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ORIGINAL ARTICLE

Epithelial–mesenchymal transition (EMT) of renal tubular cells in canine glomerulonephritis

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Abstract Tubulo-interstitial fibrosis in dogs may result from primary injury to the interstitium or develop secondary to other renal diseases. As in human renal pathology, tubular epithelial cells (TEC) are believed to actively participate in the mechanisms of renal fibrosis. In this study, we examined the changes in the tubular epithelial component in two specific canine diseases. Immunohistochemistry showed the expression of the epithelial marker cytokeratin, the smooth muscle marker α-SMA, the mesenchymal marker vimentin and PCNA in 20 dogs with membranous glomerulonephritis and membrano-proliferative glomerulonephritis. Results showed that the loss of the epithelial marker in TEC was directly correlated to the grade of tubulo-interstitial disease present and independent of the type of glomerulonephritis. Varying degrees of vimentin positivity were detected in tubular epithelium in areas of inflammation, and low numbers of scattered α-SMA-positive cells were also observed. Immunohistochemistry showed that epithelial tubular cells lose their cytokeratin staining characteristics and transdifferentiate into cells exhibiting key mesenchymal immunophenotypic feature of vimentin-positive staining in both diseases investigated. The integrity of the tubular basement membrane is likely to be fundamental in maintaining the epithelial phenotype of TEC. Animal models provide opportunities for investigating the pathogenesis of renal fibrosis in humans.

Keywords Cytokeratin · Dog · Interstitial fibrosis · Tubular epithelial cells · Vimentin

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Introduction

In dogs, membranous and membrano-proliferative glomerulonephritis are acknowledged to have a variety of aetiologies. Protozoal, bacterial and viral infections are the best-described causes, whilst yet other cases are considered idiopathic. As the glomerular lesions progress, the tubulo-interstitial (TI) compartment becomes involved, and TI damage (TID) is a common outcome of all chronic progressive renal diseases regardless of aetiology or site of primary injury [12, 14]. Histologically, these end stage lesions are characterized by interstitial fibrosis with inflammatory cell infiltrates and loss of peritubular capillaries, whilst tubules undergo concurrent degenerative, atrophic and regenerative changes [4]. Decline in renal function (reduced glomerular filtration rate) in chronic renal disease appears to be correlated more closely with

interstitial lesions than with glomerular damage [8, 15]. Interstitial fibrosis is linked to a pathological imbalance between extra-cellular matrix deposition and degradation, which is stimulated by a variety of cytokines and growth factors [13]. The fibrogenic response and the inflammatory infiltrates progressively reduce the number of functional nephrons leading to renal failure [9].

Previous studies carried out on kidneys from humans, rats and cattle have shown that, during renal injury, tubular epithelial cells (TEC) are able to acquire features typical of mesenchymal cells [16, 20, 22]. This process has been named epithelial–mesenchymal transition (EMT). EMT has been demonstrated in lung fibrosis as well as in some type of tumours, and it is considered a physiologic process in embryogenesis [21]. Renal tubular EMT, by definition, is a process in which tubular cells lose their epithelial phenotype and acquire characteristic features of mesenchyme [7] such as the expression of vimentin, the characteristic cytoskeleton protein of mesenchymal cells. Proteinuria and chronic hypoxia both appear to play significant roles in EMT, and several authors have suggested the importance of reduced local oxygen tension, resulting from peritubular capillary destruction, in development of the process [5, 11].

This current study aims to use immunofluorescence and immunohistochemical techniques to detect and quantify phenotypic changes in TEC undergoing EMT during the course of two specific canine glomerular renal diseases.

Materials and methods

Tissue samples

Twenty cases exhibiting renal lesions of one of two specific glomerular diseases were selected from archives of the Department of Animal Pathology (University of Turin) from a period spanning 4 years (2003–2007). The glomerular diseases chosen were membranous glomerulonephritis and membrano-proliferative glomerulonephritis as defined in the WHO classification of glomerular diseases. Kidney tissue samples were obtained by renal biopsy (Trucut method) in routine diagnostic cases (eight biopsies), whilst the remaining tissue was collected from dogs subjected to post-mortem examination (12 dogs). Controls were five young dogs (age range 2–6 years). These dogs were strays from dog shelters that were euthanised for aggressive behaviour. No significant lesions in systemic organs were found. Renal clinical examinations were normal. Signalment and clinical data from the dogs considered in the study are shown in Table 1.

