Renal Fibrosis:
What? How Much? Why?
Diagnostic/Pathogenetic Features, Quantification, and
Clinicopathologic Implications

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Introduction

Accumulation of interstitial extracellular matrix (ECM) is a common feature of chronic kidney diseases, and believed to contribute to the loss of function. Kidney interstitial fibrosis (IF) can be defined as the accumulation of abnormal amounts of collagen and related molecules in the interstitium of the cortex, which serve as structural scaffolding.

What? Pathogenetic Features: Cellular and Molecular Mediators

Dr. Alison Eddy has divided the process of interstitial fibrosis and tubular atrophy development into four phases in rat models: (1) cellular activation and injury phase; (2) fibrogenic signaling phase; (3) fibrogenic phase; and (4) renal destruction, with obliteration of tubules and capillaries. The first 3 phases are active, involving inflammatory and parenchymal cells, matrix proteins, soluble factors including growth factors, cytokines. Eventually, a dense organized scar develops. Data suggests that fibrosis may be reversible in the early phases, opening the door for possible intervention.1-3

Cellular mediators of fibrosis include fibroblasts4, 5 and myofibroblasts.5-10 Fibroblasts may acquire a myofibroblastic phenotype, likely a crucial event in IF.4, 9, 11-15 Fibrocytes are another cell type thought to be important to IF. These are thought to be distinct from fibroblasts.10, 16-24 The ultimate origin of interstitial myofibroblasts is uncertain with candidates being fibroblasts, pericytes,10, 25-27, endothelium,28, 29 and perivascular fibrocytes.10, 25, 30-32 Some have been mixed with regard to endothelial-mesenchymal transition.10, 25, 30-32 Inflammatory cells are important in the genesis of
IFTA, including lymphocytes,\textsuperscript{33-36} monocyte/macrophages,\textsuperscript{24, 37-43} dendritic cells,\textsuperscript{5, 35, 44, 45} and mast cells.\textsuperscript{46-57}

Tubular cells are postulated to contribute to the increased ECM through the process of epithelial to mesenchymal transition (EMT), defined as the stepwise loss of epithelial markers, such as E-cadherin, and the acquisition of mesenchymal markers, such as vimentin and SMA\textsuperscript{4, 58, 59} and the development of increased motility with traversal of the basement membrane into the interstitium.\textsuperscript{60} There is evidence both for and against EMT.\textsuperscript{61-63} The Banff Conference on Allograft Pathology in 2011 had a symposium on EMT, concluding that the in situ epithelial response exists but suggesting a name that does not imply emigration of tubular cells to the interstitium, which can be termed the “epithelial mesenchymal phenotype” (EMP).\textsuperscript{64}

Tubular epithelial cells undergo marked changes in acute injury\textsuperscript{65} and appear to be an important factor in IF.\textsuperscript{4, 13} In the process of EMP, there appear to be increased intermediate filaments (vimentin and nestin) in injured tubular epithelium.\textsuperscript{66} This may be associated with increased collagen type I and III expression.\textsuperscript{67} EMP marker production may be include changes in E-Cadherin production.\textsuperscript{68, 69} Transcription factors such as the zinc-finger transcription factor snail homolog 1 (Snai1)\textsuperscript{70, 71} appear to be important to EMP. An important component in the regulation of genes in EMP and IF may include micro(mi)RNAs.\textsuperscript{72-83} Processes that may be important include endoplasmic reticulum stress\textsuperscript{84-90}, “lipid nephrotoxicity”,\textsuperscript{91-97} and autophagy, the process whereby cells undergo “self digestion”.\textsuperscript{4, 98, 99}

The ECM components [other than collagen, the primary molecule in fibrosis] appears to have a crucial role in IF.\textsuperscript{100-104} Tissue transglutaminase (tTG) crosslinks
proteins, stabilizing ECM and making it resistant to protease degradation. Matrix metalloproteinase (MMP) enzymes are comprised of proteolytic enzymes that can degrade all matrix protein components. Tissue plasminogen activator (tPA) is proteolytic but can worsen IF through its ability to induce MMP-9 gene expression, leading to tubular basement membrane disruption and EMP promotion. ECM production can be affected by the renin/angiotensin system. Plasmin, a serine protease, can activate MMPs, leading to matrix protein degradation. Laminin-α4, a component of glomerular and PTC basement membranes, may be decreased in IFTA.

Vessels altered in IF include peritubular capillaries (PTCs) and lymphatic vessels. The importance of the renal vasculature in the development of IF is evidenced by studies on molecules such as vascular endothelial growth factor (VEGF). It has been demonstrated that inhibition of angiogenesis and inflammation with sirolimus can prevent IF. Hypoxia promotes fibrosis through such mechanisms as hypoxia-induced factor-1α (HIF-1α).

There are large number of molecular mediators of IF. Transforming growth factor (TGF) is an important mediator of IF. TGF-β acts through Smad and jagged/notch pathways for downstream signaling. TGF-β activation blockade is being investigated. Other important IF mediators include bone morphogenic protein (BMP), toll-like receptors (TLRs), the TGF-β-inducible integrin α5β6, integrin-linked kinases, hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF). The complement system appears to be important in the development of IFTA.
How Much? Diagnostic and Quantitative Evaluation of Interstitial Fibrosis

Evaluation of interstitial fibrosis can be conducted in both a qualitative and quantitative manner. Qualitatively, the patterns of IF differ and probably do not have identical causes or consequences. For example, broad scars with loss of tubules as the sequelae of severe focal injury and destruction of parenchyma, such as in pyelonephritis and infarcts. The patchy, “striped” pattern of interstitial fibrosis with corresponding tubular atrophy has been described with calcineurin inhibitor use. This is due to the apparent preferential involvement of the medullary rays. However, this pattern may also be seen with hypertensive kidney disease. This “striped” fibrosis occurs in addition to the other changes of chronic calcineurin-induced nephrotoxicity, including hyaline arteriopathy and nonspecific glomerulosclerosis. Chronic obstruction extrinsic to the ureter can lead to IF/TA with relative glomerular sparing, atubular glomeruli, dilated tubules, and intratubular Tamm-Horsfall protein casts will extravasation into the interstitium. In contrast, another pattern, which is likely far more common in renal biopsies, is diffuse or patchy fine interstitial fibrosis which surrounds tubules which are either atrophic or normal. This is associated with either diffuse or focal disease of glomeruli, tubules or vessels. Many studies have shown a reciprocal correlation between kidney function and the extent of fibrosis.

