<tr>
<td>Exon 5</td>
<td>5'-GACGTTGGCAAGGCAG-3'</td>
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<tr>
<td>Exon 8</td>
<td>5'-AATCGCTAGAGGAGCC-3'</td>
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VN, vitronectin. *The RT-PCR products generated with these primers are shown in Fig. 6.*
Powerwave XS plate reader. Controls included each of the following conditions: omitting the SPARC coating, omitting VN, omitting both proteins, omitting primary antibody, and directly coating the well with VN to test IgG affinity.

Assay of cell spreading. Two types of cells were used in these assays. Normal urothelial cells were propagated from human ureter and used as primary cultures (4). From these parental cultures, cells were immortalized by transformation with human papillomavirus type 16 E6/E7, as described previously (10). After 96-well tissue culture plates were coated with 50 μg/ml VN or VN(c), cells were seeded at 5 × 10⁴ cells/well and incubated at 37°C with 5% CO₂. The serum-free culture medium was DK-SFM (Invitrogen). Each condition was run in quadruplicate. The plate was removed at each quarter-hour for the first hour and then subsequently at 2, 4, and 6 h, and a random field in each well was photographed. Photographs were scored for cell shape by placing each visible cell into one of three categories: 1) highly refractile round cells, 2) partially spread or moderately refractile cells, or 3) fully spread cells with a cobblestone appearance. A rounding index (RI) number was assigned to each condition using the following formula (5): RI = [(3xa) + (2xb) + (1xc)]/(a + b + c). Therefore, a condition with a RI value of 3 would contain only round refractile cells, whereas a RI value of 1 would indicate only fully spread cells.

An additional cell-spreading assay was run in 24-well plates in which wild-type recombinant SPARC (5) in 1× Hanks’ Balanced Salt Solution that contained 10 mM HEPES (pH 7.4) was added to DK-SFM (Invitrogen) medium in concentrations of 0, 5, 10, 25, or 50 μg/ml each in triplicate. Cells were seeded at 2 × 10⁴ cells/well and incubated at 37°C with 5% CO₂. The plate was removed at 2, 4, and 6 h for analysis and calculation of the RI.

Statistical analysis. For RIs, after the index of each well was calculated (n = 4), the mean ± SE was reported. For titration experiments between SPARC and VN, the mean ± SE (n = 3) was reported.

RESULTS

Distribution of vitronectin in the urinary bladder wall. Immunostaining with VN-specific IgG revealed the presence of the protein throughout the bladder wall including the urothelium, lamina propria and smooth muscle layer (Fig. 1). The distribution of immunoreactive signals was not even in that the loose connective tissue underneath the urothelium, termed here as the superficial lamina propria, exhibited weak to absent signals compared with other areas. In contrast, the superficial, intermediate, and basal layers of the urothelium each displayed strong immunoreactive signals (Fig. 1B). The lack of immunoreactivity in the superficial lamina propria suggests a demarcation between VN in the urothelium and VN extravasated from the bloodstream.

The immunoreactive signals for VN in smooth muscle bundles were considered to be remarkable since two patterns of signals were observed. Small, punctate regions ~1–3 μm in diameter were observed on the periphery of all smooth muscle cells (Fig. 1D, red arrows). Larger focal regions ~3–10 μm in diameter were observed at the ends of smooth muscle cell fibers (Fig. 1D, yellow arrowheads). Therefore, the distribution of VN in smooth muscle cells is consistent with the protein serving an adhesive function to maintain tissue integrity in the face of constant bladder cycling. Smooth muscle cells surrounding blood vessels also exhibited the same small punctate and larger focal regions of VN immunoreactivity as did regions distal from blood vessels (not shown).

Distribution of VN in the bladder mucosa. Immunoreactive VN signals in the bladder mucosa were consistently observed to fall into three categories: 1) distinct patterns in the urothelium, 2) absent or very weak signals in the superficial lamina propria, and 3) intense signals in the lamina propria, especially around blood vessels. Figure 2B displays these three categories.

