Hyaluronan Regulates Transforming Growth Factor-β1 Receptor Compartmentalization

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Transforming growth factor-β1 (TGF-β1) is a key cytokine involved in the pathogenesis of fibrosis in many organs. We previously demonstrated in renal proximal tubular cells that the engagement of the extracellular polysaccharide hyaluronan with its receptor CD44 attenuated TGF-β1 signaling. In the current study we examined the potential mechanism by which the interaction between hyaluronan (HA) and CD44 regulates TGF-β receptor function. Affinity labeling of TGF-β receptors demonstrated that in the unstimulated cells the majority of the receptor partitioned into EEA-1-associated non-lipid raft-associated membrane pools. In the presence of exogenous HA, the majority of the receptors partitioned into caveolin-1 lipid raft-associated pools. TGF-β1 increased the association of activated/phosphorylated Smad proteins with EEA-1, consistent with activation of TGF-β1 signaling following endosomal internalization. Following addition of HA, caveolin-1 associated with the inhibitory Smad protein Smad7, consistent with the raft pools mediating receptor turnover, which was facilitated by HA. Antagonism of TGF-β1-dependent Smad signaling and the effect of HA on TGF-β receptor associations were inhibited by depletion of membrane cholesterol using nystatin and augmented by inhibition of endocytosis. The effect of HA on TGF-β receptor trafficking was inhibited by inhibition of HA-CD44 interactions, using blocking antibody to CD44 or inhibition of MAP kinase activation. In conclusion, we have proposed a model by which HA engagement of CD44 leads to MAP kinase-dependent increased trafficking of TGF-β receptors to lipid raft-associated pools, which facilitates increased receptor turnover and attenuation of TGF-β1-dependent alteration in proximal tubular cell function.

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that is involved in maintenance of normal homeostasis. It is important in development and tissue differentiation and normal wound healing through its effects on cell proliferation, migration, differentiation, and apoptosis (1). In addition, it is a critical regulator of the normal inflammatory response as illustrated by the death of TGF-β1 −/− mice of a multifocal inflammatory syndrome soon after weaning (2). Its growth inhibitory effect explains its role as a tumor suppressor, although its expression by tumor cells contributes to cancer progression and metastasis at later stages of disease (3, 4). TGF-β1 is also important in the pathological process of fibrosis and subsequent organ failure, leading to the concept of fibrotic disease as “the dark side of tissue repair” (5, 6). These different actions of TGF-β1 are related to altered epithelial cell responses to it in different cellular contexts (7, 8).

Sustained production of TGF-β1 is a key determinant of tissue fibrosis (6), including renal tubulointerstitial fibrosis (9). In chronic renal disease the severity of tubulointerstitial fibrosis is an excellent predictor of progression to end-stage renal failure (10, 11). Recent studies demonstrate the expression of three isoforms of TGF-β in the kidney (TGF-β1, 2, and 3), and although all three isoforms alter mesangial, interstitial fibroblast, and proximal tubular epithelial cell function the effects of TGF-β2 and TGF-β3 are mediated at least in part by stimulation of TGF-β1 protein synthesis (12). Much of our work has focused on the mechanisms that regulate TGF-β1 generation and function in the kidney and, more specifically, in renal proximal tubular epithelial cells.

TGF-βs elicit their signaling effects by binding mainly to three cell-surface receptors: types I (RI), II (RII), and 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 members of the intracellular receptor-regulated Smads (R-Smads) signal transduction pathway. For TGF-β1 these include Smad2 and 3. This causes dissociation of the R-Smads from the receptor, stimulates the assembly of a heteromeric complex between the phosphorylated R-Smad and the Co-Smad Smad4, and induces the nuclear accumulation of this heteromeric Smad complex (reviewed in Ref. 13).