Tubular atrophy (TA) is defined as loss of specialized transport and metabolic capacity and typically manifested by small tubules with cells with pale cytoplasm or dilated, thin tubules. TA is usually associated with IF (often abbreviated IFTA). However, IF and TA can be separated as distinct processes, as shown by the profound
tubular atrophy in renal artery stenosis, which characteristically has little or no fibrosis.\textsuperscript{186}

Quantitatively, the extent of IF is predictive of renal function and renal allograft outcome and may be considered a surrogate marker.\textsuperscript{187-191} Several applications require accurate IF measurement including research focused on therapeutic inhibition of IF and comparison of protocol biopsies in studies of renal allografts.\textsuperscript{105, 153, 192, 193} Visual assessment of trichrome-stained slides is often standard practice in many institutions,\textsuperscript{194} but studies have shown that this approach may have poor reproducibility.\textsuperscript{195, 196} According to Banff criteria\textsuperscript{197}, trichrome is typically used since the recommendation for slide preparation is seven slides containing multiple sequential sections, 3 with H and E, 3 with PAS or silver stains, and 1 with a trichrome stain. Under Banff working classification of renal allograft pathology, fibrosis is scored as follows:

<table>
<thead>
<tr>
<th>Table 1: Banff Quantitative Criteria for Interstitial Fibrosis (‘ci’)\textsuperscript{197}</th>
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<tbody>
<tr>
<td><strong>ci0</strong></td>
</tr>
<tr>
<td><strong>ci1</strong></td>
</tr>
<tr>
<td><strong>ci2</strong></td>
</tr>
<tr>
<td><strong>ci3</strong></td>
</tr>
</tbody>
</table>

Several morphometry techniques are used to assess IF, including morphometry of slides stained with trichrome;\textsuperscript{198, 199} Sirius Red, specific for collagen types I and III under polarized light;\textsuperscript{7, 200, 201} and collagen immunohistochemistry, particularly type III collagen.\textsuperscript{202-204} Sirius Red imparts a pink stain to most tissues when observed under white light. The dye molecule intercalates into the tertiary groove in both types I and III collagen molecules. When observed under polarized light, collagen types I and III are strongly birefringent.\textsuperscript{7} Computer-assisted morphometry has shown utility in the analysis of studies employing trichrome, Sirius Red, and collagen III immunohistochemistry; and
analysis in some of these studies have shown correlation with glomerular filtration rate (GFR).\textsuperscript{7, 186, 199-211}

The measurement of IF has intrinsic limitations, some of which are due to sampling. For example, one study estimated that repeat biopsies show a decrease in the measured level of fibrosis, presumably due to sampling, in 12\% of cases.\textsuperscript{212}

Overall, there is no consensus regarding the best way to assess IF. Efforts to reach a consensus or at least provide recommendations are currently underway under the auspices of the Banff Conference of Allograft Pathology.\textsuperscript{213}

**Why? Clinicopathologic Implications**

Not all fibrosis is “equal” or the “same” in quality and thus aggregate quantity. For example, “active” or “young” IF may have greater potential for remodeling. Broad scars may have different consequences than diffuse, fine IF. Inflammation in areas of IF has also been noted in several studies to be an adverse risk factor for progression of renal disease.\textsuperscript{9, 191, 212, 214-217} For this and other reasons, quantitative criteria for mononuclear cell interstitial inflammation (‘ti’) in total parenchyma (scarred and unscarred) is sometimes assessed according to the criteria below. This designation has not been officially incorporated into the Banff criteria yet.

<table>
<thead>
<tr>
<th>ti0</th>
<th>No or trivial interstitial inflammation (&lt;10% of parenchyma)</th>
</tr>
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<tbody>
<tr>
<td>ti1</td>
<td>10–25% of parenchyma inflamed</td>
</tr>
<tr>
<td>ti2</td>
<td>26–50% of parenchyma inflamed</td>
</tr>
<tr>
<td>ti3</td>
<td>&gt;50% of parenchyma inflamed</td>
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</table>
It is desirable to find the etiology/pathogenesis so that the problem can be alleviated for the patient. At the 8th Banff Conference on Allograft Pathology held in Edmonton, Canada from July 15-21 2005, major developments included the elimination of the non-specific term ‘chronic allograft nephropathy’ (CAN) from the Banff classification for kidney allograft pathology and the recognition of the entity of chronic antibody-mediated rejection.\(^{185}\)

Fibrosis can be important to assess in renal donor biopsies to predict subsequent allograft behavior,\(^{190}\) as shown below:

<table>
<thead>
<tr>
<th>Measure</th>
<th>Predictive Value of Feature</th>
<th>N / Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banff ci &gt; 0</td>
<td>(\uparrow) Risk of adverse outcome @ 6 months, 1.9x beyond prediction from age alone</td>
<td>78 (^{218})</td>
</tr>
<tr>
<td>Morphometric interstitial volume</td>
<td>Graft function @ 1 year</td>
<td>43 (^{219})</td>
</tr>
<tr>
<td>Banff ci</td>
<td>No predictive value, not reproducible</td>
<td>199 (^{220})</td>
</tr>
</tbody>
</table>

In the Oxford Classification of IgA nephropathy, interstitial fibrosis and tubular atrophy was found to be an important parameter. Six pathologic variables that were used to interrogate prognostic significance independent of the clinical data in IgA nephropathy: (1) mesangial cellularity score [percentage of glomeruli showing] (2) segmental sclerosis, (3) endocapillary hypercellularity, or (4) cellular/fibrocellular crescents; (5) percentage of interstitial fibrosis/tubular atrophy; and finally (6) arteriosclerosis score. Of these, mesangial-cell cellularity, segmental sclerosis or
adhesion, endocapillary hypercellularity, interstitial fibrosis and tubular atrophy were independent histopathological features with reproducibility and predictive power in survival analyses using the rate of eGFR decline and a 50% decrease in eGFR/ESRD as outcomes.\textsuperscript{221, 222}

Assessment of interstitial fibrosis can be important in predicting renal function. Multiple studies have been conducted to assess fibrosis, some of which have used computerized morphometry, as shown below:

