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Decrease of Trefoil factor 2 in cats with feline idiopathic cystitis

Stephanie I.K. Lemberger, Roswitha Dorsch, Stefanie M. Hauck*, Barbara Amann†, Sieglinde Hirmer†, Katrin Hartmann and Cornelia A. Deeg†

*Clinic of Small Animal Medicine, and †Institute of Animal Physiology, Department of Veterinary Sciences, LMU University of Munich, and *Department of Protein Science, Helmholtz Center Munich – German Research Center for Environmental Health (GmbH), Neuherberg, Germany*

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OBJECTIVE

To obtain new insights into aetiological backgrounds, and to search for diagnostic biomarkers by assessing the difference in urinary proteins between cats with spontaneous feline idiopathic cystitis (FIC) and healthy controls.

MATERIALS

Urine supernatants of 18 cats with FIC and 18 healthy control cats, and bladder biopsies of two FIC diseased cats and four healthy controls were included in the study.

METHODS

The Bradford method was used to determine protein quantity in urine supernatants. Urine was separated by two-dimensional (2-D) gel electrophoresis. Selected protein spots were excised from two-dimensional gels and analysed with tandem mass spectrometry. Validation of Trefoil factor 2 expression was realized with Western blot and immunohistochemistry. Western blot signal intensities were quantified with IMAGE QUANT software.

RESULTS

Eleven differentially expressed protein spots were identified between the 2-D gels of cats with FIC and control cats. Ten spots (only visible in the FIC gel) were identified as albumin and one spot (only visible in the control gel) was identified as Trefoil factor 2.Using quantification of Western blot signal intensities and immunohistochemistry a decrease in Trefoil factor 2 (TFF2) in cats with FIC could be revealed for the first time.

CONCLUSION

Deficiency in TFF2 possibly leads to impaired repairing abilities and immune response of the urothelium. The result could be a greater susceptibility to injury, inflammation and relapse. Therefore TFF2 deficiency might be an important event in FIC pathogenesis. Detection of a decrease in urinary TFF2 could serve as diagnostic biomarker, facilitating diagnosis. As FIC can serve as an animal model for human painful bladder syndrome/interstitial cystitis, the findings of this study might also be valuable for interstitial cystitis research and should be further investigated.

KEYWORDS

Feline idiopathic cystitis, Interstitial cystitis, urinary biomarker, Trefoil factor 2, impaired bladder defence

INTRODUCTION

Feline lower urinary tract disease (FLUTD) is a common, spontaneous disease in domestic cats. The most frequent cause of FLUTD is feline idiopathic cystitis (FIC), accounting for 55% to 69% of cases [1–3]. FIC shows many similarities to the so called painful bladder syndrome/interstitial cystitis (IC) in humans [4,5]. Clinical signs of FIC are similar to IC, and on presentation are commonly the same for cats and humans with any kind of lower urinary tract disease, irrespective of the aetiology (pain, dysuria, haematuria, pollakiuria, stranguria, urgency) [1,4]. Moreover, FIC as well as IC are characterized by relapses and chronicity [6,7]. Histologically, FIC resembles the non-ulcerative form of FIC, with mucosal petechiae (glomerulations), denudation, tears and thinning of the transitional cell epithelium [5]. However, there has also been a case report about a Hunner's ulcer in a domestic cat by Clasper in 1990 [8]. For neither of the two diseases, FIC and IC, has a consistent aetiology yet been established [4,9,10]. Moreover, diagnosis is still made by the exclusion of other diseases of the lower urinary tract, and because of the uncertain aetiology there is no causal therapy [9,11]. As a result, the disease is often protracted and entails great distress and pain to the patients.

In IC research, three urinary biomarker candidates were discovered in 1996 by Keay *et al.* [12], the anti-proliferative factor (APF), the heparin binding epidermal growth factor-like growth factor (HB-EGF), and the epidermal growth factor (EGF). These proteins revealed new information about pathophysiological events in IC and have been shown to clearly distinguish between IC, healthy controls and patients with bacterial cystitis [13]. Following this interesting finding in human medicine, the aim of this study was to search for new proteins that are differentially expressed in the urine of cats with FIC compared with healthy controls. It was suspected that these proteins might provide new ideas about pathophysiology and aetiology, leading to a better understanding of FIC. Differentially expressed proteins could also serve as urinary biomarkers, facilitating diagnosis in the future. Regarding the commonalities between FIC and IC, new insights into the aetiology of FIC might also be relevant for the understanding of IC.