A pattern of strong immunoreactivity was observed at the interface between basal cells and their underlying basement membrane in a manner consistent with VN-containing focal adhesions that comprise a principal means by which basal cells anchor urothelium to an underlying substratum (Fig. 2D, black arrowheads). Additional intense signals were observed at the interface between the 1) apical face of basal cells and the basal face of intermediate cells and 2) the apical face of intermediate cells and the basal face of superficial cells (Fig. 2D, red arrowheads). This pattern was also retained in ureteric urothelium but with additional immunoreactivity along the basolateral faces of intermediate cells (not shown). The apical face of the superficial layer often exhibited a continuum of distinct immunoreactive signals (Fig. 2B, red arrowheads).

On occasion, a VN-positive capillary was observed at the urothelial basement membrane (Fig. 2B, red arrows) in a manner consistent with a role in supporting the adhesion of the endothelial cell. In most cases, however, tissue surrounding

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Fig. 1. Distribution of vitronectin (VN) in the bladder wall. Shown are immunoreactive signals derived from complexes of VN IgG (brown) and hematoxylin-nucleic acids (blue). A and C: controls in which primary antibody rabbit anti-human VN was omitted. B and D: antibody treatments. A and B are images with low magnification, and overall VN distribution can be seen in urothelium and lamina propria. C and D show VN in smooth muscle tissue (SM). L, lumen; U, urothelium; LP, lamina propria; SLP, superficial lamina propria. Bar = 80 μm in A and B and 20 μm in C and D. Red arrows, punctate regions ~1–3 μm. Yellow arrowheads, focal regions ~3–10 μm.
blood vessels exhibited strong VN signals, whereas the bloodstream, the source of liver VN, showed little to weak variable signals (Fig. 2B). The specificity of the antibody is such that it recognizes mainly the activated multimeric matrix form as opposed to the inactive monomeric form found in blood (Fig. 2B). This observation demonstrates that VN undergoes an extravasation-dependent conformational conversion from a monomeric to multimeric configuration upon trafficking from the vascular lumen into the subendothelial matrix.

A zone of diminished or negative VN immunoreactivity in the superficial lamina propria. Weak to nil VN signals in the superficial lamina propria of the ureter also formed a zone of diminished immunoreactivity that persisted in the bladder (Figs. 1B and 2B). This zone was common in many specimens and likely represents a demarcation to separate urothelial derived from liver-derived VN. This phenomenon was not artifactual with respect to fixation or immunohistochemical procedures because abnormal bladder specimen, classified as prune belly syndrome and processed in parallel with all specimens in this study, served as an important negative control in that this specimen was devoid of VN immunoreactivity (data not shown).

Detailed visualization of VN in the bladder mucosa by electron microscopy. Immunoelectron microscopy with secondary antibodies conjugated to gold particles revealed additional information about the distribution of VN on both sides of the urothelial basement membrane. Immunoreactive signals were detected in urothelial cells and basement membranes (Fig. 3, B and C).

The control experiment of Fig. 3A lacked gold particles because primary antibodies were omitted. In Fig. 3B, the superficial lamina propria was also devoid of gold particles but for a different reason; the superficial lamina propria is devoid of VN (black arrow). The lack of detectable immunoreactive VN in the superficial lamina propria was a finding confirmed by the immunohistochemical data of Figs. 1B and 2B. Signals for VN were observed in the basement membrane and in urothelial cells, suggesting that VN was secreted in a polarized manner at this location. In Fig. 3C, a black arrowhead indicates the intense VN signal deposits at the interface between the

Fig. 2. Distribution of VN in bladder mucosa. Shown are immunoreactive signals derived from complexes of VN-IgG (brown) and hematoxylin-nucleic acids (blue) in urothelium from normal bladder. A and C: no primary antibody controls. B and D: antibody treatments. BV, blood vessel. See Distribution of VN in the bladder mucosa for arrowhead designations. Bar, 20 μm.