\textbf{Table 4: Studies to Assess Interstitial Fibrosis}

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Measure</th>
<th>Ref.(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR CA</td>
<td>SR image analysis predicted long-term renal allograft function</td>
<td>Cortical IF correlated with time to graft failure ($r = 0.64, P &lt; 0.001$) at 6 months post transplant</td>
<td>186</td>
</tr>
<tr>
<td>SR CA</td>
<td>SR image analysis predicted long-term renal allograft function</td>
<td>Positive correlation ($r = 0.62, P &lt; 0.001$) between SR fibrosis and decreases in GFR</td>
<td>223</td>
</tr>
<tr>
<td>SR CA</td>
<td>SR image analysis quantitation corresponded to light microscopic semiquantitative measurements ($r = 0.439, P = 0.0003$ overall and $r = 0.704, P &lt; 0.0001$ for just baseline specimens) in kidney allografts</td>
<td>Semiquantitative methods correlated best with long-term graft function (serum creatinine at 8 – 10 years ($P = 0.010$) and late graft loss ($P = 0.0445$))</td>
<td>201</td>
</tr>
<tr>
<td>SR CA</td>
<td>IF in non-heart-beating donor kidneys and conventional heart-beating donor kidneys</td>
<td>No significant difference in IF between the two groups</td>
<td>224</td>
</tr>
<tr>
<td>SR CA</td>
<td>IF scoring predicts survival in lupus nephritis</td>
<td>Fibrillary collagen was predictive of creatinine doubling ($P = 0.01$) and relapse ($P = 0.06$)</td>
<td>225</td>
</tr>
<tr>
<td>SR CA</td>
<td>Development of an image analysis-based application (Fibrosis HR) for interstitial fibrosis and glomerular morphometry</td>
<td>Intra- and interoperator variability was present in manual segmentation of IF, mesangial matrix, and glomerular areas but interactive identification didn’t have this variability</td>
<td>226</td>
</tr>
<tr>
<td>SR CA</td>
<td>IF measurements using digital imaging coupled with point counting correlated with GFR</td>
<td>Direct relationship between interstitial volume fraction and renal function ($r^2 = 0.54$)</td>
<td>225</td>
</tr>
<tr>
<td>Method</td>
<td>Description</td>
<td>Measure</td>
<td>Ref(s)</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>SR CA</td>
<td>Sirius red measurement of fibrosis combined with ultrasound measurements of renal artery resistance index helped predict “chronic allograft nephropathy”</td>
<td>Positive correlation ($r = 0.62$, $P &lt; 0.001$) between picrosirius red-stained cortical fractional if volume and decreased GFR</td>
<td>227</td>
</tr>
<tr>
<td>SR and collagen CA</td>
<td>Renal IF correlates with the presence of TGF-β, decorin, smooth muscle actin, and interstitial collagens</td>
<td>In all samples with IF, up-regulation of TGF-beta was observed in combination with reduced decorin expression</td>
<td>206, 226, 230</td>
</tr>
<tr>
<td>Masson TC CA</td>
<td>IF morphometry correlates with serum creatinine in IgA nephropathy and membranoproliferative glomerulonephritis</td>
<td>IF occupied &gt; 10% of the interstitium in all 10 cases and &gt; 20% in 6 and IF morphometry correlated with serum creatinine</td>
<td>231</td>
</tr>
<tr>
<td>Masson TC</td>
<td>Cyclosporine (CsA) therapy effects on quantitative fibrosis morphometry</td>
<td>IF measured by morphometry was significantly higher in the CsA group only in renal allografts 6 months posttransplant ($P &lt; 0.04$)</td>
<td>232, 233</td>
</tr>
<tr>
<td>Masson TC</td>
<td>Quantification of IF in patients receiving cyclosporine</td>
<td>Quantitative IF grade correlated with worsened creatinine clearance between 1 and 3 years</td>
<td>199</td>
</tr>
<tr>
<td>Light green TC</td>
<td>Quantitative IF morphometry in patients randomized to cyclosporine or conversion to sirolimus</td>
<td>No difference in groups with respect to fibrosis but GFR improved significantly in the conversion group</td>
<td>199</td>
</tr>
<tr>
<td>Light green TC</td>
<td>Quantitative IF in sequential renal biopsies</td>
<td>IF evolution correlated with eGFR</td>
<td>207</td>
</tr>
<tr>
<td>CIII IHC CA</td>
<td>IF measurements by a semiautomatic system correlate with GFR in protocol renal transplant biopsy specimens</td>
<td>Area fraction of immunostained collagen III of &gt; 40% @ 6 months associated with decreased GFR @ 24 months compared with ≤ 40% ($r=-0.32$, $P=0.03$)</td>
<td>204</td>
</tr>
<tr>
<td>CIII IHC CA</td>
<td>Fibrosis measurements by a semiautomatic system correlate with GFR in protocol renal transplant biopsy specimens</td>
<td>GFR correlated negatively with interstitial volume fraction @ 6 months ($P = 0.05$)</td>
<td>254</td>
</tr>
<tr>
<td>Masson TC, SR, and SMA IHC CA</td>
<td>Quantitative (CA) and semi-quantitative assessments (VA) were performed on late allograft biopsies</td>
<td>IF by VA predicted Banff '97 ci scores ($p &lt; 0.0001$) and correlated with GFR, creatinine, and urine total protein ($r = -0.48$, $p = 0.0007$; $r = 0.46$, $p = 0.0009$; $r = 0.51$, 0.0009, respectively). Of the CA methods, Only the SR-nonpolarized score correlated with GFR and urine total protein ($r = -0.29$, $p = 0.05$; $r = 0.29$, $p = 0.05$, respectively)</td>
<td>239</td>
</tr>
<tr>
<td>VA</td>
<td>IF, inflammation, and glomerulopathy correlated with poor allograft outcome</td>
<td>By multivariate Cox analysis, IF and inflammation lead to poorer survival (HR = 8.5, $p &lt; 0.0001$); fibrosis alone had less effect (HR = 4.8, $p = $ not significant)</td>
<td>191</td>
</tr>
</tbody>
</table>

Summary

The molecular mechanisms leading to IFTA are complex and typically interrelated with the primary processes leading to renal injury. Further elucidation of these mechanisms could lead to targeted inhibitors to alleviate the terminal scarring that may occur. Furthermore, many methods are available for assessing fibrosis; and efforts are underway to improve the way pathologists assess fibrosis.

Acknowledgements

The author/presenter would like to thank Drs. Robert Colvin, Paul Grimm, Michael Mengel, and many others for their collaboration on the issue of renal fibrosis.

Statement of Competing Financial Interests

The author has no relevant competing financial interests to disclose.
REFERENCES


Renal Fibroblasts: Origins, Activation and Their Role in Renal Fibrosis

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Please be noted that this syllabus is largely based on my recent review (Nat Rev Nephrol 7: 684-696, 2011).
Introduction

Although many types of cells in renal tubulointerstitium, such as fibroblasts, tubular epithelial cells and subsets of macrophages, are capable of producing extracellular matrix (ECM), fibroblasts are commonly regarded as the principal matrix-producing cells that produce a large amount of interstitial matrix components including fibronectin, type I and type III collagens. Activated fibroblasts in diseased kidneys often express a molecular signature, α-smooth muscle actin (α-SMA), and are also referred as myofibroblasts. In this context, one of the fundamental issues in renal fibrosis field is to delineate the origin, activation and regulation of these matrix-producing myofibroblasts.1-4

There are at least 5 different sources have been proposed to contribute to myofibroblast pool in diseased kidneys. These include activation of interstitial fibroblasts and pericytes, phenotypic conversion of tubular epithelial and endothelial cells, and recruitment of circulating fibrocytes.5 The relative contribution, and even the very existence, of each particular myofibroblast-generating pathway to renal fibrosis is a matter of intense debate and highly controversial. This is largely due to the inherent difficulty in identifying and tracking fibroblasts owing to the lack of specific markers for this cell type. Another major problem is that fibroblasts exhibit enormous phenotypic heterogeneity, probably reflecting their diverse origins, and change their phenotypes over the activation status, localization and times in renal fibrogenesis.

Activation of interstitial fibroblasts and pericytes

In normal adult kidneys, fibroblasts situate in the interstitial space between the capillaries and the epithelia.6 Morphologically, these cells are stellate shaped and exhibit abundant rough endoplasmic reticulum, collagen-secreting granules and actin filaments. They possess multiple cell processes, which connect to the tubular and capillary basement membranes.6 In the resting, quiescent state, interstitial fibroblasts express CD73, also known as ecto-5’-nucleotidase, in their plasma membrane, and produce erythropoietin.6,7 They also express platelet-derived growth factor receptor β (PDGFR-β),8-10 and fibroblast-specific protein-1 (Fsp1), a small cytoskeleton-associated and calcium-binding protein also
known as S100A4. Upon activation, fibroblasts acquire a myofibroblast phenotype by expressing α-SMA and producing a large amount of ECM components.

Several markers have been used to characterize fibroblasts and myofibroblasts in the kidneys such as α-SMA, desmin, FSP1, CD73, PDGFRβ. Unfortunately, none of these markers is specific. In addition, they are rarely expressed by all fibroblasts/myofibroblasts or hardly ever present all the times. Even α-SMA, a classic hallmark for myofibroblast activation in organ fibrosis of all types, is not without problem. The expression of α-SMA is not exclusive for myofibroblasts, as it is also present in vascular smooth muscle cells. In addition, not all activated fibroblasts express α-SMA all the time.

