What is the best way to measure renal fibrosis?: A pathologist’s perspective

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Interstitial fibrosis is a hallmark structural correlate of progressive and chronic kidney disease. There remain many uncertainties about how to best measure interstitial fibrosis both in research settings and in evaluations of renal biopsies performed for management of individual patients. Areas of uncertainty include determination of the composition of the matrix in a fibrotic parenchyma, the definition of how the interstitium is involved by fibrosing injuries, the choice of histologic stains for evaluation of renal fibrosis, and the reproducibility and robustness of measures currently employed by pathologists, both with and without the assistance of computerized imaging and assessments. In this review, we address some of these issues while citing the key studies that illustrate these difficulties. We point to future approaches that may allow a more accurate and meaningful assessment of renal interstitial fibrosis.

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WHAT IS THE BEST WAY TO MEASURE RENAL FIBROSIS?: A PATHOLOGIST’S PERSPECTIVE

It is commonly accepted that interstitial fibrosis (IF) is a key, and perhaps the key, structural correlate of progressive and chronic kidney disease. It is therefore surprising that there remain many fundamental uncertainties about how to best measure fibrosis and about whether all forms of fibrosis are equally detrimental to the kidney and whether the various approaches available for measurement of fibrosis are robust and reproducible. The review will identify some of the issues underlying these uncertainties, cite some key studies that give us a basis for choosing some approaches over others, and suggest ways in which we may move forward, but regretfully will not resolve the fundamental uncertainties that we will discuss.

Chronic kidney injury is manifested by a variety of structural alterations, including the accumulation of extracellular matrix (ECM). Most of what is considered ECM is colloquially termed IF. Tubular atrophy (TA) often accompanies IF and, when occurring together, IF and TA are collectively termed IFTA.¹-⁸ Taken in isolation, IF is not necessarily a marker of the degree of intactness or function of nephron units. However, studies have shown that IF quantification can help prognosticate renal outcome in renal allografts and in such native kidney diseases as IgA nephropathy, and may be considered the best available histologic marker of chronic kidney injury.⁹-¹⁴

As many investigators and practitioners ascribe a great deal of importance to the issue of IF, accurate IF measurement is often needed in a variety of applications, including research focused on the therapeutic inhibition of IF, comparison of protocol biopsies in studies of renal allografts,¹,¹⁵,¹⁶ and for clinical prognostication as is the case with IgA nephropathy and lupus nephritis.¹⁴,¹⁷-²⁰ However, to do this, one must understand the qualitative and quantitative issues related to the topic of IF. The qualitative issues relate to the actual composition and distribution of the IF (that is, ‘what?’ and ‘where’). The quantitative issues, on the other hand, relate to the amount present (that is, ‘how much?’). In addition, one must understand the systems currently used for IFTA assessment and the implications (that is, ‘who uses this?’ and ‘why’).
FIBROSIS QUALITY: WHAT IS IN A SCAR?
Composition of matrix
The cortical interstitial volume normally ranges from 5 to 20% with a mean of 12%, and this volume reportedly increases with age. The normal cortical interstitial volume is estimated at 5% in the rat. The renal interstitium ECM contains sulfated and non-sulfated glycosaminoglycans, such as biglycan and decorin. Types I and III collagen, and fibronectin. Type VI collagen is also present, particularly in rodents. IF is typically considered to be an excess accumulation of fibrillar collagen, and the role of other matrix molecules such as proteoglycans and other non-collagenous proteins has not been comprehensively investigated. Knowing the composition of a fibrotic matrix is important because matrix components may determine the susceptibility of a matrix to undergo degradation by proteases and possibly undergo regression, and may determine the local tethering and/or activation of growth factors and cytokines that mediate IFTA.

Interstitial cells and their interplay with epithelial cells and vasculature
Fibroblasts constitute a large proportion of renal interstitial cells and are the major cells maintaining constituent ECM, which can be considered the kidney ‘skeleton.’ Fibroblasts lack a good cell type-specific marker, making their study difficult. Fibroblasts and other cells may acquire a myofibroblastic phenotype, likely a crucial event in expansion of the ECM. Lymphocytes appear to have important roles in the development of IFTA. The classes of infiltrating or resident monocyte/macrophages are heterogeneous, displaying a variety of phenotypes. Some macrophages may be preferentially pro-fibrotic, whereas other classes of monocyte/macrophages may actually attenuate fibrosis. Other cells also contribute to IFTA, including pericytes, dendritic cells, mast cells, and fibrocytes. Measures of IF rarely take into account the cellularity of the fibrotic areas, and how this may reflect the age of the fibrotic process or its potential for reversibility or other biologic features of the fibrotic process.

