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Research Paper

BMP-7 and Proximal Tubule Epithelial Cells: Activation of Multiple Signaling Pathways Reveals a Novel Anti-fibrotic Mechanism

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Purpose. Bone morphogenic protein-7 (BMP-7) is a member of the transforming growth factor β (TGFβ) superfamily involved in organogenesis. Recent work suggests that BMP-7 can reverse the fibrotic effects of TGFβ but the underlying mechanism is unknown. We sought to determine BMP-7 signaling and its modulation of TGFβ induced fibrotic outcomes in adult human proximal tubule epithelial cells (PTECs).
Methods. The effect of BMP-7 on phospho-p38 was assessed by Western blotting, p38 ELISA and Bio-plex phospho-protein assay. Secreted fibronectin (Fn) was measured by ELISA.
Results. BMP-7 had a concentration-dependent effect on intracellular signaling activating Smad 1/5/8 at higher concentrations and p38 mitogen activated protein (MAP) kinase at lower concentrations in both primary and transformed PTECs; BMP-7 caused phosphorylation of p38 at 2.5 ng/ml and Smads at 200 ng/ml. Similarly, nuclear accumulation of phospho-p38 and Smad were observed at these respective concentrations. These results suggested an inverse relationship between activation of Smads and p38 MAP kinase in this context. Consistent, with this BMP7 at 200 ng/ml reduced TGFβ-induced p38 MAP activation and the p38-dependent TGFβ-induced Fn secretion by PTECs.
Conclusion. We have shown novel p38/Smad signaling along a BMP-7 gradient and demonstrated BMP-7 regulation of TGFβ MAP kinase signaling and fibrotic outcomes.

KEY WORDS: BMP-7; fibronectin; p38 MAPK; Smads; TGFβ.

INTRODUCTION

Progressive renal interstitial fibrosis is one of the major causes of established renal failure and is characterised by tubular atrophy and tubulo-interstitial fibrosis (1). TGFβ1 plays an important role in renal fibrosis where the PTEC is a key target for its pro-fibrotic effects (2,3). TGFβ classically acts via type I receptor (Alk 5) in epithelial cells and can activate either the Smad pathway (Smad 2, 3) or MAP kinase pathways such as p38 (4,5). The p38 MAP kinase pathway is involved in secretion of matrix proteins such as Fn, which contribute to the progression of renal fibrosis (6,7).

BMP-7; formerly called osteogenic protein-1 is one of a large family of structurally and functionally related BMPs and a member of the TGFβ superfamily. It has been recognised that BMP-7 is a key growth factor involved in embryogenesis and morphogenesis. BMP-7 expression is particularly critical for development of the kidney. Its expression has also been detected in healthy and injured adult human and rat kidney (8–11). In the adult BMP-7 expression is maintained primarily in the kidney with expression in glomerular and distal tubule epithelial cells (12). However, its precise role in the adult kidney remains uncharacterised.

Recently, BMP-7 has been described as a potential anti-fibrotic agent (13). In an animal model of diabetic nephropathy there is loss and decline of BMP-7 and its receptors respectively (14). Recent work in an animal model of renal fibrosis has demonstrated the striking reversal of TGFβ-driven fibrosis by BMP-7 in the mouse, even demonstrating a restoration of e-cadherin expression (15). BMP-7 has also been shown to antagonise TGFβ dependent fibrogenesis in mesangial cells (16).

BMP-7, like TGFβ, binds to a type II/type I receptor complex. On binding BMP-7 induces phosphorylation of the type I receptor by the constitutively active type II receptor. On activation, the type I receptor phosphorylates BMP receptor Smads 1, 5 and 8 which are then able to dimerise with Smad 4, translocate to the nucleus, and regulate gene transcription (9).

There is also some evidence for BMP-7 regulating p38 MAP kinase activation in different cell types. Interestingly, the concentration of BMP-7 appears to be the key factor determining whether activation of MAP kinases is observed. In mouse collecting duct cells activation of p38 MAP kinase occurs at low concentrations of BMP-7, but at higher concentrations the activation is putatively suppressed by Smad 1 (17). However and importantly the above phenomenon is not true in all cell lines. In murine mesangial cells low

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ABBREVIATIONS: BMP-7, Bone morphogenic protein-7; Fn, Fibronectin; HKC-8, HKC-clone 8; MAP, Mitogen activated protein; PTECs, Proximal tubule epithelial cells; TGFβ, Transforming growth factor β.

dose BMP-7 fails to activate p38 MAP kinase (18); however in a new study using a murine mesangial cell line, high dose BMP-7 significantly activated p38 MAP kinase (19).

The aim of this work was to investigate the ability of BMP-7 to modulate p38 MAP kinase activity and p38 mediated events in adult human PTECs. PTECs are known targets for the actions of both TGFβ and BMP-7, expressing receptors for both. The key role that PTECs have in the development of tubulo-interstitial fibrosis would suggest that understanding this process will greatly improve our knowledge of how BMP-7 may work as an anti-fibrotic agent. This may have a potential therapeutic benefit by targeting these pathways and reversing renal fibrosis (20).

