

Review

Epithelial mesenchymal transition in the progression of renal disease in dogs

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Summary. Chronic kidney disease (CKD) in dogs is the final common pathway resulting from persistent renal injury and is characterized by progressive tubulointerstitial damage (TID). Pathogenesis of CKD is divided into an initial inflammatory phase with a predominantly mononuclear infiltrate followed by a fibrotic phase with increased numbers of fibroblasts and extracellular matrix deposition that causes a progressive reduction of functional parenchyma.

Proteinuria is a common manifestation of renal diseases in dogs, and its role in the pathogenesis of CKD is still uncertain. Nevertheless, the degree of proteinuria in dogs correlates with TID progression. Increased protein filtration may have direct effects on tubular epithelial cells (TECs) that induce them to express the major histocompatibility complex type II, and thereby contribute to lymphocyte recruitment. Thus, an active pro-inflammatory role is proposed for TECs in TID progression. Moreover TECs are believed to actively participate in the mechanisms of renal fibrosis. Epithelial-Mesenchymal-Transition (EMT) of TECs in canine TID has been studied in the last decade. Down-regulation of adhesion molecules and loss of epithelial markers in TECs directly correlate with the severity of TID and with de novo expression of mesenchymal markers. Tubular basement membrane (TBM) disruption is an early EMT event. Increased activity of Matrix Metalloproteinase-2 and its co-localization with TBM splitting suggests an active role for the enzyme in

inducing EMT.

Processes occurring in canine CKD share many similarities with its human counterpart, making the dog a good model in which to examine the mechanisms of TID progression.

Key words: Animal model, Chronic kidney disease (CKD), Dog, Epithelial-mesenchymal transition (EMT)

Introduction

Chronic kidney disease (CKD) is the final common pathway resulting from persistent renal injury. In dogs, CKD is morphologically characterized by a variety of pathological changes including glomerulosclerosis, inflammation, tubular atrophy and tubulointerstitial fibrosis. Glomerular diseases are the leading cause of CKD in dogs and are important causes of morbidity and mortality in this species (Polzin and Cowgill, 2013). Other less frequent causes (e.g., renal dysplasia, pyelonephritis) have also been reported. Recently published data on the prevalence of different categories of pathologic diagnoses identified in dogs with suspected glomerular disease (Schneider et al., 2013) indicate that immune-complex glomerulonephritides are the largest single category of canine glomerular diseases (about 48% of cases). However, all other diagnostic categories taken together accounted for about half (52%) of all cases, although no other category (e.g., primary glomerulosclerosis, amyloidosis, etc.) alone accounted for more than about 20% of all cases (Schneider et al., 2013). Recently, many advances in the diagnosis of renal

biopsies have been achieved in veterinary medicine through the greater use of diagnostic modalities such as optical microscopy, transmission electron microscopy and immunofluorescence, which are routinely used in human nephropathology, and more detailed data are expected to be forthcoming (Aresu et al. 2008a; Cianciolo et al., 2013). However, in dogs it is well known that most chronic nephropathies share common pathogenic mechanisms that contribute to disease progression, regardless of the original cause of disease, and that renal fibrosis is an inevitable and consequential feature of all kinds of progressive CKD. Indeed, the degree of tubular atrophy and interstitial fibrosis observed in canine renal biopsies have been better indicators of the severity of renal functional impairment than the extent of glomerular sclerosis (Nabity et al., 2012; Benali et al., 2013).

In this review, the pathogenesis of CKD in dogs will be summarized with emphasis on the mechanisms of renal fibrosis and the role of the proteinuria in the progression of CKD. The mechanisms of the tubular epithelial-mesenchymal transition (EMT) in dogs will also be described.

Renal tubulo-Interstitial damage in dogs

The tubulointerstitial compartment of renal tissue consists of tubular and vascular structures that are embedded in and supported by a moderately cellular connective tissue matrix (interstitium). Interstitial cells are a heterogeneous population including fibroblasts, dendritic, inflammatory and hematopoietic progenitor cells that are primarily involved in the fibrogenic process. The entire tubulointerstitial compartment accounts for more than 80% of the total kidney volume, and involvement of each of the cell types has been correlated to CKD in various ways (Eddy, 2005). In general, the typical features of chronic tubulointerstitial (TI) damage are the presence of interstitial inflammation and fibrosis associated with the loss of tubules and peritubular capillaries (Eddy, 2005). Fibrosis is mostly due to an imbalance between the production and degradation of ECM, leading to excessive accumulation and deposition of collagenous ECM. Increased internal renal pressure is also a possible cause of tubular atrophy and dilatation. Multiple phases of the fibrotic process can be distinguished and considered in chronologic order, but each phase is part of an overall attempt by the kidney to repair its injury and recover from the damage (Eddy, 2000). The first to occur is the inflammatory phase, wherein lymphocytes, mostly T-lymphocytes, monocytes and plasma cells are distributed in the interstitial compartment and accumulate progressively (Aresu et al., 2012). Subsequently, resident kidney cells are activated, leading to the production and secretion of proinflammatory cytokines (Eddy, 2000). Interstitial inflammatory cells are found in all forms of canine glomerular diseases and an association between inflammatory index (number of inflammatory cells per