Table 1 Clinical features and TID grade

Case number	Age (years)	Sex	Glomerular lesion	Cause	TID grade
1	8	M	MPGN[b]	*Leishmania spp.*	2
2	7	M	MPGN[b]	Idiopathic	1
3	5	F	MPGN[b]	Canine adenovirus 1	1
4	6	M	MPGN[b]	Idiopathic	1
5	4	F	MPGN[b]	Canine adenovirus 1	2
6	8	M	MPGN[b]	*Borrelia burgdorferi*	2
7	8	M	MPGN[b]	*Leishmania spp.*	2
8	3	F	MPGN[b]	*Leishmania spp.*	3
9	10	M	MPGN[a]	Chronic pancreatitis	3
10	11	M	MPGN[a]	*Dirofilaria immitis*	2
11	12	M	MPGN[a]	Neoplasm	2
12	16	F	MPGN[a]	Neoplasm	2
13	5	F	MGN[b]	*Leishmania spp.*	1
14	6	F	MGN[b]	*Dirofilaria immitis*	2
15	5	M	MGN[b]	*Dirofilaria immitis*	1
16	7	M	MGN[b]	Idiopathic	1
17	5	M	MGN[a]	Neoplasm	1
18	2	M	MGN[a]	Idiopathic	3
19	11	M	MGN[a]	Pyometra	2
20	12	F	MGN[a]	*Dirofilaria immitis*	2
21	5	M	Control	–	0
22	6	M	Control	–	0
23	2	F	Control	–	0
24	3	M	Control	–	0
25	2	M	Control	–	0

[a] Autopsy
[b] Biopsy

Histology

Renal samples for histological examination were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 4 μm in thickness and stained with: haematoxylin–eosin, periodic acid-Schiff (PAS), acid–fuchsin orange G, Masson's trichrome and phosphotungstic acid haematoxylin.

To quantify the interstitial changes in the two forms of glomerulonephritis, the severity of TID was graded. Interstitial fibrosis and inflammation were assessed at ×200 magnification and scored as follows: normal tubulointerstitium (score=0), mild tubular atrophy and interstitial oedema or fibrosis affecting up to 25% of the field of view (score=1), moderate TI fibrosis affecting 25–50% of a given field (score=2), severe TI fibrosis >50% of a field

(score=3). Score data are shown in Table 1. The samples were scored by a veterinary pathologist and a human renal pathologist in a blinded fashion.

Immunofluorescence

For immunofluorescence staining, unfixed renal tissue was OCT-embedded, snap-frozen in liquid nitrogen and stored at −80°C. Six-micrometer-thick sections were fixed with acetone for 15 min. After washing with PBS (two passages), slides were incubated with fluorescein isothiocyanate-labelled anti-goat IgA, IgG, IgM and complement C3 antibodies specific for the dog (Bethyl Laboratories, Montgomery, AL, USA). Primary antibodies were omitted as negative controls.

Immunohistochemistry

Immunoperoxidase staining was performed on renal sections of formalin-fixed, paraffin-embedded renal samples. An immunohistochemical panel was performed to assess changes to the epithelial tubular cells. Antibody against cytokeratin (CK) AE1/AE3 (Dako, Glostrup, Denmark) was used to detect cells expressing an epithelial phenotype. Mesenchymal markers were stained with antibodies against vimentin (Dako) and α-smooth muscle actin (α-SMA, Dako). Proliferation cell nuclear antigen (PCNA; Clone PC10, Dako) was used as index of cellular proliferation.