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MATERIALS AND METHODS

PATIENTS

All cases included in the study, were spontaneously diseased cats treated at the Clinic of Small Animal Medicine, LMU, Munich, Germany, from January 2008 until March 2009. The urine supernatants of 36 cats were examined. Patients were randomly selected, in such a way that every cat presented to the Clinic of Small Animal Medicine that met the inclusion criteria for FIC or healthy controls was included in the study, until the aimed for sample size of 18 animals was complete. The study included two groups: the FIC group and the healthy control group. Inclusion criteria for the FIC group were clinical micturition abnormalities, such as haematuria, dysuria, and pollakiuria and periuria. FIC cases were excluded from the study if there was marked crystalluria, bacteriuria, urolithiasis, evidence of neoplasia, or a positive bacterial culture. Cases with (n = 10) and without (n = 8) obstruction of the urethra were equally included. Inclusion criteria for healthy controls were a physiological micturition, and an unremarkable urinalysis, including urine specific gravity, dipstick and sediment on the day of inclusion. Any history of prior urinary tract disease led to exclusion.

SAMPLES

Urine sample collection was performed by means of cystocentesis (FIC n = 11, controls n = 18) or catheterization (FIC n = 7), within 24 h after the onset of clinical signs in cats with FIC. In healthy control cats urine was obtained before the initiation of any treatment. Urine was centrifuged immediately after urine retrieval to obtain supernatants. Supernatants were immediately divided into aliquots and stored at −80 °C until further processing. Bladder biopsies were obtained from two cats with FIC and four cats with a healthy urinary tract. The biopsies were obtained within 30 min after death and fixed in Bouin's solution (Sigma–Aldrich, Munich, Germany) and embedded in paraffin (Microm International, Walldorf, Germany). One of the cats with FIC was euthanized because of concomitant decompensated hypertrophic cardiomyopathy and the other cat because the owner refused treatment. Control cats were dissected at the Institute of Veterinary Pathology, LMU University Munich as a result of diseases unrelated to this study.

URINALYSIS

A hand refractometer (ATAGO Co. Ltd, Tokyo, Japan) was used to determine urine specific gravity. Urine was analysed with the semi-quantitative urinalysis sticks Combur-9 (Roche Diagnostics, Roche Germany Holdings GmbH, Grenzach-Wyhlen, Germany) for protein, glucose, ketones, bilirubin, urobilinogen, nitrite, blood/erythrocytes and pH. Unstained sediments were examined microscopically.

PROTEIN QUANTIFICATION AND TWO-DIMENSIONAL GEL ELECTROPHORESIS (2DE)

Urine supernatants were subjected to protein quantification with the Bradford assay (Sigma). A total of 36 urine supernatants (18 FIC, 18 healthy) were solubilized in 2DE lysis buffer (9 M urea, 2 M thiourea, 1% dithioerythritol (DTE), 4%, 3-3-cholamidopropy-dimethylammonio-1-propanesulfonate (CHAPS) and 2.5 μM each ethylene glycol tetraacetic acid (EGTA) and EDTA).

Samples for the healthy control group and the FIC group, each containing 80 μg of protein, were immersed overnight on separate Immobiline dry strips NL pH 3–11, 11 cm (GE-Healthcare, Munich, Germany) in lysis buffer. The pH 3–11 NL strip covers most proteins found in prokaryotic and eukaryotic cells. The pH gradients at the extreme ends of the pH scale are non-linear, to distribute the proteins evenly over the gel to obtain maximum resolution.

In addition, 1% pharmalyte (GE-Healthcare) and 0.5% bromophenol blue were added to the strips. Isoelectric focusing (IEF) was done on a Multiphor (GE-Healthcare) for 15 kV at 20 °C, followed by the separation on gradient SDS-PAGE gels (9–15%) at constant 45 V per gel. Subsequently, gels were stained with colloidal Coomassie Blue (Kang *et al.*, 2002) and protein spot pattern was compared macroscopically.

MASS SPECTROMETRY

Spots macroscopically different between healthy control gels and FIC gels were selected for identification with mass-spectrometry. The spots were excised, destained and processed by proteolysis with trypsin as described before [14,15] and analysed by MALDI-TOF (matrix assisted laser desorption ionization–time of flight) peptide mass fingerprinting and MS/MS on a MALDI-TOF/TOF tandem mass spectrometer (ABI 4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA, USA). Combined peptide mass fingerprinting (PMF) and MS/MS queries were performed using MASCOT database search (Matrix Science, London UK; http://www.matrixscience.com) embedded into GPS-Explorer Software on the Uniprot database (http://www.uniprot.org). A protein was regarded as identified, if the probability-based MOWSE (molecular weight search) score was significant with protein scores greater than 70 (P < 0.05), if the matched peptide masses were abundant in the spectrum and if the theoretical masses of the significant hit fitted the experimentally observed values. The MOWSE score, as indicated by MASCOT (Matrix Science) is the negative common logarithm for the probability that the hits are an incidental event. Once the MOWSE score for a certain protein is significant, it is regarded as identified. Based on the probability score, theoretical molecular weight and pH are calculated. The MOWSE score and the protein score are identical.

