Chronic Renal Allograft Dysfunction: The Role of T Cell–Mediated Tubular Epithelial to Mesenchymal Cell Transition

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Abstract. Chronic graft dysfunction is now the leading clinical problem after renal transplantation. The principal histopathologic lesion seen in this disease is tubular loss with concurrent interstitial fibrosis. Although the severity of acute rejection often correlates with that of subsequent chronic dysfunction, a direct link between these processes has remained elusive. This study was designed to test the hypothesis 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 a motile population of activated fibroblasts. A study of renal allograft tissue sections showed the presence of the S100A4 marker of epithelial to mesenchymal transition in some tubular epithelial cells; the expression of this antigen was heterogeneous both within and between individual tubular cross-sections. Significantly, S100A4-expressing epithelial cells were frequently associated with infiltrating CD8+ T cells, and many coexpressed the Ki67 marker of proliferation. A parallel study of human renal cortical epithelial cells in primary culture demonstrated that S100A4 was induced by stimulation for 72 h with TGF-β, and by direct contact with membrane-associated TGF-β on MOLT-16 cells, a model intraepithelial T-cell line. Further experiments demonstrated that induction of transition coincided with a significantly increased potential for human renal epithelial cells to invade the tubular basement membrane. These data are consistent with a model in which intratubular T cells can present TGF-β and directly induce adjacent tubular epithelial cells to transform to proliferating fibroblasts that migrate across the tubular basement membrane, producing fibrotic lesions within the renal interstitium.

Despite modern multidrug immunosuppression, at least one episode of acute rejection occurs in up to 40% of kidney allograft recipients (1). The extent of tubulitis during this process correlates with graft function and forms the basis of the Banff system for rejection diagnosis (2). The apparent importance of tubulitis during acute rejection is consistent with the observation that many cytotoxic T cells (CTL) derived from a rejecting graft kill renal epithelial cells but spare other donor cell types. A proportion of intratubular T cells express the αE(CD103)β7 integrin (3), which allows specific adhesion to the counter-receptor E-cadherin present on the basolateral surfaces of epithelial cells. This provides an explanation for the specific cytolysis of epithelial target cells during acute rejection (4).

Studies performed in vitro have shown that allo-activated CD8+ T cells acquire CD103 after division in the presence of active TGF-β (3); the tubular epithelium is known to produce a range of cytokines, including chemokines (5) and, critically, TGF-β (3). A model has been proposed (6) in which activated allospecific T cells traffic from the blood to graft renal tubules under the influence of chemokines such as RANTES. After penetration of the tubular basement membrane, the cells encounter immobilized TGF-β and differentiate to the CD103 phenotype characteristic of intraepithelial T cells. Significantly, the number of CD103+ T cells within renal tubules increases with the severity of acute rejection (3, 7).

Although intratubular T cells have a potential to lyse adjacent renal epithelial cells during acute rejection, they can persist for long periods after the resolution of acute rejection (8). Renal epithelial cells produce the antiapoptotic and pro-memory factor IL-15, which is upregulated in the presence of IFN-γ and/or T cell–mediated CD40 ligation (9). It is known that intratubular T cells express the definitive α-chain of the IL-15 receptor and have a potential for chronic proliferation (10). Furthermore, in vitro studies have shown that TGF-β and IL-15 synergize to increase the number and survival of CD103+CD8+ allospecific T cells but to decrease their expression of perforin. Hence, the tubular microenvironment can actively support the long-term survival of a relatively quiescent memory T-cell population in a manner similar to that proposed for the maintenance of immunologic memory within the gut (11).

Approximately 6% of renal allografts are lost each year after the first, with the majority of this failure attributed to chronic graft fibrosis. There is an association between the number and/or severity of acute rejection episodes and decreased probability of long-term graft survival (2). As the number of resi-
dent intratubular T cells is likely to reflect the severity of acute rejection, it is possible that these cells constitute a direct link between acute and chronic rejection processes within the graft. In support of this model, antagonism of the RANTES receptor blocks both early allograft infiltration and chronic graft dysfunction (12), whereas returning an allograft to the donor animal immediately after the development of tubulitis prevents further acute rejection but fails to prevent chronic graft failure (13).

