Decorin suppresses transforming growth factor- β -induced expression of plasminogen activator inhibitor-1 in human mesangial cells through a mechanism that involves Ca^{2+} -dependent phosphorylation of Smad2 at serine-240

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Transforming growth factor- β (TGF β) is a key mediator of extracellular matrix (ECM) accumulation in sclerotic kidney diseases such as diabetic nephropathy. One of the main target cells for TGF β in the kidney are glomerular mesangial cells, which respond by increasing expression of ECM proteins, such as collagens, laminin and fibronectin, while suppressing the expression of ECM-degrading proteases and increasing the synthesis of ECM protease inhibitors, including plasminogen activator inhibitor-1. Previous studies have shown that exposure of mesangial cells to chronic high-glucose conditions, such as those seen in diabetes, increases ECM deposition in a mechanism involving glucose-mediated up-regulation of TGF β expression. Naturally occurring inhibitors of this TGF β -dependent fibrotic response include decorin, a small leucine-rich proteoglycan. While the mechanism by which TGF β stimulates gene expression via the Smad signal-transduction pathway is becoming clear, the precise mechanism by which decorin may impinge upon TGF β

activity remains to be established. In this study, for the first time we provide evidence that decorin can disrupt glucose- and TGF β /Smad-dependent transcriptional events in human mesangial cells through a mechanism that involves an increase in Ca^{2+} signalling, the activation of Ca^{2+} /calmodulin-dependent protein kinase II and ensuing phosphorylation of Smad2 at Ser-240. We show that decorin also induces Ser-240 phospho-Smad hetero-oligomerization with Smad4 and the nuclear localization of this complex independently of TGF β receptor activation. Thus, in human mesangial cells, the mechanism of decorin-mediated inhibition of TGF β signalling may involve activation of Ca^{2+} signalling, the subsequent phosphorylation of Smad2 at a key regulatory site, and the sequestration of Smad4 in the nucleus.

Key words: diabetes, fibrosis, growth factors, matrix, nephropathy.

INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal disease and a major contributing cause of morbidity and mortality in patients with diabetes. The disease is characterized by the progressive accumulation of the extracellular matrix (ECM) in the glomerular mesangium, mesangial expansion and glomerulosclerosis [1,2]. Disordered mesangial cell synthesis and catabolism of the components of the ECM under high-glucose conditions are the immediate cause of this glomerulopathy [3,4]. There is increasing evidence indicating that mesangial autocrine activation of transforming growth factor- β (TGF β) mediates the effects of high glucose concentrations [5–7], and that TGF β is a determinant in the development of human and experimental diabetic glomerulosclerosis [8,9]. Therapeutic approaches to down-regulate TGF β signalling under high-glucose conditions provide one strategy to inhibit the progression of diabetic nephropathy. One such approach is the use of the endogenous proteoglycan decorin [10], which is referred to as the naturally occurring inhibitor of TGF β [11].

Decorin is a secreted 45 kDa proteoglycan with a core protein comprised primarily of leucine-rich repeats. It is a component of the ECM in many tissues and, due to the amphipathic leucine-

rich repeats, is thought to interact with several other proteins and lipid molecules. It appears to be involved in the regulation of fundamental biological functions, such as matrix assembly [12] and the modulation of fibronectin- and thrombospondin-mediated cell adhesion [13,14]. More importantly, there appears to be a role for decorin in the regulation of TGF β activity, which is a key factor in tissue fibrosis [10,15,16], and also in promoting cell proliferation [17].

It has been shown that decorin binds to and causes rapid phosphorylation of the epidermal growth factor (EGF) receptor, with concurrent activation of the mitogen-activated protein kinase signal pathway [18,19]. Patel et al. [17] reported that activation of the EGF receptor by decorin results in elevation of intracellular $[\text{Ca}^{2+}]$ in single A431 squamous carcinoma cells. The effects of decorin persisted in the absence of extracellular Ca^{2+} but were blocked by AG1478, an EGF-specific tyrosine kinase inhibitor, and also by down-regulation of the EGF receptor. Thus Ca^{2+} signals may be important in mediating decorin-induced growth inhibition, and may also mediate some of the effects of decorin on renal fibrosis *in vivo*.