Double immunohistochemical staining

On selected representative cases, CK AE1/AE3 and vimentin double antigen-immunoperoxidase labelling was performed using a commercial kit (Vector Laboratories, Burlingame, CA, USA), according to manufacturer's instructions. Peroxidase activity was demonstrated firstly by DAB+Ni^{2+} (grey to black chromogen reaction) for Vimentin and secondly by 3-amino-9-ethylcarbazole (AEC, red chromogen reaction) for CK AE1/AE3.

Quantification of immunohistochemistry and statistical analysis

Quantification of results in each case was achieved by counting the numbers of TEC per ten high power fields (HPFs, ×200) that expressed cytoplasmic staining for the mesenchymal marker. Loss of cytoplasmic staining for the epithelial marker CK was evaluated by counting the number of negative-stained cells per HPFs. Data were analysed using Minitab 14, and results were expressed as means, standard deviations and the numbers of TEC for each fields. To demonstrate that CK and vimentin expression were statistically correlated to the TID grade, a number of paired comparisons were performed.

Results

Tubular-interstitial damage was a common finding in membranous and membrano-proliferative glomerulonephritis (Fig. 1d). All three different grades of TID were present amongst the samples examined. The quantitative analysis data corresponding to the grade of TID are summarized in Table 1. Renal samples, independent of the diagnosis, showed variable intensity of positive immunostaining in the TEC for all markers.

A diffuse and statistically significant increase (p=0.001) in the number of PCNA-positive cells was noted in nuclei of tubules within foci of nephritis (3.5±1.5 TEC/field in TID grade 3; Fig. 1c).

In control kidneys, TEC exhibited strong and uniform cytoplasmic expression of the epithelial marker CK (98.7±1.3 TEC/field in controls; Fig. 1a). In contrast, expression of CK was reduced in tubules associated with mild or severe interstitial fibrosis and completely lost in atrophic regions (54.5±4.8 TEC/field in TID grade 3; Fig. 1b).

The mesenchymal markers (vimentin and α-SMA) showed no staining in TEC from control kidneys. However, expression of vimentin was demonstrated in TEC of degenerate, atrophic tubules and tubules within areas of inflammation (39.5±13.9 TEC/field in TID grade 3; Fig. 2a,d). Some α-SMA-positive myofibroblasts were found in contact with intact tubules both in areas with no interstitial inflammation and in areas of severe TID (0.5± 0.1 TEC/field in TID grade 3). In fibrotic regions, within areas of inflammation, there was occasional positive staining of α-SMA in cells considered to be peritubular myofibroblasts. Both the size and the number of capillaries, next to the zone of cortical interstitial fibrosis and tubular atrophy, were subjectively reduced. Vimentin-PAS immunohistochemical staining showed thickening of the tubular basement membrane (TBM) with occasional multifocal membrane rupture. A high percentage of the tubules with vimentin positivity exhibited preservation of TBM integrity (Fig. 2c).

Statistical results

A linear regression model was generated using the TID grade as predictors and CK and vimentin as dependent variables (Fig. 3). The Pearson correlation coefficient confirmed the existence of a linear relationship. The two