Fig. 3. Immunoelectron microscopy localized VN to cells and cell-cell and cell matrix interfaces. Shown is a micrograph derived from a ureteric specimen that included urothelial cells (U or UC), basement membrane (BM), and LP. Black dots represent VN-IgG complexes that were detected with secondary antibodies conjugated to 10-mm-diameter gold particles. A: no primary antibody control. B and C: antibody treatments that show VN localization on basement membranes and in basal urothelial cells (UC).
with urothelial cells synthesizing and secreting their own to attach and spread on glass, a finding that is inagreement human urothelial cells exhibited a robust and repeatable ability logs (data not shown). Cultured in the absence of serum, used were determined to be specific for human VN and did not human urothelial cells grown in vitro (Fig. 4). The antibodies sion of immunoreactive signals for VN was examined in in vivo exhibit marked and consistent signals for VN. It is noted that VN was also detected in immortalized cultures of urothelial cells (Table 2) but not in the commercial unconditioned medium in which cells were grown (data not shown).

The apparent molecular size of urothelial VN was assessed by SDS-PAGE, Western immunoblotting, and subsequent Rf analysis. Normal urothelial cells plated on glass exhibited two protein bands that correlated with molecular sizes of 77 and 71 kDa, in close agreement with isoforms that we isolated from plasma (Fig. 7A).

Two bands of slightly smaller molecular size were obtained when urothelial cells were grown within the three-dimensional SIS scaffold (Fig. 7A); these two bands were also recognized by mouse anti-human VN IgG.

In situ hybridization detection of VN mRNA in urothelial cells. To further confirm the expression of the VN gene in urothelial cells, the detection of VN mRNA was accomplished by in situ hybridization techniques. Urothelial cells in vitro exhibited marked hybridization signals at the nuclear periphery in a manner consistent with ribosomal localization (Fig. 5C).

Strong hybridization signals were also observed for urothelial cells of urothelium (Fig. 5F). Signals were either absent or barely above background in the lamina propria. The small punctate dot signals in Fig. 5E are derived from hybridization of the sense probe with the chromosomal VN gene locus. The collective evidence indicates that urothelial cells synthesize VN both in vivo and in vitro.

Dideoxy sequencing of VN mRNA from urothelial cells in vivo and in vitro. VN mRNA from urothelium and urothelial cell cultures was isolated, reverse transcribed, and amplified by PCR. The sizes of resulting PCR-DNA products matched the predicted sizes of 252 and 520 bp (Fig. 6A). Because the location and sequence of the oligonucleotide primers were designed to cross intron/exon borders, the observed sizes indicate that the PCR-DNA products were amplified from

The amount of VN synthesized and secreted by cells in culture was determined by an ELISA antibody sandwich assay (Table 2). Normal urothelial cells synthesized and secreted VN into the conditioned medium at levels ~200 times less than liver HepG2 cells did, reflecting discrete biological functions of these two cell types. Liver hepatocytes function to synthesize and secrete a variety of proteins, including VN, into the bloodstream. Therefore, it is consistent that HepG2 cells derived from hepatocellular carcinoma hepatocytes would synthesize and secrete abundant quantities of VN in vitro. In contrast, urothelial cells would not be expected to secrete abundant quantities of VN from the apical or lateral plasma membranes but instead secrete the adhesive protein through the basal plasma membrane into the extracellular matrix (Table 2).

It is noted that VN was also detected in immortalized cultures of urothelial cells (Table 2) but not in the commercial unconditioned medium in which cells were grown (data not shown). The collective evidence indicates that urothelial cells synthesize VN both in vivo and in vitro.

