Published in final edited form as:

J Urol. 2008 February ; 179(2): 764–769. doi:10.1016/j.juro.2007.09.022.

Abnormal Expression of Differentiation-Related Proteins and Proteoglycan Core Proteins in the Urothelium of Interstitial Cystitis Patients

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Abstract

Purpose—The expression of proteoglycan core proteins biglycan, decorin, perlecan and syndecan-1 and differentiation-related markers of keratins 18 and 20 were examined to determine the origins of the loss of the glycosaminoglycan (GAG) layer and to investigate more fully the altered differentiation of the urothelium in IC.

Methods—Formalin-fixed biopsies from 27 IC patients and 5 controls were immunohistochemically labeled for the above proteins and scored using a modification of previous scoring for other markers. Inflammation was scored from H&E-stained slides. Combining previous with the new data, cluster analysis displayed the relationships among the markers and samples.

Results—The IC specimens clustered into 4 groups ranging from most biomarkers abnormal to most biomarkers normal, but all clustered separately from the normal controls. One group of IC specimens mainly showed aberrant expression of E-cadherin, which might represent an early abnormality. The biomarkers fell into 2 major groupings. One consisted of chondroitin sulfate, perlecan, biglycan, decorin and the tight junction protein ZO-1. A second cluster consisted of uroplakin, the epithelial marker keratin 18 and 20, and the morphology of the layer. E-cadherin and syndecan-1 showed little relation to the other two clusters or to each other. Inflammation correlated moderately with syndecan-1, but no other marker.

Conclusions—The findings strongly suggest abnormal differentiation in the IC urothelium with loss of barrier function markers and altered differentiation markers being independent and occurred independently of inflammation. The loss of the GAG layer was associated with loss of biglycan and perlecan on the luminal layer.

Keywords

interstitial cystitis; biochemical markers; urinary bladder; cell differentiation

Introduction

Although the exact sequence of events remains obscure, it is clear that the pathophysiology of interstitial cystitis involves epithelial dysfunction^{1,2}. Numerous studies have identified histopathologic^{2,3}, gene expression⁴, and molecular changes involved with loss of the barrier function of the urothelium⁵. The symptoms of pain, urgency and frequency are thought to result from the physiologic sequelae of loss of the barrier function.

In previous studies we demonstrated that biopsies from interstitial cystitis patients showed abnormal polarity of the urothelium, loss of luminal chondroitin sulfate (the “GAG layer”) and aberrant expression of adhesion molecules². We also speculated that the urothelium in the IC bladder seemed to be following an altered differentiation program, a finding that also has been suggested by other investigators^{4,6}. In this communication we have more extensively determined the expression of proteoglycan core proteins and differentiation markers to more clearly identify the molecular changes responsible for the loss of glycosaminoglycan on the luminal surface and its apparently inappropriate expression within the urothelial layer as well as to find additional evidence for an aberrant differentiation program that could be associated with epithelial dysfunction.

Materials and Methods

Patient population

The same urothelial specimens that were collected for our previous study were used for this study.² The samples were obtained from 27 IC (21 females and 6 males) patients and 5 controls. As previously described, informed consent was obtained from each patient and specimens were collected from IC patients meeting the current criteria for entrance of patients into clinical studies of IC as established National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), with moderate to severe disease symptoms of greater than 6 months duration, with an average age of 38.2 years (range= 23-63 years old) and undergoing therapeutic cystoscopy and hydrodistention. Five female patients with an average age of 46.1 years of age (range= 21-66 years old) and known to be free of bladder mucosal disease and urinary tract infection, undergoing bladder suspension procedure for stress urinary incontinence, underwent bladder biopsy and served as controls.

Specimen Collection

IC patients underwent cystoscopy and hydrodistention (90 cm H₂O for 5 min. with occlusion of the urethra), followed immediately by biopsy with cold-cup rigid biopsy forceps of posterior bladder wall through a 22 French rigid cystoscope. The control samples were obtained in the a similar fashion from patients undergoing suspension for stress incontinence without hydrodistention at 90 cm for 5 min. All samples were immediately fixed in formalin and were subsequently mounted in paraffin.

