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Hyaluronan Attenuates Transforming Growth Factor-β1-Mediated Signaling in Renal Proximal Tubular Epithelial Cells

Takafumi Ito, John D. Williams, Donald Fraser, and Aled O. Phillips

From the Institute of Nephrology, University of Wales College of Medicine, Cardiff, Wales

Increased expression of hyaluronan (HA) has been associated with both acute renal injury and progressive renal disease, although the functional significance of this remains unclear. There is overwhelming evidence that transforming growth factor (TGF)-β1 is critical to the development of progressive renal disease. Recent studies suggest an interaction between HA and TGF-β signaling in cancer cell biology. The aim of this study was to examine the potential role of HA as a modulator of TGF-β1 function in renal proximal tubular epithelial cells (PTC). Under resting conditions, co-localization of the principal receptor for HA, CD44, and both the TGF-β type I and type II receptors was demonstrated by immunoprecipitation and western analysis and further confirmed by immunocytochemistry and confocal microscopy. Stimulation of PTC with TGF-β1 led to increased synthesis of both type III and type IV collagen assessed by Western analysis. Addition of HA did not alter collagen synthesis, but abrogated TGF-β1-mediated increase in type III and type IV collagen. This effect was blocked by the addition of a blocking antibody to CD44 and also by inhibition of MAP kinase kinase (MEK) activity. Furthermore HA decreased TGF-β1 activation of a luciferase-SMAD responsive construct, and decreased translocation of SMAD4 into the cell nucleus. We have previously demonstrated an anti-migratory effect of TGF-β1 in a scratch wounding model. As with HA antagonism of TGF-β1 extracellular matrix generation, HA reduced the anti-migratory effect of TGF-β1 in a CD44-dependent manner. In contrast to the effect of TGF-β1 on collagen synthesis, which is SMAD-dependent, the anti-migratory effect of TGF-β1 in this model is known to be dependent of activation of RhoA. In the presence of HA, TGF-β1-mediated activation of RhoA was also abrogated in a CD44-dependent manner. The results suggest that co-localization of CD44 and TGF-β receptors facilitate modulation of both SMAD and non-SMAD-dependent TGF-β1-mediated events by HA. Our results therefore suggest that alteration of HA synthesis may represent an endogenous mechanism to limit renal injury. *(Am J Pathol 2004, 164:1979–1988)*

Progression of renal disease is known to correlate with the degree of renal interstitial fibrosis, and much interest has focused on the role of the renal proximal tubular epithelial cell (PTC) in its pathogenesis. PTC may contribute to the pathogenesis of renal fibrosis directly by alterations in the production of components of extracellular matrix (ECM), and indirectly by the production of pro-fibrotic cytokines.[1–5]

Transforming growth factor-β1 (TGF-β1), which is the prototypic member of the TGF-β superfamily, exerts a broad range of biological activities. It plays pivotal roles during embryonic development where it is involved in induction of cell differentiation and organogenesis. TGF-β1 has been implicated in the pathogenesis of renal fibrosis in both experimental and human disease.[6–10] A major function of TGF-β1 is to regulate the expression of genes, the products of which contribute to the formation and degradation of ECM.[11–15] Generally, TGF-β1 leads to the accumulation of ECM by decreasing the synthesis of proteases and by increasing the levels of protease inhibitors.[16] It also increases the expression of integrins through which ECM proteins such as fibronectin and collagen interact with cells.[17,18] *In vitro* studies also suggest that TGF-β1 induces phenotypic alterations in PTC using intermediate filament markers and reorganization of the cytoskeleton with cells as indicators of a "fibroblastic" phenotype.[19] Studies using normal rat PTC also suggest that TGF-β1 is a key mediator regulating differentiation of PTC into α-SMA positive cells.[20] Not only is there strong evidence that TGF-β1 is a key mediator of progressive renal fibrosis, but attenuation of its action has been postulated to be a target for therapeutic intervention in numerous disease models.[7,8,21,22] Understanding the mechanisms, which regulate TGF-β1-dependent responses, is therefore an important goal.

Hyaluronan (HA) is an ubiquitous connective tissue polysaccharide which *in vivo* is present as a high molec-

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Address reprint requests to Dr. A.O. Phillips, Institute of Nephrology, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN. E-mail: phillipsao@cf.ac.uk.

ular mass component of ECM. In the normal kidney HA is expressed in the interstitium of the renal papilla only, and alteration in papillary interstitial HA has been implicated in regulating renal water handling by affecting physiochemical characteristics of the papillary interstitial matrix and influencing the interstitial hydrostatic pressure.[23] Although HA is not a major constituent of the normal renal corticointerstitium, it is known to be expressed around PTC following renal injury caused by diverse diseases.[24–27] Increased deposition of interstitial HA has also been correlated with renal function in progressive renal disease associated with IgA nephropathy.[28] A recent study suggest that HA promotes the signaling interaction between the principal cell surface receptor for HA, CD44, and the TGF-β type I receptor in metastatic breast tumor cells. The functional significance of alterations in the expression of HA associated with renal injury, however, is not clear. The aim of the current study was to define the relationship between CD44 and TGF-β type I receptor and the potential modulating influence of HA on TGF-β1-dependent signaling and function in PTC.

Materials and Methods

Materials

Antibodies for Western blot analysis, immunoprecipitation, and immunocytochemistry and the final working dilution were as follows.

For Western blot analysis and immunoprecipitation, rat polyclonal anti-CD44 antibody (dilution 1:1000) was from Calbiochem (Nottingham, UK); rabbit polyclonal anti-TGFβ type I receptor antibody (dilution 1:500) was from Santa Cruz Biotechnology (Wiltshire, UK); rabbit polyclonal anti-TGFβ type II receptor antibody (dilution 1:500) was from Santa Cruz Biotechnology; goat polyclonal anti-type III collagen antibody (dilution 1:250) was from Chemicon International (Temecula, CA); rabbit polyclonal anti-type IV collagen antibody (dilution 1:500) was from ICN Pharmaceuticals (Basingstoke, UK); Rhotekin Rho-binding domain (RBD; recombinant protein expressed in *Escherichia coli*) was from Upstate Biotechnology (Buckinghamshire, UK); mouse monoclonal anti-RhoA antibody (dilution 1:400) was from Santa Cruz Biotechnology. All HRP conjugated secondary antibodies (1:10,000) were from Sigma (Poole, UK).