![Image](http://example.com/image.jpg)

**Distribution of VN in urothelial cells in vitro.** The expression of immunoreactive signals for VN was examined in human urothelial cells grown in vitro (Fig. 4). The antibodies used were determined to be specific for human VN and did not cross-react with the bovine (serum) or murine (Matrigel) analogs (data not shown). Cultured in the absence of serum, human urothelial cells exhibited a robust and repeatable ability to attach and spread on glass, a finding that is in agreement with urothelial cells synthesizing and secreting their own VN that ultimately provides an extracellular matrix conducive with cell matrix adhesive processes (Fig. 4, B and C).

When cultures were propagated in Matrigel, a solubilized extract of the basement membrane of the murine Engel-Horm Swarm tumor, cells continued to exhibit immunoreactive signals (Fig. 4, D and E). When cultures were propagated in SIS, a porcine small intestine submucosa extract that is popular with surgeons as a decellularized scaffold, cells also exhibited immunoreactive signals (Fig. 4F). The patterns and intensities of VN signals on Matrigel and SIS were less defined than those grown on glass (Fig. 4C), on observation likely explained when one considers that Matrigel and SIS already contain a plethora of adhesive proteins. Despite the inclusion of bovine serum in the growth medium of human cells grown on Matrigel or SIS, the antibodies used in this study did not react with bovine VN, as documented by Western immunoblot analysis of bovine serum (data not shown). This control rules out any cross-immunoreactivity with our anti-human VN antibodies with bovine VN. The collective data of urothelial cells grown in three-dimensional models supports the hypothesis that the VN gene is active in vitro even in the face of exogenously supplied matrices and scaffolds.

### Table 2. Quantification of VN protein levels in normal urothelial cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Conditioned Medium</th>
<th>Soluble Lysed Cell Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal urothelial</td>
<td>1.1</td>
<td>11.4±0.3, 13.7±0.7</td>
</tr>
<tr>
<td>Immortalized urothelial</td>
<td>1.9</td>
<td>ND</td>
</tr>
<tr>
<td>HepG2</td>
<td>232</td>
<td>1.2±0.3</td>
</tr>
</tbody>
</table>

ND, not determined. Values are in ng per approximately 1 × 10^6 cells; all cultures were serum free. Lysis with NP-40 as described in EXPERIMENTAL PROCEDURES. Cellular elution with M-Per (Mammalian Protein Extraction Reagent; Pierce).
mRNA and were not amplified from any genomic DNA contamination.

PCR-DNA products were then subjected to dideoxy sequencing and sequence analysis against the VN reference sequences for the gene at chromosome 17q11 (Gene ID no. 7448, accession no. NC_000017.9) and the mRNA (accession no. NM_000638.3). Figure 6B confirms that the sequence of the PCR-DNA products was that of VN. Oligonucleotide primer walking with subsequent PCR and dideoxy sequencing yielded sufficient sequence that allowed us to determine the full-length mRNA sequence of urothelial VN. This sequence exhibited 100% identity with the reference mRNA sequence (accession no. NM_000638.3). Our experimental analysis data did not address the nature of the differences: 1) isolation by affinity chromatography (Fig. 7A), 2) positive reaction with anti-VN antibodies in Western blot and ELISA analyses (not shown), 3) positive identification as VN by mass spectrometry (below), and 4) support and promotion of cell attachment and spreading for both primary and immortalized cell cultures (Fig. 7B).

The peptide mass fragmentation patterns following tryptic digestion matched the VN precursor (accession no. P04004) within a Δ of 0.31–0.39. Mass spectrometry-mass spectrometry analysis of the VN and VN(c) chromatomers yielded Mowse scores of 119 and 112, with a score of >36 indicating identity to a peptide. Therefore, the isolated chromatomers were confirmed as VN.