MATERIALS AND METHODS

Cell Culture and Model. Experiments were performed on HKC-clone 8 cells (HKC-8), a virally transformed adult human PTEC line, kindly provided by Dr Lorraine Racusen of the Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD (21). The cells were grown on uncoated plastic T75 Flasks (Falcon, BD Biosciences) with DMEM: Ham's F12 (1:1) media, supplemented with 18 ng/ml hydrocortisone, 20 ng/ml tri-iodothyronine, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Invitrogen, Paisley, UK; Sigma, Poole, UK) in presence of 5% heat inactivated foetal calf serum (Sigma). HKC-8 cells were subcultured by treatment with 2.5 mg/ml porcine trypsin (Sigma) and 0.5 mM EDTA in phosphate buffered saline (PBS; 137 mM NaCl, 7.9 mM NaH_2HPO_4, 2.7 mM KCL and 1.5 mM $KH2PO_4$, adjusted to pH 7.4) for 2–3 min. The cells were then seeded at 100,000 cells per 35 mm plastic dish (Falcon, BD Bioscience).

Primary human PTEC were isolated from tissue excised from nephrectomy kidneys for treatment for renal cell carcinoma. Ethical approval was obtained from the London-Surrey Borders Local Research Ethics Committee and written informed patient consent obtained on all occasions. Healthy cortex tissue was identified and dissected by the histopathologist.

The method for the isolation of primary human PTEC is essentially that of Phillips *et al.* (22). In brief, cortex tissue was passed through a series of sieves (Endecotts, UK) and the tubule fragments on the 45 μm sieve were collected, washed twice, resuspended in collagenase type IV (175 U/ml in PBS, Worthington Biochemical Corporation, Lorne Laboratories, UK) and incubated for 10 min at 37°C. This suspension was centrifuged at 250×*g* for 6 min, and cell pellet resuspended in DMEM: Ham's F12 (1:1) supplemented with 36 ng/ml hydrocortisone, 4 pg/ml tri-iodothyronine, 10 ng/ml epidermal growth factor, 16 mM *N*-(2-hydroxyethyl) piperazine-*N*-(2-ethanesulfonic acid), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated foetal calf serum (all purchased from Sigma). The cells were plated on collagen IV coated dishes (BD Bio coat, BD Biosciences) at a seeding density of 3–4 g of initial cortex tissue mass/25 cm^2 surface areas and maintained at 37°C, 5% CO_2/95% air. Isolated primary human PTEC were subcultured in a similar manner to HKC-8 cells and were seeded on collagen IV coated dishes at 20,000 cells/cm^2.

Primary PTEC lines were characterised by morphology, expression of cytokeratin and presence of the proximal tubular brush border enzymes γ-glutamyl transferase and leucine aminopeptidase (23). Primary human PTEC were also purchased from Cambrex Bio Science Company (Walkersville, MD, USA). The cells were cultured according to the supplier instructions.

Prior to any experiment all cells were made serum free for a period of 24 h. In all the experiments the cells were approximately 80% confluent at the beginning of treatments.

Western Immunoblotting

Following experimental conditions, cells were washed with ice cold PBS. The cells were then lysed in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.4, with 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 1.0 mM sodium orthovanadate, 50 mM NaF, 200 μM phenylmethanesulfonyl fluoride with 40 μl/ml protease inhibitor cocktail, Sigma). Centrifugation at >14,000×*g* at 4°C for 10 min was used to clear the lysates and the supernatant recovered. Bicinchoninic acid (BCA) protein assay kit (Pierce; via Perbio Science UK, Cramlington, Northumberland, UK) was used to measure protein concentration. Under reducing conditions, equal amounts of total protein were subjected to SDS/PAGE in 10% Bis–Tris-containing polyacrylamide gels using Xcell *SureLock*™ western Immunoblotting system (Invitrogen) according to manufacturer's instructions. After electrophoresis the proteins were transferred to PVDF membranes and the blots were blocked with TBS-T (Tris-buffered saline/20 mM Tris/HCl, 150 mM NaCl and 0.1% Tween 20) and 5% fat free milk prior to incubation with the appropriate primary antibodies. The primary antibodies were prepared in TBS-T with 5% BSA at 4°C over night. Primary antibodies used to detect phospho-Smad 1/5/8 at (1/1,000 dilution) and phospho-p38 (at 1/1,000 dilution) were purchased from Cell Signalling Technology, New England Biolabs, UK, Ltd. The blots were then incubated with horseradish peroxidase-labelled secondary antibody for 60 min at room temperature (Cell Signalling Technology). The protein bands were detected with ECL Plus® enhanced-chemiluminescence Western-blotting detection system and visualised using Hyperfilm ECL photographic film (GE Healthcare, Amersham; Little Chalfont, UK). The blots were re-probed for α-tubulin as a loading control.

