Functional characterization of podocan, a member of a new class in the small leucine-rich repeat protein family

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Abstract An important component of the extracellular matrix is the group of non-collagenous proteins belonging to the small leucine-rich repeat (SLR) protein family. A new SLR protein, podocan, with structural characteristics different from the known classes of the SLR protein family has been identified recently from the kidney. In this study, we examined the functional characteristics of this SLR protein expressed in cultured cells. Podocan was clearly observed intracellularly and was also detectable in the supernatant. Treatment of the expressed protein with various glycoenzymes suggested that podocan is a glycoprotein containing N-linked oligosaccharides but not a classical proteoglycan. Moreover, podocan was found to bind type 1 collagen. Cells transfected with podocan showed reductions in cell growth and migration, concomitant with increased p21 expression. Podocan mRNA was detected by reverse transcription polymerase chain reaction not only in the kidney, but also in other tissues including the heart and vascular smooth muscle cells, suggesting that podocan may have a potential role in growth regulation in cardiovascular tissues.

Key words: Podocan; Leucine-rich repeat; Growth; Migration; Collagen binding

1. Introduction

The extracellular matrix is a complex mixture of macromolecules including collagens, fibronectins, proteoglycans, and other related molecules, which interact with each other and other bioactive compounds, and comprise up to 30% of total body proteins. One of the components of the extracellular matrix is a group of non-collagenous glycoproteins known collectively as the small leucine-rich repeat (SLR) protein family [1]. A unique feature of the members of this family is the fact that they share a common structural motif, the leucine-rich repeat (LRR) domain, which is 20–29 amino acids long, contains asparagine and leucine residues in conserved positions, and has a general consensus sequence LXXLXLXXNXL/I [2].

Previous studies have demonstrated that the SLR proteins may play an important role in the pathophysiology of a variety of diseases including atherosclerosis and renal disease [3,4]. For example two major SLR proteins, biglycan and decorin, have been implicated in the control of cell growth, collagen deposition, and the activation and inactivation of cytokines and growth factors [1,2,5]. Expression of these SLR proteins in the kidney and vasculature is controlled by growth factors, cytokines, and vasoactive hormones, and is affected by drug treatment [6–8].

Recently, a novel member of the SLR protein family, podocan, which is expressed in the sclerotic glomerular lesion of experimental human immunodeficiency virus-associated nephropathy (HIVAN) has been identified [9]. Previously, the SLR protein family was divided into four classes based on the number of LRRs, the composition of the N-terminal cysteine cluster, and the number of exons. Podocan differs from the known SLR proteins in all three parameters, and can therefore be considered the first member of a new (fifth) class of the SLR protein family.

At present the function of this novel SLR protein is unclear. Recent studies have shown not only that the SLR proteins may play a major role in the pathogenesis of vascular and renal disease, but also that these molecules are potential candidates for the gene therapy of proliferative and fibrotic diseases [10,11]. The aim of this study was therefore to examine if podocan has functional effects on cell growth and migratory properties when expressed in cultured cells, and to examine if podocan is expressed in extra-renal tissues.

2. Materials and methods

2.1. Identification of the podocan gene and construction of recombinant podocan-tag fusion protein

The human biglycan protein was used as a query sequence in the screening of the human genome g scan protein database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to identify novel SLR proteins, and the best candidate sequence was found to be identical to the sequence of podocan which has been recently identified by representational difference analysis of cDNA in kidney podocytes from mice with and without HIVAN [9], and to an unidentified IMAGE consortium clone image 7489773R from a human brain library in pCMVSPORT6 (American Type Culture Collection (ATCC)). Human podocan cDNA was subcloned into the pcDNA3.1 V5/His-Topo vector (Invitrogen) after polymerase chain reaction (PCR) amplification of the podocan full-length coding region with the primers 5′-TTCCATCAGCCCT-3′ and 5′-TCTTGTTTCCTC-TTCCTCC-3′ using exTaq polymerase (Takara). In-frame ligation of the podocan cDNA into the pcDNA3.1 V5/His-Topo vector (Invitrogen) was performed according to the manufacturer's instructions, and the construct was sequenced using the ABI Prism Big Dye Kit (Applied Biosystems) to ensure fidelity.