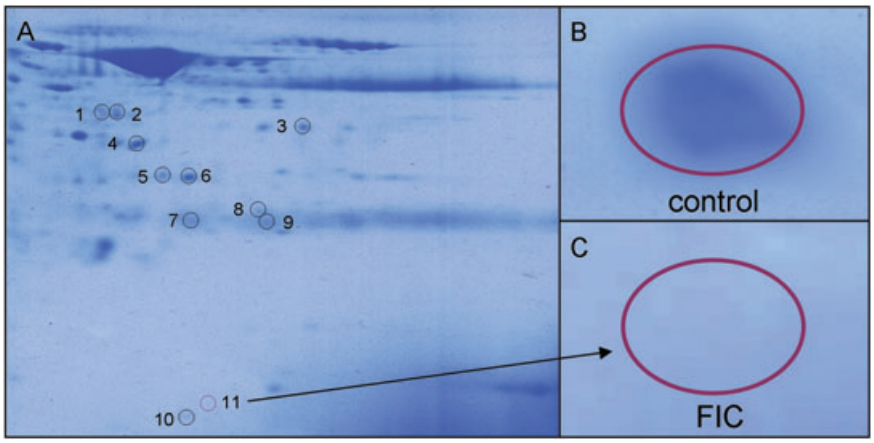
WESTERN BLOTS

For Western blots, four one-dimensional gels were performed containing urine samples of 18 FIC diseased cats and 18 control cats. One urine sample was applied per one slot in such a quantity that a protein amount of 5 μg was achieved in each slot. Western blots were then performed using PVDF (polyvinyldifluoride) membranes (GE-Healthcare). Unspecific binding was blocked with 1% PVP (polyvinylpyrrolidone) in PBS-Tween 20 (PBS-T) (1 h). Blots were subsequently incubated overnight at 4 °C, with primary antibody, mouse anti-human trefoil factor 2 (anti-human TFF2) (Abnova, Heidelberg, Germany) at a dilution of 1:1500. Then blots were washed and incubated in horseradish peroxidase-conjugated secondary antibody, anti-mouse IgG (Sigma), in a dilution of 1:3000. Signals were detected with enhanced chemiluminescence (ECL) on radiographic films (GE-Healthcare).

IMAGE ANALYSIS AND PROTEIN QUANTIFICATION

Quantification of the Western blot signals of all patients was performed with IMAGE QUANT software after scanning the films on a transmission scanner with LAB SCAN 5.0 software (all GE-Healthcare).

FIG. 1. (A)Two-dimensional gel of urine of feline idiopathic cystitis (FIC) patients. Differentially expressed spots identified with mass spectrometry are encircled and numbered according to Table 1, where identifications are given. Spot numbers 1–10 were identified as albumin. The purple circle and spot number 11 mark the place of the spot for TFF2, which could only be identified in the two-dimensional gel of the healthy controls. (B) Magnification of TFF2 spot (within the purple circle) in the healthy control gel. (C) Magnification of the TFF2 spot-area in FIC patients, no spot visible.



HISTOLOGY AND IMMUNOHISTOCHEMISTRY

For histological examination of pathological lesions in FIC, urinary bladders were excised in total, dissected into smaller parts, fixed in Bouin' solution and embedded in paraffin. Slices of all areas of the bladder were cut and stained with haematoxylin and eosin (Merck, Darmstadt, Germany). The expression pattern of TFF2 was evaluated in healthy and diseased bladders, by immunohistochemistry using the same polyclonal antibody specific for TFF2 as in Western blots. Antigen retrieval was performed at 99 °C for 15 min in 0.1 M EDTA-NaOH buffer, pH 8.8. For visualizing TFF2 in fluorescence microscopy, a secondary anti-mouse-IgG antibody, coupled to Alexa 488 (Invitrogen, Darmstadt, Germany) was employed. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindol; Invitrogen). Histological sections were examined microscopically and photographed with Leica microscope and intra-microscopically installed camera (Leica Microsystems GmbH, Wetzlar, Germany). Visualization was performed with Adobe Photoshop CS3 software. Immunohistochemistry was photographed on the Axiovision M1 microscope and edited with the AXIOVISON software program (Carl Zeiss AG, Jena, Germany).