For immunocytochemistry, rat polyclonal anti-CD44 antibody (dilution 1:100) was from Calbiochem; rabbit polyclonal anti-TGFβ type I receptor antibody (dilution 1:100) was from Santa Cruz Biotechnology; rabbit polyclonal anti-Smad4 antibody (dilution 1:100) was from Santa Cruz Biotechnology. All FITC and TRITC conjugated secondary antibodies (dilution 1:100) were from Sigma.

Other reagents used were: recombinant TGF-β from R&D Systems (Oxford, UK); mouse monoclonal anti-CD44 blocking antibody from Ancell (Bayport, MN); MAP kinase kinase (MEK) inhibitor PD98059 from Calbiochem. Hyaluronan (Lot No. F1750762) in the form of freeze-dried white powder was kindly provided by Denki Kagaku Kogyo K. K., Japan.

Cell Culture

HK-2 cells (human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus (HPV) 16 E6/E7 genes[29]) were cultured in DMEM/Ham's F12 (Gibco BRL, Paisley, UK) supplemented with 10% FCS (Biological Industries Ltd, Cumbernauld, UK), 2 μmol/L L-glutamine (Gibco BRL), 20 mmol/L HEPES buffer (Gibco BRL), 5 μg/ml insulin, 5 μg/ml transferring (Sigma), 40 ng/ml hydrocortisone (Sigma), and 5 ng/ml sodium selenite (Sigma). Cells were grown at 37°C in 5% CO_2 and 95% air. Fresh growth medium was added to cells every 3 to 4 days until confluent. Cells were grown to confluence and serum deprived for 48 hours before experimental manipulation. In all experiments cells were stimulated with either recombinant TGF-β1 and/or HA, under serum-free conditions.

In all aspects of cell biology that we have studied previously, HK-2 cells respond in an identical fashion to primary cultures of human proximal tubular cells.[30–33] They are therefore a good model from which general conclusions can be drawn in terms of proximal tubular cell biology.

Immunoblotting/Western Analysis

Briefly confluent monolayers were washed once with cold PBS (Gibco BRL), scraped, and rinsed into 5 ml of cold PBS. After centrifugation at 2500 rpm for 10 minutes, cell pellets were extracted in buffer (150 mmol/L NaCl, 50 mmol/L Tris-Cl, 0.01% NaN_3, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/ml leupeptin, 25 μg/ml aprotinin) containing 1% Triton X-100 for 30 minutes on ice. Samples were centrifuged at 12,500 rpm for 30 minutes and then the supernatant (Triton-soluble components including membrane and cytosolic fraction) was transferred to a separate tube and kept at −70°C until use.

Analysis of type III and IV collagen in the culture supernatant was performed by standard methodologies. Briefly, equal amounts of culture supernatant were prepared in SDS sample buffer (2% SDS, 10% v/v glycerol, 60 mmol/L Tris, and 0.05% v/v mercaptoethanol) and boiled for 5 minutes before loading onto 10% SDS-PAGE gels. Electrophoresis was carried out under reducing conditions according to the procedure of Laemmli.[34] After electrophoresis the separated proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia, Biotech UK Ltd, Buckinghamshire, UK). The membrane was blocked with Tris-buffered saline containing 5% nonfat powdered milk for 1 hour and then incubated with the primary antibody in Tris-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 (Tris-buffered saline-Tween) overnight at 4°C. The blots were subsequently washed in Tris-buffered saline-Tween and then incubated with an appropriate HRP-conjugated secondary antibody (Sigma) in Tris-buffered saline-Tween. Proteins were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Immunoprecipitation

Immunoprecipitation was performed by standard methodologies. Briefly, cell protein samples (200 μg) were pre-cleared with 25 μl of packed protein A cross-linked 4% beaded agarose (Sigma) at 4°C overnight. The beads were removed by centrifugation (13,000 rpm, 10 minutes) and the supernatant collected. Primary antibody (2 μg/ml) was added to the cleared supernatant and incubated at 4°C with constant mixing for 4 hours. The immune complex was captured by the addition of packed agarose protein A beads (50 μl) overnight at 4°C. Beads were washed with RIPA buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 10 mmol/L $MgCl_2$, 0.1% SDS, 1% Triton X-100), 30 μl of sample buffer was then added before boiling for 5 minutes. Separation of the beads was achieved by centrifugation (13,000 × *g* for 10 minutes) and the supernatant removed. Subsequently samples were subject to immunoblot/Western analysis as described above. Specificity of immunoprecipitation was confirmed by negative control reactions performed with either no primary antibody, or IgG control.

Assessment of RhoA Activation

Briefly, following addition of TGF-β1 (10 ng/ml), HA (25 μg/ml), or the combination for up to 2 hours, cell monolayer was rinsed twice with ice-cold Tris-buffered saline (TBS). Cell lysate was obtained by adding Mg^{2+} buffer (25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA and 10% glycerol) onto cell monolayer and scraping with cell scraper, followed by centrifugation at 14,000 × *g* for 5 minutes at 4°C. Supernatant was transferred to a microfuge tube and further incubated with 30 μg of rhotekin RBD-agarose slurry for 45 minutes at 4°C. Agarose beads were then pelleted by brief centrifugation, washed with Mg^{2+} buffer three times, and subjected to western blot analysis as described above. Activated GTP-RhoA was detected by immunoblotting with specific RhoA antibody.

Immunocytochemistry

Cells were grown in eight-well multichamber slides (Gibco BRL) under serum-free media for 48 hours and then stimulated with either recombinant TGF-β1 (10 ng/ml), HA (25 μg/ml) or the combination of both stimuli. At each time point, cells were rinsed three times in PBS for 5 minutes each, before fixation in 3% paraformaldehyde for 15 minutes at room temperature and subsequent permeabilized with 0.1% Triton in PBS for 5 minutes at room temperature. Following a blocking step (1% BSA/PBS for 1 hour), cells were incubated with the primary antibodies overnight at 4°C, followed by the incubation of FITC-conjugated and/or TRITC-conjugated secondary antibodies. After washing with PBS, cells were mounted with fluorSave reagent (Calbiochem) and analyzed by confocal microscope (Leica TCS 40, Leica Microsystems, Cambridgeshire, UK).

Transient Transfection

The SMAD-responsive promoter $(SBE)_4$-Lux was a gift from Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden).[35] For transfection of the reporter construct, 80×10^3 cells/well were seeded onto a 12-well plate (this density of cells produced a 80% confluence monolayer the following day). The next day cells were transfected with 0.5 μg of the SMAD responsive promoter-luciferase construct, using the mixed lipofection reagent FuGene 6 (Roche, Lewes, UK) at a ratio of 1.5 μl of FuGene to 0.5 μg of DNA in serum-free and insulin-free medium. Transfection efficiency was monitored by co-transfection with a β-galactosidase reporter plasmid. 24 hours after transfection, cells were stimulated with either TGF-β1, or the combination of TGF-β1 and HA. Following lysis of the cells in Reporter Lysis Buffer (Promega, WI) luciferase content was quantified by glow-type luminescence assay (Bright-Glo, Promega), and β-galactosidase activity determined by commercial assay (Promega). Luciferase activity was normalized to β-galactosidase activity.