Immunohistochemical (IHC) analysis of marker proteins and inflammation

A 5 µm section was cut from each specimen, de-waxed with a graded xylene and ethanol series and re-hydrated with a graded ethanol water series. IHC labeling was performed with the following primary antibodies: Keratin-20 (Dako, M7019, mouse monoclonal, citrate retrieval, 1:100), Biglycan (R&D Systems, MAB2667, mouse monoclonal, no retrieval, 1:100), Perlecan (Chemicon, MAB1948, rat monoclonal, no retrieval, 1:100), Keratin-18 (Novacastra, NCL-C51, mouse monoclonal, citrate retrieval, 1:50), Syndecan-1 (Abcam, ab714-500, mouse monoclonal, citrate retrieval, 1:100), Decorin (Calbiochem, PC673, goat polyclonal, no retrieval, 1:100). The following secondary antibodies were used: goat anti-mouse (Pierce,

31800), goat anti-rabbit (Pierce, 31820), rabbit anti-goat (Zymed, 61-1640), goat-anti-rat (Santa Cruz, sc-3826).

The tissue sections were blocked for nonspecific binding (Blocking Solution, Zymed) and were incubated with the primary antibody (diluted with Common Antibody Diluent, BioGenex) for 1 hour at room temperature in a humidity chamber, followed by washing (Automation Buffer, Biomedex). The appropriate antibody dilution was determined experimentally by titration. The slides were then incubated with a biotinylated secondary antibody (1:100) for 30 minutes at room temperature, followed by washing. Labeling was performed by incubation with streptavidin peroxidase for 10 minutes, followed by washing, and then incubation with AEC chromogen for 3-10 minutes (Histostain kit, Zymed). After washing, sections were counterstained with hematoxylin for 3 minutes (Zymed, 00-8011), washed, and mounted (crystal mount, M03, Biomedex). Positive assay controls consisted of normal bladder sections. Images of stained sections were captured using a Nikon Microphot FXA microscope equipped with a Hamamatsu Color Chilled 3CCD camera. Inflammation was scored on sections stained with hematoxylin and Eosin (H&E).

Method of scoring results from IHC

The tissue sections were scored for the distribution of each marker and for the morphology of the urothelium. The scoring system combined previously published information on the distribution of these markers in bladder^{2,3,6} and reports from other tissues as well as our observations of the specimens. A scoring system of -2, -1, +1, +2 was used, ranging from most abnormal to normal. The details of the scoring system shown in Table 1, and examples of each score are shown in Fig. 1. Other than scoring for inflammation (by BLB, a board-certified pathologist specializing in uropathology) sections were scored by two observers (PJH and REH) and discrepancies resolved by consensus.

Clustering

Normal and IC specimens were assigned unique ID labels unrelated to patient identifiers. Hierarchical clustering was applied to both specimens and stains by using Cluster 3.0 program (<http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster>), the next generation of Cluster program developed by M. B. Eisen. Best results were achieved via complete linkage clustering with a correlation (uncentered) similarity metric. The results were visualized with Java TreeView program (<http://jtreeview.sourceforge.net>), also the next generation of TreeView program originally developed by M. B. Eisen.

Results

Abnormal expression patterns were observed in 50-70% of IC biopsy samples

As shown in Figure 1, in normal control specimens the expression patterns of perlecan, decorin, and biglycan looked almost identical to the pattern previously reported for chondroitin sulfate². Dense staining on the surface of umbrella cells was observed, which decreased in expression from the luminal to basal layers, the latter usually stained lightly or was absent. In a portion of the samples from patients with IC, the most commonly observed abnormalities were uniform staining throughout the urothelium and absence of strong luminal staining; a patchy distribution was only occasionally detectable.

The expression of syndecan-1, a transmembrane proteoglycan, was usually restricted to the basal and intermediate layers in normal controls, where a majority of cells were outlined with light to moderate staining. Expression was strongest on the cell surface with little to no staining in the cytoplasm. In some of the specimens from IC patients, intense staining of the basal and intermediate layers, diffuse expression in the cytoplasm, or a patchy distribution was detected.