Endocytosis of cell surface receptors is an important regulatory event in signal transduction. TGF-β1 receptors internalize into both caveolin- and EEA-1-positive vesicles and reside in both lipid raft and non-raft membrane domains (14). Clathrin-dependent internalization into the EEA1-positive endosome promotes TGF-β1 signaling. In contrast, the lipid raft-caveolar internalization pathway contains Smad7-bound receptor and is required for receptor turnover. Segregation of TGF-β1 receptors into distinct compartments, therefore, regulates TGF-β1 receptor signaling and turnover.

Many studies have demonstrated an association between alteration in the generation of the extracellular matrix polysaccharide, hyaluronan (HA), and renal injury diseases (15–18). The functional significance of this, however, is not clear. HA promotes the signaling interaction between the principle cell surface recep-
tor for HA, CD44, and the TGF-β type I receptor in metastatic breast tumor cells (19). Our studies with renal proximal tubular cells (PTC) have recently demonstrated that co-localization of CD44 and TGF-β receptors facilitates modulation of both Smad- and non-Smad-dependent TGF-β1-mediated events by HA (20). This suggests that alteration of HA synthesis may represent an endogenous mechanism to limit renal injury. The aim of the current work was to examine the effect of HA on TGF-β receptor compartmentalization in PTC. More specifically, we have examined the hypothesis that HA-mediated down-regulation of TGF-β1 signaling was the result of increased segregation of TGF-β1 receptors into a lipid raft-caveolar compartment away from the endosomal signaling compartment.

MATERIALS AND METHODS

Materials—Antibodies for Western blot analysis and immunoprecipitation and the final working dilution were as follows: rabbit polyclonal anti-TGF-β type I receptor antibody (dilution 1:500; Santa Cruz Biotechnology); rabbit polyclonal anti-Smad7 antibody (dilution 1:500; Santa Cruz Biotechnology); goat polyclonal anti-p-Smad2/3 antibody (dilution 1:500; Santa Cruz Biotechnology); mouse monoclonal anti-caveolin-1 antibody (dilution 1:500; Transduction Laboratories, Oxford, UK); mouse monoclonal anti-EEA-1 antibody (dilution 1:500; Transduction Laboratories, Oxford, UK); rabbit polyclonal anti-TGF-β1 antibody (dilution 1:5000; Invitrogen); mouse monoclonal anti-CD44 blocking antibodies (dilution 1:10,000; Sigma). Other Reagents—Other reagents were: recombinant human TGF-β1 (R&D Systems, Oxford, UK); mouse monoclonal anti-CD44 antibody (Acris, Bayport, MN); MAP kinase kinase (MEK) inhibitor PD98059 (Calbiochem); nystatin (Sigma); cholesterol, water soluble (Sigma); [125I]TGF-β1 (Amersham Biosciences); HyaHyaronan (MW 2 × 105) 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 16 E6/E7 genes) (21) were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 (Invitrogen) supplemented with 10% fetal calf serum (Biological Industries Ltd., Cumbernauld, UK), 2 μg/ml glutamine (Invitrogen), 20 μg/ml HEPES buffer (Invitrogen), 5 μg/ml insulin, 5 μg/ml transferrin (Sigma), 40 ng/ml hydrocortisone (Sigma), and 5 ng/ml sodium selenite (Sigma). Cells were grown at 37 °C in 5% CO2 and 95% air. Fresh growth medium was added every 3–4 days until confluent. Cells were grown to confluence and serum-deprived for 48 h prior to experimental manipulation. In all experiments cells were stimulated with recombinant TGF-β1 and/or HA under serum-free conditions.

In all aspects of cell biology that we have previously studied, HK-2 cells respond in an identical fashion to primary cultures of human proximal tubular cells (22–25). HK-2 cells are therefore a good model from which general conclusions can be drawn in terms of proximal tubular cell biology.