The primary lesion responsible for chronic graft dysfunction is tubulointerstitial fibrosis associated with a loss of nephrons; some small arteries can also show signs of intimal thickening, but it is not known whether this causes ischemic damage (2). Proliferating interstitial activated fibroblasts constitute the primary effector cell type responsible for renal fibrosis, producing collagen types I and III together with an excess of heparan sulfate proteoglycans. The origin of these activated fibroblasts has been controversial, with some arguing for a circulating bone marrow–derived precursor (14). However, a recent series of papers has demonstrated that the majority of these cells, in the mouse at least, are generated from damaged and cytokine-stimulated renal tubular epithelial cells by a process of epithelial to mesenchymal transition (or transformation [EMT]) (15).

Recent evidence suggests that the fibrogenic properties of TGF-

In the current study, we investigated the possibility that expression of S100A4 can be used to define a process of EMT occurring during the fibrogenesis associated with chronic allograft dysfunction. Furthermore, a combination of dual-color immunohistochemistry and in vitro modeling was used to determine how the presence of infiltrating intratubular T cells might contribute to the process of EMT after renal transplantation.

Materials and Methods

Normal and Transplant Biopsy Renal Tissue

Normal renal tissue was sampled from three healthy donor kidneys that were anatomically unsuitable for transplantation. Samples of this tissue were formalin-fixed and processed to paraffin. Twenty-two diagnostic renal transplant biopsies and two nephrectomy specimens were obtained randomly from the Newcastle transplant tissue archive in accordance with local Ethics Committee approval. The tissue samples were representative of all stages of posttransplantation pathology. In most cases, routine background immunosuppression at the time of biopsy was based on cyclosporine, but one patient received tacrolimus. Biopsies taken within 2 wk of transplantation were representative of all stages of tubulointerstitial rejection. The standard antirejection therapy given during episodes of biopsy-confirmed acute rejection was high-dose methyl prednisolone. Later biopsies showed evidence of “regenerative” changes, sometimes coexisting with ongoing rejection or a “chronic infiltrate.” The two nephrectomy samples and all biopsies taken >1 yr posttransplantation showed evidence of chronic damage. None of the biopsies showed evidence of infection.

Primary Antibodies and Immune Reagents

Polyclonal rabbit anti-human S100A4 (A5114), monoclonal anti-human CD8 (C8/144B), and cytokeratin (MNF116) all were obtained from DakoCytomation. Monoclonal Ki67 antibody (NCL-Ki67-MM1) was from Novacastra Laboratories Ltd., monoclonal anti-human α-smooth muscle actin (α-SMA; A2547) was from Sigma, and monoclonal anti-human E-cadherin (67A4) was from Immunotech. Biotinylated rabbit anti-mouse immunoglobulins (E0354), biotinylated swine anti-rabbit immunoglobulin (E0353), and FITC-conjugated rabbit anti-mouse immunoglobulins (E0413) were obtained from DakoCytomation. FITC-conjugated sheep anti-rabbit IgG and rabbit IgG were from Sigma. The biotin blocking system (X0590), streptavidin biotin peroxidase complex (K0377), and alkaline phosphatase Envision (K1395) kits all were obtained from DakoCytomation.

Immunohistochemical Investigation of Renal Biopsy Sections

Immunohistochemistry was performed on 4-μm paraffin sections of renal tissue using minor modifications of a method described previously (3). After microwave antigen retrieval in citrate buffer, the sections were incubated overnight at 4°C with rabbit anti-human S100A4 at 1:400 or, for dual labeling, with a combination of rabbit anti-human S100A4 and mouse anti-human CD8 (1:50) or Ki67 (1:50); all reagents were diluted in 20% normal lamb or swine serum. Bound anti-S100A4 was detected with biotinylated swine anti-rabbit immunoglobulins; anti-CD8 or Ki67 was detected using biotinylated rabbit anti-mouse immunoglobulins. Visualization was accomplished using streptavidin biotin peroxidase and nickel-enhanced diaminobenzidine (Sigma) to give a black product. Alternatively, the Envision...
alkaline phosphatase-labeled polymer system was visualized with Vector Red substrate (SK-5100; Vector, UK). For negative control, the primary antibody was substituted with rabbit IgG or nonimmune serum. Sections in which a biotinylated secondary antibody was used were pretreated with a biotin-blocking system to minimize endogenous biotin activity. Levamisole (Vector) was added to the Vector Red substrate solution to block any endogenous alkaline phosphatase activity. Labeled sections were analyzed using Leica Image Analysis and QWin software.

