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Original Research

Human podocytes responses to alternatively spliced Extra domain A Fibronectin in culture

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Abstract: The interactions of the extracellular matrix (ECM) proteins with cells strongly regulate cell behaviour. The glomerular basement membrane (GBM) is a dynamic structure made up of protein secreted by endothelial cells and podocyte. These proteins could regulate the behaviour of these cells in health and diseases. Extra Domain A + Fn (EDA+Fn) is an alternatively spliced form of Fibronectin (Fn) recently identified in GBM and a recognised marker of various pathologies. In this study for the first time, we have investigated the responses of human podocytes cultured on different composition of GBM proteins which are cellular Fn (EDA+), plasma Fn (EDA-) and collagen IV. Conditionally immortalised human podocyte were grown on the dishes coated with different matrices; collagen IV (Col IV), cellular fibronectin (CFn) containing the EDA Exon, plasma fibronectin (PFn), which lacks the EDA Exon (EDA-Fn). We have performed western blotting to characterise the expression of the different proteins, real time PCR and RT-PCR to look for gene expression and alternative splicing of EDA+Fn. We have used TGFβ1 as a stimulator. We have used HEK-Blue-hTLR4 cells to determine the biological activity of cellular Fn. Conditionally immortalised human podocyte show marked differences in their morphology grown on the dishes coated with different matrices; Col IV, CFn, and PFn. CFn was biologically active as it activated the TLR4 signalling in HEK-Blue-hTLR4 cells. Different matrices effects basal as well as TGFβ1 mediated alternative splicing of EDA+Fn. TGFβ1 was active on different matrices as it induced phosphorylation of pSmad3 however it did not affect phosphorylation of pAkt and p38. Interestingly, different cellular matrices affected basal phosphorylation of pAkt. CFn downregulated gene expression of synaptopodin and increased gene expression of collagen I and Fn. CFn increased cell death in detached human podocytes. Alteration of the constituents of the GBM is likely to significantly alter podocyte cellular responses to growth factors involved in podocytopathies, such as TGFβ.

Key words: Extra Domain A; Fibronectin; Podocyte; TGFβ1; Alternative splicing; Glomerular basement membrane; Extra cellular matrix.

Introduction

Glomerular basement membrane (GBM) is the 300-350 nm thick Extra cellular matrix (ECM) layer present between the endothelial cells and podocytes. GBM along with the neighbouring endothelial cells and podocyte cells forms the glomerular filtration barrier (GFB) in the glomerulus. GBM is a dynamic structure made up of proteins actively secreted by podocytes and endothelial cells. These proteins of GBM are capable of changing the responses of neighbouring cells in health and diseases. Podocytes are spectacular cell type which remains in tight contact with GBM and plays a critical role in filtration by forming slit diaphragm with foot processes of adjacent podocytes (4). In the adult, both podocytes and endothelial cells regularly add and assemble the matrix constituents of GBM, maintaining a hydrated mesh-like structure made up of collagen, laminin, entactin, agrin, perlecan and Fn (1,2,3). Change in quality and quantity of GBM proteins has been shown to be associated with different glomerular nephropathies. Collagen IV mutation is well characterised in Alport syndrome. Mutation in laminin leads to Pierson syndrome. Thickening of GBM results in podocyte effacement has been shown in diabetic nephropathy (3, 4).

Integrins are transmembrane heterodimer proteins which are present on the surface of the cell in the combination of αβ subunits; the combination is cell type specific. Integrins are shown to mediate outside-in signalling and inside-out signalling. Outside-in signalling involves the clustering of various kinases such as focal adhesion kinases (FAK) and integrin-linked kinases (ILK) (6–12). Selective deletion of ILK in podocytes is demonstrated to cause the aberrant distribution of SD protein nephrin which leads to early foot process effacement and morphological abnormalities (10). Podocytes have shown to express α3β1 integrins which have been described to bind with GBM proteins. Selective deletion of the α3 and β1 subunit in mice podocytes has shown to induce the podocyte abnormalities and proteinuria (6,13).

