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Abnormalities in Expression of Structural, Barrier, and Differentiation Related Proteins and Chondroitin Sulfate in the Urothelium of Cats with Feline Interstitial Cystitis Mimic Those Seen in Human Interstitial Cystitis

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Abstract

Purpose—The urothelium of cats diagnosed with feline interstitial cystitis (FIC) was analyzed to determine if abnormalities in protein expression patterns could be detected, and whether the pattern of expression was similar to that observed in human Interstitial Cystitis/Bladder Pain Syndrome (IC) patients. The proteins that were analyzed are involved in cell adhesion, barrier function, comprise the glycosaminoglycan (GAG) layer, or are markers of differentiation.

Methods—Formalin-fixed biopsies from 8 cats with FIC and 7 healthy controls were labeled using immunohistochemistry and scored using a modified version of a system previously used for human samples. Cluster analysis was performed to investigate relationships between the markers and samples.

Results—The results showed that 89% of the FIC bladders displayed abnormal protein expression and chondroitin sulfate (CS) patterns, whereas only 27% of the normal tissues exhibited slight abnormalities. Abnormalities were found in most of the FIC samples, biglycan (87.5%), CS (100%), decorin (100%), E-cadherin (100%), keratin-20 (K20, 100%), uroplakin (50%), ZO-1 (87.5%). In the FIC bladders, about 75% of the CS, biglycan, and decorin samples

displayed absence of luminal staining or no staining. Results from the cluster analysis revealed that the FIC and normal samples fell into two clearly separate groups, demonstrating that the urothelium of cats with FIC is altered from normal.

Conclusions—FIC produces similar changes in luminal GAG and several proteins as is seen in human patients, suggesting some commonality in mechanism and supporting the use of FIC as a model for human IC.

Keywords

interstitial cystitis; biochemical markers; urinary bladder; cell differentiation

INTRODUCTION

Feline interstitial cystitis (FIC) is a naturally occurring disorder of domestic cats that is similar in many ways to Interstitial Cystitis/Bladder Pain Syndrome (IC) in human beings.¹ IC is a chronic pain syndrome that is characterized by pain associated with bladder filling, urinary urgency and frequency, and variable combinations of comorbid disorders.² Although the cause(s) of IC remains uncertain, dysfunction of the urothelium usually is associated with IC.³⁻⁹ The bladder of patients with IC may have increased permeability to urinary solutes, which could enter the urothelium and produce irritation and inflammation. The increased permeability might result from defects in the bladder's permeability defenses, which reside in a mucous layer and tight junction proteins on the surface of the apical cells of the urothelium.^{7, 8, 10} The mucous layer consists of glycosaminoglycans (GAG) attached to proteoglycans on the surface of the urothelium. These molecules have been proposed to act as a barrier to prevent solutes, bacteria, potassium, etc. from entering the urothelium.¹¹ Previously, we examined the urothelium in bladder biopsies from patients with IC and identified abnormalities in markers of differentiation, components of the "GAG layer", and in cell to cell adhesion molecules that may play a role in maintaining a protective barrier in the urothelium.^{5, 12}

A major problem in IC research is the lack of an animal model that duplicates the human disorder.¹³ In this report, to test the FIC model, the similarity of the urothelium of cats with FIC was compared to that of humans with IC with respect to the expression patterns of proteins and chondroitin sulfate (CS) in the urothelium that are involved in cell adhesion, comprise the GAG layer, or are markers of differentiation.^{5, 12}

MATERIALS AND METHODS

Animals

Bladder tissues from 8 cats with FIC and 7 healthy control cats were used in this study. All cats with FIC were obtained as donations from clients, and FIC was diagnosed at The Ohio State University Veterinary Teaching Hospital using established criteria.¹⁴ Healthy, age-matched control cats were obtained from commercial vendors and determined to be free of disease and signs referable to the lower urinary tract according to the same diagnostic criteria used for cats with FIC. All cats were housed in stainless steel cages at the Ohio State University animal facility and allowed to acclimate to their environment for at least 3

months before being studied. Bladder tissue was obtained from deeply anesthetized (98% O₂ / 2% isoflurane) cats. Anesthesia was determined to be adequate for surgery by periodically testing for absence of the withdrawal reflex to a strong pinch of the hind paw and absence of an eye blink reflex to tactile stimulation of the cornea. After removing the tissue, cats were euthanized while anesthetized using an overdose of sodium pentobarbital (80 mg/kg intravenously). All procedures were conducted in accordance with institutional animal care and use committee policies at The Ohio State University.