2.2. Transfection of podocan into cultured cells

COS-7 cells and CHO-K1 cells were purchased from ATCC. For transient transfection assays, COS-7 cells were transfected with

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pcDNA3.1 V5/His-Topo vector containing podocan-V5 tag fusion cDNA, or with empty vector using lipofectamine [12]. For stable transfections, CHO-K1 cells were transfected either with pcDNA3.1 V5/His-Topo vector containing podocan-V5 tag fusion cDNA for immunocytochemical studies and immunoblot analyses, or with pCMVSPORT6 containing full-length native brain podocan cDNA for analysis of the effects of the protein on cell growth and migration. Control cells were transfected with vector alone. Stable transfectant were isolated by selection with geneticin (400 μg/ml).

2.3. Immunocytochemistry

Transfected COS-7 and CHO-K1 cells were incubated on coverslips in culture medium for 24 h. Cultures were fixed in ethanol at −20°C, washed, and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min. Cells were labeled with anti-V5 antibody (Invitrogen) at a concentration of 1:200 for 1 h at room temperature, washed, and then labeled with fluorescein isothiocyanate-conjugated anti-mouse goat IgG (Chemicon) diluted at 1:500. After the final wash, the cultures were embedded in Vectashield (Vector) and visualized with confocal microscopy (Karl Zeiss).

2.4. Enzymatic deglycation

The transfected CHO-K1 cells in six-well plates were washed and scraped into 200 μl of 0.5% Nonidet P-40 in PBS. After homogenization, the samples were cleared by centrifugation and subjected to glycoenzymatic digestions. Samples were digested with 10 mU chondroitinase ABC (Seikagaku Kogyo) in 1×chondroitinase ABC buffer (33 mM Tris–HCl, 33 mM sodium acetate, 0.08 mg/ml BSA) or 10 mU heparitinase I in 1×heparitinase buffer (0.1 M sodium acetate, 10 mM calcium acetate, pH 7.0) or 10 mU keratanase II in the same buffer at 37°C overnight. For *N*-glycosidase F digestion, the samples were boiled at 100°C for 3 min in 0.1% sodium dodecyl sulfate (SDS), 0.1 M β-mercaptoethanol, cooled on ice, then 0.05 M EDTA was added prior to digestion with 2 U *N*-glycosidase F (Hoffman-La Roche) overnight at 37°C. Samples were run on 4–12% SDS–polyacrylamide gel electrophoresis (PAGE) gels and transferred to nitrocellulose membrane (Amersham Bioscience), and subjected to Western blotting using anti-V5 antibody (1:500, Invitrogen) using previously described protocols [7].

2.5. SDS–PAGE of ^{35}S-labeled proteins

Transfected COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS) for 24 h and the medium was changed to sulfate-free medium supplemented with [^{35}S]sulfate (Amersham) (10 μCi/ml) for 48 h. The [^{35}S]sulfate-labeled medium was collected and digested with or without chondroitinase ABC as described above, and the ^{35}S-labeled proteoglycans were precipitated with 1.3% potassium acetate in ethanol. The precipitate was resuspended in 1×Laemmli buffer and run on 8–12% SDS–PAGE gels. The gels were fixed in 10% methanol, 20% glacial acetic acid, 70% water, vacuum-dried, then the labeled proteoglycans were visualized using a BAS 2000 image analyzer (Fuji).

2.6. Collagen binding assay

Collagen binding assays were performed using a modification of a method described by Svensson et al. [13]. CHO-K1 cells expressing podocan-tag fusion protein in six-well plates were washed twice with PBS and the cell layer was scraped in 200 μl of 0.5% Nonidet P-40 in 1×chondroitinase ABC buffer (33 mM Tris–HCl, 33 mM sodium acetate, pH 8.0, 0.08 mg/ml BSA). The collected cell layer was homogenized and centrifuged, then 0.1 ml of the lysate was incubated for 5 h at 37°C with or without 15 μl of acid-solubilized bovine type 1 collagen (1 mg/ml, Sigma). The incubated sample was centrifuged for 5 min at 10 000×*g*. The supernatant was removed and the precipitate was washed once with PBS. The precipitate, the supernatant, and a control uncentrifuged sample were subjected to Western blotting as described above using anti-V5 antibody as the primary antibody.

