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Tumour necrosis factor-stimulated gene (TSG)-6 controls epithelial–mesenchymal transition of proximal tubular epithelial cells

Girish Bommaya, Soma Meran, Aleksandra Krupa, Aled Owain Phillips [1], Robert Steadman [*,1]

Institute of Nephrology, School of Medicine and Cardiff Institute of Tissue Engineering and Repair, Cardiff University, Heath Park, Cardiff, CF14 4XN, Wales, UK

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ABSTRACT

Progressive renal disease is characterized by accumulation of extracellular matrix in the renal cortex. Proximal tubular cells (PTC) may contribute to disease through a process of epithelial–mesenchymal-transition (EMT): phenotypic change, disruption of the tubular basement membrane and migration into the interstitium. Hyaluronan (HA) synthesis and its extracellular organization by hyaladherins affect cell fate in other systems: this study investigated the role of the hyaladherin, tumour necrosis factor-stimulated gene (TSG)-6, in PTC EMT triggered *in vitro* by transforming growth factor (TGF)β1.

TGFβ1 triggered the loss of PTC epithelial phenotype with 60% decreased expression of E-cadherin and 2–3-fold induction of alpha-smooth muscle actin (α-sma). It also increased the expression of TSG-6, HA-synthase-(HAS)2 and the HA-receptor, CD44, to a peak at 8–12 h, remaining elevated thereafter. Immuno-localization of HA demonstrated that unstimulated PTC assembled HA in cables and that treatment with TGFβ1 initiated cable disassembly with formation of dense HA-pericellular coats. Stable knockdown of TSG-6 with short-hairpin-RNA increased E-cadherin and HAS2 expression, produced loose HA-pericellular coats, HA cables were absent and cell migration was slowed. Treatment of transfectants with TGFβ1 did not induce α-sma, alter E-cadherin, pericellular-HA or migration but did induce HAS2. This was dependent on the expression of CD44 and was inhibited by CD44-specific siRNA.

In summary, TSG-6 was central to EMT through effects on HA macromolecular structure and through CD44-dependent triggering of cell responses. These findings suggest that controlling the assembly of HA by proximal tubular cells may be a novel approach towards intervention in renal disease.

1. Introduction

Fibrosis in most solid organs results from excessive accumulation of extracellular matrix (ECM), leading to disruption of normal tissue architecture and function. Myofibroblasts are the cells that synthesize this expanded ECM and hence determine progression of disease. They have a contractile phenotype, express alpha-smooth muscle actin (α-sma) as a characteristic phenotypic marker and their presence is one of the earliest marker of poor prognosis in a range of fibrotic diseases (Desmouliere and Gabbiani, 1995; Gabbiani, 2003; Hinz and Gabbiani, 2003).

In renal disease, the progression of fibrosis, regardless of aetiology is directly correlated with pathological changes in the cortico-interstitium and the appearance of myofibroblasts (Bohle et al., 1987, 1990; Desmouliere et al., 2003; Essawy et al., 1997; Goumenos et al., 1994; Mackensen-Haen et al., 1981; Roberts et al., 1997). Proximal tubular epithelial cells (PTC) undergoing epithelial to mesenchymal transition (EMT) are a major potential source of myofibroblasts in the cortical interstitium (Iwano et al., 2002; Ng et al., 1998; Okada et al., 2000; Strutz, 1995; Strutz et al., 1996; Strutz and Muller, 2006; Strutz, 2009; Zeisberg et al., 2001). PTC respond to injury by losing polarity, undergoing morphological changes (acquiring actin stress fibers), and migrating along and possibly through the basement membrane. This is accompanied by the down regulation of epithelium-specific genes such as those involved in junctional assembly, for example E-cadherin, and up-regulation of genes specific to mesenchymal cells and myofibroblasts, for example vimentin and α-sma (Cheng and Grande, 2002; Grande et al., 2002; Ng et al., 1998; Yang and Liu, 2001; Zeisberg et al., 2001). The principal growth factor implicated in the phenotypic transition of EMT and in progressive disease *in vivo* is transforming growth factor (TGFβ1) (Cheng and Grande, 2002; Grande et al., 1993, 2002; Tian and Phillips, 2002; Tian et al., 2003). Recent studies from our laboratory have identified an important causal link between both the induction and the maintenance of TGFβ1-dependent phenotypic change and the accumulation of the linear, long-chain polysaccharide hyaluronan (HA) in both PTC and