Transient Transfection—The Smad-responsive promoter (SBE), Lux (26) and constitutively active Alk5 (27) were gifts from Aristidis Mousta-

RESULTS

HA Increases TGF-β1 Receptor Segregation into Lipid Rafts, Associated with Caveolin-1—Previous studies have demonstrated co-localization of TGF-β receptors into both caveolin- and EEA-1-containing compartments. Caveolin associates with cholesterol- and sphingolipid-rich membrane domains called lipid rafts (31). To examine alterations in receptor partitioning, we adopted two experimental approaches: affinity labeling of endogenous TGF-β1 receptors on HK-2 cells with [125I]TGF-β1 and affinity labeling of HK-2 cells transiently transfected with the constitutively active TGF-β type I receptor Alk5 (TGF0/β1-

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The effect of HA on trafficking of TGF-β receptors was assessed by comparison of distribution in HK-2 cells exposed to either 250 μg/ml radiolabeled TGF-β1 (T) alone or radiolabeled TGF-β1 in the presence of 25 μg/ml HA molecular weight 2 × 10^6(T + HA). In all experiments subcellular fractionation was performed 24 h after addition of the stimuli. To disrupt lipid rafts cells were pretreated with 50 μg/ml nystatin at 37 °C for 1 h prior to the addition of radiolabeled TGF-β1 together with HA for a further 24 h (T + HA + N). Further characterization of the contribution of lipid rafts was examined by pretreatment of cells with 50 μg/ml nystatin for 1 h at 37 °C prior to the addition of radiolabeled TGF-β1 together with HA in the presence of 25 μg/ml cholesterol for a further 24 h (T + HA + N + C). An equal volume from each fraction was analyzed by SDS-PAGE electrophoresis followed by autoradiography. Following nystatin pretreatment and addition of [125I]TGF-β1 together with HA to cells in the absence of nystatin a HA-dependent increase in TGF-β receptor partitioning into the raft fractions. Pretreatment of cells with nystatin prior to addition of [125I]TGF-β1 together with HA resulted in only 31% of the total TGF-β receptor being associated with the raft fractions, which was no different from that seen when [125I]TGF-β1 was added in the absence of HA. As with expression of endogenous receptors the effect of nystatin was dependent on cholesterol sequestration because it was prevented by addition of cholesterol (25 μg/ml) together with nystatin to cells at 37 °C for 1 h prior to addition of TGF-β1 together with HA. This resulted in 52% of the total TGF-β receptor partitioning into the raft fractions. Statistically this was no different from the amount of receptor detected in the raft fraction when [125I]TGF-β1 together with HA were added to cells in the absence of nystatin.

Alterations in TGF-β Type 1 Receptor Associations—To determine the functional consequence of alteration in receptor partitioning, we stimulated cells with TGF-β1 (10 ng/ml) in the presence and absence of HA (25 μg/ml). Subsequently, we examined the association of TGF-β type I receptor with either EEA-1 or Cav-1 by immunoprecipitation of the TGF-β receptor and Western analysis of the latter two proteins.

Stimulation of cells with recombinant TGF-β1 (10 ng/ml) and immunoprecipitation of TGF-β type I receptor led to an increase in association of EEA-1 with the receptor (Fig. 3A). This increase in association of TGF-β1 type I receptor with EEA-1 following addition of recombinant TGF-β1 was not seen when cells were stimulated by recombinant TGF-β1 in the presence of HA (25 μg/ml). Scanning densitometry of three separate experiments confirmed a statistically significant increase in association of EEA-1 with TGF-β type I receptor, which was abrogated by the presence of HA (Fig. 3B).
HA Modification of TGF-β1 Signaling

