

Introduction

The regenerative potential of a particular tissue or treatment modality is often defined as its inherent capacity to re-establish appropriate function *in vivo* by direct replacement of lost or damaged cells. Of equal importance, however, is the ability of a cell to stimulate regeneration and attenuate the progression of disease through indirect mechanisms. In previous studies, we have shown that intra-renal delivery of bioactive kidney cells into a Lewis rat model of chronic kidney disease preserves kidney functions, resulting in significant reductions in glomerular and tubulointerstitial fibrosis and attenuation of pro-fibrotic pathways when compared to untreated controls¹. In the present study, we employed *in vitro* cell-based assays to investigate potential paracrine mechanism(s) by which bioactive kidney cells could modulate fibrosis through mediators such as Plasminogen Activator Inhibitor-1 (PAI-1).

Materials and Methods

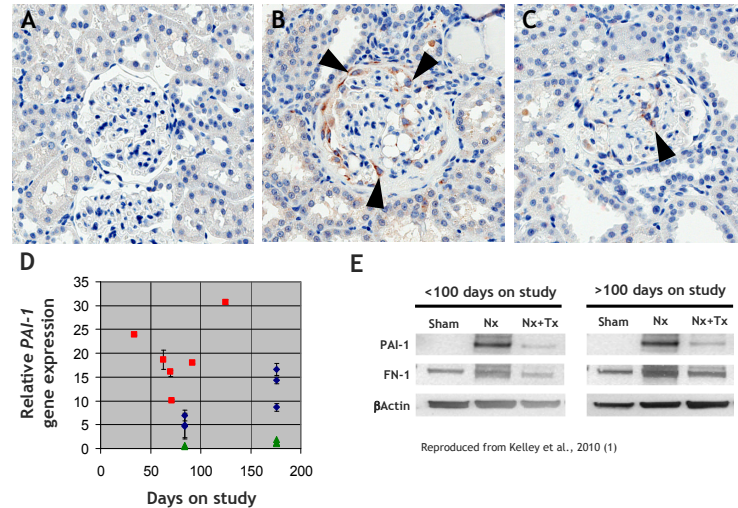
Conditioned media was collected from rat and human cultures of bioactive kidney cells^{2,3} under serum- and supplement-free conditions and utilized for *in vitro* assays. Commercially available human-derived renal mesangial cells were used as surrogates for host-response tissues in the *in vitro* assays because mesangial cells are a source of PAI-1 production in injured or diseased kidneys⁴. PAI-1 gene and protein expression were assayed by quantitative RT-PCR and Western blot, respectively. Vesicular particles shed by cells into the culture media (exosomes) were collected by high-speed centrifugation⁵ and total RNA was extracted from the pellet with TRIzol reagent (Invitrogen). RNA content of the vesicles was screened using PCR-based arrays of known microRNA sequences (Qiagen)

Figure 1. Working model for how extracellular matrix might accumulate in chronically-diseased tissues



In a normal kidney there is a balance between ECM synthesis and degradation. Interruption of this balance can occur at the onset of disease and, if balance is not restored, can lead to chronic kidney disease (CKD). Plasminogen activator inhibitor (PAI-1) and Transforming Growth Factor beta 1 (TGFβ1) affect each other in a positive feedback loop and are associated with processes that could result in inappropriate matrix accumulation and glomerulosclerosis. This study looks at the association of TGFβ1 and PAI-1 with CKD tissue pathology *in vivo* and the *in vitro* response of these genes to conditioned medium from rat bioactive kidney cells in mesangial cell cultures in the context of this working model.

Figure 2. Intra-renal delivery of bioactive kidney cells reduces the expression of fibrotic markers *in vivo*

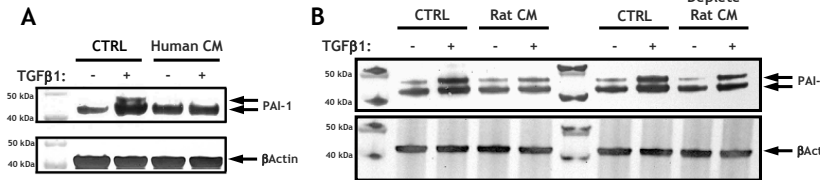


Representative immunohistochemistry images of PAI-1 (A-C) in Lewis rat kidneys that have undergone unilateral nephrectomy (A), 5/6 nephrectomy (B), or 5/6 nephrectomy with intra-renal delivery of bioactive kidney cells (C). Accumulation of PAI-1 in the glomerulus (arrowheads) as a result of the 5/6 nephrectomy procedure (B) was reduced as a result of treatment (C).