2.7. Assays of cell proliferation, thymidine incorporation, and cell migration

Subconfluent CHO-K1 cells transfected with native podocan cDNA were made quiescent by changing to serum-free medium for 48 h then stimulated with 2% FCS. To assess the cell numbers on days 0, 2, and 4 after the stimulation, cell proliferation was assessed using a water-soluble tetrazolium cell counting kit (Promega). To assess changes in [^{3}H]thymidine incorporation, [^{3}H]thymidine (New England Nuclear, 1 μCi/ml) was added 24 h after stimulation, then the cells were harvested for the quantitation of [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable material after a further 24 h [5]. Cell migration assays were performed using a modified Boyden's chamber method as described by Nozawa et al. [14] with minor modifications. In brief, cells were suspended in serum-free DMEM+0.1% BSA and added to chambers containing a 8 μm pore size polycarbonate filter (Chemotaxicel, Kurabo). 2% FCS was added to the lower chamber in DMEM+0.1% BSA. The chambers were incubated at 37°C for 12 h, then the filters were removed, fixed in methanol, and stained with Mayer's solution. Cells from four randomly chosen high-power fields on the lower surface of the filter were counted.

2.8. Assays of p21 expression, cdk2 expression, and Rho A activity

For the assay of p21 expression, CHO-K1 cells transfected with native podocan cDNA or vector were lysed in ice-cold RIPA buffer (1×PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, 1 mM sodium orthovanadate). Lysates were normalized for protein content, then subjected to Western blot analysis using anti-cdk2 and anti-p21 antibodies (Santa Cruz Biotechnology). Rho A activity was assessed by GTP-Rho affinity precipitation assays as described by Ren et al. [15]. Serum-starved cells were lysed in 50 mM Tris–HCl buffer, pH 7.2, containing 500 mM NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride. To detect GTP-associated active Rho A, lysates containing equal amounts of protein were incubated with Rhotekin Rho binding domain agarose (Upstate Biotechnology) at 4°C for 45 min. The bound protein were eluted with 1×Laemmli sample buffer, and detected by immunoblotting using a monoclonal antibody against Rho A (Upstate Biotechnology, Lake Placid, NY, USA). Total Rho expression was assessed by immunoblotting of an 1/20 aliquot from the supernatant prior to affinity precipitation. The relative intensities of the bands were assessed using computer densitometry software (Scion Image).

2.9. Reverse transcription (RT) PCR analysis of podocan mRNA expression in human tissues and cultured vascular smooth muscle cells (VSMC)

A commercial human multiple-tissue cDNA panel (Clontech) containing cDNAs from eight human tissues was used to screen for podocan mRNA expression. VSMC were obtained from rat aorta, and RNA purified by the acid guanidine–phenol–chloroform method as described previously [6]. Amplification reactions were carried out using AmpliTaq polymerase (Perkin-Elmer) with 30 amplification cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, and final elongation at 72°C for 10 min. The sense and antisense primers (5′-ACCTGCAAAACAACCGCCTG-3′ and 5′-TGTTCTTGAGGTG-CAGCTTG-3′) for studies on the human tissues resulted in a 366-bp product corresponding to bases 535–900 in the human sequence. The primers for studies on VSMC (5′-TGTCCAGCAACAACCTGT-3′ and 5′-GGGCTGGTGATGCGGTTGTA-3′) were designed from areas of high homology in the human and mouse sequences and resulted in a 373-bp product corresponding to bases 1042–1414. In some experiments, the reaction products were subcloned into pcDNA3.1 V5/His-Topo vector (Invitrogen) and sequenced to confirm identity with podocan. Commercial glyceraldehyde-3-phosphate dehydrogenase primers were obtained from Clontech.