Bio-Plex Phospho-protein Assay

HKC-8 cells were exposed to experimental conditions for various times. Cells were washed with ice-cold sterile PBS and cell lysates prepared in 50 μl ice-cold lysis buffer. Protein concentration of cell lysates was determined using micro BCA protein assay (Perbio Science, UK) and determination of phospho-p38 was carried out using a Bio-Plex™ suspension array system with a phospho-5-Plex assay (BioRad UK, Ltd) and reagent kit according to the manufacturer's guidelines.

Immunocytochemistry

Immunocytochemistry was carried out on the HKC-8 cells seeded onto collagen IV coated chamber slides at a density of

16,000 cells/cm^2. The cells were treated with BMP-7 (R and D Systems, MN, USA) for 15 and 60 min. Following ligand stimulation cells were washed with ice-cold PBS; cells were then fixed and permeabilised by immersion in pre-cooled 100% methanol at −20°C, 10 min. Cells were washed again thrice with ice-cold PBS and incubated with a blocking solution (1% BSA and 10% goat serum in PBS) for 60 min at room temperature. Cells were washed again and treated with primary Smad 1 (Santa Cruz Biotechnology, CA, USA) and monoclonal phospho-p38 (Cell Signalling Technology) antibody (0.1% BSA) for 60 min at 4°C. Cells were washed and treated with fluorochrome conjugated secondary (Santa Cruz Biotechnology) antibody (TBS, 1% BSA) for 60 min at room temperature. Cells were washed again and air-dried prior to mounting. The distribution of fluorochrome was examined by fluorescence microscopy (Axioskope 40) under ×400 magnification and the images were captured with Carl-Ziess camera.

ELISA for Phospho-p38

The production of phospho-p38 following treatment with BMP-7 in the HKC-8 cells was quantified using a commercially available enzyme-linked immunosorbent assay (Sigma, Saint Louis, MO, USA), following manufacturer's instructions. A phospho-p38 standard curve was constructed for the assay, and a curve-fitting software program was used to quantify phospho-p38 protein concentration in the lysed cells. All data were corrected for cell protein content, measured by BCA protein assay kit.

ELISA for Fibronectin

Secreted Fn was measured in HKC-8 cells following incubation for 48 h and treatment with vehicle, TGFβ (Sigma), BMP-7 or BMP-7 + TGFβ using a commercially available ELISA (Chemicon International, Temecula, CA, USA). All data were corrected for cell protein content, measured by BCA protein assay kit.

Statistical Analysis

Results are expressed as mean and standard deviation (SD). Statistical analysis was performed using GraphPad PRISM version 3.00 (GraphPad Software Inc., San Diego, CA, USA). Data was analysed by repeated measure ANOVA with post hoc *t*-testing using Bonferroni–Dunn correction. A p value of less than 0.05 was considered significant.

RESULTS

BMP-7 Dose Response in Relation to p38 and Smad 1/5/8 Activation

To investigate the concentration of BMP-7 that would activate the BMP-7 receptor complex in adult human PTECs a dose response was carried out. HKC-8 cells at 80% confluence were treated with BMP-7 at 6.6, 20, 66 and 200 ng/ml. The canonical BMP-7 Smad pathway was used as an indicator of receptor/signaling activation. Western blot technique was used and Smad 1/5/8 activation reached significant activation at 200 ng/ml of BMP-7 (Fig. 1a).

