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Epithelial-to-Mesenchymal Transition and Chronic Allograft Tubulointerstitial Fibrosis

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Abstract

Chronic allograft tubular atrophy/interstitial fibrosis (TA/IF) is a major cause of late allograft loss. A major challenge to the future of kidney transplantation is to dissect out the identifiable causes of chronic allograft TA/IF and to develop cause-specific treatment strategies. Emerging evidence suggests that epithelial to mesenchymal transition (EMT) is an important event in native and transplant kidney injury, including chronic allograft TA/IF. During EMT, tubular epithelial cells are transformed into myofibroblasts through a stepwise process including loss of cell-cell adhesion and E-cadherin expression, *de novo* α -smooth muscle actin (α -SMA) expression, actin reorganization, tubular basement membrane disruption, cell migration and fibroblast invasion with production of profibrotic molecules such as collagen type I and III, and fibronectin. This review examines the molecular and cellular pathways of EMT and their involvement in chronic allograft tubulointerstitial fibrosis. We examined the role of alloimmune T cells and oxidative stress in this context and evaluated EMT as a marker of disease progression. Potential therapeutic options are discussed. In conclusion, there is enough evidence demonstrating that EMT is involved in the pathogenesis of chronic allograft tubulointerstitial fibrosis. However, the extent of its contribution to allograft fibrogenesis remains unknown and only interventional trials will enable us to clarify this question. Furthermore, additional data are required to determine whether EMT may be used as a surrogate marker of disease progression in kidney transplant recipients.

1. Introduction

Chronic allograft tubular atrophy/interstitial fibrosis (TA/IF) is a major cause of late allograft loss¹⁻⁴. It is a chronic, progressive, non-specific and irreversible histopathological entity that is common, occurs early after transplantation^{1;2;5} and is associated with significant patient morbidity and mortality^{1;6-8}. A major challenge to the future of kidney transplantation is to dissect out the identifiable causes of chronic allograft TA/IF and to develop cause-specific treatment strategies⁴. Interstitial fibroblasts are the principal source of kidney fibrosis^{9;10}. Under stress, interstitial fibroblasts expand by cell division and generate profibrotic molecules. Up to a third of all disease-related fibroblasts can originate from tubular epithelia at the site of injury through epithelial-to-mesenchymal transition (EMT)¹⁰.

Emerging evidence suggests that EMT is an important event in native¹⁰⁻¹⁴ and transplant kidney injury, including chronic allograft TA/IF¹⁵⁻¹⁸. During EMT, tubular epithelial cells

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are transformed into myofibroblasts through a stepwise process including loss of cell-cell adhesion and E-cadherin expression, *de novo* α -smooth muscle actin (α -SMA) expression, actin reorganization, tubular basement membrane disruption, cell migration and fibroblast invasion with production of profibrotic molecules such as collagen type I and III, and fibronectin 10-14.

2. EMT: extracellular modulators

EMT is regulated by numerous growth factors, cytokines, hormones, and conditions often combined. However, transforming growth factor beta-1 (TGF- β 1) is the most important profibrotic factor in the kidney as it can initiate and maintain EMT by activating signaling pathways and transcriptional regulators involved in tissue fibrosis 19. Other molecules and conditions including interleukin one (IL-1) 20, epidermal growth factor (EGF) 21, fibroblast growth factor (FGF)-2 22, matrix metalloproteinase protein-2 (MMP-2) 23, advanced-glycation end-products (AGE) 24, reactive oxygen species (ROS) 25, angiotensin II 26 and hypoxia 27 were also able to induce EMT in kidney tubular epithelial cells (Table 1).