* Corresponding author at: Institute of Nephrology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, Wales, UK. Tel.: +44 2920748467; fax: +44 29208470.
E-mail address: steadmanr@cf.ac.uk (R. Steadman).
[1] These authors made an equal contribution to this work.

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fibroblasts. For example, HA binding to CD44, its major cell surface receptor, enhances PTC migration (Ito et al., 2004a). The interaction of HA with CD44 also triggers cross-talk between CD44 and the TGFβ1 receptor (Ito et al., 2004b,c).

HA is synthesized as a high molecular weight polymer composed solely of repeating disaccharides of D-glucuronic acid and *N*-acetyl-D-glucosamine, which, unlike other glycosaminoglycans, is not sulfated or attached to a protein core. In vertebrates it is synthesized and extruded by one of three HA synthases (HAS) located in the plasma membrane (Meyer and Kreil, 1996; Spicer et al., 1996; Spicer and McDonald, 1998). While HA is present as a constituent of many ECM, it is not a component of the healthy renal cortex. Its expression is induced, however, in both acute and chronic disease (Lewington et al., 2000; Lewis et al., 2008; Sano et al., 2001; Sibalic et al., 1997; Wells et al., 1990, 1993), with a peri-tubular localization. This increased deposition correlates with both increasing proteinuria and a progressive decline in renal function, although the functional significance of the accumulation is unclear.

Increasing HA synthesis in PTC through overexpression of HAS2 induces a migratory phenotype and the accumulation of an HA pericellular matrix (Selbi et al., 2006a). In contrast, overexpression of HAS3 favours the accumulation of HA in cables between and across cells and has no effect on cell phenotype (Selbi et al., 2006b). HA undergoes macromolecular reorganization following release from the cell and this is controlled by interactions with HA binding proteins (hyaladherins), which assemble it into extracellular and peri-cellular matrices see (Day and Prestwich, 2002) for review. Many cell types *in vitro* surround themselves with HA in an organized peri-cellular matrix or "coat" in which the HA is anchored to the cell-surface by its receptor, CD44 (Banerji et al., 2007; Day and de la Motte, 2005; Evanko et al., 2007; Knudson et al., 1996; Selbi et al., 2006a,b). Studies from this laboratory have investigated the role of the inter-α-inhibitor (I-α-I) family of hyaladherin proteins, together with tumour necrosis factor-stimulated gene (TSG)-6 and the HA-binding proteoglycans bikunin and versican in the macromolecular assembly of HA by PTC (Selbi et al., 2004, 2006a,b). The results demonstrated that the TSG-6-mediated formation of I-α-I heavy chain-HA complexes was critical for the formation of a peri-cellular HA matrix. While recent studies in fibroblasts have linked the expression of TSG-6 and the formation of a pericellular HA matrix to the control of cell phenotype, the importance of the macromolecular organization of this coat and the role of TSG-6 in PTC is not known. The current study aimed to further characterize the role of HA in the initiation of TGFβ1-triggered phenotypic change by defining the role of the expression of TSG-6 and other hyaladherins (I-α-I, bikunin and versican) and the HA pericellular coat in the control of PTC phenotype.

2. Materials and methods

2.1. Reagents

All reagents were from Sigma or Invitrogen unless otherwise stated. Reverse transcription, siRNA transfection reagents and QPCR primers and reagents were purchased from Invitrogen and Applied Biosystems (Cheshire, UK).