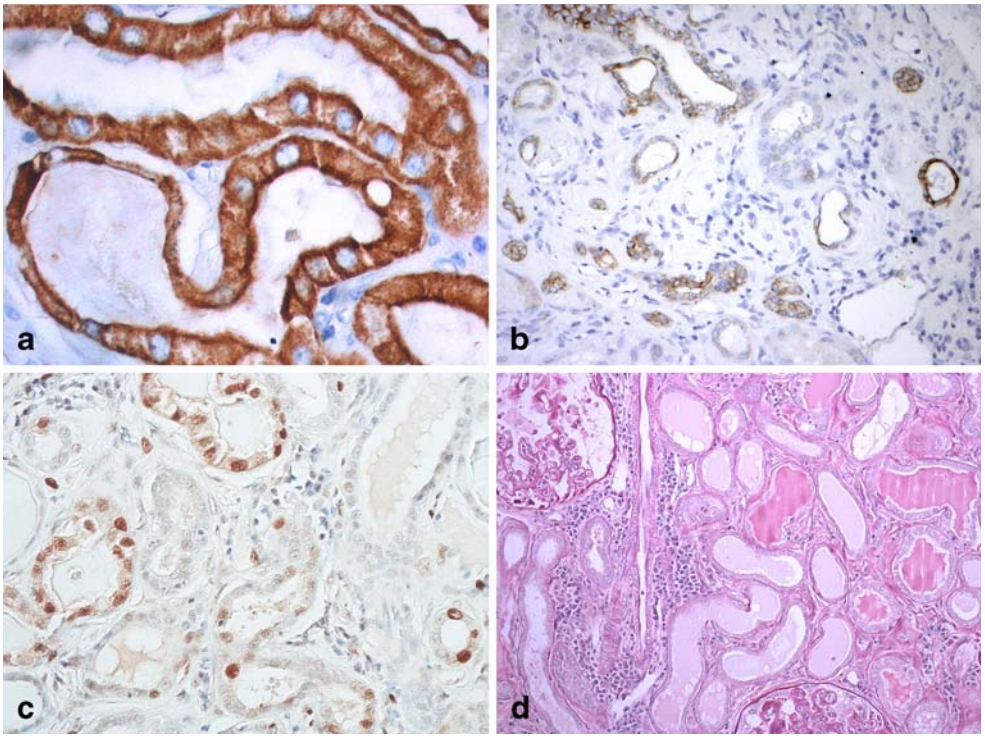

Fig. 1 **a** Normal CK expression in the cytoplasm of TEC in control kidney (×500). **b** Loss of CK expression in most of tubular cross sections inside the inflammation, TID grade 2 (×200). **c** Positive expression of scattered nuclei in epithelial tubular cells inside the inflammation, TID grade 2 (×300). **d** Area of interstitial nephritis, characterized by inflammatory cells (lymphocytes and plasma cells), tubular atrophy and minimum extent of fibrosis, TID grade 3 (PAS, ×200)

lines, with the confidence band at the level 95%, exhibited the behaviour between the indices: as the TID grade increases, the proportion of CK-positive TEC reduces (in fact, the slope is significantly negative); as the TID grade increases, the proportion of vimentin-positive TEC increases (the slope is significantly positive).

Discussion

The present results suggest that TEC are able to lose the typical cytoskeletal intermediate filament of epithelial cells (CK) and transdifferentiate into cells exhibiting the typical cytoskeletal intermediate filament of mesenchymal cells