Immunohistochemical (IHC) analysis of marker proteins

IHC labeling was performed using the following primary antibodies: Biglycan (R&D Systems, MAB2667, mouse monoclonal, no retrieval, 1:100), Chondroitin 6-Sulfate (Millipore, MAB2305, mouse monoclonal, no retrieval, pre-treatment with chondroitinase, 1:100); Chondroitin 6-sulfate analysis was performed with an antibody against the stub resulting from chondroitinase digestion of sections with 250 µl of 3 mU/ ml chondroitinase ABC (Sigma, C3667) in buffer (40mM Tris pH8, 40mM sodium acetate, 0.05% BSA) for 15 minutes at room temperature. The 6-sulfate isomer was analyzed instead of the previously analyzed 4-isomer because the price of the antibody had increased to unacceptably high levels and produced some nonspecific staining that was absent with the anti-C6S antibody. The distribution of staining of the two was indistinguishable. Decorin (Calbiochem, PC673, goat polyclonal, no retrieval, 1:100), E-cadherin (Invitrogen, 18-0223, citrate retrieval, mouse monoclonal, 1:50), Keratin-20 (Dako, M7019, mouse monoclonal, citrate retrieval, 1:100), Uroplakin (pan-uroplakin antibody was a kind gift from Dr. Tung-Tien Sun, New York University, rabbit polyclonal, no retrieval, 1:2,000), ZO-1 (Invitrogen, 61-7300, rabbit polyclonal, no retrieval, 1:75). For ZO-1, after deparaffinization was preformed, sections were pre-treated with Protease XXIV (Sigma, P8038, 1 mg/ml in PBS) for 10 minutes at 37°C, rinsed, and then blocked. The following secondary antibodies were used: goat anti-mouse (Invitrogen, 856643), goat anti-rabbit (Pierce, 31820), rabbit anti-goat (Zymed, 61-1640).

A 5 µm section was cut from each paraffin embedded specimen, de-waxed with a graded xylene and ethanol series and re-hydrated with a graded ethanol water series. The tissue sections were blocked for nonspecific binding (Blocking Solution, Zymed) and incubated with the primary antibody (diluted with Common Antibody Diluent, BioGenex) for 1 hour at room temperature (or overnight at 4°C in a humidity chamber for Chondroitin Sulfate and ZO-1), followed by washing (Automation Buffer, Biomed). Positive assay controls consisted of normal bladder sections. 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 (Histostain kit, Zymed) for 10 minutes, followed by washing, and incubation with AEC chromogen (Zymed) for 3-12 minutes. After washing, sections were counterstained with hematoxylin (BioGenex, HK100-9K) for 3 minutes, washed, and mounted (Clear Mount, 17985-17, Electron Microscopy Sciences). Images of stained sections were captured using a Nikon Optiphot microscope equipped with a Canon EOS Rebel T3i/EOS 600D camera. Morphology/

polarity were scored on sections stained with Hematoxylin (VWR, 95057-858) and Eosin (VWR, 95057-848).

The labeling was scored as previously described¹² on a scale of +2, +1, -1 and -2, with +2 being the most normal and -2 the most abnormal. Examples of the appearance of scores for each marker are shown in Fig S1. The entire visible urothelium was evaluated independently by three scorers (PJH, SVG and REH), and each scorer assigned a final score representing the predominant score. A variability score was also assigned to cover the range of scores seen within the urothelium of a given section. For example if a section showed a predominance of +2 but some +1 areas were seen, the variability score would be 1; if some -1 areas also were observed, the variability score would be 2. In general, there was little disagreement among reviewers (never more than one point), and consensus was usually reached easily. The criteria for scoring were similar to those used previously for scoring human bladder sections and are listed in Table 1. After the tissue was examined the scores were compared, and discrepancies were resolved before moving on to the next tissue.

The results were analyzed using Cluster 3.0, an updated version (by Michiel de Hoon) of the original program written by Michael Eisen. The program is available at: <http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>. The results are displayed by TreeView, a Java-based program available at the same site.