2.10. Statistics

Statistical analyses were performed by analysis of variance using Statview software. Results are presented as mean ± S.E.M.

3. Results and discussion

3.1. Identification of the podocan gene in silico

We used the BLASTP algorithm to search for sequences homologous to biglycan in the human genome g scan protein database, and found a sequence identical to podocan, a SLR protein independently identified from the kidneys of mice with HIVAN [9].

As reported by Ross et al. [9], the four amino-terminal

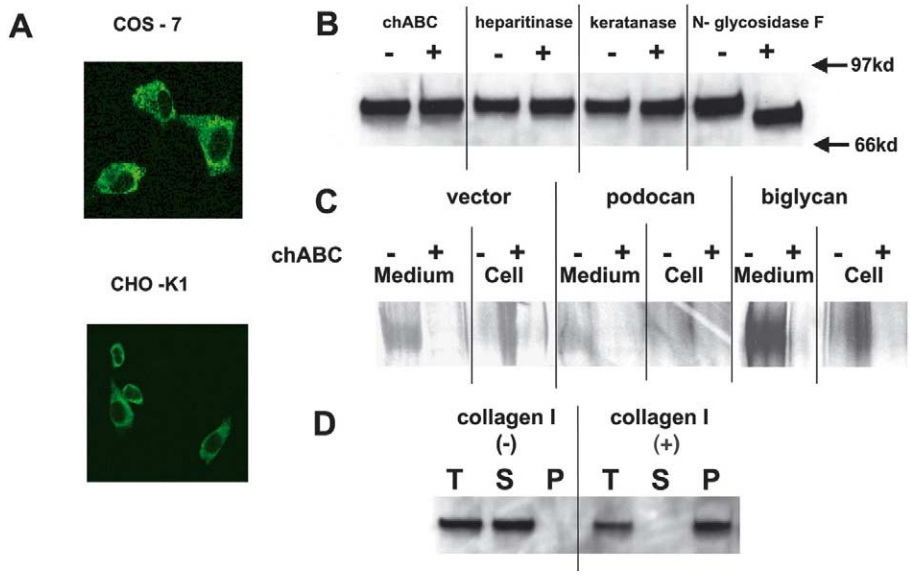

Fig. 1. A: Immunocytochemical visualization of recombinant podocan fusion protein in COS-7 and CHO-K1 cells. Cells were transfected with pCDNA3.1 V5/His-Topo podocan plasmid and immunostained by anti-V5 antibody as described in Section 2. B: Glycoenzymatic digestion of recombinant podocan-tag fusion protein. Recombinant podocan-tag fusion protein expressed in CHO-K1 cells was digested with or without the enzymes chondroitinase ABC (chABC), heparitinase, keratanase or *N*-glycosidase F. Digested products were subjected to Western blotting using anti-V5 antibody. C: SDS–PAGE of ^{35}S-labeled podocan-transfected COS-7 cells. COS-7 cells were transiently transfected with pCMVSPORT6-podocan or pcDNA 3.1-biglycan (positive control) or vector alone. The transfected cells were labeled with [^{35}S]sulfate and samples from the medium and the cell layer were subjected to SDS–PAGE. D: Collagen binding assays of podocan. Recombinant podocan-tag fusion protein was incubated with or without collagen type 1, centrifuged, and subjected to Western blotting as described in Section 2. T: total podocan, S: podocan in supernatant, P: podocan in precipitate.

cysteines of podocan show a CX3CXCX7C pattern, which is unlike any other characterized member of the SLR protein family. The podocan gene was localized in silico to human chromosome locus 1p23. Comparison of cDNA and genomic sequences shows that the gene spans over 23.3 kb, and consists of 13 exons. Since the composition of the amino-terminal cysteines, the number of LRRs (20), and the number of exons is different from known SLR proteins, podocan may be considered the first member of a new class in the SLR protein family.

