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Interstitial fibrosis is associated with increased *COL1A2* transcription in AA-injured renal tubular epithelial cells *in vivo*

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ABSTRACT

Accumulation of type I collagen is a key event in renal interstitial fibrosis. As there is no effective treatment, understanding the site where collagen is transcribed and the factors driving it in response to disease *in vivo* is critical for designing future therapies. The present research investigated the transcriptional activity of the *COL1A2* gene in a mouse model of progressive fibrosis induced by aristolochic acid (aristolochic acid nephropathy, AAN). To achieve this we genetically modified mice to express a reporter gene (LacZ) and CCN2 (connective tissue growth factor) under the transcriptional control of the *COL1A2* promoter /enhancer sequences. Using these mice we asked where is collagen actively transcribed and secondly, what is the role of CCN2 in AAN. Here, we report that *de-novo* transcription of the *COL1A2* gene occurred predominantly in damaged tubular epithelial cells during progressive interstitial fibrosis *in vivo*. The activation of *COL1A2* was studied by detection of the reporter gene *LacZ* and *COL1A2* mRNA in interstitial, glomerular, vascular, and tubular epithelial tissue from laser capture microscopy. We also demonstrated that *LacZ*-positive cells co-express E-Cadherin a marker of epithelial origin which is consistent with an epithelial phenotype which is capable of collagen expression during injury. There was no evidence of detachment of these cells from tubules to become myofibroblasts. Moreover, we showed that the transgenic mice show a modest enhancement of CCN2 expression; however fibrosis induced by AA is the same in transgenics and controls suggesting that CCN2, at this level of expression, is not sufficient to enhance fibrogenesis. Overall our study provides a better understanding into the expression patterns and roles of two major extracellular matrix proteins: type I collagen and CCN2.

1. Introduction

Since renal fibrosis is a disease that lacks an effective treatment, it is essential to study the underlying molecular mechanisms in order to inform new therapeutic treatments. Aristolochic acid (AA) is a Chinese herbal extract that has been identified as a kidney toxin. Dietary intake of AA induces progressive interstitial fibrosis which then leads to death in both humans and rodents; this disease is known as AA-nephropathy (AAN). The primary target for the nephrotoxin is the proximal tubular epithelial cell (Lebeau et al., 2005). AA-induced fibrosis is a useful mouse model in which to study the relationship between tubular degeneration and interstitial fibrosis. It is generally accepted that fibroblasts are responsible for excessive extracellular matrix (ECM) deposition leading to interstitial fibrosis (Lewis and Norman, 1998; Eckes et al., 1999; Eddy and Neilson, 2006). When activated by injury they turn into myofibroblasts which are characterised by their abundant production of fibrillar collagens and by the *de novo* synthesis of α-smooth muscle actin (αSMA) (Qi et al., 2006; Hinz et al., 2007). Type I collagen, one of the major fibrillar collagens, is involved in fibrosis but the mechanisms of transcriptional activation of the gene in kidneys remains unclear. Type I collagen is a heterotrimer made up of two COL1A1 and one *COL1A2* molecules. Both of these genes are highly expressed during development; however their expression is switched off in adulthood. Interestingly, type I collagen is re-activated during disease and participates in wound healing. However, uncontrolled collagen expression can lead to sclerosis of several organs, including the kidney. The knowledge of the exact location of *COL1A2* transcription and its key regulators of transcription could be used in order to design therapeutic strategies to prevent fibrosis. We have recently shown that CUX1, a CCAAT displacement transcription factor, binds to *COL1A2* and suppresses its expression (Fragiadaki et al., 2011). In this study we investigated the site of *COL1A2* transcription during fibrogenesis in the kidney. To locate the site of transcription of *COL1A2*, we use transgenic

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approaches combined with laser capture microdissection and PCR as well as immunohistochemical staining. We use heat shock protein 47 (Hsp47), which is a collagen-binding stress protein, as a marker of collagen production since Hsp47 expression always correlates with type I and III collagen expression during normal and pathological conditions (Nagata, 1998).

The profibrotic cytokines transforming growth factor-β (TGF-β) and CCN2 (connective tissue growth factor, CTGF) are likely to be involved in fibroblast activation in renal fibrosis and lead to collagen production (Desmouliere et al., 1993; Yokoi et al., 2001). Increased CCN2 levels were demonstrated in human biopsies of various fibrotic kidney diseases (Ito et al., 1998) and additionally CCN2 activates the tropomyosin receptor kinase complex, TrkA, reducing Smad7, thus enhancing TGF-β signalling (Wahab et al., 2005). Canonical TGFβ signalling via the activation of Smad3, is implicated as a mediator of AA-induced changes in the kidney since Smad3 knockout mice are protected from AAN (Zhou et al., 2010). We therefore hypothesized that fibrosis, and type I collagen transcription, may be increased in AAN in the presence of increased CCN2 levels. To investigate this we studied collagen transcription and fibrosis in CCN2 genetically modified mice. These mice express CCN2 and a LacZ reporter gene under the transcriptional control of the *COL1A2* promoter and the upstream enhancer ($CCN2^{COL1A2}$mice)(Sonnylal et al., 2010). LacZ reporter gene expression provides a means of identifying the site in which the *COL1A2* promoter is activated during the development of AAN.