STATISTICAL ANALYSIS

Paleontological statistics (PAST) software (http://folk.uio.no/ohammer/past/index.html) was used to calculate statistical significance. For the volume intensities in Western blots the Kolmogorov–Smirnov test was employed, to test the data distribution. As the data were not distributed normally, the Mann–Whitney *U*-test was used to calculate the statistical significance of the data. A *t*-test was performed for comparison of amounts of protein in urine supernatants. Data were regarded significant if $P \leq 0.05$.

RESULTS

PATIENT DATA

Median age was 5.50 years, (range 1–15 years) in cats with FIC, and 10 years (range 1–18 years) in healthy controls, the majority of cats were male castrated (FIC 12; controls 10) and European short hair cats (FIC 14; controls 12). All cases of the FIC group were recently diseased with severe clinical signs in only two cats, moderate signs in seven cats and mild signs in nine cats. Ten animals had their first episode of FIC, four cases were suffering from their second episode, two cats presented with their third episode and two cats had a history of several years of chronic relapsing FIC. For only one cat the owner reported concomitant disease (hypertrophic cardiomyopathy, HCM). One of the cats had had bacterial cystitis 2 months previously. All 18 cats were macroscopically haematuric. Fourteen cats suffered from stranguria, six cats displayed pollakiuria and five cats were reported to have urinated in inappropriate places. Cats of the healthy control group did not show any abnormalities in voiding urine.

Cats with FIC from which bladder biopsies were taken were 5 years and 8 years old, male castrated European short hair cats. Both cats were presented with their first episode of FIC, one with mild and one with severe clinical signs. The four control cats, from which bladder biopsies were obtained, were a median of 3.5 years old (range 1–8 years). Two cats were male castrated, one was female and one was male; all four were European short hair cats. These cats had been referred for post mortem examination to the Clinic of Pathology of the LMU Munich and were suffering from hepatic lipidosis, feline panleukopenia, brain tumour and congestive heart failure but did not exhibit any pathology of the urinary tract.

URINALYSIS

In cats with FIC urine specific gravity (USG) had a range of 1.030 g/g to >1.050 g/g, and a median of 1.043 g/g. Microscopic haematuria was seen in all 18 cases. Erythrocytes were >100/high power field (HPF) in sediments of all cats. Nine cats had 5–12 leukocytes/HPF in their urine sediment. Four cats had 0–4 leukocytes and five cats had no leukocytes. No other abnormalities were detected.

The control cats had a median USG of 1.040 g/g (range 1.030 g/g to 1.050 g/g). Except for mild microscopic haematuria (0–20 erythrocytes/HPF) in eight cats that was attributed to cystocentesis, the remainder of the urinalysis was unremarkable.

HIGHER PROTEIN QUANTITY IN URINE OF CATS WITH FIC

Protein measurement in urine supernatants showed a significant higher concentration of protein in the urine of FIC diseased cats than in healthy controls ($P < 0.001$), with a median of 1.57 mg/mL (range 0.64–4.82 mg/mL) compared with 0.43 mg/mL (range 0.19–1.17 mg/mL).

DIFFERENT PROTEIN SPOTS IN CATS WITH FIC COMPARED WITH HEALTHY CONTROLS

The 2DE gels of FIC patients and healthy controls were compared manually. Eleven spots were considered to be different between the two gels. Ten spots (Fig. 1, spots 1–10) were only visible in the FIC gel and one

spot (Fig. 1A, Spot 11 and Fig. 1B, higher magnification of same area in gel loaded with normal urine; Fig. 1C higher magnification of area in FIC urine), was visible much more intensive in the healthy control gel and only very slightly visible in the FIC gel.

IDENTIFICATION OF TFF2 WITH MS

Ten spots (Fig. 1, Spots 1–10) of the FIC gel were analysed and identified with mass spectrometry. These spots were unambiguously identified as albumin (Table 1, spots 1–10). The interesting spot number 11, which was much more intense in the control gel than in the gel of FIC diseased cats, where it was hardly visible at all, was identified as Trefoil Factor 2 (Fig. 1a spot 11, Fig. 1b; Table 1, spot 11). After studying literature concerning TFF2, it was felt that TFF2 might be of great interest for FIC pathogenesis, so further investigation of this interesting candidate was initiated.