Activated fibroblasts are often characterized by two key features: proliferation and myofibroblastic activation. The latter is illustrated by α-SMA expression and matrix production. Both fibroblasts and myofibroblasts have the capacity to proliferate in response to cytokine cues, leading to the expansion of fibroblast population and interstitial space in diseased kidneys. Several fibrogenic growth factors including PDGF, transforming growth factor-β (TGF-β), basic fibroblast growth factor (FGF-2) and connective tissue growth factor (CTGF) are the well-known mitogens for fibroblasts. In addition to these classic cytokines, tissue-type plasminogen activator (tPA) is another critical player promoting fibroblast survival, proliferation and myofibroblastic activation.

Studies suggest that vascular pericytes are a major source of myofibroblasts in fibrotic kidneys. Pericytes are a subset of stromal cells that partially cover capillary walls, thereby stabilizing endothelium. Following kidney injury, pericytes are detached from the endothelium, undergo migration and proliferation, and differentiate into myofibroblasts. The story of pericytes is quite interesting, because pericyte detachment and differentiation into myofibroblasts under pathological conditions not only result in destabilization of microvasculature, but also contribute to myofibroblast activation, leading to interstitial fibrosis.

**Phenotypic conversion of epithelial and endothelial cells**
Another source of matrix-producing cells could come from tubular epithelium through EMT, a cell phenotypic conversion process occurs in embryonic development, tumor metastasis and organ fibrosis.\textsuperscript{28-32} Similarly, fibroblasts/myofibroblasts may derive from capillary endothelium by endothelial to mesenchymal transition (EndoMT).\textsuperscript{33,34} The contribution of EMT to renal fibrosis is controversial and is the focus of several recent reviews and debates.\textsuperscript{26,35-41}

There is a broad agreement that tubular epithelial cells \textit{in vitro} can undergo EMT, characterized by loss of epithelial feature and acquisition of mesenchymal markers, under the bombardment of various pro-fibrotic cytokines, particularly TGF-\(\beta\)1. However, whether this merely represents an \textit{in vitro} artifact or it does occur \textit{in vivo} is the center of argument. Using a genetic lineage-tracking, fate mapping technique, an early study demonstrates that more than one third of the Fsp1\textsuperscript{+} interstitial fibroblasts are derived from tubular epithelia in obstructive nephropathy.\textsuperscript{42} Likewise, two independent studies also show significant contribution of endothelial cells to the generation of fibroblasts/myofibroblasts via EndoMT in a variety of CKD.\textsuperscript{33,34} However, these results are challenged by a number of similar cell fate mapping studies in which no epithelial or endothelial origin of fibroblasts is evident.\textsuperscript{27,43} Thus far, the reason behind these discrepancies is unsettled.

EMT is a dynamic program in which epithelial cells and fibroblasts represent two extremes of a continual spectrum of a variety of intermediate cell phenotypes. The frequency that epithelial cells that complete the entire EMT course and ultimately become fibroblasts probably is limited, and heavily depends on the disease models, stages and persistence of elevated cytokine pressure in the inflamed milieu.\textsuperscript{44} In most circumstances, tubular cells undergo a partial EMT, in which epithelial cells only change one or two phenotypic markers, while the transcriptional program of EMT is activated. Such a partial EMT, however, is closely associated with poor outcomes and predicts the progression toward interstitial fibrosis in humans.\textsuperscript{45} Not surprisingly, blockade of EMT by a variety of agents ameliorates renal fibrosis and preserves kidney function.

\textbf{Recruitment of circulating fibrocytes}
Fibrocytes are a subset of bone marrow-derived, circulating monocytes with fibroblast-like features in the peripheral blood. They are spindle-shaped, express hematopoietic cell marker CD45, and are capable of producing type I collagen. Fibrocytes also express certain chemokine receptors such as CCR7. In response to kidney injury, fibrocytes mobilize and infiltrate into renal parenchyma and participate in fibrogenesis.

The relative importance of fibrocytes in renal fibrogenesis is another area full of controversy. Because of the lack of specific markers for these cells, clear discrimination of them from monocytes, macrophages, fibroblasts and myofibroblasts is a great challenge. In addition, fibrocytes appear to exhibit different subpopulations. Thus far, experimental results on the involvement of fibrocytes, and bone marrow-derived cells in general, in renal fibrosis are inconsistent. Clarification of this issue needs more investigations.

The different origins of fibroblasts likely contribute to their phenotypic heterogeneity. The relative contribution of each lineage to the myofibroblast pool may depend on the disease model and specific stages. It is conceivable that myofibroblast activation from fibroblasts, pericytes or fibrocytes is an early event, while EMT often takes place at a late stage after a sustained injury (Figure 2). The pathological impact of the early activation of fibroblasts versus EMT to renal fibrosis may be different. While fibroblast activation is important for the onset of renal fibrosis, EMT could be a major determinant for fibrosis progression and irreversibility (Figure 2).

**Molecular machinery that integrates fibrogenic signals and orchestrates matrix production**

The expression and synthesis of ECM proteins by activated fibrobleasts is primarily controlled at the gene transcriptional levels in response to various extracellular fibrogenic cues. Key fibrogenic factors include TGF-β1, PDGF, FGF-2, CTGF and angiotensin II, while HGF and BMP-7 inhibit matrix production primarily by antagonizing TGF-β1 action. Through their respective receptors and specific downstream intracellular signal cascades, these fibrogenic cytokines activates a host of transcription factors that act on the cognate elements in the promoter regions of the collagen and
fibronectin genes to activate their transcription. Such signal transduction cascades and expression of matrix genes are also regulated by a variety of microRNA.\textsuperscript{56-60}

Activated fibroblasts contain stress fibers and display abundant transmembrane connections, also known as fibronexus,\textsuperscript{61} between extracellular fibronectin-containing matrix and actin microfilaments, suggesting that an increased interaction of ECM and integrins.\textsuperscript{9} Integrins transmit their signals by activating the downstream effector kinase, FAK and ILK, as they possess no enzymatic or actin-binding activity. Extensive studies indicate that ILK, a scaffolding/adaptor protein and a serine/threonine protein kinase, is especially suited to serve as a molecular platform that integrates various fibrogenic signals.\textsuperscript{62}

It becomes increasingly clear that integrins/ILK and their associated proteins constitute a fibrogenic molecular machinery that orchestrates matrix production and its extracellular assembly. Analogous to the concept of inflammasome,\textsuperscript{63} we propose this multi-component, integrin-associated protein complex as ‘matrisome’, which is molecular platform activated upon injury that integrates various fibrogenic signal inputs and triggers the production and assembly of matrix components. It appears that almost all key fibrogenic cues promote matrix production somehow by regulating, directly or indirectly, this molecular machinery.

**Summary**

Renal fibrogenesis is an enormously complex, dynamic process in which activation of fibroblasts is arguably the most important event leading to excessive ECM production and scar formation. We now have a better appreciation for the diverse origins and phenotypic heterogeneity of the matrix-producing fibroblasts, as well as their roles in renal fibrogenesis. As a variety of strategies targeting fibroblast activation are effective in animal models, we are optimistic that some of these remedies will become clinically relevant for CKD patients in the future.
References


SAM Questions

1) There is no specific marker that is exclusively expressed in activated fibroblasts. But investigators often use all of the following to identify interstitial fibroblasts, except:

   a. α-smooth muscle actin
   b. Desmin
   c. CD31
   d. PDGFRβ

Correct answer: CD31. Fibroblasts do not express CD31 in any circumstances. CD31 is a marker for endothelial cells, although it is also found on platelets, macrophages, granulocytes, T / NK cells, lymphocytes, megakaryocytes, osteoclasts, neutrophils.