The mechanisms by which TGF β mediates intracellular signalling events have been clarified. Specific cell-surface receptors for TGF β possess intrinsic serine/threonine kinase activity.

Abbreviations used: Cam kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; CTGF, connective-tissue growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, haemagglutinin; HMC, human mesangial cell; PAI-1, plasminogen activator inhibitor-1; TGF β , transforming growth factor- β ; THMC, transformed HMC; RT-PCR, reverse transcriptase PCR.

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Signalling is initiated by the type I and type II receptors, forming a ligand-inducible active heteromeric complex [20]. Transcription factors of the Smad family are direct substrates for the TGF β receptor kinase and responsible for mediating most of the effects of TGF β (reviewed in [21]). To date, eight members of the Smad family have been described, which can be segregated into three functionally distinct sub-groups. Upon activation, the TGF β receptor complex induces phosphorylation of the TGF β /activin/bone morphogenic protein pathway-restricted R-Smads (Smads 1, 2, 3, 5 and 8). In all cases, the phosphorylated R-Smads then associate with a common-mediator co-Smad (Smad4). These heteromeric complexes are translocated to the nucleus, where they regulate gene transcription by either association with DNA-binding proteins or direct binding to promoter sequences in target genes. Site-specific phosphorylation of Smad proteins has been shown to be an important mechanism for regulation of their functional properties. Thus sequential phosphorylation of two C-terminal serine residues is a triggering event for activation of receptor-regulated Smads by receptors [22,23]. In marked contrast, phosphorylation of R-Smads in the linker region by mitogen-activated protein kinase at multiple -PXSP- consensus sites can inhibit both nuclear translocation and transcriptional activity [24,25]. We have also recently defined a novel cross-talk mechanism by which Ca²⁺/calmodulin-dependent protein kinase II (Cam kinase II), activated downstream of multiple tyrosine kinase receptors, can phosphorylate Smad2, leading to inhibition of cell responses to TGF β [26].

In this study, we have investigated whether decorin can disrupt the TGF β /Smad signalling pathway in human mesangial cells (HMCs) and show that this can occur through a mechanism that involves an increase in Ca²⁺ signalling, the subsequent activation of Cam kinase II and the phosphorylation of Smad2 at Ser-240, an important negative regulatory site [26]. We also show that decorin can induce Ser-240 phospho-Smad hetero-oligomerization with Smad4 and the nuclear localization of this complex, resulting potentially in the restriction of normal TGF β signalling through Smad4.

EXPERIMENTAL

Materials and cell lines

Primary normal adult HMCs were purchased from BioWhittaker (Wokingham, Berks., U.K.), and an immortalized transformed HMC (THMC) cell line was generated as described previously [27]. These cells were maintained in RPMI 1640 containing 5% fetal calf serum supplemented with 4 or 30 mM glucose. Recombinant TGF β 1 was obtained from R&D (Abingdon, Oxon, U.K.), thapsigargin was from Calbiochem, KN-93 was from Sigma, rat monoclonal anti-haemagglutinin (HA) antibody was from Roche (used for Western blotting), mouse monoclonal anti-HA antibody was from Clontech (used for immunoprecipitation) and monoclonal anti-Cam kinase II- α antibody from Gibco Life Technologies. Human decorin was purified from fibroblast culture supernatants and was kindly provided by Dr H. Kresse (Institute of Physiological Chemistry and Pathobiochemistry, Munster, Germany). All DNA expression constructs used in this study have been described previously [26]. The 3TP-lux reporter plasmid was provided by Dr Jeffrey Wrana (Samuel Lunenfeld Research Centre, Toronto, Canada). Cells were transfected using Fugene (Roche) or by electroporation (BioRad Gene-Pulsar).