TABLE 1 Identification of proteins with MALDI-TOF/TOF (matrix-assisted laser desorption/ionization/time-of-flight/time-of-flight)

Spot ID	Protein name	Species	Accession number	MW	pI	Protein score
1	Albumin	*Felis catus*	P49064	70 611	5.46	301
2	Albumin	*Felis catus*	P49064	70 611	5.46	502
3	Albumin	*Felis catus*	P49064	70 611	5.46	330
4	Albumin	*Felis catus*	P49064	70 611	5.46	294
5	Albumin	*Felis catus*	P49064	70 611	5.46	161
6	Albumin	*Felis catus*	P49064	70 611	5.46	256
7	Albumin	*Felis catus*	P49064	70 611	5.46	260
8	Albumin	*Felis catus*	P49064	70 611	5.46	111
9	Albumin	*Felis catus*	P49064	70 611	5.46	143
10	Albumin	*Felis catus*	P49064	70 611	5.46	208
11	Trefoil factor family peptide 2	*Felis catus*	B4 × 8D8	14 943	6.77	81

Ten protein spots of the two-dimensional gel-electrophoresis from the FIC patients and one spot from the healthy controls were analysed via mass spectrometry (MALDI-TOF/TOF). The 10 spots (1–10) from the FIC patients could be identified as albumin. Spot number 11, which was the spot from the healthy control gel, was identified as the interesting candidate TFF2. The spot ID is the number of the excised spot used for identification. The accession number is used to obtain complete information about the protein on the Uniprot server (http://www.uniprot.org). pI is isoelectric point. The protein score is identical to the MOWSE (molecular weight search) score. A protein was regarded as identified, if the probability-based MOWSE score was significant with protein scores greater than 70 ($P < 0.05$), if the matched peptide masses were abundant in the spectrum and if the theoretical masses of the significant hit fitted the experimentally observed values.

DOWNREGULATION OF TFF 2 IN CATS WITH FIC

A downregulation of TFF2 expression was suspected in the urine of cats with FIC and it could be verified and quantified by Western blots of urine supernatants. On Western blots, obvious difference in signal intensity of TFF2-antibody complex was visible, when comparing FIC samples and controls. TFF2 signal intensities, as quantified with the IMAGE QUANT program, were statistically significantly ($P = 0.03$) lower in patients with FIC (Fig. 2, light grey box plot on the right), with a median of 4050 pixel (range 1377–26840 pixel), compared with a median of 12099 pixel in healthy controls (Fig. 2, dark grey box plot on the right). (range 835–48655 pixel). In all but one case pixel intensities of the FIC group were lower than the mean pixel intensity of the control group. When comparing the signal intensities between obstructed and non-obstructed cats with FIC, no statistical significant difference could be detected.

TFF2 DEFICIENCY IN SUBEPITHELIAL, INTRAMUCOSAL, AND INTRAMUSCULAR BLADDER WALL LAYERS OF FIC DISEASED CATS

Histology of full-thickness urinary bladder biopsies revealed a loss of the physiological structure of the bladder wall in cats with FIC (Fig. 3A, H&E staining of healthy cat bladder; Fig. 3B bladder of cats with FIC). The transitional cell epithelium was injured or lost in some parts (Fig. 3B, e) and there was subepithelial (Fig. 3B, a), intramucosal (Fig. 3B, f) and intramuscular bleeding, and oedema. Fibrosis was present in the muscle layer (Fig. 3B, e) as well as in the vasculature. The TFF2 expression pattern was then studied directly in the tissue by immunohistochemistry. In bladders of controls, TFF2 was arranged subepithelially (Fig. 3C, a) intramucosally (Fig. 3C, b and c), and intramuscularily (Fig. 3C, d) (TFF2 stained a bright green colour). In diseased bladders, hardly any TFF2 was detectable within the bladder wall layering at all (Fig. 3D).

