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Contribution of Epithelial Plasticity to Renal Transplantation–Associated Fibrosis

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Abstract

Every year in the United States, 5000 renal transplant recipients start or restart dialysis because of the unusual propensity of these allografts to develop interstitial fibrosis and tubular atrophy (IF/TA). Although IF/TA often follows one or more identifiable events, our capacity to specifically treat, prevent, or even detect IF/TA at an early stage is poor. These limitations are largely related to our lack of adequate tools to assess graft failure over time. Data accumulated over the past 5 years have demonstrated that tubular epithelial cells may react to certain fibrogenic stimuli to engage in the process of epithelial-to-mesenchymal transition (EMT). In this review, we highlight the current view of EMT with a focus on both its role in the context of renal transplantation and the potential for utilizing markers of EMT to identify patients undergoing early IF/TA.

Introduction

Across the world, about 40% of renal grafts are lost within 10 years of transplantation. In the United States alone, about 5000 renal transplant patients every year start or restart dialysis due to late graft failure.¹ In 2007, a consensus emerged within the renal community that the term *chronic allograft nephropathy* (CAN) was inappropriately being used as if it were a specific disease entity rather than a general term for graft scarring.² After much debate, the term *interstitial fibrosis and tubular atrophy* (IF/TA) was developed to replace CAN and better characterize the etiology of graft failure. Accumulating evidence over the past few years actually suggests that there is often an identifiable cause for IF/TA. First, in a 10-year (1996–2006) longitudinal study of patients transplanted at the Mayo Clinic, analysis of the clinical and histological data of the patients who lost their graft *because* of IF/TA allowed for the identification of a specific cause in most cases.³ Second, the extensive analysis of graft biopsies performed between 2004 and 2007 in an unselected Canadian population of

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kidney transplant patients led to the conclusion that antibody-mediated microcirculation injury was a major finding in grafts subsequently lost (63% of late kidney failures).⁴ These studies suggest that IF/TA is probably not an idiopathic entity, but rather the consequence of accumulated injury related to immune alloreaction, recurrent glomerular disease, or polyoma virus nephropathy, among others. The consensus that IF/TA is not idiopathic is somewhat reassuring. However, this paradigm shift does not solve the problem. In any context, IF/TA is obviously of high prognostic value. The identification of robust and early biomarkers for fibrogenesis is urgently needed.^{5, 6}

Interestingly enough, the presence of IF/TA on biopsy rarely leads to a change in clinical management. Thus, although most grafts will be lost because of the development of fibrosis and very rarely as a direct consequence of rejection or recurrent glomerulonephritis, we continue to use only the latter endpoints in our clinical decision making. There are several explanations for this behavior: first, the presence of IF/TA in a core biopsy may reflect a sampling error or may have been present in the donor kidney before transplantation. Second, the current dogma is that organ fibrosis occurs as an end-stage complication of tissue injury. The significance of identifying IF/TA within a graft shortly after transplantation is unclear, yet we know that fibrosis can develop within the first years of transplantation: Cosio et al demonstrated that mild fibrosis was frequently identified in living donor kidneys grafts at 1 year after transplantation; furthermore, this fibrosis occurred at a similar frequency to that seen with deceased donor grafts, even in patients without obvious risk factors.⁷ Finally, and perhaps most importantly, even if we are confident in the histological assessment, we are profoundly ill equipped to act on the finding of early fibrosis.

Fibrosis is defined by an excessive accumulation of extracellular matrix proteins (mostly collagen) that are locally produced by activated interstitial fibroblasts. The origin of these activated fibroblasts is still debated, but accumulating evidence suggests that they are not merely derived from resident fibroblasts. In the context of allotransplantation, it was shown in 2001 that a significant proportion of mesenchymal cells present in the renal vasculature and interstitium actually originated from the recipient.⁸ This finding also is supported by recent experimental findings⁹; progenitor mesenchymal cells from the recipient may thus circulate and invade the graft.

More recently, tubular epithelial cells have been shown to give rise to myofibroblasts via a process known as epithelial-to-mesenchymal transition (EMT). The objective of this review is to provide a qualitative and quantitative assessment of the role of EMT in renal graft fibrogenesis and to explain how EMT markers may be beneficial in clinical decision making at the bedside. Although the concept of EMT has met some resistance, data amassed from basic and patient-related research studies over the past 5 years suggest that it plays an important role in tissue fibrosis.