**Cell Culture**

Primary human renal cortical epithelial cells (RCEC; Clonetics, Bio Whittaker, UK) were cultured in 25-cm² flasks with appropriate medium (Clonetics) for a maximum of three passages before plating 2 × 10⁵ cells in 500-μl aliquots into each chamber of four-chamber slides (Lab-Tek, Nunc). After 24 h, TGF-β₁ (R&D Systems) was added to some chambers at a final concentration of 1 or 10 ng/ml; some cells were maintained in unsupplemented medium for control.

The MOLT-16 T-cell line (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; ACC29) was established in 1984 from a patient with lymphoblastic leukemia. These cells were passaged in RPMI1640 medium supplemented with 10% FCS (Sigma). The intraepithelial-like phenotype of these cells (25) was verified by immunofluorescence flow cytometric detection (FACSscan; Becton Dickinson) of cell surface CD3 and CD103 (UCHT1 and BerACT 8, respectively; DakoCytomation). Conditioned medium was recovered from cultures that contained 2.5 × 10⁶ cells/ml and was centrifuged at 1000 × g for 10 min to remove cell debris before passage through a 0.22-μm filter. Aliquots containing 10⁵, 2 × 10⁴, and 10³ viable MOLT-16 cells or 50 μl of conditioned medium were added to RCEC in chamber slides.

**Immunocytochemical Investigation of Cultured RCEC**

After 72 h of culture in the presence of 10 ng/ml TGF-β₁, MOLT-16 cells, MOLT-16 conditioned medium, or MOLT-16 cells combined with an anti–TGF-β blocking antibody (100 μg/ml 1D11; R&D Systems), the RCEC were fixed in 4% phosphate-buffered paraformaldehyde and permeabilized with 1% Triton X-100. The cells were then treated with 5% normal lamb serum in PBS for 1 h to minimize nonspecific antibody binding and were incubated with primary antibodies specific for S100A4 (1:25), cytokeratin (1:50), E-cadherin (1:10), or α-SMA (1:250) in normal lamb serum for 2 h at room temperature. After washing with PBS, the slides were treated with appropriate FITC-conjugated secondary reagents. Final incubation was carried out for 1 h at room temperature in the dark before mounting in fluorescence mounting medium (DakoCytomation). The primary antibody was substituted with rabbit IgG or nonimmune serum for negative control.

FITC-labeled cell preparations were scanned at 488-nm emission at 1-μm intervals to create a Z series of optical sections throughout the depth of the adherent cell layer using a Leica TCS SP2 UV confocal system with QWin software. Subsequent Z-series projection allowed imaging of the protein of interest within the RCEC. Comparison of protein expression between cells cultured in the presence of different stimuli was achieved by measurement of mean fluorescence intensity per cell for the different preparations; the RCEC were delineated electronically to exclude MOLT-16 cells where appropriate (Scion Image, www.scioncorp.com).

**Assays of the Migration and Invasion of Cultured RCEC**

RCEC either were cultured in a resting state or were activated by incubation for 72 h in medium supplemented with 10 ng/ml TGF-β₁. These cells were harvested by brief treatment with trypsin-EDTA (Sigma), washed by centrifugation, and seeded at a density of 1.5 × 10⁵ cells onto 8-μm-pore-diameter cell culture inserts (35-3097; Falcon, Becton Dickinson) or 8-μm-pore-diameter, growth factor–reduced Matrigel invasion chambers (35-4483; Falcon Biocoat). After incubation for 24 h, the cells were removed from the upper surface of both filter types by scraping, each preparation was fixed with 4% paraformaldehyde, and the cells were visualized using Mayer’s hematoxylin. After dehydration, the filters were removed from their support structure and mounted for examination. Cell migration was assessed in terms of mean number of cells per high-power microscope field; a minimum of five fields were examined on five replicate filters for each experimental variable.