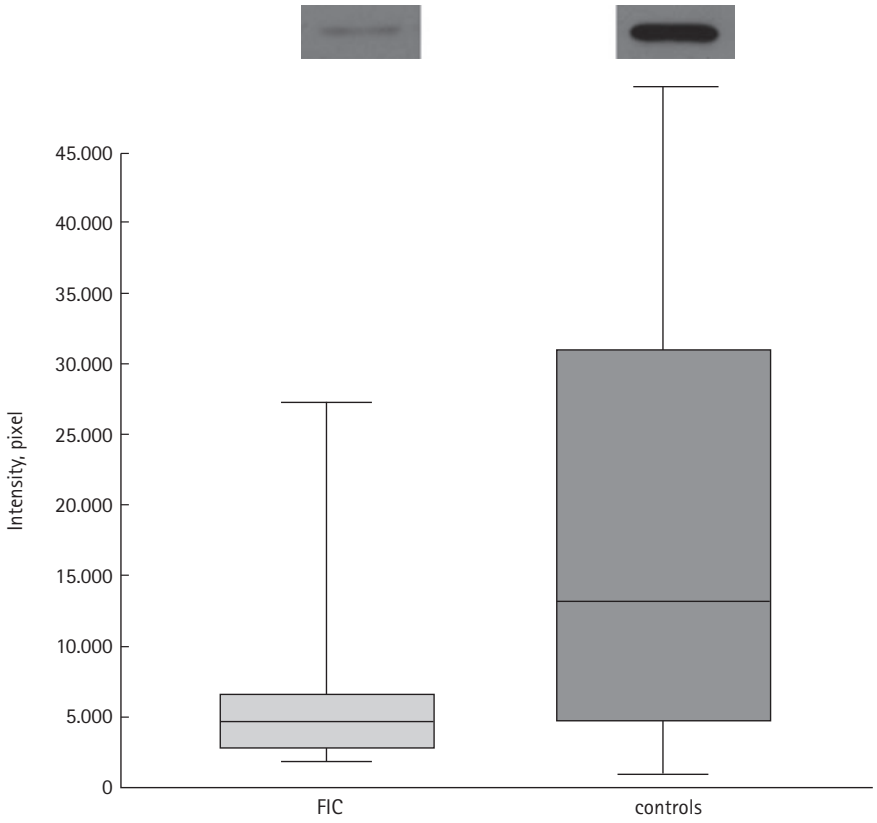
DISCUSSION

Feline idiopathic cystitis is a spontaneous disease, which is often very painful and protracted. No common consent has been found about distinct aetiological backgrounds, specific diagnostic means and therapy of FIC. As a result, new conclusions about FIC are most desirable. To date, there are no studies on the urinary protein pattern in cats with FIC in comparison with healthy controls. In this study, the aim was to search for urinary proteins that provide new perspectives on the cause of FIC. Moreover, such urinary proteins may be used as urinary biomarkers in the future, facilitating diagnosis. Bearing in mind the similarities of the two diseases, results may also be assignable to painful bladder syndrome/interstitial cystitis.

In this study, the majority of cats were male castrated European short hair cats and showed urethral obstruction. This is thought to be because many FIC cases without urethral obstruction are probably missed by owners because of less obvious signs especially in outdoor cats. In addition, male cats block more easily owing to the different anatomy of the urethra, with a very narrow point in the penile part. In painful bladder syndrome/interstitial cystitis obstructive symptoms such as dribbling, slow stream and straining are often reported [16,17], one study included even 48% patients with obstruction [17].

To identify protein patterns in cats with FIC and control cats, urine supernatants were

FIG. 2. TFF2 expression quantified with Western blots. The amounts of TFF2 in urine of cats with feline idiopathic cystitis (FIC), as analysed by IMAGE QUANT software, were significantly lower (P = 0.03) than in healthy controls. The light grey box plot (left) represents FIC cases, the dark grey box plot (right) represents healthy controls; results are given as band volume intensities of Western blot signals. Black lines in the boxes represent medians. The boxes represent the mean 50% of data, while the whiskers represent the lower and the upper 25% of the data. The left insert represents the Western blot band of a FIC patient. The right insert represents a Western blot band of a healthy control patient.



separated with 2D-gel electrophoresis. This type of electrophoresis separates proteins according to molecular size and isoelectric point, which results in a higher resolution, so that many more modifications of proteins become visible. This approach was successful and 11 differentially expressed spots (Fig. 1A) could be identified. Ten spots were identified as albumin (Table 1, spots 1–10). Albumin is the main plasma protein [18] and it very likely derives from blood in haematuric urine. Increased expression was observed for several albumin fragments. The function of these fragments is unknown, so the meaning of their increased expression remains unclear. However, increased presence of albumin fragments in the urine of cats with FIC may indicate increased proteolytic activity in this disease. An increase in proteolysis could also be one of the reasons for the catabolic state of FIC bladders, with marked tissue lesions.