2) Several different sources have been proposed to contribute to (myo)fibroblast pool in diseased kidneys. During renal fibrogenesis, an activated fibroblast can, in theory, come from:

   a. Interstitial fibroblasts and vascular pericytes
   b. Tubular epithelial cells and endothelial cells
   c. Circulating fibrocytes
   d. All of them

Correct answer: all of them. Based on published literature, activated fibroblasts (myofibroblasts) can come from a wide variety of cells, including quiescent interstitial fibroblasts, vascular pericytes, tubular epithelial cells, endothelial cells and circulating fibrocytes.

3) All of the following statements regarding matrix production in renal fibrosis are true, except:

   a. The expression of ECM proteins by activated fibrobleasts is primarily controlled at the gene transcriptional levels in response to various extracellular fibrogenic cues.
b. All growth factors such as TGF-β1, PDGF, CTGF, HGF and BMP-7 are fibrogenic and promote matrix production by fibroblasts.

c. Matrix genes are also regulated by a variety of microRNA.

d. Integrins and its downstream ILK signaling are important for matrix production and deposition.

Correct answer: b. While TGF-β1, PDGF and CTGF are well known fibrogenic cytokines, HGF and BMP-7 have been demonstrated to be anti-fibrotic and inhibit matrix production.
Role of Microcirculation in the Pathogenesis of Kidney Fibrosis

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Renal microcirculation:

While large arteries are essential for delivering blood flow into the kidney, renal microcirculation, namely glomerular capillaries and peritubular capillaries (PTC) are responsible for delivering oxygen and nutrients to the renal parenchymal cells, thus also required to sustain sufficient glomerular filtration rate for clearing waste products and production of urine. Postglomerular capillary blood flow exits the glomerulus via efferent arterioles and divide to form PTC plexuses interacting with the tubules of the nephron and interstitium.1

Renal glomerular and PTC endothelial cells are highly specialized cells with a flattened cell shape and transcytoplasmic perforating fenestrations. Glomerular endothelial cells line the inner aspect of the glomerular basement membrane and an essential part of the glomerular filtration barrier. Therefore, primary or secondary injuries targeting glomerular or PTC endothelium can lead to direct detriment to the nephrons and renal function.1

Paradigms of nephron loss and kidney fibrogenesis

Kidney fibrosis is the final common pathway of chronic progressive kidney diseases and therefore a surrogate marker for end-stage renal failure, both in native and transplanted kidneys.2,3 Kidney fibrosis (=interstitial fibrosis and tubular atrophy), rather than structural glomerular damage (i.e. glomerulosclerosis), is the best histological correlate of declined function of native or transplanted kidneys, regardless of the underlying disease type.4-6 Several paradigms were generated to explain how nephron loss and fibrosis progress after sustained and/or non-recoverable injuries. Despite intense interest, the mechanisms of nephron loss and kidney fibrogenesis are not fully understood. The postulated mechanisms include hemodynamic alterations and resultant increased intraglomerular pressure, toxic effects of proteinuria on tubular epithelium, transition of epithelium, endothelium or pericytes to fibroblasts, migration of
bone-marrow derived fibroblasts, accelerated senescence, and chronic tubular hypoxia secondary to loss of PTCs.\textsuperscript{2,7-14}

Advanced chronic kidney disease is characterized with a common pathology phenotype shared by almost all progressive renal diseases, which includes extensive interstitial fibrosis with chronic nonspecific inflammation, tubular atrophy and loss, glomerulosclerosis, and PTC loss.\textsuperscript{2} These histopathological features are closely correlated with each other, but their interdependence and causal priorities are unknown. Recently, lineage-tracing studies determined pericytes as the major source of interstitial myofibroblasts in the fibrotic kidney in a rodent model, soliciting that research on renal fibrosis should be refocused on vascular injury mechanisms.\textsuperscript{10}

**Evidence for contribution of microvascular loss to progressive kidney fibrosis: animal models**

The degree of glomerulosclerosis and interstitial fibrosis is correlated with glomerular and PTC loss in progressive models of renal disease.\textsuperscript{14} It has been postulated that mechanisms underlying the loss of renal microvasculature are mediated by impaired angiogenic responses by either incomplete endothelial proliferation and/or local alterations of angiogenic (i.e. VEGF) and anti-angiogenic (i.e. thrombospondin 1) factors in the kidney.\textsuperscript{15}

Numerous animal models of glomerular or non-glomerular progressive kidney diseases and aging, showed that interstitial fibrosis is correlated with reduced PTC density, but none of these studies demonstrated that PTC structural loss precedes interstitial fibrosis.\textsuperscript{15-24} Furthermore, some of the data stemming from animal models contradict with each other. For example, in contrast to other rodent remnant kidney models, Pillebout et al showed proliferation of PTCs with increased PTC density accompanied to severe tubulointerstitial fibrosis in mice after 75\% surgical nepron reduction.\textsuperscript{25} On the other hand, there is convincing growing evidence that tubular hypoxia precedes development of tubulointerstitial fibrosis.\textsuperscript{19 17}

Thus, functional PTC changes may be preceding nephron loss and fibrosis via inducing a chronic ischemic milieu at least in some models, but structural PTC loss appear as a late feature, possibly occurs after nephron loss as a drop-out of disuse mechanism, and correlates with advanced kidney fibrosis.
Evidence for contribution of microvascular loss to progressive kidney fibrosis: human studies

In contrast to animal models, a few clinical studies yet examined PTCs in human kidneys, which generated contradicting data. The difficulty is that most of these human studies (5 out of 9) were conducted in end-stage kidneys with severe scarring affecting all renal compartments, thus making the interpretation quite difficult (in the end, all renal parenchymal structures (glomeruli, tubules, larger vessels, PTC) become scarred and eventually disappear). Bohle et al. were the first to describe reduced PTC density in correlation with interstitial fibrosis in chronic renal failure secondary to diabetic nephropathy, amyloidosis, hypertensive nephropathy, and chronic interstitial nephritis. Studies of explants of native or transplant kidneys well documented reduced microvascular density. A study by Ishii et al. also reported PTC loss in late kidney allografts biopsied after 6.8 years post-transplant with extensive scarring and chronic graft dysfunction. More recently, Steegh et al. reported reduced PTC in 3-month protocol biopsies after kidney transplantation, which negatively correlated with inflammation and predicted higher fibrosis/atrophy and lower renal function at 12-month. In contrast, Ozdemir et al. reported angiogenesis and increased PTC density in kidney allograft biopsies with acute rejection, and interestingly this angiogenic response was related with more fibrosis in follow-up biopsies.

Therefore, whether PTC structural loss is a cause or a consequence of nephron loss remains an open question.

Is microcirculation loss a cause or consequence of nephron loss?