RNA extraction and reverse transcriptase PCR (RT-PCR) analysis

Total RNA was extracted using the RNazol B method [AMS Biotechnology (Europe), Abingdon, Oxon, U.K.]. Equal amounts of total RNA (3 μ g) from each sample were first reverse-transcribed into cDNAs with SuperScript II RNase H⁺ reverse transcriptase (Gibco BRL) and random primers. Equal amounts (1 μ l) of the reverse transcriptase reaction (20 μ l) were in turn subjected to PCR amplification for 30 cycles. To quantify PCR products comparatively and to confirm the use of equal amounts of the RNAs, we co-amplified a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amount of reverse transcription reaction used for the amplification (1 μ l) was selected as being non-saturating for the PCR product of GAPDH after 30 cycles of amplification. The sequences of primers were all designed from the published sequences of the human genes: plasminogen activator inhibitor-1 (PAI-1; sense), 5'-GTATCTCAGGAAGTCCAGCC-3'; PAI-1 (antisense), 5'-TCTAAGGTAGTTGA ATCCGAGC-3'; GAPDH (sense), 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH (antisense), 5'-TCCACCACCCTGTTGCTGTA-3'.

Transfection of HMCs/THMCs and luciferase assays

3TP-lux reporter plasmid and p β gal control vector, or HA-Smad2 vector were transfected into mesangial cells; 25×10^6 cells in 0.5 ml of electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM D-glucose and 21 mM Hepes, pH 7.1). Briefly, the cells and plasmid DNA were incubated at room temperature for 10 min and electroporated at 250 V and 500 μ F, using the Bio-Rad Gene Pulsar. After an additional 10 min at room temperature, the cells were seeded into 5 ml of growth medium containing either 4 or 30 mM D-glucose and growth factors or inhibitors as indicated. The transfected cells were incubated at 37 °C in 5% CO₂ for 48 h, after which cells were lysed. For luciferase assays, cells were transfected in six-well plates with 2 μ g of 3TP-lux or ARE-lux and/or pCMV1-Cam kinase II₁₋₂₉₀, as indicated in the Figure legends. Cells were maintained in medium containing 0.5% fetal calf serum and, 48 h after transfection, were treated for 2 h with 1% DMSO or 1 μ M thapsigargin (100 μ M stock solution in DMSO). In some instances cells were also pre-treated with the specific Cam kinase II inhibitor KN-93 at a concentration of 10 μ M (10 mM aqueous stock solution). Luciferase activity was measured following 15 h of treatment with 5 ng/ml TGF β using the dual-reporter system (Promega, Chilworth, Southampton, U.K.).

Immunoprecipitation and immunoblotting

Cells were lysed at 4 °C with 1 ml of lysis buffer (50 mM Hepes, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100 and 50 mM NaF). Lysates clarified by centrifugation (14000 g for 10 min) were then incubated with 10 μ g of anti-HA or anti-FLAG M2 monoclonal antibodies together with 20 μ g of polyclonal rabbit anti-mouse IgG and 30 μ l of pre-washed Protein A-Sepharose. After 2 h at 4 °C, immunoprecipitates were washed four times with 1 ml of wash buffer (lysis buffer + 0.1% Triton X-100). Samples were separated by SDS/PAGE and exposed to Kodak X-Omat film. For immunoblotting, proteins were transferred on to nitrocellulose and probed with anti-HA, anti-FLAG or anti-green fluorescent protein antibodies that were visualized using the ECL[®] system (Amersham Bioscience).