Involvement of Epithelial Cells in Graft Fibrogenesis

The idea that epithelial cells contribute to organ fibrosis is difficult to conceptualize. In the kidney, for example, the role of the epithelium is to participate in the maintenance of water and electrolyte homeostasis. Synthesizing matrix proteins or participating in extracellular

matrix remodeling is clearly not related to this function. Nevertheless, analysis of renal transcripts from allograft biopsies with varying degrees of dysfunction has revealed that impaired allograft function is accompanied by a decrease in kidney parenchymal transcripts and in an increase in developmental transcripts.¹⁰ Why would epithelial cells silence the functions that characterize their phenotypic state? Perhaps the natural response to injury is to revert to a more primitive cellular program that will avoid cell death and facilitate tissue repair. During kidney development, tubular epithelial cells derive from mesenchymal cells within the metanephric bud. This developmental relationship between mesenchymal and epithelial cells makes the concept of EMT within the kidney somewhat more plausible. EMT might represent a reverse embryogenesis. It is hard to imagine how fibroblasts dispersed within the interstitium could account for the thickened tubular basement membrane that is often visible on routine staining of renal biopsies from patients with chronic renal failure. Rather, it is more likely that tubular epithelial cells themselves lay down the circular matrix once they have begun to undergo EMT.

Definition of EMT

EMT refers to the process by which epithelial cells undergo profound phenotypic and functional changes that allow them to dissociate from their neighbors, digest the basement membrane on which they rest through the action of matrix proteases, and migrate outside the epithelial structure (Fig 1).^{11–14} EMT is a reversible process that not only plays a pivotal role in embryogenesis and organ development but was first described in this setting. More recently, EMT has been identified as a mechanism by which adult tissues regenerate and scar following injury, and tumors of epithelial origin invade and metastasize. The role of EMT in tissue regeneration stems from the finding that proteins characteristic of fibroblasts are expressed by tubular epithelial cells under stress.^{15, 16} Today, a new classification takes into account these different circumstances of EMT. Types 1, 2, and 3 refer to EMT in the settings of developmental biology, adult tissue repair and fibrosis, and cancer dissemination, respectively.¹⁴ Importantly, EMT is reversible, and, in a mesenchymal-to-epithelial (MET) process, cells may theoretically switch back to their initial phenotype.

Regulation of EMT and MET occurs primarily at the transcriptional level.¹⁷ In the face of a biological stress such as ischemia or inflammation, cells initiate an EMT program as a survival tactic.¹⁸ Physiological tissue repair occurs when this process occurs transiently and is followed by restitution. If the process is perpetuated unchecked, however, pathological tissue fibrosis may occur. For some, these tubular epithelium-derived fibroblasts actually represent a significant proportion (one-third) of total interstitial fibroblasts after unilateral ureteral obstruction, as shown in a mouse model of renal interstitial fibrosis¹⁶; moreover, in a vicious cycle, the appearance of fibrosis might further enhance the capacity of cells to undergo EMT.¹⁹ Others, however, using the same animal model, have refuted any contribution at all of epithelial cells to the population of interstitial fibroblasts.²⁰ These contradictory results have fueled a vivid debate in the kidney world over the last 10 years.²¹

Evidence for (and against) the Occurrence of EMT in Renal Grafts

In 2005, Vongwiwatana et al studied the expression of epithelial and mesenchymal markers in tubular epithelial cells from human renal allografts with and without IF/TA (with a score of interstitial fibrosis >2).²² It was found that IF/TA was associated with a loss of several epithelial markers (E-cadherin, cytokeratin) and acquisition of mesenchymal markers (vimentin, HSP47, and S100A4—the human orthologue for FSP-1) in roughly 30% of tubular cells. It was subsequently hypothesized that markers characteristic of EMT are expressed prior to the development of IF/TA.²³ Two were studied in particular: (1) the translocation of β -catenin from the basolateral membrane where it co-localizes with E-cadherin to the cytoplasm and (2) the de novo expression of vimentin (of note, the abnormal expression of vimentin had previously been found to correlate with the extent of graft damage²⁴). It was proposed that the presence of these two markers in more than 10% of tubules was pathological.²² Using this threshold, 40% of the transplants demonstrated EMT on surveillance biopsies 3 months after engraftment. EMT-positive grafts had both a longer cold ischemia time and significantly higher Banff t and i scores even in the absence of subclinical rejection. Factors such as ischemia (or the memory of it) and even mild inflammation could thus trigger an EMT of tubular cells.