Migration

Cell migration was examined using a monolayer wounding system as previously described.[36] Briefly quiescent cell monolayers were injured by scraping with a sterile 1-ml pipette tip to generate an intersecting area of denuded cells. The monolayer was washed twice with PBS and then incubated with serum-free medium or serum-free medium to which either TGF-β1 or the combination of both TGF-β1 and HA were added. Closure of the denuded area was monitored using an Axiovert 100 mol/L inverted microscope fitted with a digital camera (ORCA-1394, Hamamatsu Photonics, Hamamatsu, Japan), and images of the wounded area captured as a digitized sequence. The rate of motility of cells was calculated as the number of cells entering the central denuded area. Cell number was expressed as cells per mm^2 of original denuded area.

Results

Characterization of the Association of CD44 and TGF-β Receptor Expression

The relationship of CD44 to TGF-β type I receptors was examined by immunoprecipitation of CD44 followed by analysis of TGF-β type I receptor by immunoblot and also by immunoprecipitation of the TGF-β type I receptor before immunoblot analysis of CD44. We have previously demonstrated the expression of numerous splice variants of CD44,[37] for these experiments we therefore used a polyclonal antibody directed against the common region of CD44.

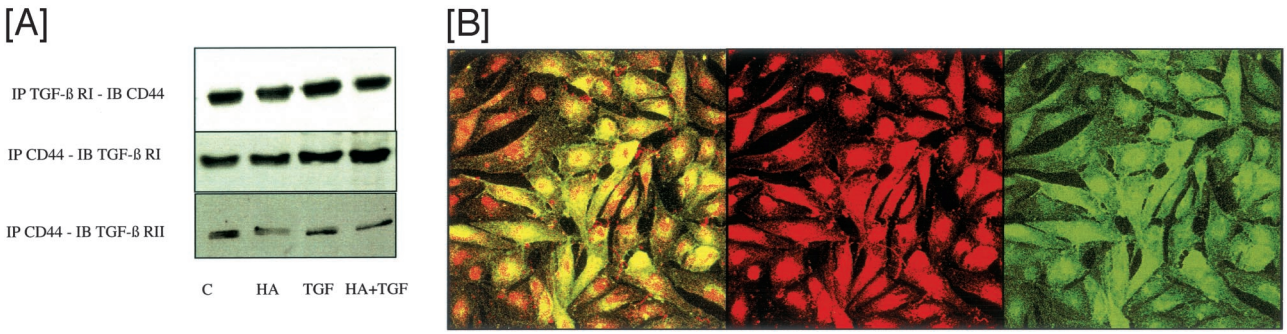

Figure 1. Co-localization of CD44 and TGF-β receptors. **A:** Western blot analysis of the association of CD44 and TGF-β receptors. Cell protein was extracted from confluent monolayers of serum deprived HK-2 cells which had been exposed to HA (molecular weight 2×10^6, final concentration 25 μg/ml, recombinant TGF-β1 (10 ng/ml) or the combination of both stimuli, all under serum-free conditions for 48 hours. Subsequently, either the TGF-β1 type I receptor (IP TGF-β RI) or CD44 (IP CD44) were immunoprecipitated and the samples subjected to immunoblot analysis with antibodies to CD44 (IB CD44), TGF-β type I receptor (IB TGF-β RI), or TGF-β type II receptor (IB TGF-β RII). In control experiments cells were exposed to serum-free medium alone for 48 hours. **B:** Immunocytochemical localization of CD44 and TGF-β type I receptor. Confluent monolayers of HK-2 cells were serum deprived for 48 hours before fixation with 3% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton in PBS for 5 minutes at room temperature. The expression of the CD44 (green) and the TGF-β type I receptor (red) were examined by immunocytochemistry and analyzed by confocal microscopy as detailed in Materials and Methods and their association examined by merging of individual images.

Immunoprecipitation of the TGF-β type I receptor resulted in isolation of both CD44 and the TGF-β type I receptor (Figure 1A). Similarly, immunoprecipitation of CD44 resulted in isolation of both CD44 and the TGF-β type I receptor in unstimulated cells (Figure 1A). Addition of either TGF-β1 or HA either alone or in combination to confluent monolayers of HK-2 cells under serum-free conditions did not influence the association of the two receptors. Furthermore, immunoprecipitation of CD44 followed by immunoblot analysis for the TGF-β type II receptor suggested that these receptor proteins were also associated.

The association between CD44 and the TGF-β type I receptor was further examined by immunocytochemistry and confocal microscopy. Double immunocytochemistry using a FITC conjugated antibody to detect CD44 and a TRITC conjugated antibody to detect the TGF-β type I receptor confirmed co-localized of the two proteins as generation of yellow staining in merged images (Figure 1B).

Modulation of TGF-β1-Dependent Matrix Generation by HA

Alteration in matrix generation was examined by determination of both type III collagen and type IV collagen in cell culture supernatants by Western analysis. Stimulation of confluence monolayers of HK-2 cells led to increased levels of both type III (Figure 2) and type IV collagen (Figure 3). In contrast incubation with HA did not influence the levels of either type III or type IV collagen. Incubation of HK-2 cells with TGF-β1 in the presence of HA led to a decrease in the amount of type III and type IV collagen detected in the cell culture supernatant compared to supernatant collected from cells stimulated with TGF-β1 alone. This decrease in TGF-β1 responsiveness in the presence of HA was prevented by adding TGF-β1 and HA in the presence of a blocking antibody to CD44 (Figures 2A and 3A).