Fn is a high molecular weight, multifunctional, ECM protein. Up-regulation of Fn has been shown in various pathologies. Fn expression is recognised as a key marker of fibrosis. Extra Domain A + Fn (EDA+Fn) is an alternatively spliced isoform of Fn which is expressed in wound healing, in developing embryo and in pathologies such as cancer, lung fibrosis, renal fibrosis, skin fibrosis, liver fibrosis (12,14–18). EDA+ region is present adjacent to RGD sequence (Arg–Gly–Asp) sequence in cellular Fn (CFn) which has been shown to interact with various cell surface integrin and can

regulate different cell signalling cascades. EDA+Fn has been demonstrated to induce the fibroblast differentiation through binding to α4β7 integrin (19). In lung cancer cells EDA+Fn causes the epithelial–mesenchymal transition (EMT) through α9β1 integrin (20,21).

In this study for the first time, I have investigated the responses of human podocytes cultured on different composition of GBM proteins which are cellular Fibronectin (CFn) which contains Extra Domain A (EDA+Fn), plasma Fibronectin (PFn) which lacks Extra Domain A (EDA-Fn) and collagen IV (COL IV).

Materials and Methods

Cell culture

Conditionally immortalized human podocyte cell culture retrovirally transfected by temperature sensitive SV40 large T-antigen (Developed by Dr Moin Saleem, a kind gift from Jochen Raiser) were cultured as monolayer at the permissive temperature of 33°C in a humidified atmosphere of 5% CO_2 and 95% air, with RPMI 1640 medium supplemented with heat-inactivated 10% Fetal Calf Serum, L-glutamine (2mM)-penicillin (100U/ml)-streptomycin (100µg/ml) antibiotics, 5mM D-Glucose and insulin (5µg/ml)-transferrin (5µg/ml)-sodium selenite (5ng/ml). The medium was changed every alternate day. Confluent cells were passaged by aspirating the media and incubating with trypsin–EDTA solution (trypsin (5 g/l), Na2-EDTA (2 g/l), NaCl (8.5 g/l) for 4 minutes at 33°C temperature. Trypsin was neutralised with normal culture medium and cells were centrifuged at 350 x g for 6 min at room temperature. The cell pellet was re-suspended in fresh culture medium. Viable cells were counted with 0.4% trypan blue dye exclusion method and were seeded at a density of 10,000cells/cm^2. For experimental studies, podocytes were grown for 4 days at 33°C (permissive condition) and then at 37°C for 14 days (Non-permissive condition). All the experiments were conducted on overnight serum starved 80-90% confluent culture of terminally differentiated podocytes passages between 3 and 25.

Stimuli and inhibitors

Serum-starved differentiated podocytes at 37°C were treated with TGFβ1 2.5ng/ml or vehicle (0.1% w/v BSA). LPS (Lipopolysaccharide) was used as a positive stimulator of NFkB signalling pathway in HEK-Blue-hTLR4 cells.

Western Blot Analysis

After the treatment, cells were washed once with ice-cold 1 x PBS and lysed by cell scraper in 70µl ice-cold lysis buffer Tris/HCl (20mM), NaCl (150mM), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM EDTA, phosphatase inhibitor cocktail (1x) and protease inhibitor cocktail (2x). Cell lysates were kept on ice for 15min and centrifuged at 10000 x g at 4°C for 10min to remove cell debris. Supernatants containing protein were collected and the pellet containing cell debris was discarded. Supernatants containing proteins were either stored at -80°C for future use or was subjected to protein quantification. Total cellular protein concentration was determined using the colorimetric bicinchoninic acid protein assay kit (BCA) following manufacturer protocol.