The expression of keratin 18 and keratin 20 was restricted to the luminal layer and was densely distributed in the normal controls. This pattern was similar to the staining pattern we previously reported for uroplakin². In the IC samples, some exhibited weak staining, intermediate layer staining, or the distribution was patchy.

Cluster analysis of biomarker expression

Cluster analysis was used to correlate the expression patterns of each marker and obtain a global view of the inter-relationships among the markers with results as shown in Fig. 2. Also included in this analysis were the biomarkers previously characterized, namely chondroitin sulfate, ZO-1, E-cadherin, and uroplakin and the morphology/polarity score² after conversion of the original normal-abnormal score to a 4-point scale. As is explained in the legend to Fig. 2, the two cluster maps differ as to whether inflammation is included. Inclusion of inflammation made little difference in the clustering of biomarkers. In the absence of the inflammation score, the 3 proteoglycans showed a high correlation with each other with Perlecan and Biglycan showing a correlation of 0.85. ZO-1 and the Morphology/polarity score also showed a high correlation with each other and formed a cluster with the proteoglycans. The two keratins and uroplakin formed a second major cluster, with keratin 18 showing a higher correlation with uroplakin than with keratin 20. Syndecan and E-cadherin expression showed little correlation with any of the other markers or with each other. The IC patients fell into 4 groups that were distinct from the normals. Two groups comprising 59% of the samples showed multiple abnormalities in all markers. The other two groups showed fewer abnormal markers and differed mainly by whether E-cadherin was normal or not. When inflammation was included, the main difference was that it showed an approximate 0.4 correlation with syndecan. The virtual disappearance of the patient group with the most normal findings occurred in part because 3 samples were eliminated due to having too many missing values. Relatively few patient samples changed their cluster membership as a result of inclusion of inflammation. It should be noted that no samples had the most severe grade of inflammation.

Discussion

In this study we mapped the extent of epithelial abnormalities at the molecular level that occur in biopsies from IC patients. Abnormalities were discovered in a majority of samples from IC patients: perlecan (68 %), biglycan (72 %), decorin (52 %), syndecan-1 (56 %), keratin-18 (56 %), and keratin-20 (68 %). Cluster analysis revealed that the IC samples fell into 3 or 4 major groups, depending on the inclusion of inflammation. Nearly 60% of the IC specimens showed multiple abnormalities (81% of all findings in the two most aberrant groups identified without using inflammation), with the remainder showing a less aberrant pattern. In this latter category, E-cadherin expression differentiated two groups. In the most affected groups defects were detected in keratin-18 (80 %), keratin-20 (87.5 %), and uroplakin (56%), which are considered to be markers of differentiation for the urothelium^{7,8}. The patients all were symptomatic at the moderate to severe level, yet some had nearly normal findings and an interesting stratification is suggested. One strong possibility commented on previously² is that the failure to detect abnormalities in all the specimens may reflect heterogeneity within the bladder. Future work including more than one specimen per patient, a wider spectrum of disease and a larger study to support comparisons between patient clinical characteristics and biomarker expression will be required to answer the important question of how these findings relate to the clinical characteristics of IC such as have been reported by Erickson.⁹

The close clustering of perlecan and biglycan with chondroitin sulfate suggest that these two proteoglycans form the “GAG Layer” previously described. ZO1 protein, which forms tight junctions, also fell within this major cluster. Perlecan is usually detected in the basement membrane of epithelial cells, and it was not detected in keratinocytes, mammary epithelial

cells, or prostate epithelial cells¹⁰, so it is surprising to find expression in the urothelium. However, microarray analyses showed that immortalized urothelial cells did express the mRNA for the perlecan core protein¹¹. The two keratins showed similar patterns of expression in the normals, being found in normal urothelium only in or on the superficial layer, but clustered differently among the IC patients. Interestingly, decorin, which shows considerable homology to biglycan, did not correlate as well with biglycan as did perlecan. Decorin bears one chondroitin or dermatan sulfate chain, while biglycan carries two of these chains. Perlecan and syndecan carry both heparan and chondroitin sulfate chains. Since decorin expression is highest in the differentiated cells of the luminal surface and decorin is known to inhibit the growth of some tumor cells¹², it suggests the possibility that decorin may function to promote differentiation in the luminal cells or at least is associated with differentiation. Therefore, altered decorin expression likely indicates failure of the urothelium in IC to differentiate properly.