RESULTS

Abnormalities were detected in 89% of FIC bladder samples

The distribution of C6S, and the two CS proteoglycans biglycan, and decorin, appeared to be remarkably similar to each other in normal cat bladders (Figure S1). Strong staining was observed near the surface of the urothelium (the GAG layer), with expression decreasing from the luminal to the basal layer. Most of the bladder samples from control cats (86%) exhibited normal biglycan and CS staining patterns. In contrast, a majority of the bladder samples from cats with FIC displayed abnormal patterns of expression for biglycan, decorin, and C6S. The results for each cat bladder are presented in Table 2. The values in the table are the overall score and the variability score following the comma. About 75% of biglycan, decorin, and C6S samples displayed absence of luminal staining or no staining, while 25% of the samples had patchy staining. Uroplakin was densely distributed in the luminal layer of the normal cat bladders, with some samples showing weak to moderate expression in the intermediate layer. Patchy expression of uroplakin was displayed in about half of the normal and FIC specimens. In some of the FIC bladders, weak uroplakin staining or mostly unstained urothelium was observed. There was no statistically significant difference in the expression of uroplakin between normal and FIC samples. Keratin-20 (K20) was densely distributed on the umbrella cells in the normal urothelium. In the FIC bladders, defects in K20 expression patterns were detected in all of the samples, with 71% showing moderate staining of the intermediate layer or absence of stain, whereas 29% were patchy. E-cadherin was concentrated in the umbrella cell layer of the normal cat bladders with weak staining present in the intermediate layer in some samples. In the FIC bladders, all of the samples exhibited anomalies in E-cadherin, 75 % displayed moderate staining throughout the urothelium; 25% were patchy. Zonula occludens-1 (ZO-1) was uniformly expressed

throughout the urothelium of normal cats, and some samples exhibited more intense staining on the surface of the umbrella cells. ZO-1 was minimally expressed in the cytoplasm and was observed almost exclusively on the outer cell membranes. In the FIC samples, ZO-1 was overexpressed in the cytoplasm or patchy in 88% of the samples.

The morphology of bladders from cats with FIC was similar to that seen in samples from humans with IC. The urothelium was somewhat thinned in the FIC bladders, but generally was at least 3 cells thick. A distinct apical layer with umbrella cell morphology was seen in the control cat bladders, whereas in the FIC bladders the distinct umbrella cell morphology was lost and the cells appeared very similar to the layer of cells immediately below. None of the cat bladders showed denuded urothelium characteristic of the Hunner's ulcer, nor were massive infiltrations of inflammatory cells seen in any of the FIC cases.

The statistical analysis of the distributions of the biomarkers in FIC and control cat bladders is summarized in Table 3. The two populations are clearly distinct, with all of the biomarkers except uroplakin, with highly statistically significant differences observed between FIC and control bladders. Although some variability in the score was found in the control bladders depending upon the location, significantly more variability in scoring ($p = 0.03$) was found in the FIC bladders than in the control bladders. The histogram in Figure 1 shows that 66% of the urothelium samples from normal cats were completely normal (score +2); 29 % had some patchy expression of the marker (score +1). In contrast, only 11% of the FIC samples displayed completely normal expression patterns (score +2), whereas 89% exhibited abnormalities (+1 or lower, usually -1 or -2).

Cluster analysis of protein and glycan expression

As shown in Figure 2, cluster analysis demonstrated that the FIC and control cat bladder expression data separated into two distinct groups without any overlap. The biomarkers clustered into three main groups, E-cadherin and decorin each sorted into separate groups. In the third group, biglycan and K20 showed significant correlation with each other, as did ZO-1 and morphology/polarity (M/P). C6S clustered with M/P and ZO-1 in the third group. Uroplakin showed the least amount of correlation to the other members in third group.

DISCUSSION

In this report, the urothelium of cats diagnosed with FIC was analyzed to determine if abnormalities in protein and glycan expression patterns could be detected, and whether the pattern of expression was similar to that seen in human (non-Hunner's ulcer) IC patients.^{5, 12} The proteins and glycans examined included components of the GAG layer (C6S, and probably biglycan and decorin), markers of differentiation (K20 and uroplakin), and cell adhesion molecules that may play a role in barrier function (E-cadherin and ZO-1). Most of the FIC bladders had aberrant expression patterns, whereas the control bladders mostly displayed normal patterns.

The staining patterns of CS, biglycan, decorin, E-cadherin, K20, and ZO-1 were similar in normal feline and human urothelium samples.^{5, 12} This is the first report to examine the expression patterns of decorin, biglycan, and K20 in the bladders of cats with FIC, and to

compare it with that of humans with IC. The expression of ZO-1 on the surface of umbrella cells in cats was previously reported.¹⁵ Uroplakin displayed slightly different expression patterns in the urothelium from normal cats and humans. In the normal cat bladders, uroplakin was densely distributed in the luminal layer, with some samples showing weak to moderate expression in the intermediate layer. In contrast, uroplakin expression was restricted to the luminal surface in normal human bladders. Comparison of the cluster analysis results from the FIC and human IC bladder shows that biglycan, C6S, and ZO-1 fell into one major cluster group, and E-cadherin comprised a second cluster group in both feline and human IC samples. In the human samples, uroplakin and K20 fell into a third cluster group, whereas in cats with FIC uroplakin and K20 were included the cluster group containing C6S, M/P, and ZO-1. The close association of biglycan and CS suggests that it comprises a major component of the GAG layer, whereas decorin may play a different role.