End-stage kidney fibrosis is associated with PTC loss, but their interdependence is unknown. We hypothesized that kidney fibrosis is dependent on PTC loss. We studied PTC density in 100 kidney transplant indication biopsies from 83 recipients (42% presenting with chronic renal dysfunction; median time post-transplant: 15-months), and compared to 40 normal control biopsies taken at time of transplantation. We labeled PTCs with CD31 immunostaining and quantified density using two methods: 1. PTC number per unit area (0.25 mm²); 2. PTC-to-tubule ratio. We also measured PTC surface area by image analysis. PTC number per unit area was lower in transplant biopsies with edema and tubulointerstitial inflammation than in controls. Surprisingly, PTC number per unit area was not reduced in biopsies with kidney scarring (interstitial fibrosis, tubular atrophy, transplant glomerulopathy, PTC multilayering) or late post-transplant time. In multivariate-analysis, interstitial edema was the only determinant of reduced...
PTC density. PTC-to-tubule ratio was higher in biopsies with interstitial fibrosis, indicating remained PTCs despite loss of nephrons. PTCs were larger in biopsies with capillaritis, but not smaller in biopsies with fibrosis/atrophy compared to controls. PTC density by both methods did not relate to renal function or survival. Contrary to our predictions, the histologic feature that correlated with reduced PTC density in biopsies was edema, not fibrosis. Edema expands the interstitium, giving a false impression of reduced PTC density in biopsies. Although postglomerular PTC hypoperfusion might still be a potential contributor, kidney transplant fibrosis is not dependent on structural PTC loss.35 (Osasan et al. manuscript in submission)

Why do our results not confirm previous experimental and clinical studies, which clearly documented that reduced PTC density correlates with advanced renal tubulointerstitial fibrosis? The answer lies within the differences between the current and previous study populations. First, most (5 of 9) human studies on PTC density were done in end-stage kidneys (failed native or transplant nephrectomies or kidneys with extensive fibrosis >50%).26-31 Analyzing end-stage kidneys reveals nonspecific results because all renal compartments become atrophic and eventually disappear within the scar tissue over time. Previous studies in the end-stage kidneys of patients maintained in chronic hemodialysis, showed nonspecific fibrous obliteration of large renal vessels, most likely representing a disuse type of change secondary to parenchymal atrophy.36

Thus previous studies documenting PTC loss in advanced chronic kidney diseases were right and their findings and the current study suggest that PTC loss occurs as a consequence of nephron loss i.e., PTCs drop-out possibly due to lack of use. For example, Ishii et al. observed that PTC density was reduced in transplant biopsies with chronic allograft nephropathy taken after a mean of 6.8 years post transplant and this was associated with the severity of fibrosis/atrophy.31 In contrast, the biopsy time in the current study was much earlier with a median 15 months post transplant. Although we studied earlier kidney transplants, majority (74%) of the cohort included fibrotic kidneys with different severity of interstitial fibrosis. We conclude that studying very late time points naturally show that PTCs are lost in addition to other renal structures, thus studies at earlier time points are crucial to analyze whether PTC loss becomes evident before later stages of chronic renal failure.

Edema expands the interstitium, giving a false impression of reduced PTC density in biopsies. Therefore, edema should be taken into account when PTC density is being analyzed in biopsy samples and requires normalization strategies when investigators compare PTC
density in biopsies with edema and without edema. Recently, Steegh et al. reported that PTCs were lost and inversely correlated with the severity of interstitial inflammation in early protocol biopsies, and predicted higher interstitial fibrosis and tubular atrophy, and reduced renal allograft function at 12-month post-transplant. We believe that this data were misinterpreted because reduced PTC density can be caused by the dilution effect of interstitial edema/inflammation in biopsy tissues. Regarding how come PTC loss could predict reduced renal function and fibrosis at one-year, it is well documented that subclinical inflammation in protocol biopsies predict worse renal outcomes and more chronic damage because subclinical inflammation causes ongoing kidney transplant injury. ⁶⁻³⁷⁻³⁸

Animal models suggest that functional PTC changes, not structural PTC loss, precede development of kidney fibrosis

There is convincing growing evidence that tubular hypoxia precedes development of tubulointerstitial fibrosis. At least two well-designed studies in rodent models of progressive kidney diseases documented that postglomerular PTC blood flow decreased after the initial injury (assessed by red blood cell velocity or marker diffusion techniques) and that PTC hypoperfusion correlated with tubular hypoxia, and preceded subsequent development of interstitial fibrosis, tubular atrophy, and PTC loss. ¹⁷⁻¹⁹ Elegant studies by Manotham et al. in the remnant kidney model suggested that tubular hypoxia, which was evident in the early days of injury, emerged secondary to PTC hypoperfusion before the development of interstitial fibrosis and tubular atrophy, and that functional PTC changes and hypoxia were dependent on activation of the renin-angiotensin system (vasoconstriction of efferent arteriole and glomerular hyperfiltration). As a proof of concept, angiotensin II receptor blocker treatment rescued both PTC hypoperfusion and tubular hypoxia, whereas untreated rats developed kidney fibrosis and PTC loss.¹⁹ Moreover, studies by Wong et al in a mouse anti-glomerular basement membrane glomerulonephritis model documented that wild-type mice with reduced postglomerular PTC flow and proteinuria developed worse renal function and more kidney fibrosis when compared to Fc receptor knockout mice with less severe disease but comparable degrees of proteinuria without reduced PTC blood flow. ¹⁷

Because PTCs are the only vessels that carry O₂ and nutrients to the tubular epithelium, it is reasonable to think that PTC hypoperfusion would trigger and/or contribute to the progressive tubular cell death, nephron loss and interstitial fibrosis. Thus, functional PTC
changes may be preceding nephron loss and fibrosis via inducing a chronic ischemic milieu at least in some models, but structural PTC loss appear as a late feature, possibly occurs after nephron loss as a drop-out of disuse mechanism, and correlates with advanced kidney fibrosis.

Summary

Human studies and animal models show that endstage kidney fibrosis is associated with PTC loss. Our studies in kidney transplant biopsies from earlier time points than in previous human kidney studies showed that contrary to our predictions, the histologic feature that correlated with reduced PTC density in kidney transplant biopsies was edema, not fibrosis. Edema gives a false impression of reduced PTC density in biopsies, thus should be corrected for in PTC quantification studies. We conclude that although postglomerular PTC hypoperfusion might still be a potential contributor, structural PTC loss is not associated with interstitial fibrosis, thus kidney transplant fibrosis is not dependent on structural PTC loss. Animal models suggest that functional postglomerular PTC hypoperfusion precedes development of nephron loss and interstitial fibrosis, which is yet to be confirmed in human diseased kidneys.
SAM Questions

1. Reduced peritubular capillary is associated with interstitial fibrosis and tubular atrophy, thus suggested to contribute progressive nephron loss in diseased kidneys. All of the following statements are correct, except:

   a. Rodent models of non-glomerular progressive kidney diseases reported peritubular capillary loss in areas of interstitial fibrosis.

   b. Clinical studies in chronic kidney diseases tissues showed that capillary loss correlates with the severity of interstitial fibrosis and tubular atrophy.

   c. Peritubular capillary loss was assumed to be related with the location of initial injury, because no reduction in capillary density was observed in experimental or clinical glomerulonephritis.

   d. Both animal models and clinical studies that analyzed peritubular capillary density were done in kidneys with advanced fibrosis.

Correct answer is C. Studies showed that PTC loss was evident in several different categories of chronic kidney diseases, both glomerular or non-glomerular with no disease-specificity.

2. The quantification of peritubular capillary density is problematic and this is due to multiple reasons, except:

   a. Peritubular capillary density measurements require that absolute capillary numbers to be divided by a constant denominator (unit microscopic area, number of tubules)

   b. The denominator is often not constant among specimens, e.g., number of tubules decrease in chronic kidney diseases.
c. Capillary density per unit area measurements are vulnerable to be affected by expansion of the interstitial space by edema and inflammation, which may cause a false impression of reduced capillary density in biopsies.

d. Peritubular capillary rarefaction has been observed as a diffuse feature, thus probably not affected by area selection bias.