3. EMT: intracellular signaling pathways

EMT is a dynamic and complex process that may require the participation and integration of multiple signal pathways at different stages. Many pathways may be required and few are sufficient for completing the entire EMT course alone. TGF- β 1 activates multiple signal transduction pathways in tubular epithelial cells, including Smad-dependent and Smad-independent pathways including RhoA, and p38 mitogen-activated protein kinase (MAPK) pathways 11. The activation of the TGF- β receptor induces phosphorylation of Smad2/3 proteins, which are then imported into the nucleus with Smad4 11. Nuclear Smads act as cofactors for other transcriptional proteins including the transcriptional protein LEF1/TCF (lymphoid enhancing factor-1/T-cell factor)- β catenin complex 28. Notably, the inhibition of GSK-3 (glycogen synthase kinase-3) phosphorylation by Wnt and small GTPases stabilizes β -catenin in the cytoplasm, keeping it available for nuclear import. Similarly, Smad downstream effector integrin-linked kinase (ILK) may play an important role in EMT 29. Activated ILK can directly phosphorylate GSK-3 30 resulting in its inhibition and leading to stabilization of β -catenin. The activation of β -catenin may lead to induction of EMT 28. Other transcription factors from the Snail family, Twist, and some FTS-1-binding proteins are also involved with their respective promoters and result in the emergence of the EMT proteome and repression of epithelial proteins 11;31. The p38 MAPK pathway may be involved in TGF- β -induced apoptosis and EMT 25;32;33 whereas RhoA and its downstream effector kinase are involved in α -SMA promoter activation, actin reorganization and cell migration 34 (Figure 1).

4. EMT: a reversible process?

There is data suggesting that EMT may be prevented or reversed in experimental models of kidney disease. Hepatocyte growth factor (HGF) 35;36, bone morphogenic protein-seven (BMP-7) 37, paricalcitol 38, angiotensin-receptor type II blockade 26 and specific inhibitors of Rho-associated coiled-coil forming protein kinase (ROCK) 39 and Erythropoietin 40 have shown early promising results (Table 2). However, these studies need to be confirmed in the clinical settings.

A better understating of EMT in the context of kidney transplant tubulointerstitial fibrosis will result in a greater knowledge of the cellular and molecular mechanisms of allograft injury. Furthermore and more importantly, because EMT is a reversible process current ongoing studies will open potential options to prevent or treat allograft fibrosis. We will review in this paper the most recent findings addressing the role of EMT in the pathogenesis of human and experimental kidney allograft injury.

5. EMT in kidney transplantation: the alloimmune response

Robertson *et al* examined the association between acute rejection and subsequent chronic dysfunction in acute cellular rejection¹⁷. They hypothesized that intraepithelial T cells recruited to the renal tubules during acute rejection can directly induce fibrosis by causing tubular epithelial cells to undergo transformation to produce activated fibroblasts. Kidney allograft tissues showed a heterogeneous expression of S100A4, a marker of EMT in some tubular epithelial cells. These S100A4-expressing epithelial cells were frequently associated with infiltrating CD8+ T cells, and many coexpressed Ki67, a marker of proliferation. Primary cell culture experiments from human cortical epithelial cells demonstrated that S100A4 was induced by TGF- β 1 and direct contact with T-cells. The authors also showed that EMT was associated with epithelial cells' invasion of the tubular basement membrane. Together, their data suggested that direct contact of tubular epithelial cells with intratubular T cells may induce adjacent tubular epithelial cells to transform to proliferating fibroblasts that migrate across the tubular basement membrane, producing fibrotic lesions within the renal interstitium. The same group later investigated the balance between TGF- β and BMP-7 signaling pathways and found that Phospho (p)-Smad2/3 was expressed at increased levels by tubular epithelial cells during acute rejection and chronic allograft injury but pSmad1/5/8 was expressed at very low levels together with induction of the EMT marker, S100A4. *In vitro* experiments demonstrated that this pattern of Smad signaling was a consequence of TGF- β signaling in the absence of BMP7, suggesting that exogenous BMP7 could restore the homeostatic balance of pSmad signaling found in normal kidneys, thereby preventing or reversing the development of chronic allograft nephropathy⁴¹.