Nuclear localization assays

Nuclear extracts were prepared as described in [28]. Confluent mesangial cells, which had been maintained under the conditions described above, were scraped from the tissue-culture dishes in PBS buffer using a cell scraper and were collected by centrifugation (1500 *g* for 5 min). The cell pellets were resuspended in hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.8 M KCl, 0.2 mM PMSF and 0.5 mM dithiothreitol) and homogenized with 10 strokes of a glass Dounce homogenizer. Nuclei were isolated by centrifugation (3300 *g* for 15 min). Nuclear proteins were extracted by suspending the nuclei in an equal volume of extraction buffer (20 mM Hepes, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 0.8 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM dithiothreitol). The protein concentration was determined using Coomassie Plus protein assay reagent (Perbio, Chester, U.K.).

RESULTS

Decorin disrupts TGF β signalling in primary HMCs to a similar extent as an increase in intracellular Ca²⁺ levels

Several recent reports have suggested that decorin can bind directly to the extracellular domain of the EGF receptor, and induce receptor dimerization and autophosphorylation. As a consequence, decorin is able to elevate cytosolic Ca²⁺, providing a possible mechanism by which decorin affects cellular growth [17]. Thus we reasoned that decorin could inhibit TGF β signalling by mobilizing Ca²⁺ and activating Ca²⁺-dependent kinases, which would in turn phosphorylate Smad2 [26] and inhibit the activity

Figure 2 Effect of decorin and Cam kinase II on the transcriptional activity of the 3TP-lux reporter in HMCs

Mesangial cells were co-transfected with 2 μ g of 3TP-lux reporter plasmid together with 2 μ g of β -galactosidase control vector, and with pCMV1-Cam kinase II₁₋₂₉₀ (grey bar) or empty pCMV1 vector, as described in the Experimental section. The cells were serum-starved for 24 h, and then incubated in medium containing 5 ng/ml TGF β (black bars) as indicated in the presence of increasing concentrations of decorin. Some cells were also treated with the specific Cam kinase II inhibitor, KN-93, at a concentration of 10 μ M. After 48 h, cells were lysed and luciferase/ β -galactosidase activities measured. The results represent means $\pm$ S.E.M. from two independent experiments.

Figure 1 RT-PCR amplification of PAI-1 transcripts in HMCs

(A) Expression of the PAI-1 gene was monitored in primary HMCs by semi-quantitative RT-PCR as described in the Experimental section. Total RNA was extracted from 4 and 30 mM D-glucose-treated HMCs. Cells were pre-treated or not with 1 mM thapsigargin or 100 ng/ml decorin, in the presence or absence of 5 ng/ml TGF β for 15 h. Expression of GAPDH was monitored as an internal control. All cells were starved for 24 h prior to treatment. (B) Analysis of the densitometry of RT-PCR bands. PAI-1 induction was normalized to GAPDH expression to correct for any differences in PCR efficiency. Cells treated with TGF β are shown by black bars. The results represent means $\pm$ S.E.M. from three independent experiments.

of the TGF β /Smad signalling pathway. Initially, to test this hypothesis we performed RT-PCR experiments to investigate the effects of decorin and Ca²⁺ signalling on the expression of PAI-1, a known TGF β -regulated gene. Addition of 5 ng/ml TGF β or exposure to a high level (30 mM) of glucose induced expression of the PAI-1 gene in primary HMCs (Figure 1A). Thapsigargin, a reagent that inhibits the Ca²⁺-ATPase pump to raise intracellular Ca²⁺, completely abolished the induction of PAI-1 by TGF β (Figure 1). In addition, decorin was able to repress the induction of PAI-1 by TGF β . Furthermore, densitometric analysis indicates that the exposure of HMCs to thapsigargin or decorin can reduce the basal expression levels of the PAI-1 gene (Figure 1B).

The same response was confirmed following transfection of THMCs with the 3TP-lux reporter, which contains TGF β response elements for both PAI-1 and collagen type 1 linked to luciferase expression. TGF β increased luciferase activity 3–4-fold and this effect was repressed by exposure to increasing concentrations of decorin (Figure 2). To confirm that this inhibition was mediated through endogenous Cam kinase II, a specific water-soluble Cam kinase II inhibitor, KN-93, was used. In the presence of KN-93, decorin-mediated inhibition of transcription was restored (Figure 2).

