Leaders

Extracellular matrix and the kidney

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Introduction

Histopathologists have long recognised that excessive accumulation of extracellular matrix in the kidney is a serious sign of irreversible damage. However, we have rarely attempted further analysis of such accumulations, beyond noting the presence of pink staining material in our haematoxylin and eosin sections, and a rough estimation of its severity. This is remiss of us, as fibrosis of the renal interstitium has repeatedly been reported to be the best single morphological correlate of renal function and prognosis.^{1,2}

The purpose of this review is to summarise selected recent developments in this field, and to consider how these developments may alter the ways in which histopathologists might assess the extracellular matrix. Although I will concentrate on the kidney, there are obvious parallels with the assessment of non-neoplastic disease in other organs.

The matrix proteins

Our ability to distinguish different extracellular matrix proteins is increasing rapidly. "Collagen" or "hyaline" is no longer sufficient. Many different collagen types have been described. Many of these can be made up of a variety of different α chains. The number of non-collagenous matrix glycoproteins continues to grow. Furthermore, a single matrix protein gene is often subject to complex splicing of its mRNA,³ which may vary in different organs and in different disease states. After translation, the level of glycosylation may also vary. The potential heterogeneity of the extracellular matrix is therefore enormous.

Type I collagen is most often assumed to be the cause of pathological fibrosis, but in the kidney it is usually only deposited late in the process and in relatively small amounts. Much more abundant is type III collagen, which produces smaller fibres and, somewhat unusually, seems to be composed of only one α chain. Type IV collagen, a non-fibrillar collagen, is the main constituent of basement membranes. Although type IV collagen is believed to auto-assemble into sheets, immunohistochemical studies show unequivocally that it can also accumulate as pathological masses which, on light microscopy, are indistinguishable from other extracellular matrix proteins. Six different collagen IV α chains have been identified,⁴ and

the human sequences for all are now known.⁵ Any one triple helix only contains two or three of these chains. Most basement membranes contain $\alpha 1(IV)$ and $\alpha 2(IV)$, but glomerular basement membranes contain the other chains too. Investigation of this area has provided an elegant explanation of the tissue specificities of Goodpasture disease, Alport syndrome, and Alport post-transplantation anti-glomerular basement membrane nephritis.⁴ The epitope responsible for Goodpasture disease is located on the $\alpha 3(IV)$ chain,⁶ but the mutations which cause Alport syndrome show it to be a heterogeneous disease, with various abnormalities on several $\alpha(IV)$ chains.^{7,8}

Many situations in which "fibrosis" is described in the kidney actually represent abnormal accumulations of non-collagenous glycoproteins. The number of glycoproteins associated with abnormal extracellular matrix deposition continues to grow.⁹ A few justify special mention.

The most abundant glycoprotein of basement membranes, laminin, is now known to exist in a variety of molecular forms,¹⁰ originating from several genes, the products of which may be further modified by post-translational splicing. This phenomenon is common in extracellular matrix proteins, and has also been extensively studied in fibronectin. The production of different splice variants is seen in different organs and can also be modified in different ways in different disease processes.¹¹ Diseases with heavy proteinuria are associated with loss of at least part of the molecule of heparan sulphate proteoglycan from the glomerular basement membrane,¹² partly accounting for the loss of the charge selective function of the glomerular filtration barrier. This molecule is also detected in abnormal accumulations of matrix in the mesangium. It is capable of binding to various growth factors¹³ and modifying their activity, a field beyond the scope of this review.