6. EMT in kidney transplantation: the role of oxidative stress

There is also evidence that oxidative stress (OS) is involved in the pathogenesis of EMT in chronic allograft TA/IF. *In vitro* studies in proximal tubular epithelial cells show that ROS play an important role in TGF- β 1-induced EMT through activation of MAPK and Smad pathways²⁵. OS was also associated with EMT in experimental chronic allograft TA/IF¹⁵. Kidney transplants were performed in 3 month-old rats, using the Fisher 344 $\rightarrow$ Lewis model. Six months post-transplant, kidney allografts displayed significant TA/IF and vascular wall thickening. Allograft recipients had significantly higher levels of serum creatinine (4.7 ± 1.3 vs 0.59 ± 0.08 mg/dl, $p=0.03$) and proteinuria (380 ± 102 vs 30.2 ± 8 mg/dl, $p=0.04$) compared to syngeneic grafts. Semi-quantitative PCR, immunoblot and immunohistochemical analyses demonstrated increased α -SMA mRNA and protein levels coupled with reduced E-cadherin mRNA and protein immunoreactivity, confirming the presence of EMT. Allograft α -SMA levels were increased as early as 1-2 weeks post-transplant. Immunohistochemical studies for collagen type I and III, superoxide anion ($O_2^{\cdot-}$), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) confirmed that tubular $O_2^{\cdot-}$, eNOS and iNOS, and interstitial collagen I, III and $O_2^{\cdot-}$ levels were significantly increased in TA/IF-associated EMT suggesting that EMT may be a link between OS and allograft fibrosis¹⁵.

Furthermore, we recently examined the role of myofibroblasts and interstitial fibroblasts in the OS pathway in human allografts with chronic TA/IF⁴². Immunofluorescent studies for α -SMA, S100A4 and gp91 were performed to study myofibroblasts, fibroblasts and the NADPH oxidase enzyme, respectively. Myofibroblasts had significantly greater intracytoplasmic gp91 expression compared to fibroblasts. A subset of tubular epithelial cells showed intracytoplasmic staining for α -SMA and gp91, suggestive of early EMT and OS. S100A4 staining was also present in atrophic tubular epithelial cells. Double-staining studies for α -SMA and S100A4 demonstrated nearly no overlap confirming previous reports that these markers separate two fibroblast phenotypes. Overall these studies demonstrated that OS and EMT were involved in the pathogenesis of chronic allograft tubulointerstitial fibrosis.

7. EMT in kidney transplantation: BK infection

BK polyomavirus nephropathy is an important cause of graft loss after kidney transplantation and is associated with TA/IF⁴³. Recent *in vitro* experiments evaluated EMT in BK virus infected human proximal tubular epithelial cells⁴⁴. There was marked upregulation of collagen type I and matrix regulatory protein TIMP4 together with EMT-associated molecules gremlin and actin filament associated protein, SMAD3 and growth factors PDGF and FGF2. Similar increases were not seen following infection with Type II adenovirus, suggesting specificity of the genes upregulated suggesting that BK infection of tubular epithelial cells results in the induction of transcripts associated with TA/IF, in part mediated by EMT⁴⁴.

8. EMT in kidney transplantation: a biomarker of progression

Vongwiwatana et al¹⁶ evaluated biopsies from 10 allograft kidneys with impaired function and TA/IF and 10 biopsies from transplants with stable function compared to their implantation biopsies. Relative to implantation biopsies, there was a loss of epithelial markers (E-cadherin, cytokeratin) in tubular epithelial cells with TA/IF together with new cytoplasmic expression of mesenchymal markers vimentin, S100A4, α -SMA and HSP-47 in deteriorating and atrophic tubules. Double immunostaining showed coexpression of cytokeratin and vimentin, S100A4 and HSP-47, indicating intermediate stages of EMT in TA/IF. These changes were nearly absent in transplants with stable function. EMT features in the TA/IF group correlated with serum creatinine (vimentin, S100A4, HSP-47), history of T-cell-mediated rejection (cytokeratin, S100A4) and proteinuria (cytokeratin), suggestive of a functional role for EMT.

These results were congruent with the findings from a prospective study by Hertig et al¹⁸ who examined EMT markers in 3 month protocol biopsies from 56 deceased-donor kidney transplant recipients with stable kidney function. EMT was evaluated in a semi-quantitative fashion using immunohistochemical staining for vimentin and translocation of β -catenin in the cytoplasm. EMT features were virtually absent in implantation biopsies, whereas 41% of the grafts were EMT-positive in the absence of advanced chronic allograft nephropathy. Thirteen patients (23%) had borderline changes or acute rejection. EMT features were more frequent in these patients than in those with normal kidney grafts. EMT in grafts corresponded with elevated serum creatinine of the donor before the recovery of kidney and longer cold ischemia time suggesting that EMT was an early and frequent phenomenon in kidney transplants and that it could be triggered by immunological and/or ischemic tubular injury. The same authors recently showed that the chronic ci (interstitial fibrosis) and ct (tubular atrophy) scores were significantly higher in EMT+ compared to EMT- grafts (ci 1.40 vs 0.45, $p=0.0002$; ct 1.44 vs 0.64, $p=0.0002$) at one year post transplant⁴⁵. Furthermore, the progression of interstitial fibrosis (delta ci) was significantly associated with the 3M EMT score suggesting that EMT features could serve as early routine biomarkers for the prediction of TA and IF in the renal graft⁴⁵.