Biglycan and decorin are small leucine-rich proteoglycans (SLRPs) that are composed of a core protein attached to one or two chains of GAGs. Biglycan is attached to two CS or dermatan sulfate (DS) chains, while decorin is attached to one CS or DS chain. In the normal feline and human urothelium, biglycan, decorin, and CS were more highly expressed near the luminal surface, with expression decreasing towards the basal layer. In contrast, 75% of the FIC samples displayed absence of strong luminal staining or no staining of biglycan and decorin in the urothelium. Interestingly, biglycan and decorin¹⁶ have been shown to mediate sterile inflammation, which can be a component of IC. Tissue injury or stress can cause biglycan to be cleaved and released in the tissue as a soluble form where it can bind to toll like receptors 2 and 4 in dendritic cells and macrophages, activate TNF α synthesis and secretion of IL1 β .^{17, 18} Also, since biglycan and decorin have been shown to exert a growth inhibitory effect on carcinoma cells, the absence of biglycan or decorin in FIC could result in impaired differentiation.^{19, 20} The common aberration in biglycan and decorin expression seen in FIC and human IC^{5, 12} strongly suggests that sterile inflammation produced through these two signaling molecules could be a factor in producing the symptoms of IC.

In our earlier study of human IC biopsies, one question that arose was how representative were the small biopsies of the whole bladder.^{5, 12} The current study, which used much larger pieces of tissue, suggests that a small biopsy cannot represent the bladder as a whole. Tissue from the cats with FIC showed significantly larger overall variability than did tissue from control cats. Additionally, some areas with normal or nearly normal expression of markers were observed in the majority of the FIC tissue sections. This finding suggests that some of the variability in overall results previously noted in human patients was due to the variability inherent within the bladders of individual patients, and that examination of more or larger biopsies might decrease the number scored as normal or near normal.

The concordance in expression patterns of C6S and the proteins investigated here suggests some commonality in the etiologies of FIC and human IC, or at least that common mechanisms lead to remodeling of the urothelium to produce a permanent permeability. In the cat, social stress is a major cause of clinical signs, and relieving the stressors causes the signs to disappear.^{21, 22} This suggests that central nervous system control can lead to alterations in the expression of proteins and glycans in the urothelium. In humans, although

stress definitely plays a role in exacerbating the symptoms, its role as a causative factor is much less clear.^{23, 24} In both humans and domestic cats, the number of co-morbidities that accompany IC seems to be a feature of a more system-wide disorder. Moreover, effective multimodal environmental modification for cats with FIC has been found to mitigate signs of comorbid disorders in both clinical and laboratory studies.^{21, 25} We speculate that similar central nervous system mediated mechanisms may be acting in patients with IC to inhibit the normal rapid recovery from urothelial damage (e.g., from the urothelial sloughing that accompanies a urinary tract infection) and/or the normal differentiation program of urothelium²⁶. Some evidence for this hypothesis has been published.^{27, 28} Uncovering these and the mechanisms that produce inflammation in the bladder could provide keys to more effective management of IC.

CONCLUSIONS

The findings that bladders from human patients and cats with their respective IC diagnoses show similar changes in expression of the GAG layer and a number of proteins further support the use of FIC as a model for human IC. The main difference between FIC and HIC was that in cats uroplakin was not statistically differentially expressed in FIC and was distributed slightly differently in cats than in humans in that it was weakly or moderately expressed in the intermediate layer of cells. The main difficulty with the feline model is that the victims are usually pets, and although it is a very common cause of pet euthanasia, the ability to perform certain experiments is limited. Nonetheless, cats with FIC can certainly serve in biomarker and therapeutic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IC	Interstitial Cystitis/Bladder Pain Syndrome
FIC	Feline Interstitial Cystitis
GAG	Glycosaminoglycan
CS	Chondroitin Sulfate
C6S	Chondroitin 6-sulfate
K20	Keratin-20
ZO-1	Zonula Occludens-1

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Figure 1.

Histogram of cat bladder scoring; the labeling was scored on a scale of +2, +1, -1 and -2, with +2 being the most normal and -2 the most abnormal. Examples of scoring are shown in Figure S1.

Figure 2.