3.2. Visualization of podocan in transfected cells

To investigate the glycosylation and functional properties of podocan, podocan cDNA was subcloned in-frame in a V5 tag-containing vector and expressed as a tag fusion protein in COS-7 and CHO-K1 cells. Immunostaining with anti-V5 antibody showed that the podocan-tag fusion protein could be clearly visualized in the cytoplasm but not on the cell membrane (Fig. 1A), suggesting that it is not a membrane-bound protein, a finding which is compatible with the absence of predicted transmembrane helices in the podocan protein motif. Moreover, the fusion protein was also detectable in the culture supernatants by immunoblotting, compatible with secretion of the protein into the supernatant (data not shown).

3.3. Glycoenzymatic characterization of podocan protein

The protein has four potential *N*-glycosylation site at Asn 214, 281, 299 and 410, defined by the consensus sequence Asn-Xaa-Ser/Thr. Treatment of podocan with *N*-glycosidase F confirmed that podocan contains N-linked oligosaccharides as shown by the change in its mobility on SDS–PAGE after treatment with *N*-glycosidase F (Fig. 1B). On the other hand, treatment of podocan with other glycoenzymes specific for glycosaminoglycan (GAG) side chains showed no mobility shift on SDS–PAGE. Moreover, COS-7 cells transfected with podocan fusion protein showed no detectable increase in ^{35}S-labeled proteins compared to vector-transfected cells, in contrast to cells transfected with the positive control SLR protein biglycan (Fig. 1C). Of interest, the observed molecular weight of podocan after *N*-glycosidase F digestion was similar to the expected molecular weight ($\sim$70 kDa) for unmodified protein based on the amino acid sequence. All these results suggest that podocan is an N-linked glycoprotein which is not expressed as a proteoglycan in these cells.

Although the majority of SLR proteins are covalently bound to GAG chains, there are exceptions such as asporin, another member of the class I extracellular matrix SLR proteins, which does not exist as a proteoglycan [16]. Although the podocan sequence contains one consensus GAG attachment motif (Ser-Gly) in its amino-terminus, it lacks the carboxy-terminal cysteine residues involved in disulfide formation. Some extracellular matrix SLR proteins with GAG side chains are known to exist in both non-sulfated and sulfated forms ('part-time proteoglycans'). For example, arterial lumican, one of the extracellular matrix SLR proteins and keratan sulfate proteoglycan, has no sulfated side chains, while corneal lumican exists in highly sulfated proteoglycan form [17,18]. Therefore, the possibility that podocan may exist as a proteoglycan in other cells and tissues cannot be ruled out.

3.4. Interaction of podocan with collagen

Recombinant podocan-tag fusion protein produced by transfected CHO-K1 cells was analyzed for its binding to type 1 collagen. When cell lysates were incubated without collagen then centrifuged, recombinant podocan protein was