However, we did observe that the VN and VN(c) chromatomers from plasma were not identical because they exhibited the following three differences in properties: 1) differential elution from a heparin column as a function of urea concentration (see below and in Mass spectrometry of VN isoforms isolated from human plasma), 2) different electrophoretic mobilities of 75 and 61 kDa for VN and 70 kDa for VN(c) (Fig. 7A), and 3) differential interaction with SPARC protein (Fig. 8B). Our experimental analysis data did not address the nature of the different Mn of VN and VN(c). Differences in glycosylation and/or differential susceptible internal protease cleavage sites are the likely mechanisms that account for these three sizes. These differences are likely to explain why only VN(c) was eluted from a heparin affinity column with 0.5 M NaCl, 0 M urea, 0.01 M sodium phosphate (pH 7.7), and 0.005 M EDTA that was originally equilibrated with 0.15 M NaCl, 8 M urea, 0.01 M sodium phosphate (pH 7.7), and 0.005 M EDTA. VN was observed to elute with 0.5 M NaCl, 8 M urea, 0.01 M sodium phosphate (pH 7.7), and 0.005 EDTA, in agreement with prior studies (21, 64).

Adhesive interactions of VN with urothelial cells and inhibition by the matricellular protein SPARC. VN is known to interact with cell surface and extracellular proteins in tissues besides urothelium. Therefore, it was of interest to determine to what extent VN would interact with urothelial cells and the extracellular matrix. Freshly plated cells from a primary culture were observed to attach and rapidly spread on VN-coated polystyrene (Fig. 7B, green line). Changes in cellular morphology over time were quantified by calculation of a RI. Over the first hour, spreading was rapid and the RI followed a slope of −1.6 (r² = 0.98). This result demonstrates that spreading was...
dependent only on interactions with exogenous VN, because there was insufficient time for the cells to synthesize and secrete other adhesive proteins such as fibronectin or laminin. From 2 through 6 h, the slope was reduced by 50% to −0.08 \( (r^2 = 0.99) \), thus completing a two-phase RI pattern. No differences were observed when cells were plated on the VN(c) chromatomer (Fig. 7B, purple line). Nearly identical VN-dependent spreading activity was observed in both parental (Fig. 7B, green and purple lines) and immortalized (Fig. 7B, blue and black lines) urothelial cell lines.

Of additional interest was the extent to which the matricellular protein SPARC abrogated the VN-dependent spreading activity of freshly plated normal urothelial cells. Figure 8A demonstrates that SPARC was able to inhibit spreading in a concentration-dependent manner (Fig. 8A). The basis for this inhibition was studied by a sandwich ELISA that was capable of detecting interactions between VN and SPARC and between VN(c) and SPARC. The assay involved the coating of a 96-well plate with SPARC or its third extracellular Ca\(^{2+}\)-binding (“EC”) domain (12), blocking with BSA, and adding either VN or its VN(c) chromatomer as a function of Ca\(^{2+}\) concentration. The presence of bound VN was then determined using protein ELISA. VN was observed to bind to SPARC in a manner inversely proportional to Ca\(^{2+}\) concentration, as described previously (43). The highest degree of interaction was observed between VN and EC-SPARC, a result that indicates that the pair of high-affinity Ca\(^{2+}\)-binding EF hands were the principal motifs of SPARC involved in this interaction, an observation supported by prior studies with synthetic peptides of the EF-hand pair (43). The VN(c) chromatomer, in contrast, did not bind SPARC at any Ca\(^{2+}\) concentration (Fig. 8B), suggesting that the conformation of VN is important for binding to SPARC.