Previous studies have demonstrated that CD44-mediated alteration in cell function may be associated with the MAP kinase signaling cascade,[38] and we have previously demonstrated activation of this cascade following addition of exogenous HA to HK-2 cells.[39] We therefore examined the role of MAP kinase signaling in HA-medi-

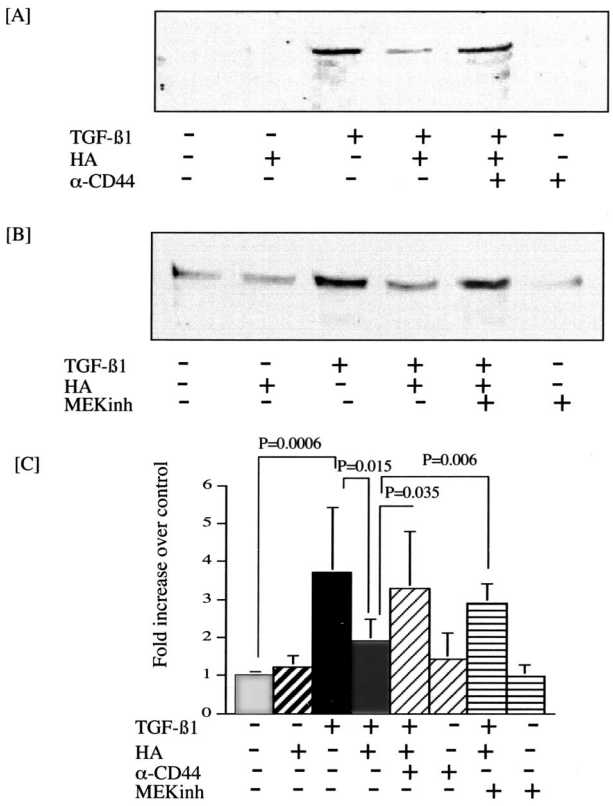

Figure 2. HA prevents TGF-β1-mediated increase in type III collagen. Confluent monolayers of serum deprived HK-2 cells were stimulated with TGF-β1 and HA as indicated in the presence or absence of either blocking antibody to CD44 (final concentration 5 μg/ml) (**A**), or the MEK inhibitor PD98059 (10 μmol/L) (**B**). Supernatant samples were collected 48 hours following addition of stimuli, and equal volumes of supernatant subjected to Western blot analysis for type III collagen. **C:** Following scanning densitometry, alteration in type III collagen was expressed as fold increase in densitometric ratios of collagen expression over control. The data represent the mean ± SD of six separate experiments.

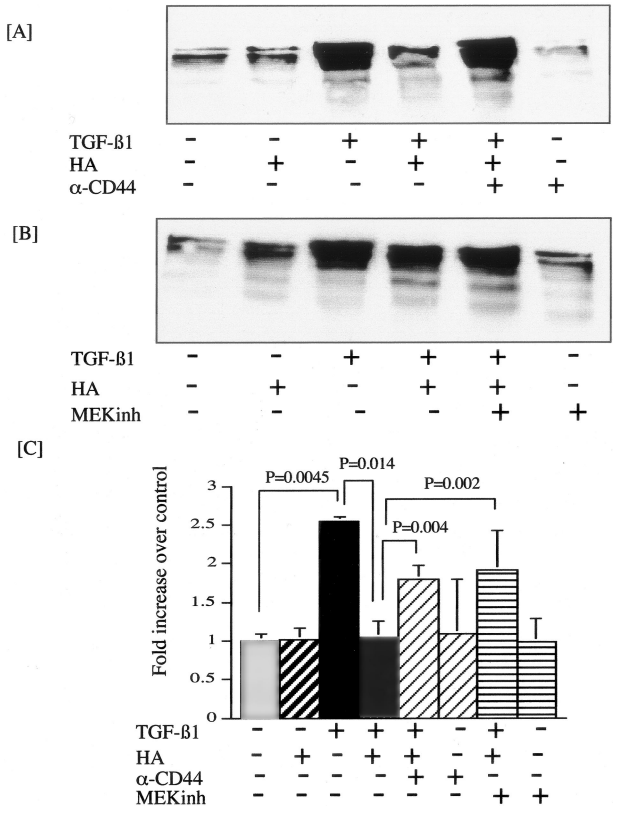

Figure 3. HA prevents TGF-β1-mediated increase in type IV collagen. Confluent monolayers of serum-deprived HK-2 cells were stimulated with TGF-β1 and HA as indicated in the presence or absence of either blocking antibody to CD44 (final concentration 5 μg/ml) (**A**) or MEK inhibitor PD98059 (10 μmol/L) (**B**). Supernatant samples were collected 48 hours following addition of stimuli and equal volumes of supernatant subjected to Western blot analysis for type IV collagen. **C:** Following scanning densitometry, alteration in type IV collagen was expressed as fold increase in densitometric ratios of collagen expression over control. The data represent the mean ± SD of five separate experiments.

ated abrogation of TGF-β1 stimulation of collagen synthesis by the addition of the MEK inhibitor PD98059 (Calbiochem). Stimulation with both TGF-β1 and HA in the presence of the MEK inhibitor prevented antagonism of the effect of TGF-β1 on both type III and type IV collagen generation (Figures 2B and 3B). Densitometric analysis of six individual experiments confirmed statistically significant stimulation of type III collagen following TGF-β1 stimulation, which was abrogated by addition of HA (Figure 2C). Furthermore, densitometry confirmed statistically significant prevention of HA-mediated abrogation of TGF-β1 stimulated collagen synthesis by either blocking antibody to CD44 or inhibition of MEK by PD98059. Similarly densitometric analysis of five individual experiments confirmed an identical pattern on the synthesis of type IV collagen following TGF-β1 stimulation and stimulation by TGF-β1 in the presence of HA (Figure 3C).

Decreased SMAD-Dependent Signaling

TGF-β1-dependent alterations in collagen generation have previously been demonstrated to be dependent on nuclear translocation of Smad3/Smad4 complexes.[40–42] Confirmation of activation of the SMAD signaling pathway was sought using the $(SBE)_4$-Lux reporter which contains 4 repeats of the CAGACA sequence identified as a SMAD binding element. Addition of TGF-β1 led to a 14-fold increase in luciferase activity of the reporter construct (Figure 4A). Addition of HA did not increase the signal above control values, but addition of HA in the presence of TGF-β1 led to a significant and dose-dependent decrease in luciferase activity (Figure 4A). At a dose of 25 μg/ml of HA, this represented a 32% reduction in luciferase activity compared to that generated by TGF-β1 alone (TGF-β, 14.05 ± 2.0 versus TGF-β+HA-9.6 ± 0.814, mean fold increase in luciferase activity over control, mean ± SD, $n = 9$, $P = 0.0001$). Interestingly this effect of HA was only seen with HA of a high molecular weight (2×10^6) while HA of much lower molecular weight (65,000) did not antagonize the effect of TGF-β1.