**Results**

**Immunohistochemical Investigation of Renal Biopsy Sections**

The distribution of S100A4 was examined in normal kidney and in transplant renal biopsy and nephrectomy sections representing the full spectrum of posttransplantation pathology, including end-stage renal failure as a result of severe chronic allograft nephropathy. Normal kidney showed a small number of S100A4-expressing fibroblasts within the interstitium, but no tubular epithelial cells expressed this antigen and CD8⁺ T cells were not observed (Figure 1a). However, S100A4 was regularly observed in the tubular epithelium of acute rejection biopsies (Figure 1b). The expression of S100A4 within epithelial cells was often heterogeneous between and within individual tubular cross-sections; in many cases, individual S100A4 expressing epithelial cells were observed adjacent to nonexpressing cells within the same tubule (Figure 1b). In some cases, bipolar fibroblast-like S100A4-expressing cells were identified beneath damaged tubular basement membrane at a site suggestive of the migration of transformed epithelial cells from disrupted tubular epithelium. Significantly, the most intense tubule-associated expression of S100A4 was observed within foci of active acute rejection defined by an intratubular and interstitial CD8⁺ T-cell infiltrate (Figure 1c).

Dual-labeling was also used to assess the relationship between tubular expression of S100A4 and tubular proliferation. Figure 1d demonstrates the frequent occurrence (arrow) of proliferating tubular epithelial cells that also expressed S100A4. There was no evidence of the proliferation of tubular epithelial cells that did not express S100A4, and proliferating tubular epithelial cells were not observed in the normal kidney samples. Examination of a matched section (Figure 1e) showed that α-SMA was not present within recognizable tubules, being restricted to established fibrotic lesions within the interstitium. As with early biopsy sections, there was also evidence of co-localization of CD8⁺ T cells and S100A4 within remnant tubules and fibroblasts at the interface with an established fibrotic lesion (Figure 1f).
Immunocytochemical Investigation of Cultured RCEC

Under normal culture conditions, the primary RCEC showed uniform “cobblestone” morphology and did not express S100A4 (Figure 2a). The epithelial nature of these cells was demonstrated by uniform expression of the characteristic markers cytokeratin (Figure 2b) and E-cadherin (Figure 2c). Treatment of RCEC with 10 ng/ml TGF-β1 for 72 h caused the cells to adopt a bipolar morphology with less cell–cell contact and uniform high-level expression of S100A4 (Figures 2d and 4); TGF-β1 at 1 ng/ml produced a smaller change and was not used in further experiments. High-magnification confocal microscopy showed that S100A4 was perinuclear and associated with the cytoskeleton. The expression of E-cadherin by TGF-β1–treated cells became heterogeneous, but cytokeratin con-

Figure 1. Representative results from immunohistochemical investigation of human renal sections. (A) Demonstration that neither CD8+ T cells nor S100A4-expressing cells were observed in normal kidney sections. (B) During acute rejection, S100A4 (red stain) was present in some tubular epithelial cells in all but two cases, both of which were graded as borderline rejection. As shown in this example, the expression of S100A4 by epithelial cells was usually heterogeneous both within and between individual tubular cross-sections. (C) During acute rejection, both CD8+ (black) T cells and S100A4-expressing (red) tubular epithelial cells were found in close proximity to each other within inflammatory foci. (D) The expression of S100A4 (red) and Ki67 (black) were frequently co-localized (arrow) within tubular epithelium in areas showing ongoing fibrogenesis. (E) Analysis of the same area of fibrogenesis shown in D demonstrated that the expression of α-smooth muscle actin (α-SMA) was principally interstitial, with little evidence of intratubular expression. (F) Demonstration that CD8+ T cells (black) and S100A4-expressing cells (red) remain in close proximity within “remnant” tubules in fibrotic specimen from a patient with chronic allograft dysfunction.
continued to define the cytoskeleton of most stimulated RCEC. Importantly, α-SMA was not detected in either resting RCEC or cells that had been treated with TGF-β1 for 72 h (data not shown).