EMT markers as IF/TA Markers

The fact that these markers are frequently expressed in nonatrophic tubules of renal grafts raises the possibility that they might serve as an early marker for renal fibrosis. In 2008, using sequential protocol biopsies performed at 3 and 12 months post-transplant, it was reported that the intensity of the expression of EMT markers correlated with subsequent development of IF/TA.²⁵ The patients with evidence of EMT at 3 months also exhibited a more rapid deterioration in graft function.

In contrast, Vitalone et al failed to identify an association between the expression of EMT markers and early allograft fibrosis.²⁶ This study included detection of EMT markers between implantation and 1 and 3 months post-transplant. At the transcriptional level, the majority of the genes associated with EMT (or MET) were found to be unmodified, or nonspecifically dysregulated in the biopsies showing tubulointerstitial lesions (score of interstitial fibrosis = 1). Likewise, at the protein level, the expression of EMT markers at 1 month was not associated with IF/TA at 3 months. Of note, different markers were used than in the other studies: EMT was defined by the loss of E-cadherin and the expression of either alpha-smooth muscle actin or S100A4 by tubular epithelial cells. The physiological expression of E-cadherin is, however, minimal in human proximal tubular epithelial cells, and even in fibrotic tissues, detecting an abundant expression of S100A4 at 3 months is rare, suggesting that this protein could be a late marker of EMT.²³ It is also critical to point out that this Australian population study consisted primarily of recipients of renal grafts harvested from living donors, and that the time window between the measurement of markers and the assessment for histopathology was narrow (2 months). Given this, the absence of detectable EMT in this study could be related to a bias in the criteria set forth for EMT, the fact that both the graft and the recipients had improved histology at the time of analysis, or a premature study endpoint. Alternatively, it could be that the complete process

of EMT does not occur in grafts. It is true that the last biological phase of EMT, namely, the migration of tubular cells outside the epithelial structure, has not been convincingly demonstrated in vivo in type 2 EMT. To escape this controversy, the term *epithelial phenotypic changes* was coined to describe the early steps of EMT observed in human renal grafts.²⁵ But this technical discussion should not complicate the facts: tubular epithelial cells do lose the expression of epithelial markers and abnormally express mesenchymal proteins. These facts argue for the occurrence of EMT. Evidence for a *causal* role of EMT in graft fibrosis is still lacking, since EMT has never been targeted in the clinic.

Biological Events Triggering EMT in the Graft

Transforming growth factor-beta (TGF β) is considered the master regulator of EMT in vitro. Binding of TGF β to its membrane receptor leads to signal transduction through the activation of various smad proteins. Emerging evidence suggests that the balance between TGF β and bone morphogenetic protein 7 (BMP7) actions may determine the rate of fibrosis progression. John Kirby and colleagues demonstrated that this balance was disrupted in the context of acute rejection.²⁷ When compared to normal kidneys, in which epithelial cells show an intense staining of both activating and inhibiting smads, a proportion of tubular epithelial cells from grafts biopsied during an episode of acute cellular rejection were found to express S100A4. Interestingly, these cells had a very strong nuclear expression of the activating smads 2 and 3 and a reduction in the expression of the inhibitory smads 1/5/8. In another publication from this group, the expression of S100A4 by epithelial cells was associated with adjacent immune infiltrate.²⁸ Roselyn Mannon and colleagues also demonstrated that the pro-EMT property of TGF β could be enhanced by connective tissue growth factor (CTGF), another cytokine that is increased both in a mouse model of kidney transplantation and also in human allografts. In vitro, CTGF and TGF β synergistically effect the expression of EMT markers by tubular epithelial cells.²⁹ Either by direct contact with inflammatory cells or by a paracrine effect, pro-EMT cytokines generated by inflammatory cells during the alloresponse could target the epithelium to adjust the standard cellular program and initiate a fibrogenic process. This scenario is probably not unique to acute rejection, but rather a generic mechanism for epithelial injury in the context of inflammation. For instance, the gene expression profile observed in grafts with polyoma-BK virus nephropathy shows a combined pattern with activation of T-cell and macrophage transcripts, but also of TGF β , extracellular matrix genes, and S100A4.³⁰