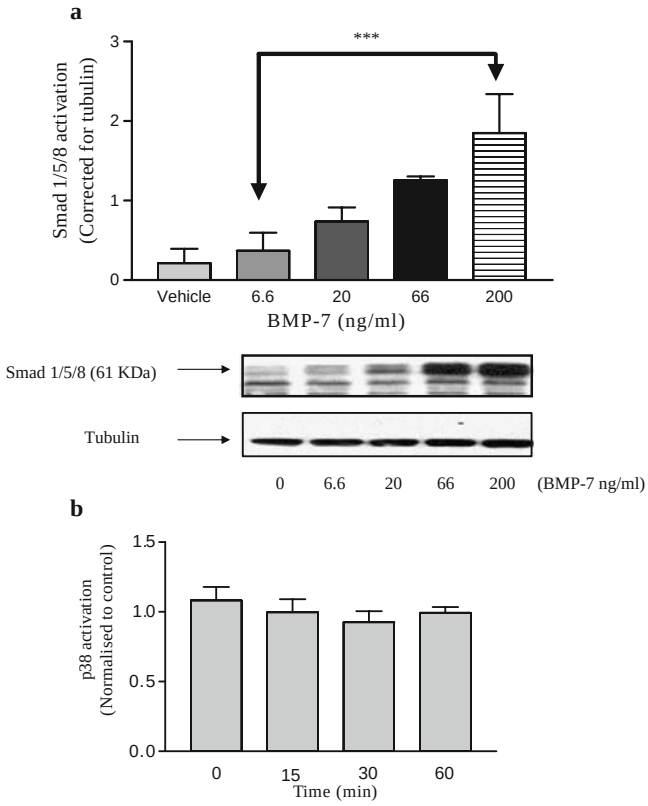

Fig. 1. Induction of Smad 1/5/8 at high concentration of BMP-7 (200 ng/ml) with no activation of phospho-p38. BMP-7 at high dose (200 ng/ml) causes clear activation of Smad 1/5/8 in HKC-8 cells with no significant activation at 6.6 ng/ml (**a**, $n=3$, $p<0.001$). Dose response of BMP-7 on Smad 1/5/8 activation is shown with a representative western blot. No activation of phospho-p38 could be demonstrated with Bio-Plex phospho-protein assay following treatment with high dose (200 ng/ml) BMP-7 (**b**, $n=3$). Mean ± SD of three independent experiments (*triple asterisk* $p<0.001$).

Bio-Plex™ suspension array system was used and a time course of BMP-7 treatment at 200 ng/ml, as established from the previous experiments described above, from 5 to 60 min showed no activation of p38 (Fig. 1b).

Work on murine inner medullary collecting duct cells had indicated p38 activation at a low concentration, 7.5 ng/ml of BMP-7 (17); consequently time course experiments were performed on HKC-8 and primary cultures at 7.5 ng/ml. It was noted that medium change alone induced an acute transient activation of p38; hence in all experiments activation of p38 was expressed relative to contemporaneous controls.

"Low" Concentration of BMP-7 (7.5 ng/ml) Results in Phospho-p38 Induction

To determine if low dose of BMP-7 (7.5 ng/ml) caused induction of phospho-p38, both HKC-8 and primary human PTECs were treated with vehicle (0.1% BSA) or BMP-7 for 5, 15 and 60 min. Western blot technique was used and in HKC-8 cells, there was a consistent induction of phospho-p38 at 15 min; however this did not reach statistical significance (Fig. 2a). Similar results were observed in primary cultures (Fig. 2b). Concurrent investigation of Smad activation in the same samples generally showed little or no activation,

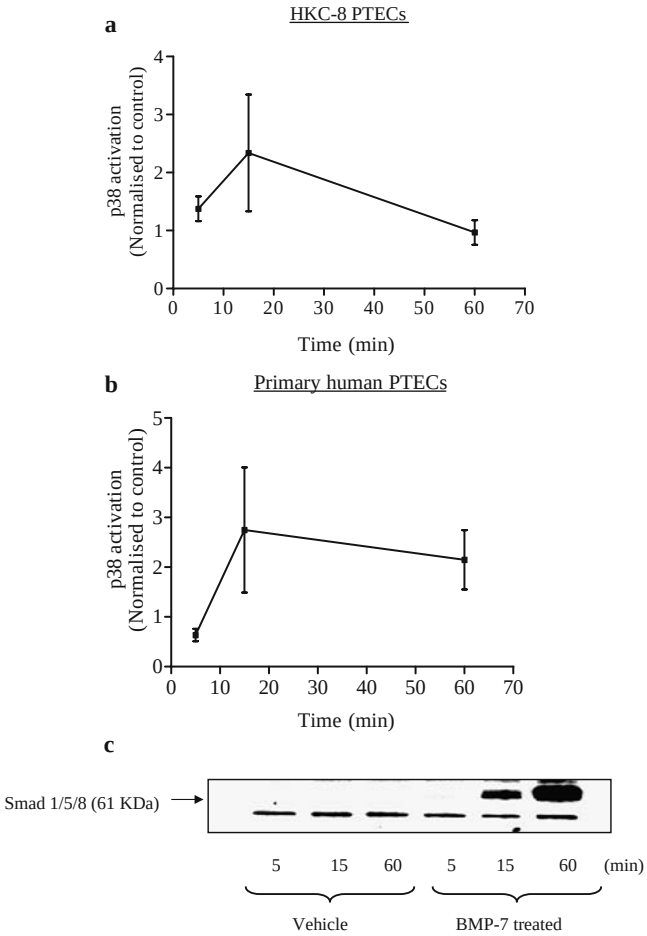

Fig. 2. Induction of phospho-p38 at low concentration of BMP-7 (7.5 ng/ml). Induction of phospho-p38 in HKC-8 cells by western blot could be seen 15 min post treatment with low dose BMP-7 (**a**, $n=5$). Induction of phospho-p38 in primary human PTECs, at 15 min and was sustained at 60 min post treatment with low dose BMP-7 (**b**, $n=6$). Results are mean ± SD and expressed as a percentage of the contemporaneous control value. Western blot showing an occasional activation of Smad 1/5/8 at 15 and 60 min post treatment with 7.5 ng/ml BMP-7 (**c**).

however in some cultures Smad activation was observed at 15 min (Fig. 2c).