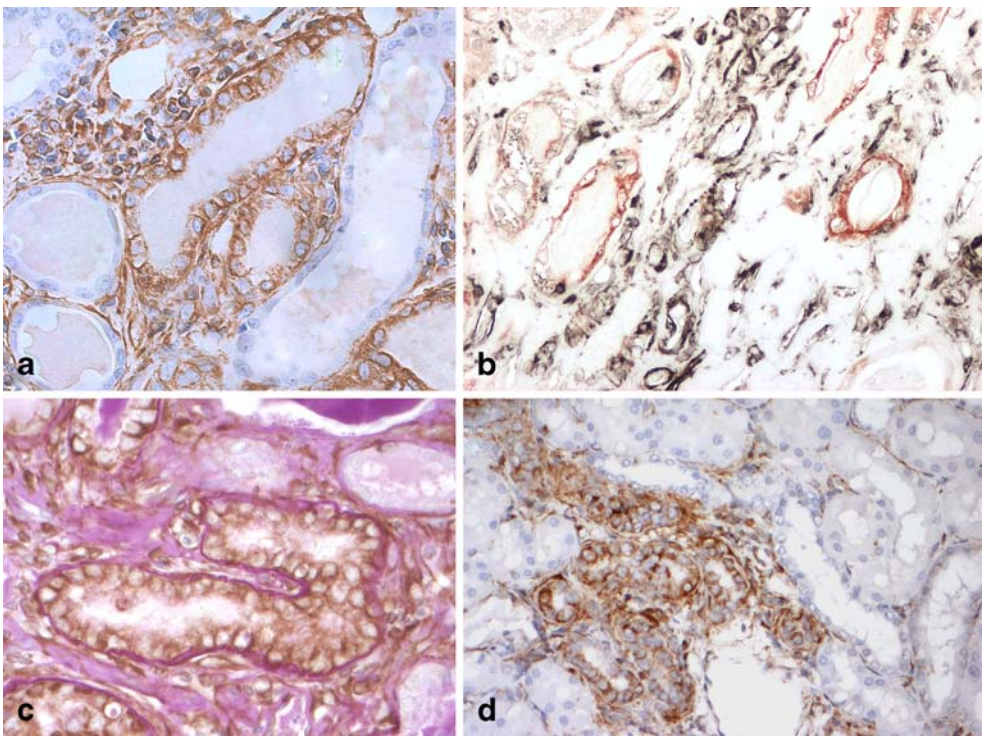

Fig. 2 **a** Diffuse expression of vimentin marker in TEC inside the inflammation, TID grade 2 (×400). **b** Atrophic-degenerating TEC in area of severe TID predominantly express vimentin (black chromogen reaction) and only in few tubules co-localization with CK (red chromogen reaction) is still present (×200). **c** Vimentin expression in tubules associated with thickening of PAS-positive TBM, TID grade 2 (×500). **d** Positive vimentin expression in TEC inside the area of inflammation and negative vimentin expression in tubules not involved, TID grade 1 (×200)

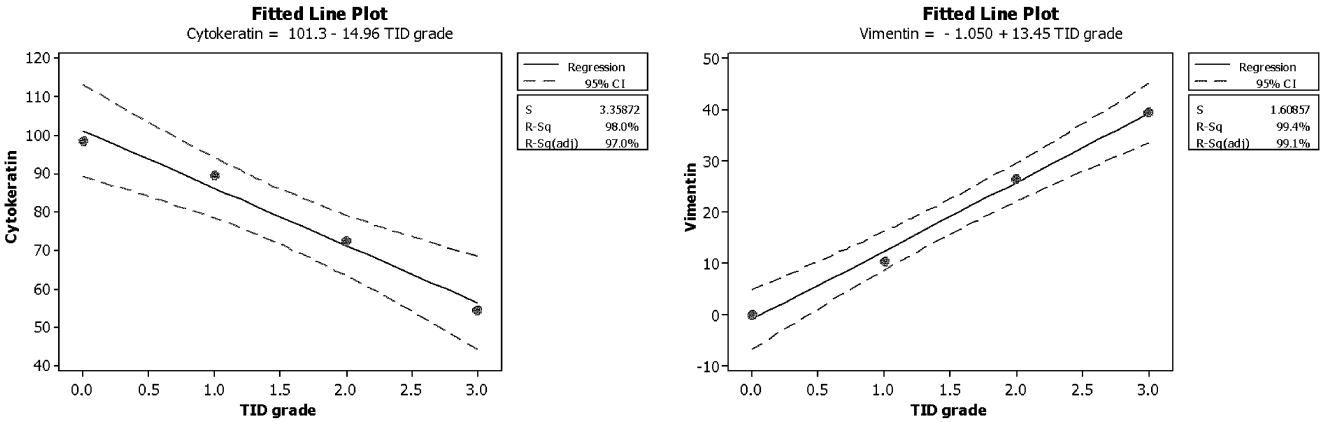

Fig. 3 Linear relationship between TID grade and CK loss (*left*) and vimentin expression (*right*)

(vimentin). This change in the cytoskeletal components named EMT was observed in both the canine renal conditions considered in this study. The process of cell shifting between epithelial and mesenchymal phenotype has long been known to play an important role in cellular transdifferentiation during organ fibrosis and tumour progression in human pathology [21] as well as in cases of interstitial nephritis in animals [16]. During TID, TEC are probably capable of these radical changes in cytoskeletal phenotype expression because of their embryonic metanephrogenic mesenchymal origin [13].