Fig. 2. HA-mediated alteration in distribution of transfected Alk5. HK-2 cells transiently transfected with the constitutively active TGF-β type I receptor, Alk5, were affinity labeled with [125I]TGF-β1 and subjected to sucrose gradient subcellular fractionation to separate lipid rafts from other cellular components. The effect of HA on trafficking of TGF-β receptors was assessed by comparison of distribution in transfected cells exposed to either 250 pM radiolabeled TGF-β1 (T) alone or radiolabeled TGF-β1 in the presence of 25 μg/ml HA molecular weight 2 × 10^6 (T + HA). Subcellular fractionation was performed 24 h after addition of the stimuli in all experiments. To disrupt lipid rafts, cells were pretreated with 50 μg/ml nystatin at 37 °C for 1 h prior to the addition of radiolabeled TGF-β1 together with HA for a further 24 h (T + HA + N). The contribution of lipid rafts was further examined by pretreatment of cells with 50 μg/ml nystatin for 1 h at 37 °C prior to the addition of radiolabeled TGF-β1 together with HA in the presence of 25 μg/liter of cholesterol for a further 24 h (T + HA + N + C). An equal volume from each fraction was analyzed by SDS-PAGE electrophoresis followed by autoradiography. B, following scanning densitometry of autoradiographs, the distribution of TGF-β receptor into the raft (fractions 5 and 6) and non-raft (fractions 7–10) was quantified and the data from three separate experiments expressed graphically.

Previously, we have demonstrated that HA-dependent inhibition of TGF-β1 signaling was inhibited by co-incubation with a blocking antibody to CD44. Furthermore, we have demonstrated that addition of HA led to CD44-mediated activation of MEK activity and that inhibition of MEK by the inhibitor PD98059 also prevented the effects of HA on TGF-β1 signaling. In keeping with these observations, HA-mediated decreased association of EEA-1 with TGF-β type I receptor following addition of TGF-β1 was prevented by co-incubation with either blocking antibody to CD44 or the inhibitor of MEK, PD98059 (Fig. 3, A and B). Similarly co-incubation of HA with blocking antibody to CD44 or PD98059 and stimulation with recombinant TGF-β1 prevented the increased association of TGF-β type I receptor and Cav-1 seen when cells were stimulated with TGF-β1 in the presence of HA in the absence of either the blocking antibody to CD44 or PD98059 (Fig. 3, A and C). Further determination of the functional significance of alteration in TGF-β1 type I receptor segregation was examined by defining the association between EEA-1 and phosphorylated Smad2/3 and the association between Cav-1 and the inhibitory Smad, Smad7 (Fig. 4).

Association of EEA-1 with phosphorylated Smad2/3 was determined by immunoprecipitation of EEA-1 and Western analysis of phosphorylated Smad2/3 (Fig. 4A). Following addition of TGF-β1 alone there was a significant increase in the association of EEA-1 with phosphorylated Smad2/3 (Fig. 4B). In contrast, stimulation of cells with TGF-β1 (10 ng/ml) in the presence of HA (25 μg/ml) prevented the increased association of EEA-1 and phosphorylated Smads. This effect of HA on EEA-1 association with phosphorylated Smads was prevented by incubation with either blocking antibody to CD44 or incubation with the MEK inhibitor, PD98059. TGF-β1 stimulation followed by immunoprecipitation of Smad7 and Western analysis of associated Cav-1 demonstrated that addition of TGF-β1 alone led to a decrease in association of Smad7 with Cav-1, whereas the addition of TGF-β1 in the presence of HA increased the association of Smad7 with the inhibitory Cav-1 (Fig. 4, A and C). When these experiments were performed in the presence of either blocking antibody to

In contrast, immunoprecipitation of TGF-β type I receptor and Western analysis of Cav-1 demonstrated that addition of TGF-β1 (10 ng/ml) decreased the association of the two proteins (Fig. 3A). HA (25 μg/ml) alone increased association of TGF-β type I receptor and Cav-1. Furthermore, when cells were stimulated with TGF-β1 in the presence of HA, increased association of TGF-β type I receptor and Cav-1 was also apparent. Scanning densitometry confirmed the statistical significance of these changes (Fig. 3C).
**Fig. 4. Phosphorylated Smads and inhibitory Smads localize to distinct subcellular fraction.** Confluent growth-arrested HK-2 cells were stimulated with recombinant TGF-β1 or HA for 24 h as indicated in the presence or absence of either blocking antibody to CD44 (αCD44) or the MEK inhibitor PD98059 (MEKi). Subsequently, either EEA-1 or Smad7 were immunoprecipitated as described under “Materials and Methods” and phosphorylated Smad2/3 or Cav-1 expression in the precipitate examined by Western analysis (A). Following scanning densitometry, alteration in phospho-Smad2/3 (B) or Cav-1 (C) was expressed as fold increase in densitometric ratio compared with control. The data represent the mean ± S.D. of three separate experiments.