Aji Djamali and colleagues have demonstrated the critical role of oxidant stress in EMT. The Fisher 344-to-Lewis rat transplant model of CAN demonstrates prominent EMT of tubular epithelial cells, with positive immunolabeling for reactive oxygen species (ROS) in tubular cells in the vicinity of macrophages.^{31, 32} Based on these findings, we may hypothesize that TGF β locally produced by inflammatory cells induces epithelial cell production of ROS. In this setting, the particular role of Nox2 seems to be prominent.³³ Interestingly, ROS have previously been shown to be capable of inducing EMT. This provides a mechanistic link between inflammation and graft fibrogenesis.

Unanswered Questions and Therapeutic Perspectives

In comparison to patients with native kidney disease, in whom EMT was also found to be associated with renal fibrosis,³⁴ recipients of a kidney transplant are characterized by an almost constant exposure to calcineurin inhibitors (CNIs). Although CNIs are widely considered to be nephrotoxic, the mechanisms by which they induce renal fibrosis are poorly understood. Constricting the afferent arteriole is undoubtedly a source of downstream ischemia, but direct toxic effects on the epithelium could also be involved. As an example, CNIs were found to induce endoplasmic reticulum stress (which may be linked to EMT³⁵) and alter the expression of molecules essential to tubular cell differentiation such as *hensin*.³⁶ Definitive proof that CNIs (whether cyclosporine or tacrolimus) induce EMT in vivo is, however, lacking. Likewise, the possibility that fibroblasts derive from the vascular epithelium (ie, the endothelium) in what is referred to as endothelial-to-mesenchymal transition (EndMT) has not been explored in the setting of kidney transplants. EndMT is, however, a relevant source of fibroblasts in mouse models of kidney diseases.³⁷

Another important question is whether or not EndMT is a potential target for therapeutic intervention. There are at least three potential sources for activated fibroblasts within the graft: resident fibroblasts, circulating mesenchymal precursors from the recipient, and tubular epithelial cells from the donor. The relative contributions of these cell types to EMT in the setting of IF/TA is completely unknown.

BMP7 has emerged as a potential inhibitor of EMT. Although BMP7 may decrease E-cadherin expression in tubular epithelial cells, it also efficiently opposes the TGF β -induced expression of mesenchymal markers^{27, 38} and is considered an anti-EMT agent. In vitro, it is also an anti-inflammatory and antiapoptotic molecule. In mouse models of EMT-associated nephropathy, BMP-7 was demonstrated to prevent kidney fibrosis^{39, 40} and, in some cases, contributed to the regression of the fibrosis. Many other molecules relevant to EMT, including those in the TGF β , integrin/ILK, and WNT/ β catenin signaling pathways, may be appropriate therapeutic targets to prevent IF/TA in the graft. Youhua Liu has written an extensive review that characterizes these pathways in EMT.⁴¹

Conclusions

Acute rejection remains a serious complication of transplantation but has thankfully become less frequent. The current challenge in managing renal transplant patients is the prevention and early diagnosis of graft fibrosis. The current evidence suggests that type 2 EMT plays an important role in the development of this fibrosis. A controversy still exists as to whether human tubular epithelial cells fully undergo EMT, but regardless of whether what we observe is incomplete or complete, EMT-related markers may help to identify patients at high risk for IF/TA before they define themselves with overt renal dysfunction. The EMT program also offers an invaluable source of new therapeutic targets that will have to be tested in animal models. With further research, a more dynamic and less pessimistic vision of graft outcome will undoubtedly ensue.

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Figure 1.

The following conditions represent potential triggers of epithelial-to-mesenchymal transition (EMT) in tubular epithelial cells: cold ischemia, alloreaction, and calcineurin inhibitor (CNI) toxicity. If intense or sustained with time, they may cause an increase in transcription factors such as hypoxia-inducible factor or smads, or increase oxidant stress. Depending on whether the tubular basement membrane is disrupted by matrix proteinases, tubules will undergo atrophy, or tubular cells may undergo EMT, move into the interstitium, and actively participate in the development of interstitial fibrosis. basement membrane, TGF β , transforming growth factor- β ; CTGF, connective tissue growth factor.