Studies carried out in human renal pathology and in experimental models in laboratory animals have confirmed that TEC actively participate in the mechanisms of injury within the interstitial compartment. These modifications act at different steps including proliferation and phenotypic changes, to eventually lead to synthesis of extracellular matrix. Furthermore, in humans as well as in dogs, the expression of class II major histocompatibility complex molecules in TEC in interstitial nephritis has been demonstrated, drawing attention to the possible role of TEC in the development of TI disease [19]. The present work focuses on the modification of TEC in the two most common canine renal diseases, membranous glomerulonephritis and membrano-proliferative glomerulonephritis. The association between the thickening of the TBM (depicted by the PAS staining) and vimentin expression by TEC suggests that direct interactions between the TEC and the extracellular matrix of the interstitium could be important in the induction of the transdifferentiation process. Other researchers have hypothesised that the critical features of EMT are the ability of epithelial cells to lose polarity, to disassemble cell adhesion systems and their strict relation with the basement membrane [1, 2, 17, 21]. The integrity of the TBM is thus likely to be fundamental in maintaining the epithelial phenotype of TECs [18].

In this study, we have described the first steps in the pathogenesis of renal fibrosis in two of the most common disease resulting in renal failure in dog. Renal tubular EMT is a phenotypic alteration that occurs after chronic injury and results in increased numbers of interstitial mesenchymal cells. As such, EMT may be involved in the initiation of renal scar formation. However, activation of resident interstitial fibroblasts would also be expected to occur after injury as part of normal wound healing by which the injured kidney attempts to repair and to recover [10]. The variable intensity of vimentin-positive staining in TEC suggests the existence of different steps in phenotype transition with the proportion of TECs undergoing transition related to the grade of TI inflammation (see Table 2). Double immunostaining demonstrated the co-expression of CK and vimentin markers within TEC in TID (Fig. 2b).

Table 2 Summary of immunohistochemical results

	Control		TID grade 1		TID grade 2		TID grade 3		*p* value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Cytokeratin	98.7	1.3	89.6	2.3	72.6	4.8	54.5	4.8	0.0012
Vimentin	0.0	0.0	10.5	3.2	26.5	8.3	39.5	13.9	0.0010
α-SMA	0.0	0.0	0.3	0.1	0.5	0.1	0.8	0.2	0.0084
PCNA	0.7	0.3	1.2	0.6	2.6	0.9	3.5	1.5	0.0010
Number of cases	5		7		10		3		

Results are reported as percentage of positive TEC.

Vimentin, the intermediate filament protein specific for mesenchymal cells [6], from our work appears to be the most suitable marker for the defining the process of EMT in the kidney. In our study, PCNA staining results showed an increase in the proliferation index of TEC within the TI compartment directly correlated with the severity of TID. This could be considered further evidence of the suggested multi-potential capacity of tubular cells in repairing damage [23]. EMT features were totally absent in the control kidneys, suggesting a link between TI inflammation and phenotypic transition of epithelial tubular cells. Histological studies of human kidneys and animal models have also shown that TI injury is associated with damage to renal arterioles and loss of peritubular capillaries [3, 5]. In our work, the mild increase in number of α-SMA-positive cells recorded in TID correlated with a minimal myofibroblasts proliferation. Glomerular inflammation and scarring have the potential to compromise blood flow in downstream peritubular capillaries and thereby result in ischemic injury to tubule cells. This mechanism may be intimately involved with EMT, with hypoxia activating fibroblasts and altering the extra-cellular matrix metabolism of resident renal cells. Within areas of fibrosis, small arteries may remain constricted causing anoxia and perpetuating the vicious cycle of TI disease, through further fibroblasts activation [11].

In summary, the crucial loss of the epithelial phenotype in EMT in dogs can be demonstrated by CK and vimentin co-expression in double immunostaining, whilst the process of EMT may contribute to the pathogenesis of fibrosis in the canine kidney. The importance of EMT in canine TI disease remains to be fully elucidated. The canine model can be used to define the evolution of the lesions, and sequential screening of the progression of the renal fibrosis at different times would be helpful in understanding the mechanism of transition.

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