CD44 or the MEK inhibitor PD98059, there was no increase in the association of Smad7 with Cav-1 following stimulation with TGF-β1 in the presence of HA.

**Effect of Inhibition of TGF-β1 Receptor Trafficking**—Previous studies have demonstrated that cholesterol depletion by the addition of nystatin shifted receptors into non-raft compartments. The results presented above also demonstrated that pretreatment of cells with nystatin prevented a HA-mediated shift of TGF-β receptor into the raft fraction. In the current study we determined the effect of nystatin on attenuation of TGF-β1-stimulated activation of the Smad signaling pathway using the (SBE)4-Lux reporter, which contains four repeats of the CAGACA sequence identified as a Smad binding element. Addition of TGF-β1 led to a 10-fold increase in luciferase activity of the reporter construct (Fig. 5). 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 decrease in luciferase activity (Fig. 5). This represented a 27% reduction in luciferase activity compared with stimulation with TGF-β1 alone (mean of n = 6, p < 0.005). In contrast, disruption of cholesterol by pretreatment of cells with nystatin (31) at 37 °C for 1 h led to a dose-dependent reversal in HA-mediated attenuation of TGF-β1 stimulation of luciferase activity, such that there was no difference in luciferase activity following addition of TGF-β1 alone and TGF-β1 in combination with HA. This effect of nystatin was dependent on cholesterol depletion because addition of cholesterol (25 μg/ml) together with nystatin to cells at 37 °C for 1 h prior to addition of TGF-β1 together with HA restored TGF-β1-dependent Smad signaling.

Next we examined the effect of inhibition of clathrin-mediated endocytosis by K+ depletion, which prevents clathrin lattice assembly and has been shown to inhibit endosomal-dependent TGF-β1 signaling (14). Activation of the TGF-β1 signaling pathway was assessed by using the (SBE)4-Lux reporter (Fig. 5B). TGF-β1-stimulated increase in luciferase activity was significantly attenuated when carried out in minimal medium. In addition, attenuation of TGF-β1 signaling by the addition of HA was enhanced when carried out in the presence of minimal medium and was significantly greater than the effect of potassium depletion by addition of minimal medium alone.

**DISCUSSION**

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 (33–37). A major function of TGF-β1 is to regulate the expression of genes, the products of which contribute to the formation and degradation of extracellular matrix (ECM) (38–42). Generally, TGF-β1 leads to the accumulation of ECM by decreasing the synthesis of proteases and by increasing the levels of protease inhibitors (43). It also increases the expression of integrins through which ECM proteins such as fibronectin and collagen interact with cells (44, 45). *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 (46). Studies utilizing normal rat PTC also suggest that TGF-β1 is a key mediator regulating differentiation of PTC into α-smooth muscle actin-positive cells (47). 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 (35, 48, 49). Understanding the mechanisms that regulate TGF-β1-dependent responses is therefore an important goal.