In a separate study, qRT-PCR (D) was conducted on kidney tissue harvested at necropsy and the relative gene expression values were plotted against days on study. 5/6 nephrectomized rats (red squares) demonstrated more robust expression of PAI-1 relative to those treated with bioactive renal cells (blue diamonds) and sham-operated controls (green triangles).

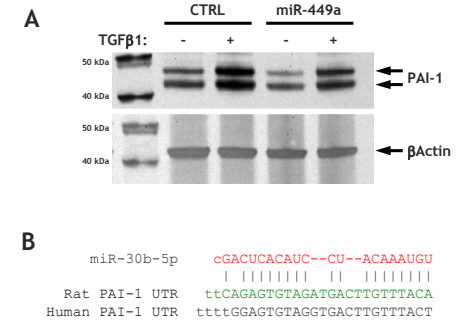
Representative Western blot analysis (E) on kidney samples taken at 3 and 6 months post-treatment. Treated tissues (Nx+Tx) of 5/6 nephrectomized rats (Nx) had reduced the accumulation of PAI-1 and Fibronectin (FN) protein.

Figure 3. Conditioned media from human and rat bioactive kidney cells attenuates TGFβ1-induced PAI-1 expression *in vitro*



In vitro experiments were designed to mimic the treatment effect of bioactive kidney cells observed *in vivo*. Human renal mesangial cells (HRMC) express increased levels of PAI-1 in the presence (+) of 5 ng/ml TGFβ1. Co-culture with conditioned media (CM) derived from human bioactive kidney cells attenuates TGFβ1-induced PAI-1 protein expression (A). PAI-1 expression at the mRNA level was unaltered by CM (data not shown). CM from rat bioactive kidney cells had similar effect on cultured HRMC induced (+) and uninduced (-) with TGFβ1. CM supernatant (Deplete Rat CM) collected after centrifugation was less effective at attenuating PAI-1 expression, suggesting that the CM component responsible for the observed attenuation of PAI-1 protein might be associated with vesicles secreted by the rat bioactive kidney cells.

Figure 4. Secreted vesicles contain microRNAs that are putative repressors of PAI-1



Secreted vesicles from human and rat bioactive kidney cell CM were collected by high-speed centrifugation and assayed for microRNA content using PCR-based arrays of known sequences. miR-449a, a putative regulator of PAI-1 (6), was identified.

HRMC were transiently transfected with miR-449a or not (CTRL). 24 hours post-transfection cells were either exposed to 5 ng/ml TGFβ1 (+) or not (-) for an additional 24 hours. Total protein was prepared and assayed for PAI-1 and βActin by Western blot (A).

miR-449a reduced steady-state PAI-1 protein levels (compare lane 1 to lane 3) and induced levels of PAI-1 protein were also lower in miR-449a transfected cultures (compare lane 2 to lane 4).

Another microRNA, miR-30b-5p, was also identified in the PCR-based array (B) and is a putative regulator of PAI-1 based on predictive algorithms (<http://mirbase.org>). Functional analysis of miR-30b-5p will be performed in future experiments.

Conclusions

In vivo and *in vitro* findings support the working model of how bioactive kidney cells might improve renal function in chronically-diseased kidneys by modulating fibrotic pathways such as the TGFβ1/PAI-1 feedback loop.

- *In vivo* PAI-1 protein levels in glomeruli decrease after treatment of CKD induced by 5/6 nephrectomy with bioactive renal cells
- Excreted vesicles from bioactive renal cell cultures contain components that attenuate PAI-1 induced by the TGFβ1/PAI-1 feedback loop
- Excreted vesicles contain miR-449a and uptake of miR-449a into mesangial cells reduces PAI-1 expression
- Taken together, these data support the hypothesis that one mechanism by which intra-renal delivery of bioactive kidney cells improves renal function might be via cell-cell transfer of components that modulate fibrotic pathways in resident kidney cells

References cited:

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