As with the effect of HA on TGF-β1-mediated alteration in collagen synthesis, addition of a blocking antibody to CD44 prevented antagonism of TGF-β1-mediated increase in luciferase activity of the SMAD responsive reporter construct (TGF-β+HA 9.6 ± 0.814, versus TGF-β+HA+CD44 antibody 13.26 ± 1.78, mean fold increase in luciferase activity over control, mean ± SD, $n = 4$, $P = 0.0001$). The luciferase activity following addition of TGF-β1, HA and blocking antibody was statistically no different to addition of TGF-β1 alone (Figure 4A).

In a separate series of experiments abrogation of TGF-β1-dependent activation of the SMAD reporter construct was also prevented by addition of the MEK inhibitor PD98059 in a dose-dependent fashion (Figure 4B). This effect was significant at all doses of PD98059 used, such that luciferase activity in the presence of TGF-β1 and HA together with PD98059 was statistically no different to activity following addition of TGF-β1 alone (TGF-β, 14.05 ± 12.0; MEKi 1 μmol/L 9.6 ± 1.9; MEKi 25 μmol/L 12.5 ± 1.52; MEKi 50 μmol/L 14.02 ± 2.23, mean fold increase in luciferase activity over control, mean ± SD, $n = 4$, $P < 0.029$).

Modulation of SMAD-dependent signaling was also examined by immunocytochemistry and confocal imaging of SMAD4. Addition of TGF-β1 led to the expected nuclear translocation of Smad4. Addition of HA together with TGF-β1 led to a decrease in the intensity of nuclear Smad4 translocation (Figure 5). As with attenuation of luciferase activity of the SMAD responsive reporter construct, stimulation of cells with TGF-β1 in the presence of HA did not completely abolish SMAD4 translocation.

Modulation of TGF-β1 Anti-Migratory Effect in the Scratch Wound System

We have previously demonstrated in a scratch wound model of cell migration that TGF-β1 has profound anti-migratory effects related to alteration in focal adhesion assembly and RhoA activation.[36] To determine whether the effects of HA were specifically related to TGF-β1-dependent SMAD signaling, or to a more general inhibition of TGF-β1-mediated signaling, we sought to examine the effect of HA on the anti-migratory effect of TGF-β1 in this experimental system.

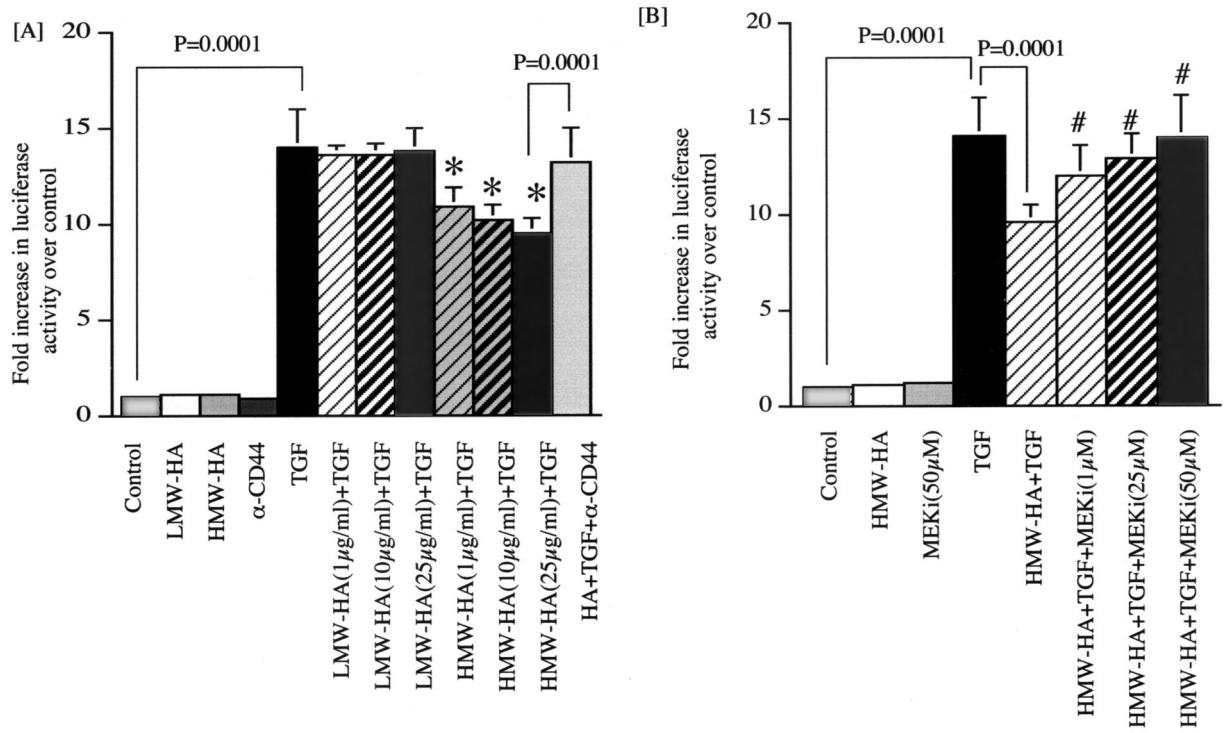

Figure 4. Antagonism of TGF-β1-mediated increase SMAD-dependent signaling. **A:** HK-2 cells were transfected with a SMAD-responsive promoter $(SBE)_4$-Lux, before stimulation with TGF-β1 (1 ng/ml) or either low molecular weight HA (LMW-HA molecular weight 65,000) or high molecular weight HA (molecular weight 2×10^6) at a concentration of 25 μg/ml, all under serum-free conditions. Cells were also stimulated with the combination of TGF-β1 (1 ng/ml) and increasing doses of high molecular HA as indicated. At the highest dose of high molecular weight HA cells were stimulated with TGF-β1 and a blocking antibody to CD44 (final concentration 5 μg/ml). **B:** In a separate series of experiments serum deprived monolayers of HK-2 cells were stimulated with TGF-β1 (1 ng/ml) and high molecular weight HA (25 μg/ml) either alone, in combination, or in combination together with increasing doses of the MEK inhibitor PD98059 as indicated. All stimuli were applied for 24 hours. Data represent mean ± SD, $n = 9$, $^*P < 0.05$ compared to TGF-β1 alone, $^{\#}P < 0.005$ compared to TGF-β1 and HA in the absence of PD98059.

As we have previously reported,[36] addition of TGF-β1 led to a marked inhibition of cell migration as compared to control experiments. Quantification of the number of cells entering the denuded area confirmed the anti-migratory effect of TGF-β1. Significant inhibition of cell migration being evident at all time points beyond 24 hours following mechanical injury of the monolayer and addition of 10 ng/ml of TGF-β1 (Figure 6A) (24 hours, control 39.4 ± 2.9 versus TGF-β1 13.5 ± 7.0 $P = 0.002$; 72 hours, control 146.0 ± 28.3 versus TGF-β1 63.5 ± 14.7 $P = 0.005$; 120 hours, control 322.5 ± 52.7 versus TGF-β1 140.3 ± 22.6 $P = 0.026$; mean cell number mm^{-2} ± SD, $n = 3$).