There has been much recent interest in tenascin, a glycoprotein which has been associated with control of cell movement and matrix remodelling^{14,15} and also with involution.¹⁶ It has been suggested that its presence correlates with "active" fibrosis rather than old scarring.¹⁷ Studies in the kidney have been few, but increases in tenascin mRNA have been reported to coincide with elevation of transforming growth factor (TGF) β mRNA in one animal

model.¹⁸ Several recently described glycoproteins exhibit a close association with the presence of specific cytokines—for example, synthesis of decorin and biglycan is said to be closely linked to local concentrations of TGF β .¹⁹ However, it is not necessarily the case that extracellular matrix is synthesised locally; in two experimental models of glomerular sclerosis, abundant accumulations of fibronectin were convincingly shown to originate from plasma rather than local synthesis.^{11,20} The mechanism of such pathological trapping remains obscure.

Matrix degradation

In normal tissues slow synthesis of extracellular matrix is balanced by slow removal. In chronic disease, therefore, a decrease in removal could result in tissue fibrosis. Consideration of the mechanisms of fibrosis must therefore include an assessment of rates and mechanisms of degradation. Unfortunately this is hard to achieve. A variety of enzymes can degrade the matrix, of which the most important is the group of zinc-requiring enzymes known as the matrix metalloproteinases (MMP) and numbered 1 to 9.²¹ Of these, MMP2 (also known as gelatinase A, 72 kDa metalloproteinase) and MMP9 (gelatinase B, 92 kDa metalloproteinase) have been most extensively studied in the glomerulus. In the tubules various other enzymes have been described which can degrade extracellular matrix,²² but it is often unclear whether this is their physiological role, or whether they are responsible for degrading peptides which have been filtered and reabsorbed. Little is known of the relative importance of these various enzymes in humans. Recently, a membrane bound matrix metalloproteinase has been described, which not only degrades matrix but also activates other MMPs.²³ This is likely to be of great relevance to penetration of tissues by inflammatory cells, and to invasion by malignant cells, but its relevance in renal fibrosis is not yet clear. The secreted metalloproteinases are released in pro-enzyme form, with a terminal peptide being cleaved by a variety of proteinases to achieve activation.

There are several specific inhibitors, known as tissue inhibitors of metalloproteinase (TIMP). TIMP1 and 2 have been extensively studied for several years; TIMP3 has been described recently but its primary sequence is already known.²⁴ TIMPs can not only inhibit active MMPs, but can bind to the pro-enzyme and inhibit its activation. Some MMPs also bind to the extracellular matrix, which may result in sequestration of their tissue activity.²⁵

All these checks and balances make studies in this area very difficult to interpret. The rate of synthesis of any component may bear little relation to its abundance, and the abundance of an enzyme or an inhibitor does not necessarily reflect the amount of the active form. Measurement of enzyme activity in tissue homogenates is not entirely reliable, as activation or inactivation could have occurred during tissue processing. Localisation of gelatinase activity is possible by observing digestion of gelatin

films coated over frozen sections, but the method is not sensitive and permits little quantitation.

The control of matrix synthesis

Matrix production is controlled by a complex cytokine network,²⁶ but the most important single molecule identified so far seems to be TGF β .^{27–29} This cytokine has many effects in all organ systems, but its main role in renal disease seems to be in extracellular matrix remodelling. TGF β in acute renal disease is probably produced mainly by infiltrating macrophages,³⁰ but it can also be synthesised by other cell types including mesangial cells, resulting in autocrine stimulation and increased synthesis of various extracellular matrix components by mesangial cells and interstitial fibroblasts.³¹ TGF β also inhibits the synthesis of matrix metalloproteinases and increases the synthesis of their inhibitors.³² Evidence of the importance of TGF β is not limited to tissue culture; increased synthesis correlates with glomerular and interstitial fibrosis in intact animals,³³ anti-TGF β ameliorates disease in experimental nephritis³⁴ and elevated TGF β is found in the urine of patients with focal segmental glomerulosclerosis.³⁵ Many factors control TGF β activity. It is secreted in an inactive form, but is readily activated by proteases (or an acid environment).²⁷ Like the MMPs, it may be inactivated by binding to the extracellular matrix.³⁶ One important mechanism in chronic renal damage may be angiotensin II; this induces TGF β synthesis *in vitro*³⁷ and angiotensin converting enzyme inhibitors limit excessive renal fibrosis in a variety of models^{38,39} and also in humans.⁴⁰