A very interesting protein identified was TFF2, which could only be found in the urine of control cats but was not present or present in only in trace amounts in cats with FIC (Figs 1,2) and is also aberrant in the bladder tissue of affected cats (Fig. 3D, lack of green staining). TFF2 is a low molecular weight peptide [19] belonging to the trefoil factor family, a protease-resistant protein family consisting of three members (TFF1–3) [20]. It is primarily expressed in mucous neck cells of the gastric mucosa [21]. Moreover, TFF1–3 expression in humans has been shown in the intestine, the pancreas, the gall bladder, the breast, the hypothalamus, the pituitary and the respiratory tract [22]. Not much is known about distribution and function of TFFs in the urinary tract. Chutipongtanate *et al.* in 2005 discovered human urinary TFF1 as novel calcium oxalate crystal growth inhibitor preventing the formation of kidney stones in humans [23]. In this study, immunohistochemistry demonstrated the presence of TFF2 in epithelial, mucosal and muscular layers of the urinary bladder from healthy control cats and therefore confirmed TFF2 expression in the feline urinary bladder, which is a novel finding. (Fig. 3C, green staining). In the bladders of FIC diseased cats, however, a lack of TFF2 could be shown using immunohistochemistry directly in the tissue (Fig. 3D, lack of green staining). All three TFFs are rapidly up-regulated in the setting of mucosal damage or injury, where they contribute to epithelial restitution [24,25]. Their healing properties primarily result from promotion of cellular motility and migration and the inhibition of apoptosis [26]. Moreover, TFF2 plays an important role in the immune response of the gastrointestinal tract and could similarly be of importance for the urinary immunosystem. TFF2 deficient mice show alterations in immune response relevant genes, such as genes involved in MHC-I molecule presentation [27]. A significant reduction of BAG-2 leads to alterations in the signalling pathway for response to cellular stress [28]. Moreover, TFF2 deficient transgenic mice exhibit over-expression of cysteine-rich intestinal peptide, which causes changed numbers of different white cell types and cytokine patterns and therefore an altered immune response [29]. In addition, TFF2 deficient T-cells and macrophages exhibit enhanced secretion of interleukins (IL) in response to IL-1R, and this *in vitro* response correlates to an increased inflammatory response *in vivo* [30].

Absent TFF2 is associated with gastrointestinal inflammation [21] and susceptibility to gastric ulceration [31] and progression of malignancies for example in the gastrointestinal tract [32], the liver [24] and the prostate [33] of humans. Studies with TFF2-deficient mice demonstrate that TFF2 knockouts have a slightly higher susceptibility to stress-induced gastric ulceration and that the damage score (depth of ulceration and degree of associated inflammation) is higher in these mice than in wild-type controls [28].

Imunohistochemistry as well as Western Blots revealed a decrease of TFF2 in the bladder of cats with FIC compared with healthy controls. This deficiency in cats with FIC may lead to severe defects in the bladder mucosa and the defence mechanisms of the urinary bladder, and therefore to a susceptibility to mucosal lesions and inflammation and their relapse. A

deficiency in such an important growth factor may lead to severely impaired repair and regeneration abilities of the urothelium. As a result, the normal remodelling processes in the bladder can not occur in a physiological way, and even small lesions could cause major urothelial defects and inflammation. Moreover TFF2 deficiency might lead to a malfunction of the immune response in the urinary bladder, causing it to be susceptible to inflammation and infection with microorganisms. These pathophysiological changes could also explain the chronic and relapsing character of the disease.

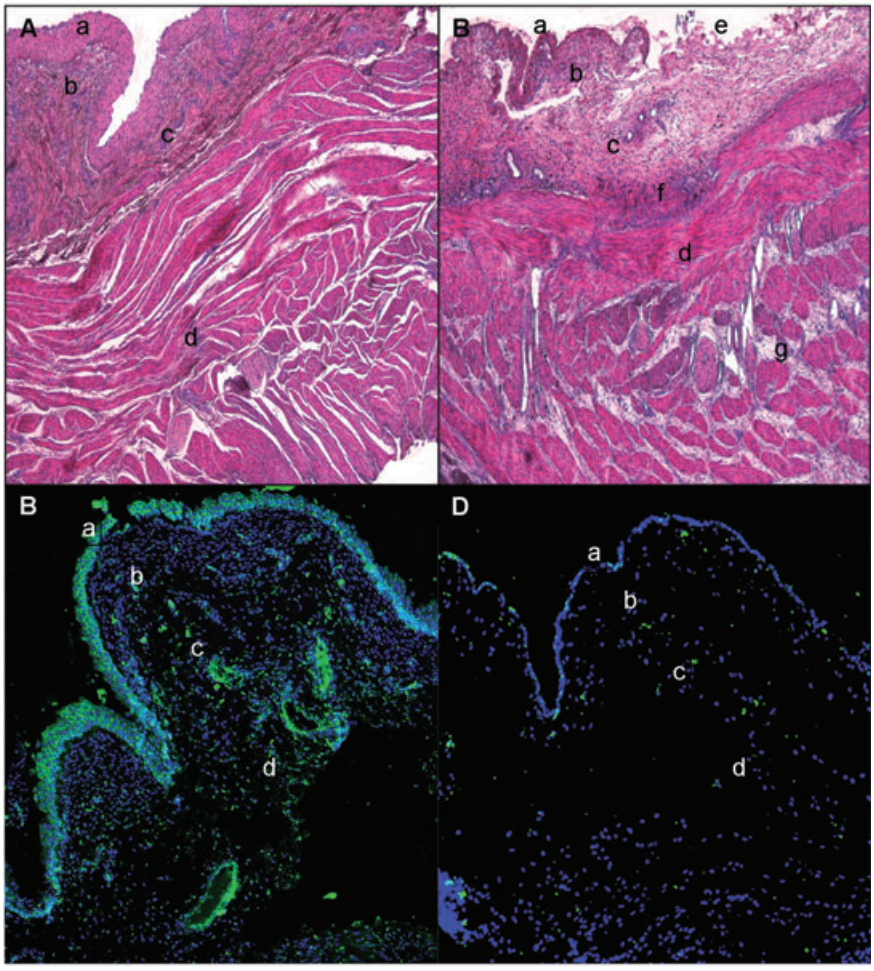
These facts suggest that TFF2 deficiency plays an important role in the pathophysiology of FIC. Currently, it is uncertain whether deficiency in TFF2 is the cause or the result of FIC, but considering the generalized deficiency in TFF2 throughout the bladder and the typical and marked mucosal lesions in FIC, TFF2 deficiency might be the cause of the development of FIC and the lack of TFF2 might be genetic.