At all time points addition of TGF-β1 in the presence of HA significantly attenuated the anti-migratory effect of TGF-β1 (24 hours, TGF-β1 13.5 ± 7.0 versus TGF-β+HA 23.7 ± 1.6 $P = 0.03$; 72 hours, TGF-β1 63.5 ± 14.7 versus TGF-β1 + HA 101.2 ± 15.8 $P = 0.019$; 120 hours, TGF-β1 140.3 ± 22.6 versus TGF-β1 + HA 193.2 ± 10.8 $P = 0.010$; mean cell number mm^{-2} ± SD, $n = 3$). This attenuation of the anti-migratory effect of TGF-β1 by HA was overcome by addition of a blocking antibody to CD44, such that at all time points studied there was no statistical difference between cell migration following addition of TGF-β1 alone as compared with TGF-β1 together with HA and blocking antibody to CD44 (Figure 6A).

In a separate series of experiments abrogation of TGF-β1-dependent cell migration by HA was also prevented by addition of the MEK inhibitor PD98059 (Figure 6B). At all time points studied there was no statistical difference between cell migration following addition of TGF-β1 alone as compared with TGF-β1 together with HA and PD98059.

We have previously demonstrated that the anti-migratory effect of TGF-β1 in the scratch wound system is related to activation of RhoA.[36] Increased expression of activated RhoA was demonstrated following GTP-Rho immunoprecipitation followed by RhoA immunoblot analysis (Figure 7). Addition of HA alone did not affect RhoA activation. Co-incubation of cells with TGF-β1 and HA inhibited TGF-β1-mediated activation of RhoA (Figure 7A). Inhibition of RhoA activation in the presence of both TGF-β1 and HA was prevented by the presence of the blocking antibody to CD44 (Figure 7B) and also by inhibition of MEK with the inhibitor PD98059 (Figure 7C). Densitometric analysis of three separate experiments confirmed statistically significant inhibition of TGF-β1-mediated activation of RhoA by HA, and that this effect could be prevented by either blocking antibody to CD44, or inhibition of MEK by PD98059 (Figure 7D).

Discussion

The importance of pathological changes in the renal cortical interstitium is now well recognized, suggesting that interstitial fibrosis represents a crucial development leading to progressive renal dysfunction.[43–48] With increasing awareness of the importance of these pathological interstitial changes, interest has focused on the role of cells,

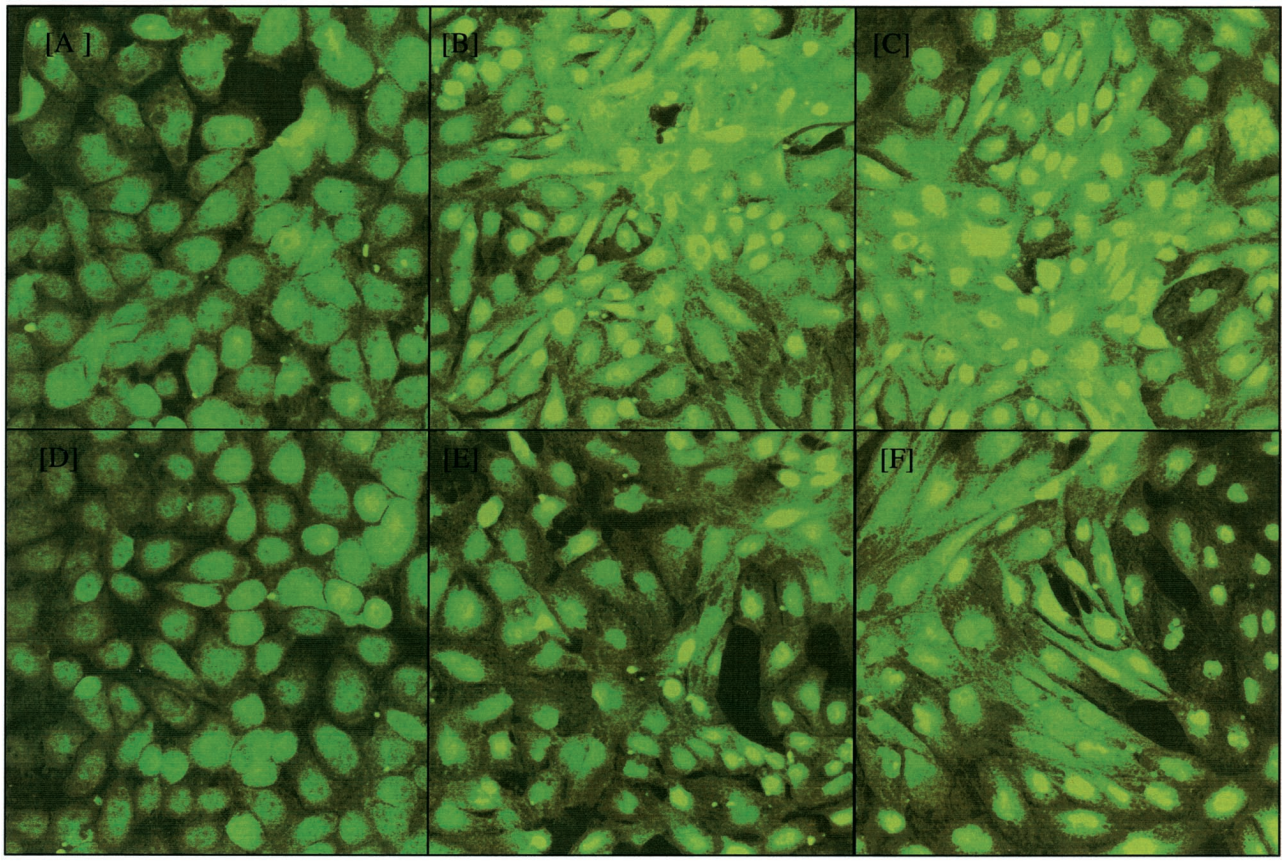

Figure 5. Nuclear translocation of SMAD 4 is attenuated by high molecular weight HA. Confluent monolayers of serum deprived HK-2 cells were stimulated with increasing doses of recombinant TGF-β1 (0, **A** and **D**; 1 ng/ml, **B** and **E**; 10 ng/ml, **C** and **F**) either alone (**A–C**) or in the presence of 25 μg/ml HMW-HA (**E** and **F**). In separate experiments cells were stimulated with 25 μg/ml of HMW-HA alone (**D**)**.** Cells were fixed by addition of 3% paraformaldehyde for 15 minutes at room temperature and permeabilized with 0.1% Triton in PBS for 5 minutes at room temperature 48 hours following application of the stimuli. SMAD 4 localization was examined by immunocytochemistry and analyzed by confocal microscopy.

such as PTC, in the initiation of a fibrotic response. We have demonstrated that PTC may contribute to the pathogenesis of renal fibrosis directly by alterations in the production of components of ECM, and indirectly by the production of pro-fibrotic cytokines such as TGF-β1.[1–5] The evidence implicating TGF-β1 in the pathogenesis of renal fibrosis is now overwhelming. In contrast although increased expression of HA has been documented in both acute injury[24] and progressive fibrosis[28] its role in the pathogenesis of these disease processes is not clear.