The protein samples were prepared for western blotting under denaturing and reducing condition by heating at 70°C for 10min in a solution containing NuPAGE LDS Sample buffer (1x) and NuPAGE Sample Reducing Agent (1x). Equal amounts of cellular proteins were subjected to SDS-PAGE. Proteins were transferred onto PVDF membrane for 4h in NuPAGE transfer buffer at 30 V using the XCell II Blot Module (Life Technology). After transfer the blots were washed with TBS-T buffer [Tris-buffered saline/20mM Tris/HCl, 150mM NaCl and 0.1% (v/v) Tween 20) 5 % (w/v)] on 3D gyratory rocker for 15 min. Blots were then blocked with TBS-T fat-free milk 5 % (w/v) for 60min. Blots were washed for 10min x 3times with TBS-T. Blots were incubated with appropriate primary antibodies (Table 1) either in TBS-T with 5% (w/v) BSA (rabbit polyclonal antibodies) or in TBS-T with 5% (w/v) fat-free milk (Mouse Monoclonal antibodies) at 4°C overnight. Blots were washed for 10min x 3times with TBS-T buffer on a 3D gyratory rocker. Blots were incubated with horseradish peroxidase HRP-labelled secondary antibody for 1h at room temperature at 3D gyratory rocker and developed with ECL Prime enhanced chemiluminescence western blotting detection system and visualised with Hyperfilm ECL photographic film developed by MI-5 X-ray film processor (VWR). Blots were stripped and re-probed for housekeeping tubulin protein as a loading control.

Western blot films were imaged by ImageQuant 300 Imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

Table 1. Antibody dilutions used for Western blotting.

Target protein	Size(KDa)	Antibody	Dilution	Cat. no	Supplier
EDA+ Fn	220	Mouse monoclonal	1:1000	ab 6328	Abcam
Total Fn	220	Rabbit polyclonal	1:2000	Sc-9068	SantaCruz biotechnology
pSmad3	52	Rabbit monoclonal	1:1000	9520	Cell signaling
spP38	43	Rabbit polyclonal	1:1000	9211	Cell signaling
pAkt	60	Rabbit polyclonal	1:1000	9271	Cell signaling
Antimouse IgG(Secondary Ab)	-	Rabbit	1:40	A9044	Sigma-Aldrich
Antirabbit IgG(Secondary Ab)	-	Rabbit	1:1000	7074	Cell signaling
α/β-Tubulin	55	Rabbit polyclonal	1:1000	2148	Cell signaling

RNA extraction, reverse transcription and PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen UK Ltd) using manufacturer's protocol. All buffers for RNA extraction were provided as part of extraction kit. All disposable plastic wares were purchased as DNase and RNase free. Total cellular RNA concentration was then quantified by measuring the absorbance at 260nm and quality was measured by determining 260 to 230 ratio using NanoDrop Spectrophotometer. The extracted RNA was subjected to Reverse Transcription (RT) to synthesise cDNA. RT was performed to synthesise cDNA using Reverse Transcriptase system (Applied Biosystem, Foster City, CA, USA). The cDNA generated after the RT-PCR was subjected to conventional PCR amplification for EDA+/-Fn. The PCR reaction was performed using the Crimson Taq Polymerase (New England Biolabs) in a total volume of 25µl and the volume of cDNA template was 1µl from 1 in 10 diluted RT reactions. Each forward and the reverse primer was used at 20µM with 1.25 U polymerase per reaction. The inclusion of EDA exon was detected using a pair of primer binding constitutively spliced exon upstream and downstream of 270bp EDA exon. The PCR leads to inclusion of EDA exon which yields two products a 104bp band fragment corresponding to EDA-Fn and a 374bp fragment corresponding to EDA+Fn detected as two separate bands after the agarose gel electrophoresis.

The primer sequence for EDA+/-Fn was 5'GGAGAGAGTCAGCCTCTGGTTCAG3' Forward, 5'TGTCCACTGGGCGCTCAGGCTTGTG3' Reverse.