BMP-7 at 2.5 ng/ml Induces Phospho-p38 Activation with no Smad 1/5/8 Induction

Phospho-p38 ELISA was performed on HKC-8 cells treated with an even lower concentration of BMP-7. More consistent activation of phospho-p38 was observed at 2.5 ng/ml compared to 7.5 ng/ml BMP-7. (Data not shown). This lower concentration of 2.5 ng/ml was used subsequently.

In order to look at p38 and Smad 1/5/8 activation in same samples, experiments were carried out on both HKC-8 and Primary human PTECs and incubated for 5, 15 and 60 min with 2.5 ng/ml of BMP-7 and vehicle (0.1% BSA). Western blot analysis showed in HKC-8 cells significant induction of phospho-p38 with 2.5 ng/ml of BMP-7 at 15 min post incubation. High dose BMP-7 at 200 ng/ml did not induce phospho-p38 at the above time point (Fig. 3a and b). This p38 induction can also be seen in primary cell lines as well (Fig. 3b). No activation of Smad 1/5/8 was seen at this concentration of BMP-7 (Fig. 3c).

Nuclear Translocation of Smad 1/5/8 and Phospho-p38 with 200 ng/ml and 2.5 ng/ml BMP-7

To investigate the effect of low and high concentration of BMP-7 on Smad 1/5/8 nuclear translocation, HKC-8 cells were plated on collagen IV slides. The slides were treated either with vehicle (0.1% BSA) or BMP-7 at 2.5 and 200 ng/ml for 15 and 60 min. Immunocytochemistry was used to demonstrate localisation of total Smad 1 and phospho-p38. In both vehicle and BMP-7 at 2.5 ng/ml, there was low-density peri nuclear localisation of Smad 1 with majority being in the cytoplasm. BMP-7 at 200 ng/ml caused a shift of the Smad 1 from the cytoplasm to the nucleus. High and low dose BMP-7

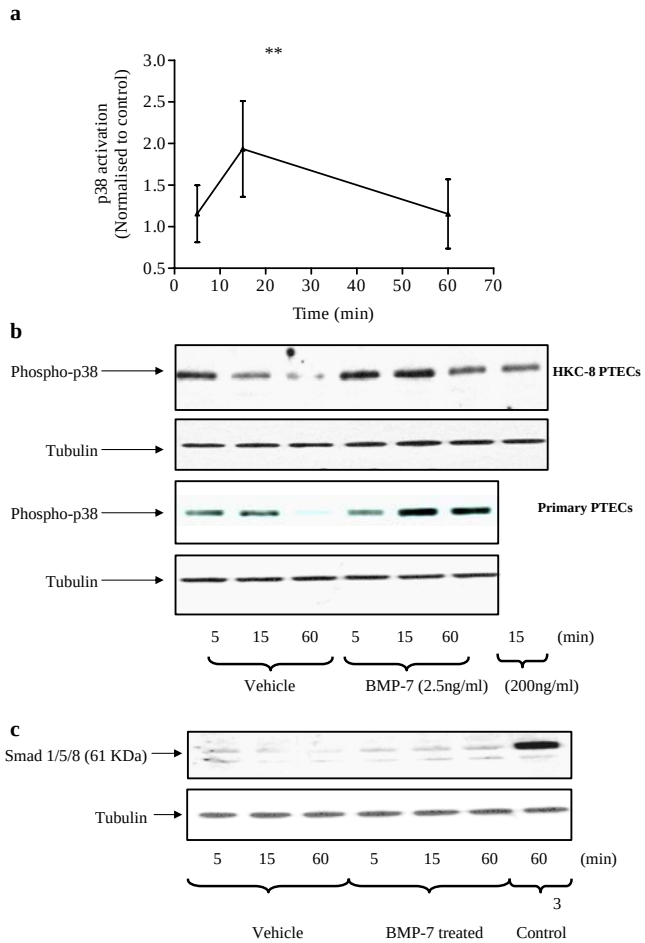

Fig. 3. Significant activation of phospho-p38 with BMP-7 at 2.5 ng/ml with no Smad 1/5/8 activation. BMP-7 at 2.5 ng/ml caused a significant induction of phospho-p38 at 15 min post treatment in HKC-8 cells. The pattern of activation is similar to treatment with BMP-7 at 7.5 ng/ml. A similar pattern was seen in primary cultures (**a**). The results are mean ± SD and expressed as a percentage of control value ($n=6$, *double asterisk* $p<0.01$). The above results are clearly seen in representative western blots in both HKC-8 and primary cells (**b**). No activation of Smad 1/5/8 was seen at this concentration of BMP-7, although activation of Smads is demonstrated with BMP-7 200 ng/ml as a control (**c**).

had the opposite effect on cellular localisation of phospho-p38. BMP-7 at 2.5 ng/ml causes a clear nuclear localisation of phospho-p38 compared to both vehicle and high dose BMP-7 at 200 ng/ml (Fig. 4).