Correct answer is D. PTC loss was especially reported around atrophic tubules and within fibrotic areas, therefore, area selection biases may introduce errors in quantitative data.

3. It has been suggested that PTC hypoperfusion, rather than structural loss of PTCs, precedes development of interstitial fibrosis. This is because:

   a. Postglomerular PTC blood flow decreased after the initial injury in rodent models of remnant kidney and anti-GBM glomerulonephritis.

   b. Tubular hypoxia, which was evident in the early days of injury, emerged secondary to PTC hypoperfusion before the development of interstitial fibrosis and PTC loss.

   c. PTC loss was reported in advanced or end-stage human kidneys, thus giving no clues about the priorities.

   d. All of the above.

Correct answer is D.
Reference List


Inflammation and Fibrosis – Interactions and Impact on the Kidney

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Introduction
Chronic kidney disease (CKD) is characterized by progressive interstitial fibrosis, tubular atrophy and glomerulosclerosis. The mechanisms of fibrosis are complex, and may involve excess synthesis of collagen with decreased degradation, in association with parenchymal injury and loss of functional tubules and glomeruli. The extracellular matrix (ECM) that makes up the fibrotic kidney has numerous sources, but in particular is contributed to by activated myofibroblasts. Regardless of the type of CKD, inflammatory cells are associated with the interstitial fibrosis. These inflammatory cells are diverse, including macrophages, various T-cells, dendritic cells, plasma cells and even granulocytes in more acute phases of injury. The impact and interaction of these cells with the parenchyma and effect on fibrosis are complex and stage specific.

Monocyte/macrophage subsets
Similar to evolution of our understanding of the complex various subsets of lymphocytes over the last many decades, monocyte/macrophages are now recognized to consist of unique subsets with varying cell surface markers, chemokine receptors and roles in injury and repair vs. fibrosis. Cells with varying expression of the cell surface marker Ly6C show functionally important differences in receptor expression of key chemokines. Monocytes that express Ly6C show high levels of the chemokine receptor CCR2 but low levels of CXCR1 (CCR2\textsuperscript{high} CXCR1\textsuperscript{low}). In contrast, monocytes without Ly6C on their surface have low levels of CCR2 and high levels of CXCR1. These CCR2\textsuperscript{high} CXCR1\textsuperscript{low} monocytes are the typical cells that infiltrate tissue and are implicated in disease processes and have been termed inflammatory or M1 macrophages. The Ly6C negative cells (Ly6C-CCR2\textsuperscript{low}CXCR1\textsuperscript{high} cells) are also termed resident monocytes and may replenish tissue macrophages and dendritic cells. Tissue macrophages also have discrete phenotypes. Classically activated macrophages, M1, and alternatively activated macrophages, M2, were initially distinguished by their responses in vitro to interferon γ (IFN-γ) and lipopolysaccharide for an M1 phenotype, with an M2 phenotype observed after treatment with interleukin (IL)-4 or IL-13. M1 macrophages are proinflammatory and secrete TNF-α, IL-1β, reactive oxygen species and nitric oxide. In contrast, the M2 macrophages are anti-inflammatory and secrete IL-6 and insulin-like growth factor 1. M2 macrophages may promote tissue healing and angiogenesis. Further subcategories of these inflammatory cells are evolving based on in vivo responses.

Various theories for the origin(s) of these subtypes have been put forth, including the Hume hypothesis of one macrophage population that may assume infinite phenotypes, vs. the Geissman hypothesis, postulating that each subpopulation has its own unique precursor, and a third model where sequential subpopulations evolve based on tissue environment and activating stimuli. More recent evidence suggests that in various settings, there is evidence to support each of these possibilities. Examination of
the impact of macrophages on parenchymal cells and ultimate response to injury are beginning to emerge from experimental models. Examination of specific activation state and subset of macrophages in human tissue in vivo is still limited. However, overall glomerular or interstitial macrophage number correlates with worse outcome in various diseases, including fibrosis and tubular atrophy. More recent studies suggest that this link may be modulated and macrophage phenotype altered with a beneficial impact on outcome.

**Activation of macrophages**

When activated by various stimuli, including pathogen associated molecular patterns (PAMPs), which activate macrophages through Toll-like receptors (TLRs) and other receptors. Signaling is activated through NF kappa-B and MAP kinase, and proinflammatory cytokines are produced by macrophages include TNFα, interleukin IL-1β, IL-12, IL-12, IL-13, and IL-16. Further inflammation and influx of other inflammatory cells is enhanced by macrophage production of chemokines including MIP-1, MIP-2, and monocyte chemotactic protein. Increased reactive oxygen species generated from activated macrophages also may contribute to injury. These activated macrophages expressed Mac2 (galactin-3) in tissue, along with NOS2 or IL-1β. In contrast to these injurious activation patterns, macrophages also can elaborate vascular endothelial growth factor (VEGEF), transforming growth factor-β (TGF-β), IL-10, angiopoietin 1, hepatocyte growth factor, fibroblast growth factor 2, which together could promote angiogenesis, wound healing and anti-inflammatory responses. Macrophages may also scavenge fragments of injured cells and abnormal matrix and thus promote tissue remodeling and regeneration following injury. In injury settings where there are no immune complexes or external pathogens, macrophages may be activated by release of factors from injured cells, which could serve as ligands for activating receptors on the monocytes. These danger-associated molecular patterns (DAMPs) are factors that may bind to Toll-like receptors and also promote macrophage activation. DAMPs include advanced glycation end products, HMGB1, adenosine and others.

Studies now indicate that macrophage polarization also exists in vivo, and not just in the artifactual in vitro situation. In addition to M1 and M2 macrophages, a regulatory macrophage appears in response to the anti-inflammatory cytokine IL-10. Serum amyloid P, also known as pentraxin-2, or apoptotic cells and immune complexes may in specific contexts result in a macrophage subpopulation that generates increased IL10 and thus suppresses further immune responses. These regulatory macrophages therefore may dampen ongoing response of other infiltrating cells.

**Macrophages, angiotensin and effects on tissue injury**

The consequence of macrophages on tissue injury has been studied in various ways, with depletion of macrophages by transgenic models where these cells expressed diphtheria toxin on their surface, or with bone marrow transplant experiments, or with chemical ablation. The renin angiotensin system is a major activator of progressive kidney disease, and antagonism and/or inhibition of its actions are the mainstay of treatment of CKD. We therefore investigated the potential role of the angiotensin type-1 receptor (AT1R) on macrophages in promoting fibrosis. To this end, we generated chimeric mice that lacked the AT1a subtype receptor only on
macrophages, by performing bone marrow transplant with AT1a deficient marrow after lethal radiation. We then exposed the reconstituted chimeric mice to injury that induced progressive tubulointerstitial fibrosis, namely unilateral ureteral obstruction (UUO). In mice with intact AT1 receptor on parenchymal cells, but with bone marrow-derived cells with absence of AT1, there was surprisingly increased interstitial fibrosis, although macrophage infiltration was decreased. These AT1 deficient macrophages showed defective phagocytosis, and impaired migratory capacity ex vivo. We postulated that a defective phagocytic scavenging by the AT1 receptor deficient macrophages could contribute to a profibrotic phenotype.