These results are consistent with the model displayed in Fig. 8D. The model proposes that the hemopexin-like domain of vitronectin (yellow 4-bladed propeller) binds to the cell-binding region of SPARC (green EF hands with orange Ca\(^{2+}\) spheres). A proposed hinged region lies at the junction between domain 2 (Fig. 8, blue) and domain 1 (Fig. 8, red). We propose two mechanisms that cause the α-helical barrel of domain 1 to move from an open structure (promotion of spreading) to a closed structure (inhibition of spreading) that masks the availability of the EF-hand pair. 1) If SPARC is secreted with only the high-affinity Ca\(^{2+}\)-binding sites of the EF hand occupied, then the protein will function to counter VN-depending spreading by directly binding to VN; this mechanism requires a regulatory function that is administered by the endoplasmic reticulum. 2) A motif of SPARC not involved in Ca\(^{2+}\) binding is involved in the interaction with VN. This latter mechanism is supported by experiments where removal of Ca\(^{2+}\) from SPARC by titration with EDTA results in decreased binding of VN to SPARC (Fig. 8C). Abrogation of VN-SPARC interactions was observed to begin at EDTA concentrations as low as 0.1 mM and persisted to 10 mM (Fig. 8C). There is further support for this latter mechanism with the existence of a heparin-binding motif in the domain 2 of SPARC, as described previously (25).

**DISCUSSION**

As a multifunctional protein, vitronectin is related to many physiological and pathological processes (35, 67). As an important component of the extracellular matrix, the cell attachment activity of vitronectin is eight to 16 times higher than that of fibronectin (23), thus making vitronectin the principal component by which cells spread in vitro in the presence of serum. Our studies in vitro, however, used a defined serum-free medium that is devoid of detectable vitronectin. The collective evidence indicates that urothelial cells synthesize and secrete vitronectin in a manner to provide a substratum for cellular attachment and spreading.

Historically, liver has been recognized as the principal source of vitronectin. Secretion into the bloodstream and extravasation into subendothelial spaces is a recognized mechanism by which liver vitronectin can gain entry into various tissues. In this report, we describe the mechanism by which vitronectin is expressed in the urothelial layers of urothelium in the human lower urinary tract. To our knowledge, this is the first report of the local synthesis of urothelial vitronectin and
adds this epithelial cell subtype to a list that includes retinal pigmented epithelia (19, 39). These findings together raise the following question. Why do urothelial (epithelial) cells need to synthesize their own vitronectin, whereas cells on the mesenchymal side of the basement membrane can rely on liver vitronectin? One potential answer is the lack of detectable blood vessels within the urothelium and the inability of multimeric vitronectin to pass through the basement membrane because of its large size (~500 kDa) and binding to heparin-like glycosaminoglycans (67) and collagens (43) in the lamina propria. Abrams et al. (1) showed that the urothelial basement membrane exhibits a network structure with an average pore diameter of 82 ± 49 nm. Howat et al. (28) also demonstrated that the pores on the basement membrane are big enough to allow immune cells to move between the epithelial and mesenchymal compartments without disruption of the basement membrane. By electron microscopy, the diameter of monomeric plasma vitronectin is only 6–8 nm, and multimeric vitronectin occurs as globular specimens with an average diameter of 15–28 nm (55). Therefore, the diffusion of vitronectin as either the mono- or multimeric form across the basement membrane could, in theory, be possible. Considering its focal adhesive function, however, vitronectin must bind to some other immobilized extracellular matrix components, for example, heparan sulfate proteoglycans, to support and immobilize cells. Therefore, the likely reason to prevent diffusion of liver vitronectin into the urothelium is that vitronectin is sequestered immediately by the extracellular matrix of the lamina propria after its extravasation from blood stream. The consequences of this sequestration are the observed zone