Mechanisms of control of matrix synthesis in diabetic nephropathy have been the subject of intense study. Induction of cytokine secretion seems to be part of this process.⁴¹ Cultured glomerular cells produce more collagen in high glucose media, but there is disagreement whether increased synthesis⁴² or decreased degradation⁴³ is the more important effect. Giving glycated albumin to mice elevates mRNA levels of TGF β and various matrix components.⁴⁴ Decreased proteinase activity has been reported in diabetic rat strains which develop nephrosclerosis, but not in one which does not; the sclerosis and the decreased enzyme activity are ameliorated by treatment with insulin. The importance of altered degradation has also been suggested in sclerosis as part of the ageing process; various strains of rat show a gradual elevation of collagen mRNA in their kidneys with age,⁴⁵ but only in strains which do not produce an accompanying increase in degradative enzymes is this accompanied by glomerular sclerosis.⁴⁶ The mechanisms are obviously very complex and are only slowly being unravelled.

Interstitial fibrosis is a constant companion of chronic renal disease of almost any cause, and correlates well with renal function.¹² Despite this, the precise mechanisms of interstitial fibrosis have been subjected to much less study than glomerular fibrosis, possibly because the

cells in the normal renal interstitium are less well characterised and less amenable to manipulation in tissue culture. One of the interstitial cell types resembles a myofibroblast, contains α -smooth muscle actin and increases in number in fibrotic renal conditions in humans.⁴⁷ Some parallels with the situation in the glomerulus are evident.

The links between sclerosis and circulating lipids are very complex. Glomerular cells bear various lipoprotein receptors, which can be modified in disease states.⁴⁸ Culture of mesangial cells with low density lipoprotein leads to increased synthesis of collagen⁴⁹ and of a peptide which acts as a macrophage chemoattractant.⁵⁰ Fatty acid deficiency reduces macrophage influx and glomerular damage in rat nephrotoxic nephritis, apparently by inhibiting the synthesis of a lipid chemoattractant.⁵¹ In the interstitium lipids are also likely to influence matrix synthesis. It has recently been suggested that in proteinuric states, some of the fatty acids carried by filtered albumin may be modified after absorption by the tubular cells to produce the lipid chemoattractant mentioned above, with consequent macrophage influx and induction of fibrosis (G E Schreiner, personal communication). This lipid chemoattractant is not made from the fatty acids found in fish oils, which could explain the repeatedly observed benefit of fish oil diets in experimental renal disease. This area is complex and developing, and has been reviewed recently.^{52,53}

Implications for the diagnostic process

It seems likely that we could now do better for our patients than merely guessing at the amount and nature of matrix accumulation. But what should we measure, and how? We should be clear what we are trying to achieve.

Attempting to identify matrix changes which are specific to a disease process has a long history,^{54,55} but has had little success. With improved understanding of the complexity of the matrix some distinctions may nevertheless be available. For example, the pattern of deposition of the six α (IV) collagen chains varies in different diseases⁵⁶; the changes are not specific, but this sort of approach might help if we need only to distinguish two morphologically similar conditions. In collagen type III glomerulopathy, a recently described hereditary glomerular disease, the type of collagen which accumulates in the glomerulus is the defining feature of the disease.⁵⁷ In diabetes the increased matrix glycosylation has traditionally been detected with a periodic acid Schiff stain, but more specific detection of glycosylated proteins can be achieved by immunohistochemistry. Chondroitin sulphate has been reported in the glomerular basement membrane of rats with diabetes only.⁵⁸ A detailed investigation of age related changes in the glomerular extracellular matrix in the rat showed patterns which were different to those seen in most glomerular diseases.⁵⁹ Distinctions have also been claimed in humans by some authors⁶⁰ but denied by others.⁶¹ It has been suggested that glomerular

accumulations of the glycoprotein tenascin are seen only in relatively recent lesions in humans⁶² but other authors have not supported this finding.⁶³ It is evident that progress in this area will take a great deal more work, and success is not guaranteed. It is a major problem that the literature abounds with suggestions and animal models, but exhaustive studies in human renal biopsy specimens are lacking.