Inhibition of Phospho-p38 with "High" Dose BMP-7

Our results suggest that high intracellular levels of phospho-Smad 1/5/8 are not consistent with elevated levels of phospho-p38. Consequently we investigated the effect of "high dose" BMP-7 on TGFβ induced phospho-p38. HKC-8 cells were treated with vehicle (0.1% BSA), TGFβ (2.5 ng/ml) or co-treatment of TGFβ with BMP-7 (200 ng/ml) for 60 min. TGFβ (2.5 ng/ml) induced a significant increase in phospho-p38 compared to vehicle, with an approximate three fold increase in band density by western blot probed with anti-phospho-p38 antibody and indexed for tubulin as a house keeping protein. However co-incubation of TGFβ with BMP-7 at 200 ng/ml, a concentration demonstrated above to induce accumulation of phospho-Smad 1, significantly reduced TGFβ induced p38 production (Fig. 5).

Anti-fibrotic Effect of BMP-7 on TGFβ-Induced Fibronectin Production

The above results suggest that high dose BMP-7 (200 ng/ml) could inhibit TGFβ induced p38; the subsequent hypothesis was that BMP-7 at this concentration could inhibit TGFβ-induced p38-mediated events such as Fn production (7).

Experiments were carried out on HKC-8 cells to investigate the effect of TGFβ and co-treatment with BMP-7 on Fn production. Cells were treated with vehicle (0.1% BSA), TGFβ (2.5 ng/ml), co-treatment with TGFβ and BMP-7 (200 ng/ml) and BMP-7 alone. The cells were treated with the above conditions for 48 h and secreted Fn measured by ELISA. The results are expressed as ng Fn/mg total protein and normalised to secreted Fn in the vehicle (Fig. 6).

TGFβ induced a significant production of Fn compared to vehicle. Co-incubation of TGFβ and high dose BMP-7 significantly inhibited TGFβ induced Fn secretion.

DISCUSSION

Tubulointerstitial fibrosis is a major prognostic indicator of chronic and end stage renal failure. BMP-7 has been proposed to play a role in regulating and controlling renal fibrosis. *In vitro* work on mouse mesangial cells has shown that BMP-7 can partially block TGFβ-induced fibrotic outcomes such as accumulation of collagen IV and fibronectin by antagonizing down-regulation of matrix metalloprotease-2 (16). Zeisberg *et al.* and Morrissey *et al.* also demonstrated that in animal models of renal fibrosis administration of BMP-7 leads to improved renal function, histology and survival associated with induction of matrix metalloprotease-2 and significant reduction in pro-fibrotic outcomes (15,24). In a rat model of diabetic renal disease, a reduction in endogenous tubular BMP-7 and BMP-7 receptor expression was observed in tubule epithelial cells which was associated with increased expression of extra-