We previously demonstrated that the association of CD44 and TGF-β1 receptors facilitated attenuation of PTC response to TGF-β1 (20). More specifically, we demonstrated a decrease in synthesis of collagen in response to TGF-β1 and decreased nuclear transllocation of Smad4 when cells were stimulated with TGF-β1 in the presence of HA. In addition to HA antagonism of TGF-β1 extracellular matrix generation, HA antagonized the effect of TGF-β1 on PTC migration. In contrast to the effect of TGF-β1 on collagen synthesis, which is Smad-dependent, the anti-migratory effect of TGF-β1 on PTC migration in this model is known to be dependent on activation of RhoA (50). In the presence of HA, TGF-β1-mediated activation of RhoA was also abrogated in a CD44-dependent manner (20). This suggests that co-localization of CD44 and TGF-β receptors facilitates modulation of both Smad- and non-Smad-dependent TGF-β1-mediated...
events by HA. Interestingly, this effect of HA was only seen with HA of a high molecular weight (2 x 10^6), whereas HA of much lower molecular weight (65,000) did not antagonize the effect of TGF-β1 (20). CD44 binds HA of high and low molecular weight and in doing so has distinct function. Antagonism of TGF-β1-mediated effects by high molecular weight HA is consistent with the assumption that, in general, high molecular weight HA represents the normal homeostatic state, whereas the generation of low molecular weight HA fragments signals a disruption of the normal homeostatic environment. In terms of renal disease, increased expression of HA is known to occur in both acute and chronic models of injury, and we have proposed that its role is to facilitate repair and limit progressive fibrotic effects that underlie progressive renal dysfunction. The aim of the work outlined in the current study was therefore to determine the mechanism by which HA-CD44 interactions down-regulate TGF-β1-dependent events.

Clathrin-coated pit-mediated endocytosis is traditionally considered a major mechanism by which cells regulate the level of cell surface receptors. For TGF-β1, however, Smad2 or -3 activation and downstream signaling occurs after endocytic vesicle formation (51). Thus, TGF-β receptor endocytosis is required to propagate post-receptor signaling events. Endocytosis via clathrin-coated pits of TGF-β receptor is constitutive, occurring in the absence of ligand (52). It has been demonstrated that this process is dependent on a short sequence (residues Ile-218-Ile-219-Leu-220) that conforms to the dileucine family of internalization signals.

In mammalian cells, internalization of cell surface proteins occurs through both clathrin-dependent and -independent pathways. Recent studies have demonstrated TGF-β Ser-Thr kinase receptor internalization through the classical clathrin-dependent pathway and also through the raft-caveolin route, with the former pathway facilitating Smad activation and the latter mediating receptor degradation (14). Furthermore, regulation of TGF-β receptor trafficking was not regulated by ligand stimulation, suggesting that the kinase activity of the receptors themselves, although regulating signal transduction, do not regulate receptor trafficking. Although the functional significance of two TGF-β receptor pools is now apparent, very little is known regarding the regulation of trafficking of TGF-β receptors between the non-raft-signaling and raft-associated degradative pools. The data in the current study demonstrate that interaction of HA with its receptor CD44 leads to an increase in the trafficking of TGF-β receptor to lipid raft-associated membrane pools. Furthermore, this result was carried out in the minimal medium. In control experiments, cells were exposed to minimal medium supplemented with 10 mM potassium chloride. Subsequently, luciferase content was quantified as described under “Materials and Methods” and the results normalized for transfection efficiency (using β-galactosidase) expressed as the fold increase above the non-stimulated control. The data represent the mean ± S.D. of four individual experiments; *, p < 0.05 compared with TGF-β1 and potassium-supplemented minimal medium.

In our previous studies, antagonism of TGF-β1-Smad-mediated events such as increased collagen synthesis was prevented by inhibition of MAP kinase (20). We speculated that this may be related to CD44-mediated activation of the MAP kinase cascade and subsequent phosphorylation of TGF-β1-regulated Smad proteins, because it is well established that phosphorylation of MAP kinase sites in the linker region of Smad proteins is known to negatively regulate their function (53). It has been previously demonstrated that the cytoplasmic domain of CD44 binds to the TGF-β type I receptor at a single site with high affinity (19). Rather than affecting Smad protein linker region phosphorylation, it seems more likely that activation of MAP kinase following engagement of CD44 by HA facilitates TGF-β-Smad-mediated events.