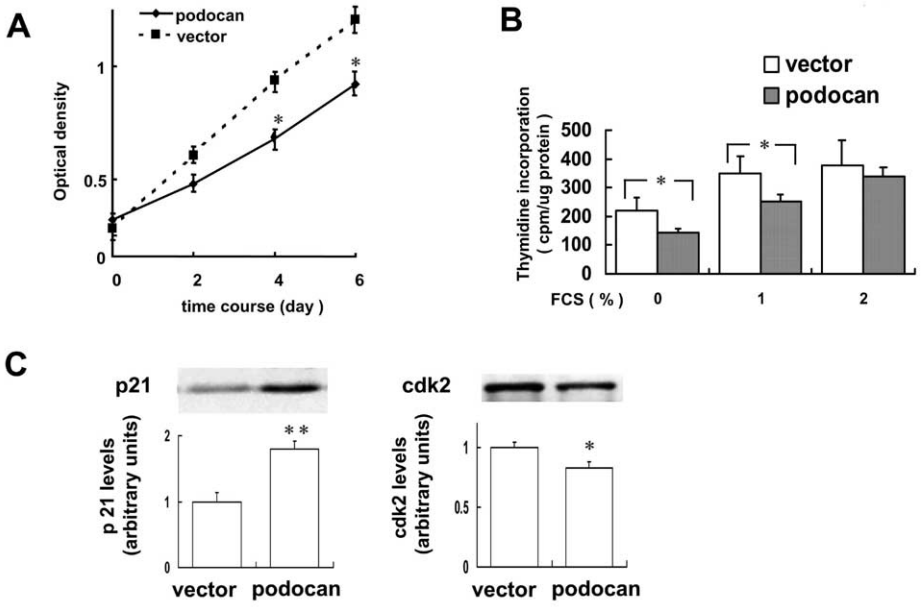

Fig. 2. Effects of podocan on cell proliferation. CHO-K1 cells were stably transfected with pCMVSPORT6-podocan or vector. A: Comparison of cell proliferation. B: Comparison of [^{3}H]thymidine incorporation. C: Comparison of p21 and cdk2 expression. Upper panel: Representative Western blot. Lower panel: Results of densitometry. Experiments were performed on cells from two independent transfections with similar results. $*P < 0.05$, $**P < 0.01$ podocan vs. vector ($n = 4$).

totally recovered in the supernatant. When the expressed podocan was coincubated with type 1 collagen, podocan recombinant protein coprecipitated with the collagen fibrils and none of the protein was detected in the supernatant, suggesting that podocan was bound to this collagen (Fig. 1D). Collagen binding is a common feature of SLR proteins, but the nature of the binding appears to depend on the sequence of each SLR protein, since decorin, one of the class I SLR proteins, binds to collagen via its LRR 4–5 on the core protein, whereas the related SLR protein biglycan does not [13,19].

3.5. *Effects of podocan on cell growth and cell cycle protein expression*

Several studies have shown that many SLR proteins have cell growth-regulatory properties [1,2]. Biglycan has an inhibitory effect on the proliferation of fibroblasts [5], while decorin inhibits cell growth through interactions with cell cycle proteins [20]. We hypothesized that podocan might also have an effect on cell proliferation because of its structural similarity to these SLR proteins. Comparison of cell proliferation and [^{3}H]thymidine incorporation showed suppression of cell growth and DNA synthesis in podocan-expressing CHO-K1 cells when compared with vector-transfected CHO-K1 cells (Fig. 2A,B). To examine if these changes were associated with changes in the expression of cell cycle proteins, we studied the expression level of the cell cycle regulatory proteins, p21 and cdk2 (Fig. 2C). Podocan overexpression was associated with an increase in p21 expression, whereas cdk2 expression in the same cells was attenuated. Activation of a cdk2–cyclin complex during the G1 phase is involved in the G1 to S phase transition and this complex is inhibited by the cdk inhibitor p21 [21]. Therefore our findings concerning p21 and cdk2 levels are consistent with the anti-proliferative effects of podocan which we found in this study. On the other hand, in order to assess the biological significance of the ob-

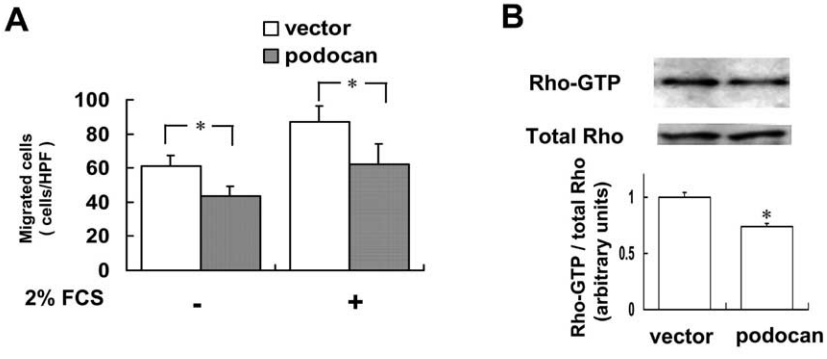

Fig. 3. Effects of podocan on cell migration. CHO-K1 cells were stably transfected with pCMVSPORT6-podocan or vector. A: Comparison of cell migration. B: Comparison of Rho activity. Upper panel: Representative Western blot. Lower panel: Results of densitometry. Experiments were performed on cells from two independent transfections with similar results. $*P < 0.05$ podocan vs. vector ($n = 4$).