Moreover studies as of oral and parenteral administration of TFF2 have revealed beneficial effects on healing of gastric ulceration in the rat [34], TFF2 might also have therapeutic value in the treatment of FIC. The findings of this study very interesting and merit further investigation.

STUDY LIMITATIONS

Limitations of this study are the fact that the majority of FIC diseased cats were obstructed, and therefore it cannot be excluded that the results of this study are representative of obstructive FIC rather than FIC itself. However, it is important to stress that the amount of TFF2 was also decreased in cats without urethral obstruction, and that comparison between signal intensities of obstructed cats and non-obstructed cats did not reveal any significant differences. Another limitation is the sample size of the bladder biopsies for histology, which is relatively small, so that significance is not ultimately ensured. Moreover, no other control group, such as urinary tract infection, urolithiasis or neoplasia was provided to compare TFF2 expression in other bladder pathologies. This study cannot clarify whether the decrease in TFF2 is the cause or a result of FIC. Regarding the transferability to painful bladder syndrome/IC, no definitive conclusions can yet be drawn as no experiments have been undertaken with human urine. Therefore, research into this promising new urinary protein is strongly warranted.

FIG. 3. (A,B) Histological sections stained with Haematoxylin and Eosin. (A) healthy control bladder: a and b represent the mucosal tunic with a, transitional cell epithelium (TCE) and b, lamina propria; c, submucosal tunic; d, muscle tunic; e, loss of TCE; f, intramucosal bleeding; g, fibrosis in muscle tunic. (B) Feline idiopathic cystitis (FIC) bladder, loss of normal bladder wall physiology: e, loss of TCE; f, intramucosal bleeding and oedema and g; fibrosis in muscle tunic. (C,D) Immunohistochemical staining of TFF2 (green). (C) Healthy control bladder. The bright green colour indicated TFF2, which is visible in all layers. (D) FIC bladder. In the FIC bladder there is a lack of green colour, which means a lack of TFF2 in its normal subepithelial, submucosal and muscular distribution compared with the distribution in healthy controls. Blue colour indicates DAPI (4′,6-diamidino-2-phenylindol), which stains cell nuclei.



CONCLUSIONS

This study found statistically significant lower amounts of TFF2 in the urine of cats with spontaneous FIC compared with healthy controls. The lack of TFF2 expression could also be confirmed directly in tissue. For the first time, physiological TFF2 expression in the subepithelial, mucosal and muscular layers of the feline urinary bladder could be demonstrated. With regard to the physiological functions of TFF2, this study suggests that TFF2 deficiency in the urinary bladder may lead to severe impairment of defence mechanisms and therefore to a susceptibility to mucosal lesions and inflammation, and their relapse.

These findings support the suggestion that deficiency in TFF2 in the urinary bladder of cats with FIC might play an important role in pathogenesis. Furthermore, the detection of TFF2 downregulation in urine of cats with FIC

could be a valuable diagnostic biomarker for the future, and oral or parenteral supply with TFF2 might be a novel approach in FIC therapy. FIC can serve as a valid model for IC in humans, and therefore this study could be basis for new research studies in human medicine.

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CONFLICT OF INTEREST

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Correspondence: C.A. Deeg, Institute of Animal Physiology, Department of Veterinary Sciences, LMU University Munich, Munich, Germany.
e-mail: deeg@tiph.vetmed.uni-muenchen.de

Abbreviations: **IC**, interstitial cystitis; **FIC**, feline idiopathic cystitis; **FLUTD**, Feline lower urinary tract disease; **TTF2**, Trefoil factor 2.