The MOLT-16 T cells expressed both CD3 and CD103 (Figure 3) and were found to bind rapidly to the RCEC, with 1 × 10^5 cells producing a significant induction of S100A4 in the renal cells after 72 h (P < 0.001; Figure 4). It is interesting that the MOLT-16 cells (arrow) also expressed S100A4 (Figure 4). Smaller numbers of MOLT-16 cells induced less expression of S100A4 and were not used in further experiments. The induction of S100A4 expression in RCEC was inhibited by addition of a blocking anti-TGF-β antibody to the MOLT-16 cells (Figure 4). Furthermore, S100A4 was not induced by the addition of medium that had been conditioned by previous culture of MOLT-16 cells (Figure 4).

**Functional Analysis of TGF-β1–Stimulated RCEC**

Comparison between the potential of resting and TGF-β1–stimulated RCEC to migrate through an 8-μm-pore-diameter filter showed that almost twice as many of the cytokine-activated cells migrated within a 24-h period (P < 0.001; Figure 5). A similar observation was made for invasion through an artificial basement membrane followed by penetration of an 8-μm-pore-diameter filter. In this system, the overall number of migrant cells was smaller than that observed for uncoated filters, but more than twice as many TGF-β1–stimulated as resting RCEC were observed on the basal surface of the filter (P < 0.0001; Figure 5). In both experiments, the migrant cells had a uniform bipolar morphology typical of TGF-β1–stimulated RCEC.
fibroblasts that have penetrated the tubular basement membrane. Significantly, the development of S100A4 in tubular cells was spatially associated with CD8+ T-cell infiltrates. Some tubular epithelial cells showing S100A4 expression also expressed the Ki67 antigen, potentially indicating a localized fibroproliferative response. This is the first demonstration of the induction of S100A4 expression during the EMT of human renal tubular epithelial cells.

The expression of α-SMA is generally considered a good indicator of the presence of myofibroblasts (29). However, this antigen was rarely associated with intact tubules, providing no useful indication of localized EMT. Indeed, data from the current study are consistent with previous studies of nephropathy that have shown that α-SMA is found only in mature regions of renal fibrosis (30). It is most likely that the acquisition of α-SMA by S100A4-expressing cells is indicative of progression from an activated fibroblast to a myofibroblast phenotype, although it has been reported that some intrarenal fibroblasts fail to make this change in vivo (31). Allografts with chronic dysfunction showed S100A4 within the cells comprising remnant tubules and within areas of mature fibrosis. It was again found that the apparent EMT of renal epithelial cells was associated with the presence of local CD8+ T cells. In previous studies, it was shown that infiltrating T cells can be maintained within renal tubules after episodes of acute rejection (8), potentially in an intraepithelial memory or immunoregulatory state, by the presence of local TGF-β and IL-15 (10).

As with the in vivo study, it was found that few primary renal epithelial cells expressed S100A4. However, these cells did express high levels of the epithelial marker antigens cytokeratin and E-cadherin. In accordance with reports of the phenotype of murine renal epithelial cells (18), it was found that the addition of TGF-β1 at 10 ng/ml induced cells within 72 h to undergo a morphologic change to a bipolar fibroblast-like phenotype and also induced the expression of high levels of S100A4, indicating initiation of the transition process. It is interesting that at this early stage, the cells maintained some expression of E-cadherin and cytokeratin but failed significantly to increase their expression of α-SMA.

Addition of the MOLT-16 T-cell line, which was used previously to model the properties of normal CD103+ intraepithelial lymphocytes (25), was also found to induce expression of S100A4 by cultured renal epithelial cells. The observed expression of S100A4 by the T-cell line is consistent with previous reports of the occurrence of this antigen in a range of T-cell lymphomas, including MOLT-16 (32). In the current study, T cells were observed to rosette around the renal epithelial cell membrane, an adhesion stabilized at least partially by interaction between the αEβ7-integrin and E-cadherin (25). This cell–cell interaction may play an important role in the transition process as renal cells did not undergo EMT in response to supplementation of the culture with T-cell conditioned medium.