**Macrophages, TGF-β and fibrosis**

When macrophages infiltrate tissue, tissue signaling interaction pathways must be intact for fibrosis to be effected. In addition, numerous therapies that decrease fibrosis, including ARBs, PPAR-γ agonists, decrease fibrosis and macrophage infiltration in parallel. Of note, inhibition of TGF-β may have complex actions. In addition to promoting macrophage infiltration and matrix synthesis, TGF-β has important immune modulatory effects. We found that inhibition of TGF-β with a pan-antibody decreased fibrosis at low doses, but higher doses were ineffective, linked to higher levels of infiltrating macrophages.

TGF-β circulates in latent form. Activation of TGF-β by cleaving it from latency-associated peptide can be mediated by e.g. thrombospondin-1 and the integrin αV β6. αV β6 integrin is expressed in epithelium in the lung, the kidney and the skin. β6 deficient mice were protected from lung fibrosis and interstitial fibrosis induced by UUO. Of interest, infiltrating macrophages were even more abundant in β6 knockout mice, but no increase in collagen content was observed after UUO, and TGF-β was not activated by UUO in these mice. However, activation of other pathways by adding exogenous angiotensin II, could restore fibrosis but through a non-TGF-β-dependent pathway, which we linked to increased plasminogen activator inhibitor-1 (PAI-1) and thymosin β4. Thymosin β4 is a G-actin sequestering protein with numerous effects on cell migration, angiogenesis, and activates PAI-1 and TGF-β. We showed that thymosin β4 is necessary for angiotensin to induce PAI-1 in endothelial cells. PAI-1 could influence fibrosis in part by effects on cell migration through vitronectin, and in part by inhibiting plasmin-dependent proteolysis of accumulated ECM proteins. Thymosin β4 promotes wound healing in skin and corneal wounds, by promoting cell migration and ECM and angiogenesis. In early stages after UUO, thymosin β4 increased injury, but at later stages thymosin β4 was protective. Our most recent data with conditional knockout of thymosin β4 in macrophages supports that expression of thymosin β4 may influence polarization of macrophages.

**Modulating macrophages to decrease fibrosis**

Additional recent exciting studies from the laboratory of Duffield shows that treatment with exogenous pentraxin-2 (serum amyloid P) also is anti-fibrotic in the UUO model. The postulated mechanism may be opsonization of cell debris with serum amyloid P, and interaction with activated Fcγ receptors on macrophages, resulting in an anti-inflammatory IL10 expression. These and other studies have led to the theoretical consideration of yet another macrophage population, a potentially fibrolytic macrophage.
In additional studies, mice were genetically manipulated to express the diphtheria toxin only on macrophages, under the CD11b promoter. Injection of the toxin then resulted in specific monocyte/macrophage depletion. Ablation of monocyte/macrophages at day 4 through 6 after UUO resulted in decreased fibrosis. Similar results were seen when macrophage ablation was induced at a later stage after UUO from day 7 to 10. Fibrosis could be restored by adoptive transfer of monocytes from normal mice. Interestingly, when resident renal macrophages were depleted there was no effect on fibrosis- only depletion of circulating monocytes and recruited macrophages blunted fibrosis. In the nephrototoxic serum nephritis model, macrophage ablation also decreased injury, with decreased interstitial myofibroblasts and fibrosis. Of note, depletion of macrophages from mice with skin wounds resulted in decreased ECM deposition and cell proliferation and delayed healing. These mechanisms were tied to interference with macrophage production of PDGF, which in turn led to decreased activation of fibroblasts and fibroblast osteopontin expression. A balance of macrophage effects therefore appears to be important in early wound healing.

A key step in macrophage modulation of fibrosis is modulation of monocyte recruitment to become tissue macrophages. The monocyte chemoattractant protein (MCP-1, or CCL2) and its receptor CCR2, are key for activation and chemotaxis. In mice genetically deficient in CCL2, macrophage infiltration was decreased and fibrosis was diminished in models of UUO and early diabetic injury. However, the specific phenotypes of resulting macrophages were not explored. Similarly, CCR2 receptor inhibitors ameliorated renal fibrosis. Interestingly, blockade of CCR1, which is present on M2 macrophages, decreased macrophages and fibrosis in the Alport mouse model, but CCR2 blockade was ineffective, while CCR1 blockade was effective in db/db mice, adriamycin nephropathy and UUO.

In addition to experimental maneuvers to deplete macrophages, infusion of modified macrophages has been examined for potential therapeutic impact on fibrosis. The impact of modulating macrophages will likely vary depending upon whether it is initiated in the injury or healing phase. When macrophages were transferred to mice at a late stage after UUO, there was attenuation of fibrosis. Manipulation of specific cell populations will influence the responsiveness. For instance, depletion of dendritic cells selectively did not affect renal fibrosis, while depletion of all monocyte lineage cells could have this beneficial effect in the UUO model. Specific injection of M2 macrophages primed with IL-4 and IL-13 ameliorated adriamycin nephropathy and reduced endogenous macrophage infiltration, whereas injection of M1 macrophages worsened injury and increased inflammatory gene expression, and unmodified macrophages did not alter the disease course.

In addition to promoting or regulating angiogenesis and myofibroblast production of matrix, macrophages have effects on parenchymal cells. Macrophages can promote activation to cell cycle entry and death in injured parenchymal cells. Entry of injured cells into cell cycle may lead to their death by pausing at DNA checkpoints and thus promoting apoptosis whereas cells with intact functionality will be able to continue through cell cycle without cell death.

Galactin-3 macrophage expression was necessary for them to transduce fibrosis. Interestingly, the kidney parenchyma itself may be important in mediating the renal
accumulation of specific macrophage phenotypes. The transcription factor Krüppel-like factor-5 (KLF-5) is expressed mostly in collecting duct epithelial cells, and haplo-insufficient mice (KLF-5 +/-) had less injury after UUO than wild type, with decreased macrophage infiltration of the M1 phenotype, and increased accumulation of M2. KLF-5, in concert with C/EBPα, mediated expression of chemotactic proteins S100a8 and S100a9, resulting in recruitment of inflammatory monocytes to the kidney and their activation into M1 type macrophages. This collecting duct-derived transcription factor thus played a major role in influencing the phenotype and consequences of inflammatory cell infiltration.

Other parenchymal receptors also contribute to tissue inflammation. Discoidin domain receptor-1 (DDR-1) is a tyrosine kinase transmembrane receptor for collagen constitutively expressed in several organs including the kidney, where it is found predominantly on vascular smooth muscle cells, mesangial cells and epithelial cells. DDR-1 is activated by binding to collagens I to VI and VIII and regulates cell differentiation, adhesion, proliferation and ECM remodeling. It is upregulated after UUO. DDR-1 deficient mice had decreased collagen and TGF-β and remarkable decrease in macrophages, but no shift in M1/2 polarization.

Summary

In summary, macrophages show diverse phenotypes that are context dependent. The ultimate effect on response to injury depends upon balance of macrophage phenotypes and their persistence or resolution, and whether injury and inflammatory stimuli persist or resolve. Inflammatory macrophages may play beneficial roles in response to transient inflammation with induction of macrophages that can suppress further inflammatory response and promote repair. Later in wound healing, profibrotic macrophages promote fibroblast proliferation and fibrogenesis with elaboration of TGF-β. Macrophages may also promote clearance of ECM, through the postulated fibrolytic macrophage phenotype. Anti-inflammatory M2 macrophages may promote tubular repair.
References:


Conway B and Hughes J: Cellular orchestrators of renal fibrosis. QJM 2011 (epub head of print)