Decorin activates the EGF receptor and Cam kinase II in HMCs

Decorin has been shown to bind and cause rapid phosphorylation of the EGF receptor and elevation of intracellular [Ca²⁺] in A431 squamous carcinoma cells [17–19]. To investigate whether decorin can mediate a similar response in HMCs, cells were incubated with 100 ng/ml decorin for periods of 5 min, 15 min, 30 min, 2 h and 24 h. In primary HMCs decorin induced the

Figure 3 Decorin-mediated activation of the EGF receptor and Cam kinase II in HMCs

(A) HMCs were incubated with 100 ng/ml decorin for the times indicated. Cells were then lysed and immunoprecipitated with anti-EGF receptor (EGFR) antibody. Immunoprecipitated proteins were resolved by SDS/PAGE (15% gel) followed by anti-phosphotyrosine immunoblotting. (B) Activation of Cam kinase II in primary HMCs was monitored using the p-Cam KII (T286) antibody. Cells were maintained in 4 mM glucose prior to treatment with 100 ng/ml decorin, 100 ng/ml decorin + 50 μM KN-93 or 1 μM thapsigargin, as indicated. Cells were lysed and resolved by gradient SDS/PAGE (4–12% gel) and Western blotting.

phosphorylation of the EGF receptor after about 30 min of incubation and the activation was sustained up to 24 h (Figure 3A). The presence of the EGF receptor on the surface of HMCs and the activation of receptor autophosphorylation by decorin was also confirmed by immunocytochemical staining (results not shown). To examine whether decorin could also activate Cam kinase II, primary HMCs were incubated in 4 mM glucose-containing media with the Ca^{2+} -mobilizing agent thapsigargin, or with 100 ng/ml decorin in the presence or absence of 10 μM KN-93, a specific water-soluble inhibitor of Cam kinase II. A phospho-specific antibody (p-CamKII, T286) was used to monitor the activation of Cam kinase II in these experiments. Both decorin and thapsigargin activated Cam kinase II, whereas the inhibitor KN-93 blocked this activation (Figure 3B). This result was confirmed by immunostaining of HMCs with p-CamKII (T286) under the same conditions (results not shown).

Decorin induces phosphorylation of Smad2 at Ser-240 in HMCs via the activation of Cam kinase II

We have shown previously using phosphopeptide-specific antisera (anti-PS-240) that phosphorylation of Smad2 at Ser-240 by Cam kinase II, activated downstream of the EGF receptor, can inhibit $\text{TGF}\beta$ /Smad signalling [26]. To investigate the Ser-240 phosphorylation status of Smad2 in THMCs after exposure to decorin, HA-tagged Smad2 was expressed and the cells were then exposed to 100 ng/ml decorin for 48 h in the presence or absence of the specific Cam kinase II inhibitor KN-93. Following anti-HA and anti-PS-240 immunoblotting, we found that decorin induced the phosphorylation of HA-Smad2 at Ser-240 and that the specific Cam kinase II inhibitor KN-93 abrogated this phosphorylation (Figure 4A). Next, the phosphorylation levels of endogenous Smad2 in THMCs were examined after treating

Figure 4 Cam kinase II and decorin induce phosphorylation of Smad2 at Ser-240 in HMCs