One possible framework for understanding the effects of loss of the GAG layer and different patterns of expression of proteoglycan core proteins in the IC urothelium is that the actions of cytokines are likely to be altered as well as the permeability being increased. Both decorin and biglycan are able to bind TGF- β and seem to be capable of regulating the availability of the ligand¹³, which could have effects on both the growth and differentiation of urothelial cells. Perlecan and syndecan, which bear heparan sulfate chains, bind heparin-binding growth factors such as FGF, TGF- β , Platelet Derived Growth Factor (PDGF), and Vascular Endothelial Growth Factor (VEGF)¹⁴. FGF-7 (Keratinocyte Growth Factor)¹⁵ and FGF-10¹⁶ are known to be important mitogens that regulate the growth of urothelial cells. FGF-7 was found to be required for bladder cells to form a stratified urothelium; when FGF-7 was absent, the urothelial cells failed to divide, and they lacked an intermediate layer¹⁷. Perlecan and syndecan may control the growth and differentiation of urothelial cells through their ability to bind and sequester growth factors such as the FGF family¹⁴. In colon carcinoma cells, perlecan was shown to be required for downstream signaling by the FGF-7 receptor, but not for binding to the receptor¹⁸. Therefore, the defects in the expression of perlecan or syndecan in IC patients, such as when perlecan expression is reduced on the luminal cells, may be responsible for creating abnormal levels of critical growth factors such as FGF-7 or for affecting downstream signaling by the receptor. This mechanism might then explain the thin urothelium and altered differentiation pattern present in the urothelium of IC patients.

Interestingly, Syndecan-1 and E-cadherin showed little correlation with any of the molecular markers. E-cadherin expression, however, was a major driver of clustering. Syndecan on cell surfaces often reflects inflammation as it is cleaved by metalloproteinases and shed in response to inflammation and injury and creates a chemotactic gradient that attracts neutrophils to the injured site¹⁹. This could play a role in the inflammation that is observed in IC patients. Syndecan showed a moderate association with inflammation, but none of the other biomarkers correlated with inflammation. Although IC is often considered to be an inflammatory disease, no patients scored -2 (severe) for inflammation and only 3 scored -1 (moderate). All others scored none or mild. It is therefore clear that stromal inflammation is not the cause of the epithelial abnormalities.

The origins of these multiple abnormalities are not clear. Several non-exclusive etiologies for IC have been proposed including newer causes such as pathology caused by the frizzled 8-related glycopeptide, Antiproliferative Factor (APF),⁴ and interactions between urothelium and nerves such that spinal cord injury produces a transient loss of barrier function and the umbrella cell layer²⁰. Whatever the ultimate cause, epithelial dysfunction clearly is a major factor and likely produces much of the symptomology. In this study, we have shown that the urothelium from IC patients displays a number of consistent abnormalities that could possibly serve to characterize patients for both treatment and diagnosis.

Conclusions

In interstitial cystitis, the loss of the GAG layer is due to loss of two proteoglycans, biglycan and perlecan. Abnormalities in expression of other proteoglycans indicate an abnormal pattern of urothelial differentiation that leads to loss of barrier function. The findings suggest that reestablishment of normal urothelial barrier function will require restoring the normal differentiation program in the long term or the barrier by exogenous means in the short term.

Acknowledgements

This work was supported in part by R01 DK069808 (REH) and by a grant from Stellar Pharmaceuticals, Ltd. (REH) and by the Department of Urology.