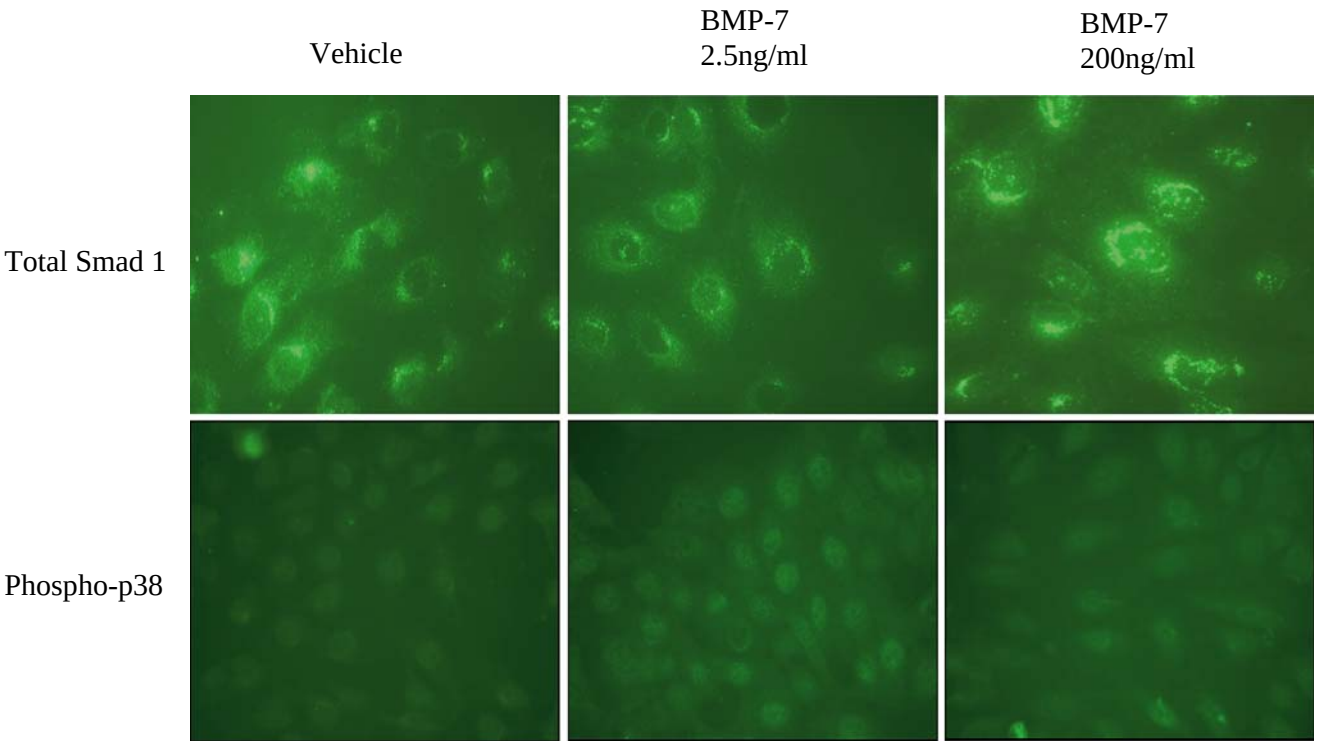

Fig. 4. Inverse relationship of high and low dose BMP-7 on Smad 1 and p38 cellular localisation. Treatment with 2.5 ng/ml BMP-7 results in no change in distribution of Smad 1 from the cytoplasm to the nucleus compared to the vehicle. In both there is peri nuclear distribution of total Smad 1. Treatment with high dose BMP-7 causes a clear translocation of Smad 1 to the nucleus. There is a clear phospho-38 nuclear localisation with 2.5 ng/ml BMP-7 compared to vehicle. BMP-7 at 200 ng/ml has minimal effect on distribution of phospho-p38 from cytoplasm to nucleus and is similar to vehicle treated group.

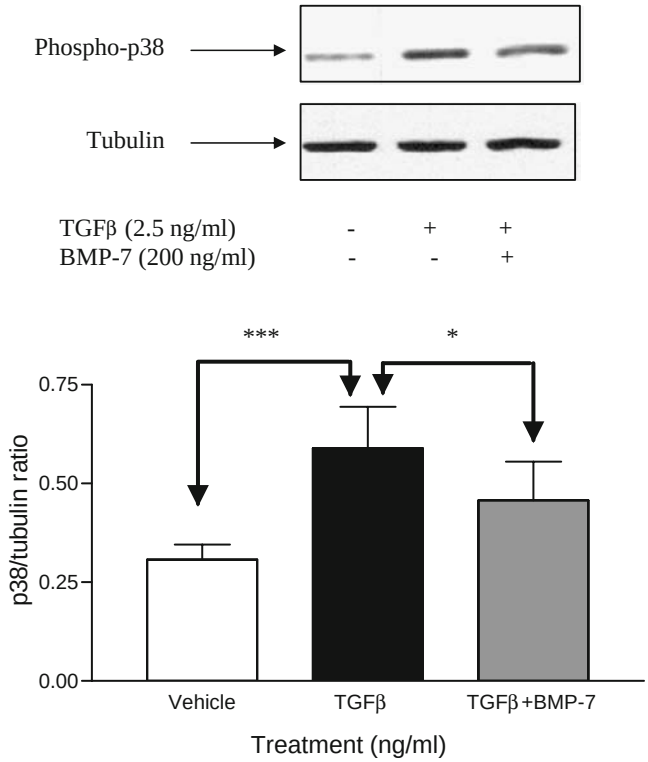

Fig. 5. BMP-7 at high dose has an inhibitory effect on phospho-p38 production. Western blot analysis shows TGFβ (2.5 ng/ml) causes a significant activation of phospho-p38 compared to vehicle ($n=6$, *triple asterisk* $p<0.001$). Co-treatment with BMP-7 (200 ng/ml) and TGFβ (2.5 ng/ml) significantly inhibited TGFβ induced p38 production (*single asterisk* $p<0.05$). The results are mean ± SD.

cellular matrix proteins (14). However the mechanism by which BMP-7 antagonises renal fibrosis remains unclear, particularly in PTECs; although it has been proposed that in mesangial cells the process was related to reduced TGFβ-induced Smad 3 signaling (18).

BMP-7, like other BMPs, can activate the canonical pathway whereupon ligand binding the type II receptor phosphorylates type I receptors (Alk 2, 3 and 6) with subsequent phosphorylation of Smad 1/5/8 and dimerization of the complex with Smad 4 (9). However, BMP-7 can activate the non-canonical pathways (MAP kinase) in a manner similar to TGFβ, although these pathways are less well understood and characterised. MAP kinase signaling pathways are important mediators of intracellular signal transduction pathways responsible for cell differentiation and cell growth.