(A) HA-tagged Smad2 was expressed in THMCs in medium containing 4 mM D-glucose or 4 mM D-glucose and 100 ng/ml decorin, as indicated. After 48 h, Smad2 was immunoprecipitated with anti-HA and samples separated by SDS/PAGE and then probed by Western blotting against anti-HA or anti-PS-240 antibodies. Equivalent expression levels were monitored in whole-cell lysates by anti-HA Western blotting. Where indicated, cells were also treated with the specific Cam kinase II inhibitor, KN-93, at a concentration of 10 μM. (B) THMCs maintained in 4 mM D-glucose were stimulated for 30 min with 5 ng/ml $\text{TGF}\beta$ or 100 ng/ml decorin. In some instances, cells were transiently transfected with constitutively active Cam kinase II₁₋₂₉₀. Cells were lysed, and samples probed by Western blotting against anti-Smad2 or anti-PS-240. Expression of Cam kinase II₁₋₂₉₀ was confirmed by specific immunoblotting (results not shown). Where indicated, cells were also treated with KN-93 (10 μM). (C) Phosphorylation of Ser-240 was monitored in primary HMCs using the PS-240 antibody. Cells were maintained in 4 mM D-glucose prior to treatment with 100 ng/ml decorin or 100 ng/ml decorin + 10 μM KN-93, where indicated. Cells were lysed and proteins resolved by gradient SDS/PAGE (4–12% gel) and Western blotting (WB). IP, immunoprecipitation.

the cells with $\text{TGF}\beta$ and decorin, or by transiently transfecting them with constitutively active Cam kinase II₁₋₂₉₀, a version of Cam kinase II which lacks a C-terminal autoinhibitory domain [29]. Decorin treatment and expression of constitutively active Cam kinase II both induced phosphorylation of the endogenous Smad2 at Ser-240, whereas $\text{TGF}\beta$ had no significant effect on Smad2 phosphorylation at this site (Figure 4B). The presence of the inhibitor KN-93 completely abolished the effect of decorin (Figure 4B). Since these results were obtained in a transformed mesangial cell line, we also confirmed these effects on endogenous Smad2 in primary HMCs isolated from human kidney. In these experiments, primary HMCs were incubated with or without 100 ng/ml decorin and in the presence or absence of the inhibitor KN-93 for 2 h. Cells were lysed and immunoprecipitated with anti-Smad2 antibody and the results confirmed that decorin could induce phosphorylation of endogenous Smad2 at Ser-240 in primary cells as well as in established cell lines (Figure 4C).

Figure 5 Nuclear translocation of Ser-240 phospho-Smad2 in mesangial cells

Primary HMCs maintained in 4 mM D-glucose were treated with 5 ng/ml TGF β , 100 ng/ml decorin or both, as indicated. After 2 h cells were lysed and nuclear extracts were prepared as detailed in the Experimental section. The proteins were resolved by gradient SDS/PAGE (4–12% gel) and samples probed by Western blotting. Phosphorylation of Smad2 at Ser-465/467 and Ser-240 was monitored using PS2 and PS-240 antibodies, respectively. Similarly, nuclear expression of Smad3 and Smad4 was investigated using specific antisera.

Nuclear translocation of Ser-240 phosphorylated Smad2 in HMCs

The translocation of Smads into the nucleus following TGF β receptor activation plays an important role in the activation of TGF β -dependent transcriptional responses (reviewed in [21]). We investigated whether the phosphorylation of Smad2 at Ser-240, in response to the addition of decorin, can prevent Smad2 translocation to the nucleus in HMCs, in a similar manner to that seen previously in Cos-1 cells [26]. HMCs were incubated in the presence of TGF β , decorin or both together. After 2 h, nuclear extracts were prepared from cells, resolved on a gradient SDS/PAGE (4–12% gel) and probed with Smad2 phosphopeptide-specific antibodies for Ser-465/467 (PS2), which are normally targeted by the activated type I TGF β receptor kinase, and Ser-240 (PS-240). Nuclear extracts were also probed with antibodies against Smad3 and Smad4. Smad2 was translocated to the nucleus in response to TGF β stimulation, together with Smad3 and Smad4, and was phosphorylated at Ser-465/467 (Figure 5). With decorin alone, Smad2 phosphorylated at Ser-240 is present in the nucleus, along with increased levels of Smad4 (Figure 5). Addition of TGF β and decorin together led to the presence of nuclear Smad2 phosphorylated at both Ser-240 and Ser-465/467 (Figure 5). In the presence of TGF β , Ser-465/467 phospho-Smad2 was present in the nucleus and there was a very low amount of Ser-240 phospho-Smad2 (Figure 5). Smad3 and Smad4 are seen in the nucleus at similar levels following TGF β stimulation in the presence or absence of decorin. However, decorin alone can induce the nuclear localization of Smad4 but not Smad3 (Figure 5).