References

1. Graham E, Chai TC. Dysfunction of bladder urothelium and bladder urothelial cells in interstitial cystitis. *Curr Urol Rep* 2006;7:440. [PubMed: 17052438]
2. Slobodov G, Feloney M, Gran C, Kyker KD, Hurst RE, Culkin DJ. Abnormal Expression of Molecular Markers for Bladder Impermeability and Differentiation in Urothelium of Interstitial Cystitis Patients. *J Urol* 2004;171:1554. [PubMed: 15017219]
3. Leiby BE, Landis JR, Probert KJ, Tomaszewski JE. Discovery of morphological subgroups that correlate with severity of symptoms in interstitial cystitis: a proposed biopsy classification system. *J Urol* 2007;177:142. [PubMed: 17162024]
4. Keay S, Seillier-Moiseiwitsch F, Zhang CO, Chai T, Zhang J. Changes in human bladder epithelial cell gene expression associated with interstitial cystitis or antiproliferative factor treatment. *Physiol Genomics* 2003;14:107. [PubMed: 12847144]
5. Hu P, Meyers S, Liang FX, Deng FM, Kachar B, Zeidel ML, et al. Role of membrane proteins in permeability barrier function: uroplakin ablation elevates urothelial permeability. *Am J Physiol Renal Physiol* 2002;283:F1200. [PubMed: 12388410]
6. Laguna P, Smedts F, Nordling J, Horn T, Bouchelouche K, Hopman A, et al. Keratin expression profiling of transitional epithelium in the painful bladder syndrome/interstitial cystitis. *Am J Clin Pathol* 2006;125:105. [PubMed: 16482998]
7. Cross WR, Eardley I, Leese HJ, Southgate J. A biomimetic tissue from cultured normal human urothelial cells: analysis of physiological function. *Am J Physiol Renal Physiol*. 2005
8. Scriven SD, Booth C, Thomas DFM, Trejdosiewicz LK, Southgate J. Reconstitution of human urothelium from monolayer culture. *J Urol* 1997;158:1147. [PubMed: 9258159]
9. Erickson DR, Tomaszewski JE, Kunselman AR, Bentley CM, Peters KM, Rovner ES, et al. Do the National Institute of Diabetes and Digestive and Kidney Diseases cystoscopic criteria associate with other clinical and objective features of interstitial cystitis? *J Urol* 2005;173:93. [PubMed: 15592040]
10. Murdoch AD, Liu B, Schwarting R, Tuan RS, Iozzo RV. Widespread expression of perlecan proteoglycan in basement membranes and extracellular matrices of human tissues as detected by a novel monoclonal antibody against domain III and by in situ hybridization. *J Histochem Cytochem* 1994;42:239. [PubMed: 7507142]
11. Dozmorov MG, Kyker KD, Saban R, Knowlton N, Dozmorov IM, Centola M, et al. Analysis of the Interaction of Extracellular Matrix and Phenotype of Bladder Cancer Cells. *BMC Cancer* 2006;6:12. [PubMed: 16412233]
12. Santra M, Mann DM, Mercer EW, Skorski T, Calabretta B, Iozzo RV. Ectopic expression of decorin protein core causes a generalized growth suppression in neoplastic cells of various histogenetic origin and requires endogenous p21, an inhibitor of cyclin- dependent kinases. *J Clin Invest* 1997;100:149. [PubMed: 9202067]
13. Drogue R, Cabello-Verrugio C, Riquelme C, Brandan E. Extracellular proteoglycans modify TGF-beta bio-availability attenuating its signaling during skeletal muscle differentiation. *Matrix Biol* 2006;25:332. [PubMed: 16766169]

14. Forsberg E, Kjellen L. Heparan sulfate: lessons from knockout mice. *J Clin Invest* 2001;108:175. [PubMed: 11457868]
15. Zhang D, Kosman J, Carmean N, Grady R, Bassuk JA. FGF-10 and its receptor exhibit bidirectional paracrine targeting to urothelial and smooth muscle cells in the lower urinary tract. *Am J Physiol Renal Physiol* 2006;291:F481. [PubMed: 16597614]
16. Bagai S, Rubio E, Cheng JF, Sweet R, Thomas R, Fuchs E, et al. Fibroblast growth factor-10 is a mitogen for urothelial cells. *J Biol Chem* 2002;277:23828. [PubMed: 11923311]
17. Tash JA, David SG, Vaughan ED ED, Herzlinger DA. Fibroblast growth factor-7 regulates stratification of the bladder urothelium. *J Urol* 2001;166:2536. [PubMed: 11696824]
18. Ghiselli G, Eichstetter I, Iozzo RV. A role for the perlecan protein core in the activation of the keratinocyte growth factor receptor. *Biochem J* 2001;359:153. [PubMed: 11563979]
19. Li Q, Park PW, Wilson CL, Parks WC. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* 2002;111:635. [PubMed: 12464176]
20. Apodaca G, Kiss S, Ruiz WG, Meyers S, Zeidel M, Birder L. Disruption of bladder epithelium barrier function after spinal cord injury. *Am J Physiol Renal Physiol*. 2003