Our results clearly demonstrated that the association of CD44 and TGF-β1 receptors facilitated attenuation of PTC response to TGF-β1. More specifically we have demonstrated both a decrease in synthesis of collagen in response to TGF-β1 and also decreased nuclear translocation of SMAD 4 when cells were stimulated with TGF-β1 in the presence of HA. HA-dependent inhibition of TGF-β1 function was blocked by both blocking antibody to CD44 and inhibition of the MAP-kinase signaling pathway. TGF-β-mediated alterations in extracellular matrix synthesis are regulated by the SMAD family of signaling intermediates.[49] As central mediators of TGF-β family signals, SMADs are subject to many different regulatory mechanisms. One mode of regulation is phosphorylation of MAP-kinase sites in the linker region.[50] Nuclear accumulation of SMAD1 are antagonized by activation of the Erk

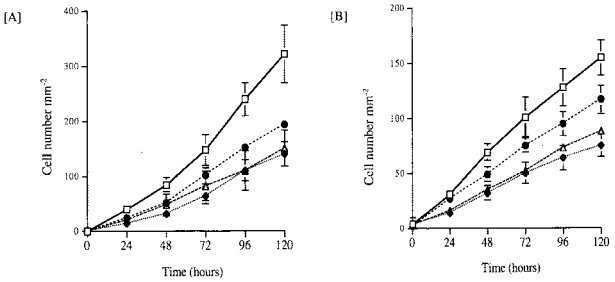

Figure 6. Quantification of inhibition of wound closure following addition of TGF-β1. **A:** Confluent monolayers of HK-2 cells were "scratched" as described in Materials and Methods. Subsequently the rate of cell migration following addition of 10 ng/ml of TGF-β1 (♦), the combination of TGF-β1 (10 ng/ml) and HMW-HA (25 μg/ml) either in the presence (△) or absence (●) of blocking antibody to CD44 was assessed. In control experiments cells were exposed to serum-free medium alone (□). Cell migration was assessed by directly counting the number of cells migrating into the intersecting denuded area at each of the time points indicated. **B:** In parallel experiments following creating of a wounded area the rate of cell migration following addition of 10 ng/ml of TGF-β1 (♦), the combination of TGF-β1 (10 ng/ml) and HMW-HA (25 μg/ml) either in the presence (△) or absence (●) of 10 μmol/L of the MEK inhibitor PD98059 was assessed. In control experiments cells were exposed to serum-free medium alone (□). Cell migration was assessed by directly counting the number of cells migrating into the intersecting denuded area at each of the time points indicated. The data represent the mean ± SD of three separate experiments and is expressed as the number of cells mm^{-2} of denuded area. $P < 0.05$ TGF-β1 versus control, and TGF-β1 versus TGF-β1+HA at all time points beyond 24 hours. P-NS TGF-β1 versus TGF-β1 + HA + blocking antibody to CD44, and TGF-β1 versus TGF-β1 + HA + PD98059.

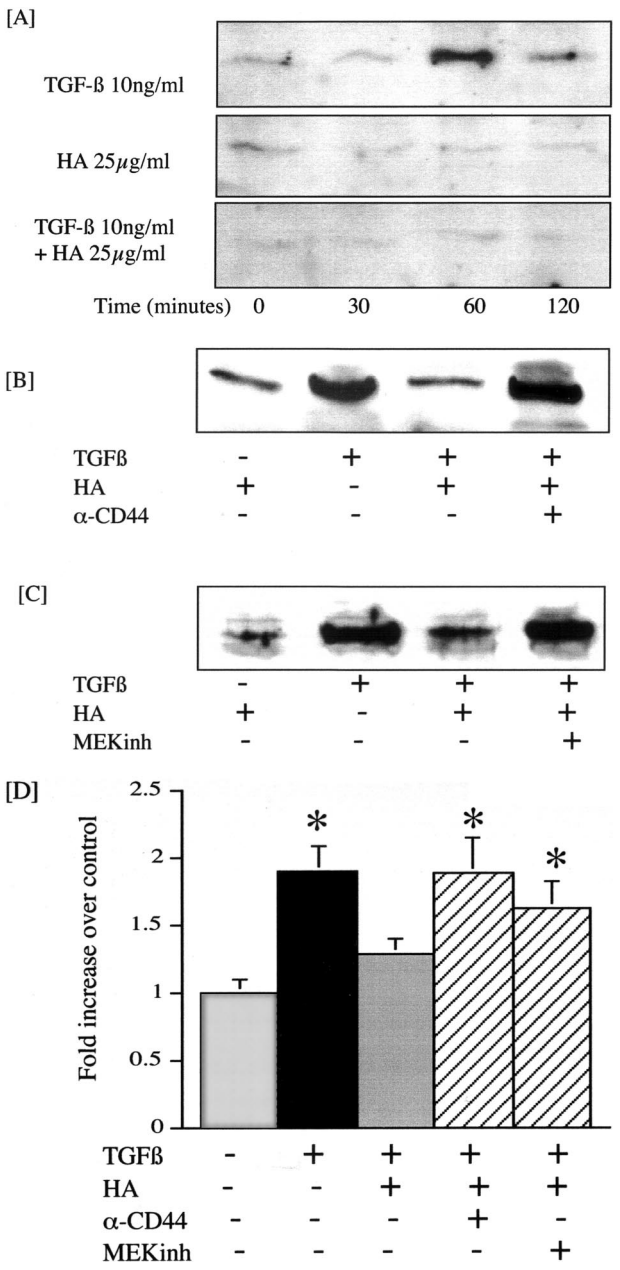

Figure 7. Modulation of TGF-β1-mediated activation of RhoA. **A:** Confluent monolayers of HK-2 cells were stimulated with either recombinant TGF-β1 (10 ng/ml), HMW-HA (25 μg/ml), or both stimuli applied together for time periods up to 120 minutes as indicated. In parallel experiments cells were stimulated with TGF-β1 (10 ng/ml), HMW-HA (25 μg/ml) or the combination of the stimuli either in the presence or absence of 5 μg/ml of a blocking antibody to CD44 (**B**), or the MEK inhibitor PD98059 (**C**) at a concentration of 10 μmol/L. Activation of RhoA was assessed by immunoprecipitation of GTP-RhoA as described in Materials and Methods. **D:** Following scanning densitometry, alteration in RhoA was expressed as fold increase in densitometric ratios of RhoA expression over control. The data represent the mean ± SD of three separate experiments $^*P < 0.05$ versus both control and TGF-β+HA.