Fig. 8. Spreading of UC on VN is inhibited by the matricellular protein secreted protein acidic and rich in cysteine (SPARC). A: UC spreading on VN is inhibited by rSPARC in a concentration-dependent manner. Rounding index (means ± SE; n = 4) of 1 and 3 represents spread and round cells, respectively, as described previously (29). B: interaction of VN and VN(c) with SPARC and EC-SPARC as a function of [Ca²⁺], as measured by protein ELISA. VN was isolated from human plasma as described in Fig. 7A (means ± SE; n = 3). C: titration of SPARC-treated wells with EDTA prior to measurement of VN binding by protein ELISA. VN was isolated from human plasma as described in EXPERIMENTAL PROCEDURES. See Fig. 7 (means ± SE; n = 4). D: model of interaction between VN and the matricellular protein SPARC. Modeled from the crystal structure for domains 2 and 3 (24, 25); structure for domain 1 remains unsolved, since it is refractory to crystallization. Domain 3 was cloned and expressed in bacteria as biologically active.
of diminished immunoreactivity in the superficial lamina propria. On the other hand, studies showed that extracellular levels of soluble vitronectin are controlled by receptor-mediated endocytosis that occurs within fibroblasts (62). In this process, the vitronectin receptor αvβ3 integrin directly mediates the internalization step (36). Only multimeric vitronectin and its exposed heparin-binding domain can enter this pathway that can be inhibited by exogenous heparin (40, 61). These studies imply that free vitronectin without the ability to bind to heparin or heparan/dermatan/chondroitin sulfate proteoglycans will be removed by fibroblast cells. The collective data in this report are consistent with these mechanisms and indicate that urothelial cells synthesize their own vitronectin J because of a need for adhesive proteins to anchor the basal urothelial layer and 2) because liver vitronectin is denied entry to the urothelium.

The mRNA and protein products of the vitronectin gene were detected in all urothelial cells of ureter and bladder tissues by in situ hybridization and immunostaining, respectively, indicating that all urothelial cells possess the ability to synthesize the protein. The lack of appreciable vitronectin in conditioned media indicates that the protein’s synthesis is dependent on the polarity established by urothelial cells in culture. Such polarity is established in vivo by the urothelium through mechanisms that separate the anchoring property of the basal layer from the barrier function of the superficial layer. The significance of vitronectin on the apical surface of the urothelium is likely to involve cross-linking of bacteria to urothelial cells via the protein’s RGD integrin-binding motif and a separate bacterial-binding domain for pathogens (53). Bacteria coated with vitronectin would thus have the ability to evade the complement attack and survive. Vitronectin would then be able to cross-link bacteria to either cells of the urothelium or the superficial lamina propria and instigate bacterial uptake. Thus, it would be expected that infected urothelium would exhibit large amounts of vitronectin between cells, much like lung and kidney parenchyma do (53). A complementary mechanism to modulate urothelial antimicrobial activity could then involve the interaction of apical SPARC (29) with the heparin-binding domain of vitronectin (43), a motif that also serves to bind bacteria (53). The extent that SPARC could provide antimicrobial activity through its marked presence in the apical surface of bladder urothelium is an intriguing hypothesis that is deserving of additional investigation. The detection of vitronectin at the apical surface of the urothelium is also consistent with its detection in urine. Secretory processes that involve the plasminogen activation system, of which vitronectin is a component (14), have been reported to be active in bladder superficial cells (13). Such urothelial secretory processes would then contribute to levels of urinary vitronectin, which have been described to rise dramatically during chronic renal failure and certain forms of glomerulonephritis and sclerosis (57).

Because it was not possible to isolate appreciable amounts of urothelial vitronectin for biochemical study, we elected to isolate vitronectin from human plasma. The isolation of two vitronectin-containing fractions from heparin affinity chromatography provided milligram amounts of protein for in vitro assays. The VN(c) chromatomer, exhibiting a Mr of 70 kDa, is consistent with the smaller size being a protease-generated form comprised of two chains held together by a disulfide bond (9, 46, 56). To the best of our knowledge, this is the first report of a novel form of multimeric vitronectin isolated from plasma by heparin affinity chromatography under denaturing conditions.