2. Results

2.1. AA toxin drives potent and progressive kidney fibrosis

Injection of mice with AA toxin induced potent fibrosis as observed by staining for fibrillar collagen with picrosirius red (PSR) which under polarised light shows fibrillar collagen deposition. We report that five injections on five consecutive days with AA toxin induced interstitial fibrosis as early as day 7 in C57BL6 mice. The disease was progressive and reached maximum at 56 days post injection (dpi) (Fig. 1A). Having established that AA toxin was capable of driving fibrosis in these mice we then went on to study αSMA expression. αSMA served as a marker for both activated fibroblasts and vascular smooth muscle cells located in vessels. We detected some αSMA-positive interstitial fibroblasts 28 dpi and we hypothesised that they might be partly responsible for the collagen production at day 28. However at 7 dpi there were no αSMA-positive fibroblasts and very few at 56 dpi (Fig. 1B). These findings raised the question as to whether cells other than αSMA-positive interstitial fibroblasts may contribute to fibrillar collagen synthesis during AAN.

2.2. COL1A2 promoter is active in the injured epithelium

To identify the site of *COL1A2* transcription we investigated *LacZ* staining after AA injections in *COL1A2* promoter/enhancer driven *LacZ* transgenics. The details of the construct used to make the transgenics are shown in Fig. 2A. *LacZ* (which indicates *COL1A2* promoter activity) was strongly expressed in tubular epithelial cells in these mice as evident by immunohistochemical staining for β-galactosidase (Fig. 2B and C for quantification). Interestingly, β-galactosidase staining was not detected in any interstitial cells. This was unexpected since it was anticipated that activation of *COL1A2* would occur predominately in interstitial fibroblasts. This finding raised the question as to whether *COL1A2* is transcribed in epithelial cells of mice with AAN.

2.3. COL1A2 mRNA is expressed by epithelial cells

Having observed that the promoter of *COL1A2* is active in epithelial cells during AAN we then went on to validate this finding in mouse renal epithelial cells during AAN. To achieve this we used TG mice with AAN (56 dpi) and employed laser-capture-microdissection (LCM) to dissect cells followed by real-time PCR (Fig. 3A). The total mRNA was isolated from interstitium, glomerular, epithelial cells and cortical blood vessels from TG mice at 56 dpi. We then studied E-cadherin and *COL1A2* mRNA expression. We report very little expression of these genes in interstitial cells. Since expression of E-cadherin and *COL1A2* was low in interstitial cells we used interstitial expression as the baseline to calculate the fold change of expression in other cells types (Fig. 3B). We then checked for expression of these two genes in cells from the glomerulus and we did

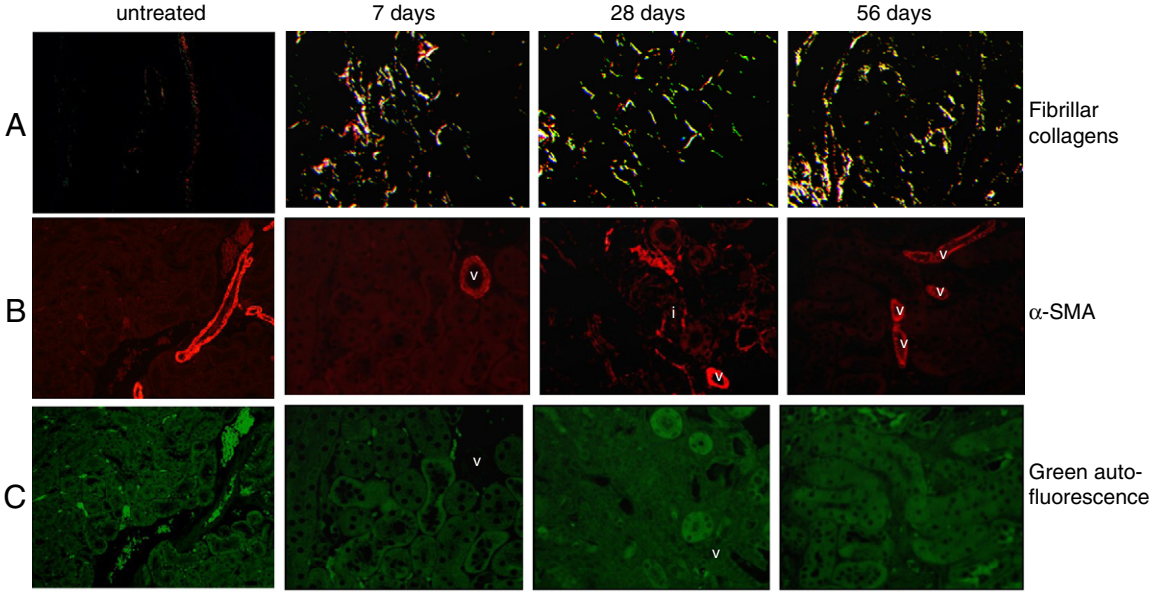

Fig. 1. Increased fibrillar collagen deposition with few interstitial fibroblasts is observed in AAN. AAN was induced in C57BL5 mice. Fibrosis was assessed by measuring fibrillar collagen deposition and α-SMA expression. A: Representative pictures of PSR staining (gold/red) are shown for mice at day 7, 28 and 56 post AA injection. B: Anti-α-SMA antibody was used to stain smooth muscle cells in vessels and also interstitial fibroblasts (middle row- red stain). Staining was performed three times with consistent results and representative pictures are shown. **C:** Green auto-fluorescence is shown to aid identification of histological structures (bottom row). V: blood vessels; i: interstitial areas. Original magnification 20×.