MAP-kinase pathway, a mechanism which may underlie the ability of fibroblast growth factor to oppose the effects of BMP2 during limb bud outgrowth or tooth development.[50] Other receptor-regulated SMADs also have potential; MAP-kinase phosphorylation sites in their linker region. As antagonism of TGF-β1-SMAD-mediated events such as increased collagen synthesis are prevented by inhibition of MAP kinase, it is interesting to speculate that this may be related to CD44-mediated activation of the MAP kinase cascade and subsequent phosphorylation of TGF-β1-regulated SMAD proteins.

In addition to the SMAD signaling intermediates, numerous other pathways of signal transduction, such as MAP kinase and Rho GTPases, have been identified following TGF-β1 receptor activation.[51–57] Our recent studies in a scratch wound model demonstrated that the addition of TGF-β1 led to a marked inhibition of cell migration, increased expression of paxillin and vinculin and their incorporation into dense focal adhesion plaques. This was associated with increased association of focal adhesion components with the F-actin cytoskeleton. The effect on migration and focal adhesion reorganization, was abrogated by inhibitors of the RhoA downstream target ROCK. The data in this paper demonstrate that HA inhibits TGF-β1-mediated activation of RhoA and that this is associated with blunting of the anti-migratory effect of TGF-β1 in the scratch wound model of cell migration. This data together with attenuation of TGF-β1-dependent SMAD-mediated stimulation of collagen synthesis suggest that the effect of HA on TGF-β1 modulation of PTC function is not restricted to one specific post-receptor signaling pathway, but results in inhibition of all post receptor-ligand binding events.

In addition to a role in chronic fibrosis, altered HA expression has been documented in association with the acute renal injury in renal ischemia.[24] Interestingly, recent studies performed in a rodent model of ischemic injury demonstrated elevation of TGF-β1 expression, seen predominantly in the proximal tubule, occurring as early as 12 hours post-ischemia, during the phase of cellular proliferation and persisted for as long as 14 days post-ischemia well into the phase of remodeling.[58] Recovery of renal function is dependent in large part on the restoration of proximal tubular cell integrity and function, a process which is initially dependent on replenishing the population of proximal tubular cells by a wave of proliferation occurring 1 to 3 days post-injury.[59] This wave of proliferation therefore occurs in the presence of elevated levels of TGF-β1which in itself has potent antiproliferative effects on PTC.[60] Following the initial proliferative phase, restoration of PTC structure and function is dependent on numerous other events such as migration along a modified tubular basement membrane.[61] This also occurs in the presence of elevated levels of TGF-β1, which in itself has anti-migratory effects on PTC.[36] It is interesting to speculate therefore that increased expression of hyaluronan around injured cortical tubules may be involved in the recovery from acute ischemic injury by opposing the effects of TGF-β1 thus allowing both cell proliferation and cell migration in the face of elevated levels of TGF-β1 which if unopposed would be expected to inhibit both of these important components of the healing process.

Our results demonstrating attenuation of TGF-β1 signaling by HA are in contrast to recent reports using metastatic breast tumor cells in which CD44 interaction with the TGF-β receptor I kinase increases SMAD2/SMAD3 phosphorylation.[62] Furthermore, in these studies,

activation of the TGF-β receptor by HA also phosphorylated CD44, which enhanced its binding interaction with the cytoskeleton modulating breast tumor cell migration. TGF-βs elicit their signaling effects by binding mainly to three cell-surface receptors: type I (RI), type II (RII), and type III (RIII). RI and RII are serine/threonine kinases that form heteromeric complexes, and are necessary for TGF-β signaling, which is initiated when the ligand induces assembly of a heteromeric complex of type II and type I receptors. The RII kinase then phosphorylates RI on a conserved glycine-serine rich domain. This activates the RI kinase, which subsequently recognizes and phosphorylates downstream signaling partners such as members of the intracellular receptor regulated SMADs (R-SMADs) signal transduction pathway,[63] and non-SMAD kinase pathways such as RhoA and p38MAP kinase.[52,54,64] It is now well established that TGF-β1 plays a complex role in carcinogenesis. In human breast cancer, decreased expression of the TGF-β type II receptor in hyperplasias correlates with increased risk of developing invasive breast cancer, and in invasive breast cancer loss of receptors correlates with higher tumor grade, suggesting that TGF-βs are tumor suppressors early in carcinogenesis.[65] However, advanced tumors overexpress TGF-β-ligand, suggesting a switch to a pro-oncogenic role in advanced disease.[66] In addition to alteration in TGF-β1 type II receptor expression, expression of a truncated form or a splice variant associated with the development of malignancy may also result in defective ligand binding and aberrant TGF-β1 response. We speculate that differences in the expression pattern of the TGF-β1 receptor subtypes and their association with CD44 may explain why HA attenuated TGF-β1 signaling in our nonmalignant epithelial cell line, while in the breast tumor cell line HA triggered a TGF-β1 like signaling cascade. One important difference between our results and those derived from the breast tumor epithelial cells may relate to the degree of co-localization of CD44 with the TGF-β1 type II receptor. In our experiments CD44 immunoprecipitation resulted in detection of both TGF-β1 type I and type II receptors in equal abundance. In contrast, in the malignant cells, CD44 was associated predominantly with TGF-β1 type I receptors and to a much lesser extent with the type II receptors.

In summary we have demonstrated in renal proximal tubular epithelial cells, through co-localization of TGF-β1 and HA signaling receptors, that HA acts to antagonize TGF-β1-mediated alterations in cell function. In terms of renal disease, increased expression of HA is known to occur in both acute and chronic models of injury, and it is interesting to speculate that its role is to facilitate repair and limit progressive fibrotic effects which underlie progressive renal dysfunction.

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