Fig. 5. Attenuation of TGF-β1 receptor signaling. A, sequestration of TGF-β1 from lipid rafts. HK-2 cells were transiently transduced with the Smad-responsive promoter (SE) 4-lux prior to stimulation with recombinant TGF-β1 (10 ng/ml) either in the presence or absence of HA (25 µg/ml, molecular weight 2 x 10^6) for 24 h. The role of lipid rafts in Smad signaling was examined by pretreatment of transfected cells with 50 µg/ml nystatin at 37 °C for 1 h or pretreatment with 50 µg/ml nystatin at 37 °C for 1 h prior to the addition of TGF-β1 together with HA in the presence of 25 µg/ml cholesterol. Subsequently, luciferase content was quantified as described under “Materials and Methods” and the results normalized for transfection efficiency (using β-galactosidase) expressed as the fold increase above the non-stimulated control. The data represent the mean ± S.D. of six individual experiments. B, inhibition of endosomal internalization. HK-2 cells transfected with the reporter construct were incubated in medium (Dulbecco’s modified Eagle’s medium/Ham’s F12) with 10 ng/ml TGF-β1, followed by incubation in minimal medium (serum-free medium containing 20 mM Hepes, pH 7.5, 140 mM sodium chloride, 1 mM calcium chloride, 1 mM magnesium sulfate, and 5.5 mM glucose) for 1 h at 37 °C prior to stimulation with TGF-β1 (10 ng/ml) either in the presence or absence of HA (25 µg/ml, MW 2 x 10^6) for 24 h. Stimulation was also carried out in the minimal medium. In control experiments, cells were exposed to minimal medium supplemented with 10 mM potassium chloride. Subsequently, luciferase activity was quantified as described under “Materials and Methods” and the results normalized for transfection efficiency (using β-galactosidase) expressed as the fold increase above the non-stimulated control. The data represent the mean ± S.D. of four individual experiments; *, p < 0.05 compared with TGF-β1 and potassium-supplemented minimal medium.
regulation of TGF-β1 signaling is not the result of modulation of Smad protein expression. It seems more likely that the role of MAP kinase activation is to regulate the trafficking of TGF-β receptors to lipid raft fractions. Furthermore, although we have previously demonstrated that both type I and type II receptors may bind to CD44, in the current study we demonstrated that addition of HA altered the trafficking of TGF-β receptors when cells were transfected with the type I receptor alone. This would suggest that formation of heterodimers of the type I and II receptors are not necessary to facilitate HA-mediated trafficking of the receptors to the lipid raft pool and that it is the regulation of the direct association of the type I receptor with CD44 that is critical for receptor trafficking.

Our results demonstrating attenuation of TGF-β1 signaling by HA are in contrast to recent reports utilizing metastatic breast tumor cells in which HA increased CD44 interaction with the TGF-β receptor I kinase and increased SMAD2/SMAD3 phosphorylation (19). It is clear that TGF-β1 has both tumor suppressor and tumor promoting activity at different stages of tumorigenesis. This dual- or multifunctionality of TGF-β1 expression has been the source of much research. Cell surface expression of certain CD44 isoforms is closely correlated to the development of numerous tumors and their prognosis. In the study utilizing breast tumor cell line (MDA-MB-231), a single CD44 isoform CD44v3 was expressed (19). In contrast, we have previously demonstrated that numerous isoforms of CD44 are expressed in PTC (54). More specifically, in addition to expression of the standard form of CD44, human proximal tubular cells also expressed epithelial CD44 (CD44 v8-v10), pMeta1 (CD44 v4-v7), pMeta2 (CD44 v6,v7) and keratinocyte CD44 (CD44 v8-v10). It is interesting to speculate that one mechanism by which TGF-β1 responses may be regulated may therefore be related to the differential expression of CD44 isoforms, with some isoforms enhancing TGF-β1 signaling and others attenuating TGF-β1-dependent responses.

In conclusion, we have demonstrated that engagement of CD44 by HA in PTC attenuates TGF-β1 signaling by increasing trafficking of TGF-β receptors to non-signaling lipid raft-associated pools, which is likely to increase receptor turnover.

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