DISCUSSION

Many studies have shown that TGF β is a profibrotic cytokine and an important mediator of kidney fibrosis [30,31]. Increased levels of glomerular TGF β mRNA in diabetic nephropathy biopsies correlate with the hyperglycaemic status of the patient [32], and glomerular TGF β protein is elevated in this disease. The small proteoglycan decorin has been used as a natural inhibitor of TGF β to slow the pathology of the disease. However, there has been little information about the mechanistic role that

decorin plays in this regulation of TGF β signalling. Since decorin was shown to raise intracellular Ca²⁺ levels by acting as a ligand for EGF receptor [17], we have investigated whether the Ca²⁺-dependent mechanisms that disrupt TGF β /Smad2 signalling, which we reported previously [26], occurs in HMCs and in response to decorin. In HMCs, raising intracellular Ca²⁺ levels with thapsigargin leads to inhibition of the TGF β -mediated expression of the PAI-1 gene and this effect can be mimicked by treatment of the cells with decorin. Transfecting HMCs with the TGF β -dependent 3TP-lux transcriptional reporter confirmed these effects of decorin and Ca²⁺ signalling on TGF β -mediated transcriptional activity. The decorin-induced reduction in 3TP-lux activity appears to be dose-dependent and acting via Cam kinase II, since use of the Cam kinase II-specific inhibitor (KN-93) restored the transcriptional activity of the 3TP-lux transcriptional reporter in these cells. These data are consistent with the hypothesis that decorin is capable of raising intracellular Ca²⁺ levels and thus, acting through Cam kinase II, can disrupt the TGF β -mediated intracellular signalling of the cell.

To further elucidate the intracellular mechanism by which decorin affects TGF β -dependent transcriptional responses, we examined changes in the functional activity of the Smad transcription factors. Smads are the major targets of the activated TGF β receptor kinase, and they translocate from the cytoplasm into the nucleus in response to TGF β stimulation where, in co-operation with other nuclear factors, they mediate specific gene responses. Phosphorylation of receptor-regulated Smad proteins at two C-terminal serines leads to their activation [22,23], whereas phosphorylation by other kinases at separate sites can inhibit Smad function [24,25]. Recently, we have defined an important mechanism by which Ca²⁺ ion flux and pathways activated downstream of receptor tyrosine kinases can inhibit Smads [26]. We have shown that Ca²⁺/calmodulin-dependent kinases block nuclear accumulation of Smad2, and induce Smad2–Smad4 hetero-oligomerization independently of TGF β receptor activation while preventing TGF β -dependent Smad2–Smad3 interactions, leading to the abrogation of TGF β -mediated transcriptional events. Furthermore, we identified Ser-240 in Smad2 as a major negative regulatory phosphorylation site, and raised specific antisera against phosphoserine-240-modified Smad2 (PS-240). In the present study, this PS-240 antibody was used to establish a connection between the decorin-mediated disruption of the TGF β pathway and Ca²⁺ signalling linked to Cam kinase II activation. We find that exogenous decorin addition to both THMCs and primary HMCs induces phosphorylation of Smad2 at Ser-240 and that this effect is mediated by Cam kinase II, since it was prevented by the specific inhibitor KN-93.