2.2. Cell culture

All experiments used HK-2 cells (CRL-2190; American Type Culture Collection, Rockville, MD), which are human PTC, immortalized by transduction with human papilloma virus 16 E6/E7 genes (36). Cells were cultured in DMEM/Ham's F12 (Life Technologies, Paisley, UK) supplemented with 10% FCS (Biologic Industries Ltd., Cumbernauld, UK), L-glutamine, insulin, transferrin, sodium selenite, hydrocortisone, and HEPES (Sigma–Aldrich, Poole, UK). For stimulation with TGFβ1 cells were growth arrested in serum-free medium for 48 h before addition of the cytokine in serum-free medium at the concentrations and for the times shown.

2.3. Cell migration

Cell migration was examined as previously described (Ito et al., 2004a) Briefly, a denuded area was generated on cell monolayers of HK-2 cells by scratching with a sterile pipette tip. The monolayer was washed twice with phosphate-buffered saline (PBS) (Gibco BRL) and then incubated with serum-free medium alone or containing TGFβ1. To quantify re-epithelialization, closure of the denuded area was monitored for times up to 72 h, using an Axiovert 100 M inverted microscope fitted with a digital camera (ORCA-1394, Hamamatsu Photonics, K.K. Hamamatsu, Japan), and images of the denuded area were captured as a digitalized sequence. The rate of motility of cells was calculated as the decrease in pixel number in the denuded area each hour and the mean decrease per hour was calculated for the time when the decrease in gradient was linear (between 6 and 24 h).

Reverse transcription (*RT*) *and quantitative PCR* (*QPCR*) – RT and QPCR were used to quantify the expression of mRNA. Cells were grown to confluence and following the appropriate treatment were washed with phosphate-buffered saline pH 7.3 (PBS) before lysis with tri-reagent and RNA purification according to the manufacturer's protocol (Sigma–Aldrich). Reverse transcription was performed using random primer High Capacity cDNA Reverse Transcription, according to the manufacturer's protocol (Applied Biosystems). A negative RT control was included. QPCR was performed using Applied Biosystems 7900HT fast real time PCR with TaqMan primers and probes (α-sma-Hs_00426835, TSG-6-Hs_00200180, HAS2-Hs_00193435, E-CADHERIN-Hs_01023894 &CD44-Hs_00153304, ribosomal RNA (rRNA)-4310875. Amplification was carried out using a cycle of 95 °C for 1 s and 60 °C for 20 s for 40 cycles. A negative PCR control was included. Ribosomal RNA amplification, using primers and probe from Applied Biosystems, was performed in parallel. The comparative *CT* method was used for relative quantification of each amplified product and this was then corrected for the appropriate value of ribosomal RNA. The corrected expression of the target gene in experimental samples relative to expression in control samples was then calculated using the equation:

$$2^{-(\Delta CT(1)-\Delta CT(2))}$$

where $\Delta CT(1)$ is the mean ΔCT calculated for the experimental samples and $\Delta CT(2)$ is the mean ΔCT calculated for the control samples.

Stable transfection with short hairpin (*sh*)*RNA*. Stable transfection of siRNA against TSG-6 was carried out using U6 siSTRIKE vector (Promega), according to the manufacturer's protocol. This system was designed for expression of short-hairpin RNA (shRNA) in mammalian cells under a U6 promoter and contains a Puromycin resistance gene to allow for stable transfection. Separate A and B oligonucleotides were designed, and then annealed to form the hairpin structure before ligation into the U6 siStrike Puromycin Vector, using the manufacturer's protocol. The complete sequences of the A and B oligonucleotides were:

(A) ACCGCACGGTCTGGCAAATACAAAGTTCTCTTGTATTTGCCAGACCGTGCTTTTTC

(B) TGCAGAAAAAGCACGGTCTGGCAAATACAAGAGAACTTTGTATTTGCCAGACCGTG