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Original contribution

# Epithelial phenotypic changes are associated with a tubular active fibrogenic process in human renal grafts☆

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**Summary** Some recently published works contest the epithelial origin of myofibroblasts, which are the major extracellular matrix producers. However, our previous studies showed that, in tubular cells, some phenotypic changes reminiscent of epithelial-to-mesenchymal transition constitute an interesting early marker that predicts the progression of fibrosis in renal grafts. We hypothesized that activated epithelial cells could directly contribute to fibrogenesis, although they remain within the tubules. Using immunohistochemistry, we studied the association between epithelial phenotypic changes (de novo expression of vimentin and intracellular translocation of $\beta$-catenin) and the production of profibrotic molecules (connective tissue growth factor, HSP47, and laminin), in tubular epithelial cells from 93 renal grafts biopsied of 77 patients. We observed the de novo production of connective tissue growth factor, HSP47, and laminin in the tubular epithelial cells displaying epithelial phenotypic changes. The score of vimentin was significantly correlated with those of connective tissue growth factor ($r = 0.785$, $P < .0001$), HSP47 ($r = 0.887$, $P < .0001$), and laminin ($r = 0.836$, $P < .0001$). The level of tubular expression of mesenchymal cell markers and profibrogenic molecules, but not graft histologic lesions according to Banff acute or chronic scores, was correlated with graft dysfunction and proteinuria at the time of biopsy ($r = -0.611$, $P < .0001$ for vimentin with estimated glomerular filtration rate) ($r = 0.42$, $P = .0006$ for vimentin with proteinuria). Our results demonstrate that the epithelial phenotypic switch is associated with an active fibrogenic process in tubular epithelial cells and with graft injury indicators. Perpetuation of this tissue injury-repair response may drive fibrogenesis in renal grafts. This "repair response" represents an interesting marker for renal graft surveillance.


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## 1. Introduction

Interstitial fibrosis and tubular atrophy (IF/TA) are universal features of failing kidneys, including renal grafts, regardless of the cause of injury. At present, no effective treatment is available to reverse fibrosis. Therefore, early markers of IF/TA are needed before fibrosis spreads, to detect those patients who will eventually lose their renal function with time. They are the ones for whom a nephroprotective approach will show the major benefit. Of all contexts, kidney transplantation is emblematic because graft loss is usual after a period that is currently around 12 years and because numerous interventions can be proposed to prevent the progression of fibrosis (eg, a better control of the alloimmune response, calcineurin inhibitors (CNIs) sparing, etc).

We have already reported that 2 tubular epithelial phenotypic changes (EPCs), reminiscent of the so-called epithelial to mesenchymal transition (EMT), were early and interesting markers, which predict the progression of graft IF/TA and the decline of graft function [1,2]: the de novo expression of vimentin, a landmark of mesenchymal cells, in tubular epithelial cells, and the overexpression and cytoplasmic translocation of $\beta$-catenin, a coadhesion molecule for E-cadherin.

EMT is an important process to disperse cells in both embryo development and cancer progression. During the last 15 years, it was also demonstrated that the EMT program could be activated in adult organs subjected to injury, as a general repair response [3-7]. Thus, tubular cells subjected to injury will lose their distinctive epithelial characteristics and acquire mesenchymal features. In animals, a full-blown EMT (ie, leading to a migratory phenotype and ultimately to the generation of de novo fibroblasts producing matrix) has been demonstrated by some, but not all groups [8-13]. This is why the Banff working group has decided to abandon EMT as a concept and to coin a new term to acknowledge that epithelial cells can acquire mesenchymal features without generating fibroblasts [14]. Definitive evidence is, however, also lacking that "transformed" epithelial cells from adult tissues really contribute to fibrosis in situ, for example, inside the tubules. Theoretically, they could be fibrogenic in 2 ways: first, indirectly, through the production of profibrotic cytokines able to activate interstitial fibroblasts; second, and more directly, through the secretion of extracellular matrix components such as collagen fibers. Supporting this hypothesis, tubular epithelial cells from native diabetic kidneys [15] or fibrosing renal grafts [16] have been shown to produce connective tissue growth factor (CTGF). CTGF is a member of the CCN family of matricellular proteins. It can be secreted into extracellular spaces and seems to be a key mediator in tissue fibrosis. In vitro and in vivo animal experiments have shown that CTGF can act synergistically with transforming growth factor $\beta$ (TGF$\beta$), a well-known profibrotic factor. The aim of the present study was to find out whether the tubular epithelial cells displaying EPCs in kidney grafts produce also CTGF as well as other fibrogenic molecules such as HSP47, a chaperone molecule reflecting collagen synthesis, and laminin, a major component of tubular basement membranes.

## 2. Materials and methods

### 2.1. Patient characteristics and control samples

Between August 2006 and October 2009, a total of 93 renal graft biopsies collected from 77 adult renal transplanted patients was available as leftover material for extensive immunostaining. All patients had given their informed consent. Seventy-three of these patients had received a graft from a deceased donor, and the other 4 from living relatives. The mean recipient age was 50.3 years at the time of biopsy, and 68.8% of them were male. The mean donor age was 58.6 years. The mean cold ischemia time was 1221 minutes. Seventy percent of patients had received basiliximab as induction therapy. Most of the patients received triple therapy including a CNI. Only 2 patients were on a CNI-free regimen and treated by rapamycin. Twenty-six biopsies were performed for graft surveillance (23 of them for a 3-month check-up), and the other 67 were biopsied due to delayed or declining graft function, suspicion of rejection, occurring 8 days to 6.5 years after transplantation. Renal histopathologic analysis was scored according to the 1997 and recently updated Banff working classification for renal graft pathology [14,17]. Thirty-two of the biopsies indicated acute rejection, only 4 from the biopsies for graft surveillance. Mean i score was 0.94 ± 0.91, t score was 0.57 ± 0.9, g score was 0.36 ± 0.7, and ptc score was 0.5 ± 0.8. Twenty-four displayed acute tubular necrosis lesions, and all of them were biopsied for a cause. Chronic Banff score for ci was 0.87 ± 0.88, and ct was 0.7 ± 0.85. As controls, we studied 3 normal kidney samples obtained from the healthy part of kidneys from nephrectomies performed due to renal carcinoma and from 5 implantation biopsies (T0). All control samples were examined for the normality of kidney tissue.

### 2.2. Immunohistochemistry for profibrotic molecules (CTGF, HSP47, laminins) and EMT markers ($\beta$-catenin and vimentin)

Immunohistochemistry was performed on paraffin-embedded tissue as previously described [2]. Target retrieval was carried out either by heating the tissue in citrate buffer (pH 6) (Dako cytomation, Trappes, France) (for HSP47, $\beta$-catenin, vimentin, and aquaporin 1) or by pretreating the tissue with proteinase K (Roche, Meylan, France) (for CTGF and laminins). The sections were incubated overnight at 4°C with phosphate-buffered saline containing anti-CTGF (Abcam, Paris, France), anti-HSP47 (Stressgen, Villeurbanne, France), anti-laminins (Dako Cytomation), anti–$\beta$-catenin