It was shown recently that regulatory T cells, up to 20% of which also express CD103 (33), both express and present to adjacent cells a membrane-bound but biologically active form

Discussion

Loss of nephrons together with the development of tubulointerstitial fibrosis is a process that leads to renal failure, which is common to a range of kidney diseases (15). A series of recent papers has shown that these two pathologic events can be linked directly by the process of aggravated transition from tubular epithelial cells to activated fibroblasts. The application of antibodies specific for the murine FSP1 antigen has allowed visualization in animal models of the earliest stages of this process, which occurs in tubular cells that still maintain a recognizable epithelial morphology (17). However, similar definitive studies of the induction of fibrogenic EMT have not been reported for human kidney specimens (26), despite existence of the human S100A4 homologue of FSP1.

Chronic renal allograft dysfunction limits the survival of the majority of human kidney allografts. The severity of this process correlates positively with the incidence of T cell–mediated acute rejection, suggesting a mechanism that involves immunologic damage of renal epithelial cells followed by repopulation of the damaged organ by fibroblasts (2). Previous studies have shown that normal tubular epithelial cells divide rarely but can undergo significant proliferation after episodes of acute rejection (27) and ischemic injury (28). Although EMT has not been examined after animal model or clinical renal allotransplantation, it is reasonable to seek evidence that this process contributes to fibrogenesis after alloimmune damage of transplanted human kidneys by examination of the expression of S100A4 in clinical biopsy specimens.

Normal human renal tissue was not infiltrated by CD8+ T cells and showed no evidence of tubule-associated S100A4. However, renal biopsies with acute rejection showed S100A4 within individual tubular epithelial cells, with some peritubular cells showing phenotypic attributes consistent with activated
of TGF-β (34). Nakamura et al. (35) reported that the activity of this form of T cell–associated TGF-β can be inhibited reproducibly by application of a high concentration of a monoclonal anti–TGF-β. Use of the same antibody-blocking system in the current study completely inhibited the induction of S100A4 expression produced in RCEC by contact with the MOLT-16 T-cell line. Hence, the requirement for EMT of close proximity between renal epithelial cells and T cells in vitro is consistent with presentation by the lymphoid cells of an active but membrane-bound form of TGF-β. A similar mechanism may account for the spatial association between these two cell types observed during EMT in renal transplant biopsy specimens.

One of the important features of an EMT mechanism for the development of interstitial fibrosis during chronic renal allograft dysfunction is the potential for transforming epithelial cells to migrate across the basement membrane from tubules into the interstitium. It was shown previously that treatment of murine renal epithelial cell lines with TGF-β1 enhances the cells’ potential to migrate through a bovine tubular basement membrane preparation, a process that is associated with the production of MMP-2 and -9 (36). The current study demonstrated that conditions that induce EMT in primary human RCEC increased the potential of these cells to migrate through 8-μm-pore-diameter filters. Furthermore, these cells showed an increased capacity to penetrate a tubular basement membrane–like gel rich in collagen IV, laminin, and heparan sulfate proteoglycans. Hence, the induction of EMT in human tubular epithelial cells can provide a mechanism for the escape of activated fibroblasts into the renal interstitium. Damage to the basement membrane during this process might also enhance EMT through the localized activity of MMP-2 (24) or by the degradation of collagen IV that normally maintains the epithelial phenotype (37).

The observations made during this study are consistent with the suggestion that intratubular T cells provide a stimulus for local epithelial cells to undergo transition to a proliferating fibroblast phenotype. These cells migrate through the basement membrane to produce the fibrotic lesions, which ultimately replace renal tubules during chronic allograft dysfunction. The persistence of intraepithelial T cells after resolution of acute rejection provides an explanation for the positive correlation between the severity of the intrarenal cell–mediated rejection process and subsequent development of chronic fibrosis and graft dysfunction. An implication of this model is the possibility that elimination of intratubular T cells, potentially through apoptotic blockade of the function of IL-15, could remove an important stimulus for EMT. It might then be possible to restore renal function by treatment with bone morphogenetic protein-7, which was shown recently to limit or reverse TGF-β–mediated transition of tubular epithelial cells to fibroblasts (38).

Acknowledgments

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References