optical microscopy field) and cortical interstitial fibrosis has been found in multiple recent studies (Aresu et al., 2012; Benali et al., 2013). The phenomenon called tubulitis, where inflammatory cells are located between tubular epithelial cells and the tubular basement membrane, has been scarcely described in veterinary medicine. However, the functional significance of this phenomenon is still unclear both in experimental animal models and humans. The next phase of the fibrotic process partially overlaps the inflammatory phase and is mainly characterized by the proliferation of fibroblasts and deposition of extracellular matrix (Eddy, 2005). Resident interstitial fibroblasts are known to be the primary source of extracellular matrix (Nahas, 2003). Fibroblasts become activated due to stimulation by cytokines, including TGF- β 1 and PDGF, which are known to be secreted by inflammatory cells (Benali SL manuscript in preparation). Fibroblasts are able to synthesize many of the constituents of the ECM, such as fibronectin and types I, III, and V collagen. They are also a major source of ECM-degrading proteases such as matrix metalloproteinases (MMPs), underscoring their crucial role in maintaining ECM homeostasis via regulation of turnover (Aresu et al., 2011a). The possible bone marrow origin of some fibroblasts and the concept of fibroblast stem cells have raised several hypotheses and novel therapeutic possibilities, but their exact contribution remains to be determined (Eddy, 2005). Correlated with the accumulation of ECM, an increased number of atrophic tubules are found in the interstitium. The morphology of the tubules is characterized by thickening or wrinkling of the basement membrane. Atrophy begins with loss of the brush border and basal interdigitating cell processes and continues with transformation of the complex proximal tubular epithelium into a simple flat epithelium. From a physiological perspective the tubule is no longer able to perform its normal secretion and reabsorption functions, one manifestation of which is the development of proteinuria (Roura et al., 2013).

The role of proteinuria in renal tubulo-interstitial damage in dogs

The role of proteinuria in the pathogenesis of CKD is still uncertain, but several studies in humans and experimental animal models report a direct link between the degree of proteinuria and the progression of the TI damage (Strutz, 2009; Erkan, 2013). In animal models other than dogs several theories have been proposed to explain this association. The ones most often cited are: 1) various toxic proteins having a direct effect in the progression of tubulo-interstitial damage and 2) the induction of an active role for TECs due to their production of proinflammatory and profibrotic mediators after protein uptake (Christensen and Verroust, 2008). The latter possibility is supported by the more convincing findings; in experimental animal models, an increased uptake of albumin and albumin-bound lipids

by proximal tubules has been shown to correlate with the pathogenesis of CKD. Different *in vitro* studies have also shown that an overload of high molecular weight proteins causes a toxic effect leading to apoptosis, as well as to the production of proinflammatory, profibrogenic growth factors and cytokines (Abbate et al., 1998, 2006; Erkan, 2013). In dogs, persistent proteinuria is a common clinical sign and is found in most renal diseases (Lees et al., 2005). In veterinary medicine, proteinuria is most often initially detected by semiquantitative methods including dipstick colorimetric tests that are inexpensive and easy to use, but have only moderate specificity in dogs (Grauer, 2011; Maddens et al., 2011). Therefore, positive semiquantitative tests for proteinuria that are borderline or questionable must be verified with other more specific tests, such as by a quantitative or semiquantitative species-specific immunoassay for albuminuria (Lees et al., 2005). However, once persistent renal proteinuria is confirmed, urine protein excretion should be quantified. The urine protein:creatinine ratio (UPC) has been shown to be a useful index of magnitude of proteinuria in dogs; UPC values from random voided specimens were highly correlated with total 24-hour urine excretion (Grauer et al., 1985). Many efforts have been made recently to identify and validate other more sensitive and specific biomarkers to detect early renal injury, but to date proteinuria remains the most sensible parameter to evaluate for this purpose (Smets et al., 2010; Nabity et al., 2012; Slocum et al., 2012).