Abbreviations

IC	Interstitial Cystitis
IHC	Immunohistochemistry
GAG	Glycosaminoglycan
FGF	Fibroblastic growth factor
TGF-β	Transforming growth factor β
PDGF	Platelet derived growth factor

Fig. 1. Examples of normal and abnormal distributions of marker molecules. (There is no photo shown for Decorin +1 because none of the samples displayed this pattern).

Fig. 2. Dendrograms of hierarchical clustering of patient samples and marker distributions. Similarity is indicated by the length of the line segments connecting the elements in the dendrogram with short line segments indicating high similarity. The numerical value of correlation is shown by the scales shown on the figures. The dendrogram on the left shows the clustering observed without inclusion of inflammation. On the right is shown clustering including inflammation. Because some of the biopsies had been exhausted, some data were missing. This was most significant for inflammation, the last marker examined, and to prevent the missing data from driving the clustering, specimens with more than 2 missing data points were excluded from clustering analysis. Interestingly, all those excluded came from the least severe group.

Table 1
Scored characteristics of different stains.

Morphology/polarity	
-2	Urothelium 1-2 cells thick, cells all the same size, umbrella cell layer absent
-1	Urothelium 3-5 cells thick, cells all the same size, umbrella cell layer absent
+1	Some polarization evident with some stratification of cell size. Umbrella cells are spotty or urothelium is thin (~3 cells thick)
+2	Urothelium is polarized with respect to cell size. Umbrella cells cover most of the urothelium uniformly, and the urothelium generally is 5 cells or more thick
Chondroitin sulfate, perlecan, decorin	
-2	Uniform distribution throughout urothelial layer
-1	Absent strong staining on luminal surface
+1	Patchy
+2	Dense luminal staining with decreasing expression from luminal to basal layers
Biglycan	
-2	Uniform distribution / No staining of luminal surface
-1	Absent strong staining on luminal surface
+1	Patchy
+2	Dense luminal staining with decreasing expression from luminal to basal layers
Syndecan-1	
-2	Intense stain throughout basal and intermediate layers
-1	Diffuse expression in cytoplasm
+1	Patchy / Weak staining
+2	Light to moderate stain in basal and intermediate layer, absent luminal layer, minimal expression in cytoplasm with each cell outlined by marker
Keratin 18 and 20	
-2	Mostly unstained
-1	Strong staining of intermediate layer
+1	Patchy / Weak staining of intermediate layer
+2	Found only on luminal layer, densely distributed
Uroplakin	
-2	Weak staining of luminal layer
-1	Staining of intermediate layer
+1	Patchy
+2	Found only on luminal layer, densely distributed
E-cadherin	
-2	Dense expression throughout urothelium
-1	Weak throughout urothelium
+1	Patchy
+2	Concentrated in the umbrella cell layer or immediately underlying the cell layer
ZO-1	
-2	Mostly unstained / Absent
-1	Expressed in cytoplasm / Not organized on cell surfaces
+1	Patchy

+2	Uniformly distributed throughout urothelium, but possibly more intense under the umbrella cell layer, with minimal expression in the cytoplasm, with each cell outlined by the marker
Inflammation	
-2	Prominent multifocal inflammatory aggregates noted as patchy or diffuse infiltrates on low power scan
-1	Focal or multifocal prominent aggregates (larger than score 2) of inflammatory infiltrates noted on low power scan
+1	Focal or multifocal aggregates of inflammatory infiltrates noted on low power scan
+2	Minimal or no inflammatory infiltrate identified primarily as single cells on low-power scan