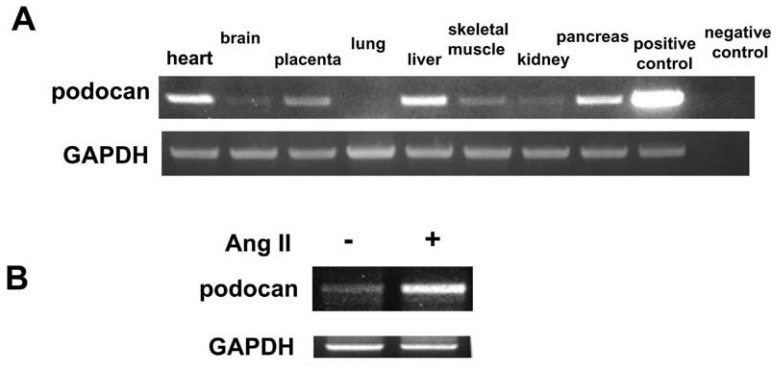

Fig. 4. Expression profiles of podocan mRNA in different tissues. A: RT-PCR analysis of a human multiple-tissue cDNA panel. B: RT-PCR analysis of podocan mRNA in cultured rat VSMC treated with or without angiotensin II (Ang II, 10^{-7} M) for 24 h.

served changes, further studies using exogenous podocan in a variety of cell lines are required, since the possibility that podocan has different effects in different cell types, or that exogenous podocan may have a different effect than endogenously overexpressed podocan cannot be completely ruled out.

3.6. Effects of podocan on cell migration and Rho A activity

Cell migration is crucial for many vascular pathological and physiological processes, such as the repair of injured blood vessels, angiogenesis, and atherogenesis. Cell movement is controlled by a variety of growth factors and extracellular matrix proteins and accompanied by the formation of stress fibers in vivo. Recently, Tufvesson showed that the small proteoglycans biglycan and decorin increase cell migration with cytoskeletal changes and the activation of Rho A in lung fibroblasts [22]. We therefore investigated whether the novel glycoprotein podocan affects cell migration and Rho activity using CHO cells stably expressing human podocan or vector. Overexpression of podocan in CHO cells resulted in significant suppression of cell migration compared to the cells transfected with the vector (Fig. 3A). Moreover, podocan-transfected cells showed a modest decrease in Rho A activity compared to vector-transfected cells (Fig. 3B).

3.7. Tissue distribution of podocan

RT-PCR analysis of a commercial cDNA panel was performed to screen for the tissue distribution of podocan in multiple human tissues. A weak but consistently detectable signal was found in the kidney. Of interest, more robust signals for podocan mRNA were also found in the heart as well as the liver and pancreas (Fig. 4A). RT-PCR analysis was also performed on cultured VSMC. Podocan mRNA was readily detectable in VSMC. Moreover, the expression of podocan mRNA appeared enhanced in cells treated for 24 h with angiotensin II (Ang II) (Fig. 4B), consistent with our previous report that Ang II is a major regulator of SLR protein expression in the vasculature [6].

3.8. Conclusion

The structural features of podocan have suggested that it is a member of a novel class of the SLR protein family. The results of this study suggest that it can be expressed in a non-proteoglycan form, associates with type 1 collagen, and exerts anti-proliferative and anti-migratory effects concomitant with changes in p21 and Rho activity. The finding of podocan mRNA expression in the heart and VSMC may be of importance, since members of the SLR protein family may play a major role in the pathogenesis of cardiovascular disease and atherosclerosis. Finally, other SLR proteins such as decorin are prospective candidates for the gene therapy of fibrotic and proliferative diseases. In view of the potential anti-proliferative effects of podocan, further studies are warranted to examine if podocan could be a useful addition to our armamentarium for the therapy of fibrotic or proliferative diseases.

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