Cluster analysis of protein expression patterns; dendrograms show clustering analysis of cat bladder urothelium 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.

Table 1

Scored characteristics of different stains

Morphology/polarity (M/P)	
-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
Variability Score	
0	Minimal differences in staining patterns present in most of the urothelium
1	Staining patterns vary by 1 scoring unit in the urothelium
2	Staining patterns vary by 2 scoring units in the urothelium
Biglycan (BGN)	
-2	Uniform distribution in luminal and intermediate layers or no staining of luminal surface
-1	Absent strong staining on luminal surface
+1	Patchy
+2	Dense luminal staining with decreasing expression from luminal to intermediate layers
Chondroitin sulfate: (CS)	
-2	Uniform distribution throughout urothelium or 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
Decorin (DCN)	
-2	Uniform distribution throughout urothelium or 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
E-cadherin (ECAD)	
-2	Dense expression throughout urothelium
-1	moderate staining throughout urothelium
+1	Patchy
+2	Concentrated in umbrella cell layer especially between the umbrella and intermediate cell layers, also may have weak staining in the intermediate layer
Keratin 20 (K20)	
-2	Mostly unstained
-1	Moderate staining of intermediate layer
+1	Patchy / weak staining of intermediate layer
+2	Found only on luminal layer, densely distributed
Uroplakin (UPK)	
-2	Mostly unstained or dense expression throughout urothelium
-1	Weak staining of luminal layer

- +1 Patchy
- +2 densely distributed on luminal layer, may have weak to moderate staining of intermediate layer

Zonula Occludens-1 (ZO-1)

- 2 Mostly unstained / absent
  - 1 overexpressed in cytoplasm / not organized on cell surfaces
  - +1 Patchy
  - +2 Uniformly distributed throughout urothelium, but possibly more intense on surface of umbrella cell layer, with minimal expression in the cytoplasm, with each cell outlined by the marker
-

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Table 2

presents all the results for each cat bladder. The values in the table are the overall score and the variability score following the comma.

	M/P	BGN	C6S	DCN	ECAD	K20	UPK	ZO-1
H1	+2,0	+2,1	+2,1	-1,2	+2,0	+2,1	+1,2	+2,0
H2	+2,1	+1,1	+2,2	+1,2	+1,1	0	+1,0	+2,0
H3	+2,0	+2,1	+1,1	+1,2	+2,1	+2,1	+1,2	+2,0
H4	+2,1	+2,1	+2,0	+2,2	+1,2	+1,3	+2,1	+2,0
H5	+2,0	+2,1	+2,0	+1,2	+1,2	+2,1	+2,1	+2,0
H6	+2,0	+2,1	+2,1	+1,2	+1,1	+2,1	+2,1	+2,0
H7	+2,0	+2,0	+2,1	+2,1	-1,2	+2,1	+2,1	+2,0
IC1	+1,1	+2,3	-1,3	-1,2	-1,2	-1,2	+1,3	-1,0
IC2	+1,2	+1,3	-1,3	-1,2	-1,2	+1,1	+2,1	-1,1
IC3	-1,0	-1,1	+1,2	-1,1	+1,1	-2,2	-1,1	-1,0
IC4	+1,2	-1,0	0	-1,1	-1,1	-1,2	-1,3	+1,1
IC5	-2,0	-1,1	-1,2	+1,2	+1,3	-2,2	+2,2	-1,1
IC6	+2,0	-2,3	-1,2	+1,1	-1,2	-1,0	+2,1	+1,2
IC7	+1,3	-1,1	+1,2	-1,1	-1,2	+1,3	+2,1	-1,1
IC8	-1,0	-2,0	-1,2	-1,0	-1,1	0	-2,2	+2,0

Table 3

Mann-Whitney analysis of biomarker distributions of FIC and control bladder sections.

Biomarker	Median Control	Median FIC	p-value (2-tailed)
All	2 (n=56)	-1 (n=64)	<0.0001
Chondroitin 6-sulfate	2 (n=7)	-1 (n=8)	0.0009
Biglycan	2 (n=7)	-1 (n=8)	0.0033
Decorin	1 (n=7)	-1 (n=8)	0.0242
E-cadherin	1 (n=7)	-1 (n=8)	0.0242
Uroplakin	2 (n=7)	1.5 (n=8)	0.4800
Keratin-20	2 (n=7)	-1 (n=8)	0.0025
ZO-1	2 (n=7)	-1 (n=8)	0.0014
Morphology-Polarity	2 (n=7)	1 (n=8)	0.0070
Heterogeneity	1 (n=56)	1 (n=64)	0.0301