Both the VN and VN(c) chromatomers were active in spreading assays. However, only VN was capable of interacting with the matricellular protein SPARC, suggesting that the motif of vitronectin involved in SPARC binding is active in certain conformations. We present here our model of how the balance between adhesion and counteradhesion of urothelial basal cells is regulated. It is proposed that the region between domains 1 and 2 of SPARC can act as a “hinge” in a Ca2+-dependent manner to allow domain 1 to either “open” or “close.” Evidence that multiple Glu residues present in domain 1 constitute low-affinity, high-capacity, Ca2+-binding motifs comes from circular dichroism studies (5, 6, 12) and from this study, where low levels of EDTA were sufficient to remove bound Ca2+ ions. Experimentally, the saturation of Glu residues with Ca2+ would induce domain 1 to close off domain 3 from interacting with vitronectin, effectively promoting cellular adhesion. This mechanism is supported by our observation that rEC (domain 3 of SPARC) is the principal motif of SPARC that interacts with vitronectin. Removal of Ca2+ from the Glu residues would then induce domain 1 to open and allow VN to bind to domain 3, effectively promoting cellular counteradhesion. Such removal of Ca2+ in vivo would then be accomplished by a yet-to-be-identified SPARC-binding protein whose interaction would either promote or inhibit this interaction. Alternatively, SPARC could be secreted with Ca2+ ions not present in domain 1. These interactions would then serve as the principal mechanism to regulate the steady balance between adhesive and counteradhesive forces that function during urothelial cell spreading.

The balance between adhesion and counteradhesion is a process that occurs every time a urothelial cell divides or, alternatively, undergoes migration. SPARC is known to function as a counteradhesive protein for urothelial cells (12, 29). Counteradhesion refers to a reversal of the adhesive process in which a cell moves from a state of stronger adherence to a state of weaker adherence that is more conducive to cell motility (18). It is thought that SPARC’s counteradhesive effects are due to its competition with adhesive extracellular matrix proteins for their cognate receptors, and this interference results in focal adhesion disassembly (65). Our previous study demonstrated that a consistent activity of rSPARC was Ca2+-dependent inhibition of urothelial cell spreading, and such activity was localized to recombinant (r) domain 3 (rEC). We reported that rEC exhibited the same activity as the wild-type rSPARC protein and that this inhibition was abolished by mutagenesis of the high-affinity Ca2+-binding EF hand no. 2 within the rEC domain (12). Our study is also consistent with our prior report where the binding characteristics of vitronectin and SPARC were initially described (43). Disruption of this steady-state balance between adhesion and counteradhesion is predicted to have serious and deleterious consequences.

Because the urinary bladder undergoes tremendous changes in shape during the constant filling and emptying of urine, it is imperative that basal urothelial cells be properly anchored to the urothelial basement membrane. These observed patterns of immunoreactive signals indicate that interactions between extracellular vitronectin and intermediate cells constitute an additional mechanism for cell-cell cohesiveness in the face of ureteric peristalsis and/or constant bladder cycling. In the
presence of cancer, the disruption of this anchorage permits invasion of malignant urothelial cells into the lamina propria. During conditions occurring in the bladder affected with interstitial cystitis where the entire urothelium is often disrupted with respect to cell-matrix and cell-cell integrity, the loss of vitronectin function may prove to be a key to better understanding this and other conditions.

Of interest to many laboratories that study urothelial cells is the extent to which immortalized cells can faithfully replicate the parental primary culture from which they were derived. Our immortalization procedures, previously described in detail (10), have resulted in the creation of a ureteric cell line that has proven reliable in mirroring the parental untransformed phenotype in the following four in vitro assays: 1) culture morphology and colony formation, 2) DNA synthesis in pathways dependent on and independent of the epidermal growth factor receptor, 3) differentiation as evaluated by positive expression of cytokeratins 7, 14, and 17, and 4) retention of karyotype, when passage number was less than 21 (or 100 days in culture). To this list, we can now add nearly identical vitronectin-dependent spreading activity in parental and immortalized cell lines. The kinetics of spreading of parental or immortalized cultures was nearly identical when cells were plated on vitronectin or the VN(c) chromatomer. These data support the utility of our immortalized cultures as a viable tool to better understand urothelial cell biology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