We have also specifically analysed nuclear extracts from mesangial cells for the presence and phosphorylation status of Smad2. As predicted, TGF β caused Smad2 phosphorylation at the main sites targeted by the type I TGF β receptor kinase at Ser-465/467. This was detected in the nucleus in response to TGF β , along with increased levels of Smad4 and Smad3 proteins. Decorin caused Smad2 phosphorylation at Ser-240 and Smad2 was also detected in the nucleus along with a similar level of Smad4; however, no induction of Smad3 translocation was detectable. When TGF β and decorin were added together to the cells, phosphorylation of Smad2 at Ser-465/467 as well as at Ser-240 was detected in the nuclear fraction, although the phosphorylation of Smad2 at Ser-465/467 was reduced. These data suggest that Cam kinase II-phosphorylated Smad2 may interact and oligomerize with Smad4 but not Smad3. However, unlike in COS-1 cells [26], this complex is still able to translocate to the nucleus. This may be significant with respect to the specific location within the cell at which Cam kinase II is able to

phosphorylate Smad2 and the cell-type-specific differences in the way that Ca^{2+} signalling might disrupt the $\text{TGF}\beta$ signalling network. In mesangial cells, and unlike in COS-1 cells, we find that decorin and/or Ca^{2+} will both activate Cam kinase II and, interestingly, induce its relocalization to the nucleus. Thus, under these conditions, Smad2 is still able to enter the nucleus. However, once in the nucleus, nuclear-translocated Cam kinase II will phosphorylate Smad2 and prevent it from activating transcription. This might occur either by inducing binding of the Smad complex to DNA sites, known to be negative transcriptional regulation sites, or alternatively by competing with the Smad2-465/467-Smad3-Smad4 complex for its positive transcriptional regulation sites ($\text{TGF}\beta$ response element). We find that decorin induces formation of the Smad2-Smad4 complex independently of $\text{TGF}\beta$ receptor activation, as well as the relocation of this complex into the nucleus. Therefore an important aspect of the mechanism by which decorin might affect the $\text{TGF}\beta$ /Smad signalling pathway is by sequestering the important co-Smad4 protein out of the cytoplasm, making it unavailable for subsequent signalling by activated cell-surface $\text{TGF}\beta$ receptors.

In summary, we conclude that an important mechanism by which decorin can play a functional role in regulating $\text{TGF}\beta$ signalling is through decorin-induced Ca^{2+} signalling and subsequent inhibition of the Smad pathway. Other mechanisms for antagonizing $\text{TGF}\beta$ activity will include the high-affinity binding of decorin to $\text{TGF}\beta$ forming an inactive complex [33,34]. Clearly the matrix remodelling changes that occur in response to high-glucose conditions with regard to alterations in $\text{TGF}\beta$ and decorin expression are complex. Up-regulation of both $\text{TGF}\beta$ and its natural antagonist decorin has been found in HMCs in response to prolonged hyperglycaemia [3], and increased levels of decorin are also seen in diabetic nephropathy [35]. Kinetic analysis of glucose-mediated $\text{TGF}\beta$ and decorin expression suggests that the early activation of $\text{TGF}\beta$ signalling and subsequent inhibition by decorin may provide a pulse of signalling activity, leading to the triggering of fibrotic events [3]. The prolonged accumulation of ECM seen in diabetic nephropathy could then be driven by an autocrine loop sustained by other $\text{TGF}\beta$ -inducible genes such as connective-tissue growth factor (CTGF) [27]. Interestingly, the expression of CTGF can be induced by either long-term exposure to high glucose or brief exposure to $\text{TGF}\beta$, and the subsequent increases in the expression of matrix components such as fibronectin and PAI-1 are then independent of $\text{TGF}\beta$ signalling events [27]. Our present findings on the mechanism by which decorin affects intracellular $\text{TGF}\beta$ signalling pathways provide another important dimension to decorin as a promising therapeutic agent. Such information may be useful in designing new therapeutic avenues that could, in combination with other strategies to antagonize the function of cytokines such as CTGF, prevent excessive $\text{TGF}\beta$ signalling in diabetic nephropathy and other fibrotic diseases.

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