Moreover, recent studies in dogs have shown that proteinuria might be a cause rather than merely a consequence of tubulointerstitial damage in this species; two studies have suggested an active role of TECs in dogs with proteinuria (Vilafranca et al. 1995; Benali et al., 2013). Vilafranca and colleagues were the first to identify expression of the major histocompatibility complex type II (MHC II) by TECs, suggesting that these cells were capable of acting as non-professional antigen presenting cells. In our recent study, we analyzed a set of renal biopsies from dogs with a variety of glomerular and tubular diseases and found a close correlation between the expression of human leukocyte antigen (HLA)-DR in TECs and degree of proteinuria. HLA-DR is a portion of MHC class II complex that is highly conserved in the dog (Yuhki et al., 2007). The complex of HLA-DR constitutes a ligand for the T-cell receptor and is constitutively expressed by canine dendritic cells, B cells, monocytes and macrophages but not by TECs in normal kidneys. In contrast, however, in glomerular diseases HLA-DR was observed in the cytoplasm of TECs and was correlated both with interstitial fibrosis and inflammatory cell number (Benali et al., 2013). Interestingly, more intense immunostaining was found in TECs adjacent to lymphocytes. These findings represent the first evidence of the ability of TECs to directly interact with inflammatory cells and suggest a possible proinflammatory role for the TECs (Benali et al., 2013). The hypothesis supported by these

observations is that tubular protein overload initiates and contributes to the progression of renal damage by invoking interstitial inflammatory cell accumulation and subsequently fibrosis in renal interstitium.

Epithelial-mesenchymal Transition (EMT) in canine renal diseases

Epithelial-Mesenchymal Transition (EMT) is defined as the phenotypic conversion of epithelial cells into cells with a mesenchymal phenotype. This represents a simplistic definition of a more complex process that occurs in both physiological and pathological biological settings. However, the key points of the transition include four events: (1) loss of epithelial adhesion properties; (2) *de novo* expression of mesenchymal markers; (3) disruption of basement membrane (TBM); and (4) enhanced cell migration and invasion (Liu, 2010). Moreover, a more detailed classification system has been proposed recently to further outline the EMT process. Type 1 EMT is characterized by changes of cellular phenotype occurring during embryonic development, such as embryogenesis and organ development, Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis, and type 3 EMT is mainly encountered in the biology of the metastasis and tumor progression (Kalluri and Weinberg, 2009; Cervantes-Arias et al., 2013). Even if a clear distinction among these three types of EMT has not been demonstrated in veterinary medicine, numerous reports agree with this classification (Bongiovanni et al., 2013).

EMT in renal fibrosis was first demonstrated by Strutz (Strutz et al., 1995) more than a decade ago, whereas it has been studied in dogs only recently. At the authors' institution, we have investigated EMT as a feature of canine renal fibrosis. Studies of the mechanisms of EMT in canine CKD were first reported in 2005 in the work by Yamate and colleagues (Yamate, et al. 2005). The aim of their study was to identify a suitable animal model in which to explore EMT in chronic TI damage. Changes in the phenotype of tubular epithelium in dogs with renal fibrosis were investigated by detecting the expression of cytoskeletal filaments: alpha-smooth muscle actin (alpha-SMA), desmin and vimentin. Canine epithelial cells in areas of fibrosis strongly expressed desmin and vimentin but were negative for alpha-SMA. Further investigations of the process of EMT in dogs with immune-mediated glomerular disease were reported in 2007 (Aresu et al., 2007). The phenotype of TECs was studied in relation to the severity of TI lesions, and cytokeratin and vimentin were used as epithelial and mesenchymal markers, respectively. Cytokeratin expression by TECs was diffusely detected in kidneys of healthy dogs or in unaffected areas in the diseased renal samples. In contrast, loss of cytokeratin expression and *de novo* expression of vimentin were detected in tubules within areas of inflammation and fibrosis. Moreover, these

observations also were correlated with the degree of fibrosis. Double immunohistochemical staining confirmed that TECs with progressive loss of cytokeratin also showed *de novo* acquisition of vimentin, suggesting the presence of a transition to a mesenchymal phenotype. The occasional identification of colocalization of the two antibodies in scattered cells within the tubules was considered to reflect an intermediate step in the EMT process. In agreement with the previous study by Yamarate et al. (2005), the TECs were alpha-SMA negative. As mentioned previously, EMT is a multistep process in which the down-regulation of junctional complexes is one of the key events. In a separate group of dogs with different types of spontaneous glomerular diseases, we observed a strong correlation between the severity of TI damage and the down-regulation of E-cadherin and Beta-catenin. Additionally, the loss of adhesion junction molecules was detected in association with the acquisition of mesenchymal markers expression by TECs (Aresu et al., 2008b).