If, however, we are attempting to measure the severity of a given disease process, there are more grounds for optimism. The greatest potential in this area is probably in assessing prognosis.

We have the experience of numerous conventional morphometric studies. The correlation of interstitial fibrosis and renal function has been mentioned, but in routine practice, conventional morphometric methods are too time consuming to be practical. Nevertheless, various alternatives are appearing. An ingenious method for using an enzyme linked immunosorbent assay to measure the collagen content of a tissue section has been described,⁶⁴ but so far has not been widely taken up. In our own laboratories we are investigating the use of immunostaining for type III collagen, without a counterstain. A digital image is then "grabbed" and the interstitial volume fraction can be measured rapidly by a semi-automated image analysis system. This takes only an additional minute of a pathologist's time per case.

We may be able to do better still. If the end stage of renal disease is fibrosis, and given our understanding of the mechanisms which cause fibrosis, should we not be looking at and measuring the earlier stages, to predict the rate of progression? The above discussion provides numerous candidates for such study. The presence of an interstitial infiltrate of inflammatory cells implies active disease; but which cells? Measurement of interstitial lymphocyte subsets is said to be of some prognostic value in membranous glomerulonephritis.⁶⁵ Would quantitation of macrophages, possibly with an immunohistochemical measurement of volume fraction as described above, provide prognostic information? Would quantitation of cytokines such as TGF β be better? Further down the sequence of events, a conventional morphometric measurement of interstitial myofibroblasts has been claimed to give prognostic information in a rather unpredictable disease, IgA nephropathy.⁶⁶ Does an excess of one matrix component give better prognostic information than another? Tenascin might be a candidate here. There is obvious potential, but much more work is needed to define what exactly is useful and practical.

Finally, the most exciting prospect in this field comes from molecular biology. We have so far considered only what is present in tissue sections; we have not considered how fast things are being synthesised. If accumulation of, say, type III collagen is known to be significant in a disease process, then it is likely (though not inevitable) that the rate of synthesis of type III collagen will give us prognostic information. The best measurement of synthetic rates we can get from a single biopsy

specimen is the level of the specific mRNA for that substance. Specific mRNA sequences in tiny biopsy specimens can be detected by in situ hybridisation or by reverse transcriptase polymerase chain reaction (RT-PCR). In situ methods have been used—for example, to demonstrate elevation of mRNA for collagens in glomerular epithelial and mesangial cells.⁶¹ However, this approach lacks the sensitivity and the potential for quantitation of RT-PCR, especially competitive RT-PCR. Although RT-PCR lacks tissue localisation, microdissection methods have been used to obtain RT-PCR samples in various tissues; in the kidney, single glomeruli⁶⁷ or short segments of tubule⁶⁸ can be used in this way. There is already evidence that measurement of collagen mRNA in a single glomerulus correlates with glomerular sclerosis.⁶⁹ Competitive RT-PCR has been used to demonstrate quite small increases in levels of mRNA of TGF β and tenascin in isolated glomeruli in diabetic mice.¹⁸ There is no reason why this approach should not be used with glomeruli plucked from the surface of routine human renal biopsy specimens. We have preliminary data suggesting that further dissection may be possible by using a gentle lysis buffer which extracts mRNA from glomerular epithelial cells while leaving the rest inside the glomerular basement membrane.⁷⁰ The potential of such new approaches is self evident.

There is much work to do, but these advances in understanding and methodology provide great scope for using our renal biopsy specimens better, not only to obtain more diagnostic information but also to gain a deeper understanding of the processes which cause disease.

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