Similar studies evaluated the EMT transcriptome in the urine of patients with chronic allograft TA/IF⁴⁶. The investigators performed mRNA profiling of urine samples from 25 renal allograft recipients with biopsy confirmed TA/IF and from 51 with normal graft biopsy and stable function. They developed pre-amplification assisted kinetic quantitative PCR assays and studied EMT by measuring TGF- β 1, α -SMA and E-cadherin and TA by uterine sensitization associated gene1 expressed in distal tubular cell (USAG1) and NaK2Cl transporter expressed in loop of Henle cells (NKCC2). They showed that urine specimens from patients with TA/IF expressed higher levels of EMT genes compared to the normal biopsy group; TGF- β /E-cadherin ratio was 16.75 in TA/IF and 6.5 in Normal Biopsy ($P=0.01$); α -SMA/E-cadherin ratio was 0.43 in TA/IF and 0.13 in Normal Biopsy ($P=0.01$).

9. Conclusion

There is enough evidence demonstrating that EMT is involved in the pathogenesis of chronic allograft tubulointerstitial fibrosis (Table 3). However, the extent of its contribution to allograft fibrogenesis remains unknown and only interventional trials will enable us to clarify this question. Furthermore, most of these recent studies are not published yet and additional data are required to determine whether EMT may be used as a surrogate marker of disease progression in kidney transplant recipients.

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Figure 1. Simplified diagram representing TGF-β1-dependent and independent signaling pathways involved in EMT.

Table 1
Extracellular inducers of EMT in kidney tubular epithelial cells

Extracellular inducers of EMT	Reference
TGF- β 1	19
IL-1	20
MMP-2	23
FGF-2	22
EGF	21
AGE	24
Angiotensin II	26
ROS	25
Hypoxia	27

TGF- β 1 is the most important profibrotic factor in the kidney as it can initiate and maintain EMT by activating signaling pathways and transcriptional regulators involved in tissue fibrosis ¹⁹. Other molecules and conditions including interleukin one (IL-1) ²⁰, epidermal growth factor (EGF) ²¹, fibroblast growth factor (FGF)-2 ²², matrix metalloproteinase protein-2 (MMP-2) ²³, advanced-glycation end-products (AGE) ²⁴, reactive oxygen species (ROS) ²⁵, angiotensin II ²⁶ and hypoxia ²⁷ were also able to induce EMT in kidney tubular epithelial cells.

Table 2
Inhibitors of EMT in experimental models of kidney disease

Inhibitors of EMT	Reference
HGF	35, 36
BMP-7	37
Paricalcitol	38
ARB	26
ROCK-inhibitor	39
Erythropoietin	40

There is data suggesting that EMT may be prevented or reversed in experimental models of kidney disease. Hepatocyte growth factor (HGF) ^{35;36}, bone morphogenic protein-seven (BMP-7) ³⁷, paricalcitol ³⁸, angiotensin-receptor type II blockade ²⁶ and specific inhibitors of Rho-associated coiled-coil forming protein kinase (ROCK) ³⁹ and Erythropoietin ⁴⁰ have shown early promising results.

Table 3

EMT and kidney transplantation

Author	Model	Context	Reference
Robertson <i>et al</i>	Human	T-cells mediate EMT	17
Tyler <i>et al</i>	Human	Signaling pathway of EMT	41
Hertig <i>et al</i>	Human	Immune response and Cold Ischemia Time associated with EMT	18, 45
Vongwiwatana <i>et al</i>	Human	EMT as Biomarker of Progression	16
Djamali <i>et al</i>	Rats	EMT as Biomarker of Progression	15
Muthukumar <i>et al</i>	Human	Oxidative Stress associated with EMT	46
Orlena <i>et al</i>	In vitro	EMT as Biomarker of Progression BK virus infection induced EMT	44