Because splitting or rupture of the TBM in the interstitium is considered one of the fundamental steps of EMT in experimental animal models, investigations have also been focused on examining the role of MMPs in canine renal fibrosis (Aresu et al., 2011a). MMPs are a large family of calcium-dependent zinc-containing endopeptidases that are responsible for tissue remodeling and degradation of the extracellular matrix (ECM), including collagen (Liu, 2004; Cheng et al., 2006; Aresu et al., 2011b). Previous studies using cell cultures and transgenic mice (Cheng and Lovett, 2003; Cheng et al., 2006) described two alternative patterns of MMP activity in the EMT process: 1) the extra-tubular pattern, characterized by migration of tubular cells with a mesenchymal phenotype into the interstitial space as a result of TBM disruption; and 2) the intra-tubular pattern, where TECs express mesenchymal markers although the TBM is preserved. Nevertheless, structural integrity of the basement membrane does not exclude the presence of damage. When MMPs and tissue inhibitors were investigated in dogs with renal damage (Aresu et al., 2011a) the hypothesis was that collagenases might impair the protective role of the TBM, thereby initiating the mechanisms of EMT. Supportive evidence was obtained by identifying MMP-2 protein located within the segments of TBM splitting. Also, an increase of MMP-2 activity was demonstrated by gel-zymography in dogs and correlated with the amount of extracellular matrix deposition. Finding MMP-2 in the TBM suggested an active role of this enzyme in the degradation of collagen type-IV structure, leading to basement membrane damage but without disruption (Aresu et al., 2011a,b). This result proved for the first time in dogs the fundamental role of TBM integrity in the maintenance of a properly regulated microenvironment for normal function of TECs. Importantly, morphological integrity of TBM was not considered sufficient for the maintenance of the

epithelial phenotype (Cheng and Lovett, 2003).

Additionally, having back-tracked the modifications that occur in the mesenchymal transition of TECs in dogs, we postulated that the ability of epithelial cells to present antigens might be an initial step of EMT. Therefore, to investigate this possibility, double immunohisto-chemistry was performed to examine the expression of EMT markers and HLA-DR in canine renal biopsies with renal fibrosis. Multiple patterns were observed in neighboring tubules and suggested the existence of at least four sequential steps. The first is when TECs constitutively express Beta-Catenin and are considered normal. The second is when TECs expressing MHC type II have a pro-inflammatory role in recruitment of lymphocytes. In the third step, TECs are able to express both MHC type II and vimentin and might be compatible with a specific phase of the transition. The last one is characterized by complete change of the phenotype with diffuse cytoplasmic vimentin expression and the complete EMT process has occurred. Observation of these presumably sequential steps simultaneously in the TECs of different tubules within a single biopsy is consistent with the heterogeneity of stages of injury that is typically exhibited among the renal tubules of dogs with CKD.

Summary and future directions

In summary, recent studies as reviewed herein have provided evidence that the process of EMT occurs in dogs with CKD and contributes to the progression of TI damage that leads to end-stage renal disease. Moreover, the processes that occur in dogs have many similarities with those in human chronic renal disease, making dogs a good model in which to examine changes in the tubulointerstitial compartment during progressive CKD. And, in comparison with murine models or studies in cell culture systems, canine renal disease models offer several distinct advantages for study of the mechanisms of renal fibrosis. These include the occurrence and availability of spontaneous (as opposed to experimentally induced) diseases for investigation, as well as the slow progression of canine CKD and the general similarity of lesions in both canine and human CKD. Nevertheless, to further validate dogs with renal disease as useful animal models for studies of CKD mechanisms, additional investigations are needed. For example, studies should be performed to investigate the possible existence of intermediate steps before the acquisition of vimentin by TECs is complete. In experimental animal models, S100A4, which is a member of the S100 superfamily of cytoplasmic calcium-binding proteins, is known to be expressed in fibroblasts in organs undergoing remodeling, including kidney, lung, and heart (Donato et al., 2013). In addition, S100A4 is commonly used as a marker to identify epithelial cells undergoing EMT. Therefore, the validation and use of additional mesenchymal markers in dogs will help to more completely identify the relative

roles of EMT versus fibroblast-related processes in canine renal fibrosis.

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Canine epithelial mesenchymal transition

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Accepted May 28, 2014