In the present study, a novel dose dependent relationship between p38 MAP kinase and Smad 1/5/8 activation by BMP-7 in adult human PTECs was observed. At high concentration of BMP-7 (200 ng/ml) there is a clear activation of the canonical pathway (Smad 1/5/8) with no p38 MAP kinase activation. Others have shown in mouse collecting duct cells that at low concentration (7.5 ng/ml) BMP-7 activated p38 but at higher concentrations no activation was observed. However, these authors also demonstrated activation of Smad 1; based on this they proposed a model of Smad 1 inhibition of p38 (17). In our work BMP-7 at 7.5 ng/ml activated p38 MAP kinase at 15 min post treatment, however in some experiments Smad 1/5/8 activation was observed at this concentration and p38 activation did not reach statistical significance. For the first time to our knowledge, lower concentrations of BMP-7 at 2.5 ng/ml were used which clearly activated p38 MAP kinase significantly, 15 min post treatment in PTECs with no Smad 1/5/8 activation. These results show inverse relationship between p38 MAP kinase and Smad 1/5/8 activity that is dependent on the dose of BMP-7.

We hypothesized that activated Smad 1 is capable of suppressing p38 MAP kinase activity; hence BMP-7 activation of Smad 1 should be able to suppress TGFβ-p38 dependent outcomes. In this study significant activation of phospho-p38 was observed with TGFβ after 60 min. Co-treatment of TGFβ with high dose BMP-7 inducing Smad 1/5/8 significantly suppressed the activation of phospho-p38. This supports our proposition that the suppression of phospho-p38 activation is Smad 1 dependent and reiterates the inverse relationship between phospho-Smad 1 and phospho-p38 in the context of BMP-7 stimulation of these cells. BMP-7 at low dose which does not activate Smad 1/5/8 had no effect on TGFβ induced Fn secretion.

TGFβ induced fibrotic outcome such as Fn production have been proposed to be both Smad and MAP kinase signaling dependent. Fukami *et al.* in diabetic rat model has proposed TGFβ induced Fn secretion to be dependent on Smad signaling (25). Recent work from our group on HKC-8 PTECs has shown TGFβ induced Fn secretion to be reduced by incubation with the p38 MAP kinase inhibitor SB202190 and to be Smad independent (7). Uchiyama-Tanaka *et al.* in mesangial cells has also proposed that TGFβ induced Fn secretion to be sensitive to both inhibition of p38 and Erk 1/2 MAP kinase signaling (26). In our work presented here, we have shown a significant induction of Fn with TGFβ. Co-incubation with high dose BMP-7 significantly reduced the amount of secreted Fn compared to the TGFβ group. These results suggest that BMP-7 at high dose is activating Smad 1, which may have an inhibitory effect on phospho-p38 induction and accumulation with a subsequent reduction in secreted Fn.

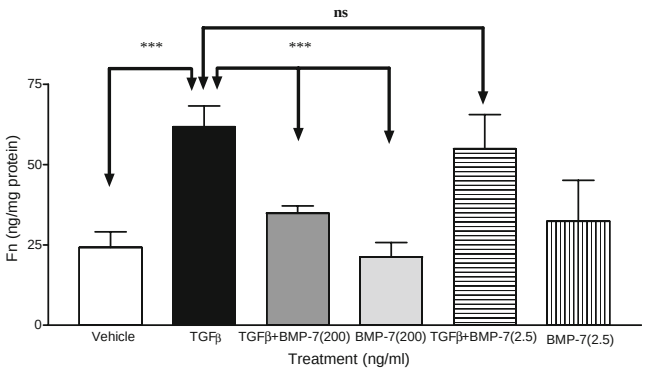

Fig. 6. Fibronectin production with TGFß and inhibition with high dose BMP-7. There was a significant induction of secreted Fn by TGFβ (2.5 ng/ml) treatment for 48 h. Co-treatment with BMP-7 (200 ng/ml) and TGFβ (2.5 ng/ml) significantly prevents this induction. BMP-7 alone is without effect ($n=3$, *triple asterisk* $p<0.001$). However co-treatment with low dose BMP-7 (2.5 ng/ml) and TGFβ (2.5 ng/ml) had no effect on the secreted Fn production. Low dose BMP-7 (2.5 ng/ml) alone is also without effect. Results are normalised to Fn secretion in the vehicle group and expressed as mean ± SD.

CONCLUSIONS

In the present study we have demonstrated a novel p38 MAP kinase activation pathway with a very low dose of BMP-7 at 2.5 ng/ml in PTECs. No activation of Smad pathway was observed at this concentration. At high dose of BMP-7 the reverse phenomena was true with activated Smad pathway and no activation of p38 MAP kinase pathway. These results would support the hypothesis that Smad 1 has an inhibitory effect on p38 MAP kinase activity. We have also presented novel results showing high dose BMP-7 may be regulating TGFβ MAP kinase signaling and fibrotic outcomes. As yet the full molecular mechanism underlying the above counter-regulation is unknown. There are currently no therapies which target Smads but there are a number targeting MAP kinase pathways. We believe that by identifying regulation of non-Smad TGFβ signaling we may identify novel targets for BMP-7 mimetics which will offer more precise therapies for renal fibrosis.

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