FIBROSIS DISTRIBUTION: WHERE IS THE FIBROSIS?
Patterns of IF vary and likely do not have identical causes or consequences. For example, the patchy, ’striped’ pattern of IF with corresponding TA has been described with calcineurin inhibitor use. It has been proposed that this is because of the apparent preferential involvement of the medullary rays; however, IF also might be the result of toxic injury to discrete segments of small arteries and arterioles with consequent diminished blood supply to those portions of the cortical parenchyma supplied by the injured vessels. Despite the use of this association as a way to identify calcineurin inhibitor effect, 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. Broad scars with the loss of tubules are the sequelae of severe focal injury and destruction of parenchyma, such as in pyelonephritis and infarcts. 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 with extravasation into the interstitium. The IF resulting from the metabolic injuries of diabetic nephropathy is both diffused and more homogeneous in distribution, although modification of the homogeneous distribution may occur as a result of concurrent vascular disease that may be of irregular severity. As kidneys age, there is often a pattern of subcapsular fibrosis, usually attributed to a marginal blood supply that is not replicated in less superficial portions of the renal cortex. Despite these associations, there is often an essentially nonspecific pattern of fibrosis in renal biopsies of patients with chronic kidney disease, including diffuse or patchy fine IF surrounding tubules, which can be either normal or atrophic. This is associated with either diffuse or focal disease of glomeruli, tubules, or vessels. Although assessment of cortical IF is often stressed, medullary IF likely parallels cortical IF and epithelial loss, as stressed in studies by Farris et al.

What about the interstitial microvasculature?
In allografts, loss of peritubular capillaries (PTCs) occurs following transplantation. One study has shown that PTCs decrease with time in allografts and are inversely related to renal function; decreased PTC density at 3 months predicts later loss of function at 1 year. Loss of PTC presumably results in a diminished supply of nutrients to the tubulointerstitium, and these PTC changes are often thought to parallel the presence of IF. However, it remains unclear whether loss of PTCs is causal in the development of IF and, conversely, whether restoration of the PTC density can lead to reversal of IF. Despite the obvious importance of PTC for a healthy tubulointerstitium, PTC density is rarely measured in preclinical studies of fibrosing injuries and is virtually never measured in clinical practice.

QUANTITATION METHODOLOGY: WHAT DO OUR HISTOLOGIC STAINS STAIN?
Trichrome staining (Figure 1) is often used in addition to other conventional histologic stains (hematoxylin and eosin, PAS, Silver Methenamine) to assess collagen content in the interstitium. Trichrome staining is quite practical for both clinical management of individual patients and for research studies, as it is widely available and inexpensive. For quantitation, visual assessment of trichrome-stained slides is the standard practice at many institutions, however, studies have shown that this approach may have poor reproducibility. Part of the reproducibility issue arises from uncertainty as to whether the definition of IF employed is based on total area occupied by the stainable collagen or based on areas containing any amount of stainable collagen (that is, ‘fine fibrosis’) as discussed further below and illustrated in studies by Furness et al. and Farris et al.
Trichrome stains may not be sensitive at milder levels of fibrosis. Trichrome dyes are sensitive to length of formalin fixation, which introduces an important variable in studies of renal biopsies, which are not handled uniformly in multi-institutional studies.

Picrosirius Red (also referred to as simply ‘Sirius Red’) is examined under both polarized and unpolarized light. Sirius Red is thought to be specific for collagen types I and III under polarized light. Because of the high specificity for binding to collagen fibers, this stain has a high signal-to-noise ratio and lends itself to computerized image analysis. However, Sirius Red is not widely used and is subject to discrepancies between polarized and unpolarized measurements. Technical considerations have a large effect on performance, and it will likely be difficult to standardize Sirius Red among different laboratories. Furthermore, studies to test the reproducibility of this methodology across institutions are currently lacking. An important consideration is...
hindering the use of Sirius Red as a standard in measuring IF is that it is more time-consuming and expensive to perform and analyze than a trichrome stain.

Collagen III immunohistochemistry is probably the least widely used fibrosis stain and thus has little clinical validation. As it also discriminates among collagen molecules, and therefore provides a very discrete signal, it has the advantage of lending itself to computerized image analysis. Technical considerations make it difficult to standardize between laboratories and even intralaboratory assays.

**Measuring fibrosis**

Sufficient data to enable us to decide how to characterize fibrosis of the tubulointerstitium are lacking for human assessment of histology slides. If we cannot agree on definitions, reproducibility will be a problem. Some people consider % IF to be the percent of overall tissue occupied by fibrous tissue, whereas others consider the percent of fibrous tissue to be the % of tissue that is abnormal (Figure 2). These are very different conceptual ways of considering % IF, and these perceived differences can lead to differences in pathologic interpretation and quantitation. For example, in a study that is both insightful and disappointing in its outcome, Furness et al. performed an interobserver variability study among 21 pathologists in 15 countries. As a first step, they circulated glass slides from 55 renal allograft biopsies and scored them. A second circulation of the same slides accompanied by feedback on how the individual pathologist deviated from the norm led to a second round of scoring. In a third step, photographs of very selected areas of the slides depicting specific pathologies were circulated. They found that international variation in histologic grading was large (that is, good reproducibility was not achieved), and persistent feedback did not improve reproducibility. For example, the kappa for IF was stated to be 0.295 for all cases circulated (compared with the highest kappa, 0.378, for intimal arteritis); moreover after feedback, the kappa actually went down from 0.306 before feedback to 0.249 after feedback. The kappa for scoring fibrosis was actually less with the use of the highly focused photographs (0.259) compared with the assessments obtained from the glass slides (0.295). In this study, it was pointed out that there is a problem in assessing the ‘area affected’ by a progressive process. Therefore, it is clear that definitions have many caveats, and many key definitions are unresolved (these include definitions that encompass the usual forms of IFTA vs. parenchymal contraction in which intervening tubulointerstitial parenchyma between obsolescent glomeruli have been lost; the difference between kidney parenchymal area occupied by fibrotic matrix vs. areas containing both fibrotic matrix and intact glomeruli and tubular structures as discussed above and illustrated in Figure 2; defining the threshold for how much matrix needs to be present to identify a region of the kidney as being involved by fibrosis; and a consideration whether our definition of fibrosis should be a ‘one size fits all’ approach (that is, are matrix accumulations of type III collagen equivalent to matrix accumulations of proteoglycans or other matrix proteins?). Current analytic approaches, in either clinical or preclinical studies, generally avoid rigorous assessment of these issues.

Computer-based morphometry techniques have been used to assess IF, partly because of the interobserver variability that has been shown in the past (Figure 1). These computer-based methods include morphometry of slides stained with trichrome, Sirius Red, and collagen III immunohistochemistry. Analysis in some of these studies has shown correlation with glomerular filtration rate; however, as shown in the studies by Farris et al., this may not improve upon assessment made by the unaided human eye.

Other methodologies could be employed in the future. Other histologic stains that may improve upon our assessments include the Movat’s pentachrome stain that allows the assessment of collagen content, proteoglycan content, and elastic tissue content with a single staining procedure. Stains to allow measurement of PTC density may also enhance our ability to measure clinically relevant changes in the tubulointerstitium linked to chronic kidney disease. Other sophisticated methods that could show promise in the future could include transcriptomics and mass spectrometry.

**Fibrosis-scoring systems currently in clinical practice**

Several diagnostic schema include fibrosis as an integral component. The National Institute of Health (NIH) lupus nephritis activity/chronicity indices include a provision for scoring fibrosis, and the International Society of Nephrology and Renal Pathology Society Working Group on the Classification of Lupus Nephritis specifies that extent of...
IF be specified in the pathology report, although a formal score is not provided. The Banff Classification for renal allograft rejection includes a provision for IF. It is termed the ‘ci’ score. However, current Banff working group studies show variability in the way that pathologists score fibrosis. The ‘MEST’ score developed in the Oxford Classification of IgA nephropathy includes a ‘T’ component for a visual estimate of the extent of IF. Specifying the extent of IF is important in these diseases, as longitudinal studies will use this information to evaluate disease progression and the effect of drugs on the disease. Having definitions and standardized approaches that are agreed upon will hopefully help to make the data more meaningful.

CONCLUSIONS AND RECOMMENDATIONS

We have touched on only some of the outstanding issues that confront investigators and clinicians alike in assessing renal fibrosis from pathology specimens. Among the issues critical to these endeavors, but not considered in this review, include sampling variation and artifacts, differences in fibrosis composition between animal models and human diseases, and the dynamics of IF and how features of fibrosis may change over time. In considering the immediate charge of drugs on the disease. Having definitions and standardized approaches that are agreed upon will hopefully help to make the data more meaningful.

DISCLOSURE

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