PCR steps were as: initial denaturation for 5 min at 95°C and 25 cycles of 30 sec at 95°C for, annealing temperature for 30 sec at 56°C, 30 sec at 68°C and final extension for 5 min at 68°C. The PCR product is separated by 1.5% agarose gel electrophoresis and visualised with ethidium bromide staining under UV light. The separated bands of EDA+/-Fn in the agarose gel were captured by ImageQuant 300 imager and ImageQuant Capture software (v1.0.0.4; GE Healthcare Life Sciences). Quantification of band density was done by Image count TL software (v1.0.0.4; GE Healthcare Life Sciences).

The 1:10 diluted cDNA synthesised after RNA extraction and reverse transcription PCR was used to study gene expressions by real-time PCR. Real-time PCR was performed by using TaqMan custom made FAM/MGB probes labelled TaqMan gene expression assay supplied by Applied Biosystems (Foster City, CA, USA). Each assay contains pre-formulated primers and TaqMan FAM/MGB probes in a 20x concentration and was supplied for 250 reactions at a 20µl reaction volume. Pre-optimized TaqMan gene expression assays containing FAM/MGB dye-labeled probes were used for the expression analysis of target gene of interest. TaqMan endogenous control for GAPDH gene as custom made primer and probe sets labelled with VIC/MGB reporter dye was used as a housekeeping gene to normalise the gene expression supplied by Applied Biosystems. To present the relative gene expression, the widely used comparative C_T method also referred to as the $2^{-\Delta\Delta C}$ method was used.

Assessment of biological activity of cellular Fn by TLR4 active HEK Blue cells

HEK-Blue-hTLR4 cells were designed and commercially available by Invivogen (San Diego, CA, USA). These cells were used for studying the stimulation of human Toll like receptor 4 (TLR4) receptor by ligand by monitoring the activation of NFkB signalling pathway. These cells were manufactured by co-transfection of the hTLR4, MD-2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene was placed under the control of an IL-12 p40 minimal promoter fused to five NF-kB and AP-1 binding sites. Stimulation with a TLR4 ligand activates NF-kB and AP-1 which induces the production of SEAP. Levels of SEAP can be determined with HEK-Blue detection or QUANTI-Blue, detection media that turn purple/blue in the presence of alkaline phosphatase. HEK-Blue hTLR4 cells and all the reagents were purchased from Invivogen and cultured as per the manufacturer's protocol. The level of SEAP was determined by measuring absorbance at 620-655nm by sunrise microplate absorbance reader using Xfluor4 software. Cell culture mediums used are as: Growth medium: DMEM, 4.5g/l glucose, 10% (v/v) fetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin, 100µg/ml Normocin antibiotic, 2mM L-glutamine. Freezing medium: DMEM, 4.5g/l glucose, 10% (v/v) fetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin, 100µg/ml Normocin antibiotic, 2mM L-glutamine, 10% (v/v) DMSO. Test Medium (used with Quanti blue): DMEM, 4.5g/l glucose, 10% (v/v) fetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin, 100µg/ml Normocin antibiotic, 2mM L-glutamine.

Cells Grown on different cellular matrices

35mm diameter cell culture plastic dishes were coated with the three different cellular matrices which are COL IV, CFn (EDA+Fn) and PFn (EDA-Fn) with the concentration of 5µg/cm^2. Cellular Fn (EDA+Fn), Plasma Fn (EDA-Fn) were diluted with a sterile balanced salt solution and Collagen IV was diluted with 10mM acetic acid to make 5µg/cm^2. Diluted solutions were added to each dish and air-dried for 60min. Remaining solutions were removed by aspiration. Podocytes were then grown on these dishes with 80-90% confluence till differentiation.

Differentiated podocytes detachment

Differentiated podocytes were detached and reattached on culture dishes or dishes coated with the different cellular matrices which are Collagen IV, Cellular Fn (EDA+Fn) and Plasma Fn (EDA-Fn) with the concentration of 5µg/cm^2. Podocytes were allowed to differentiate for 14 days. Completely differentiated podocytes were detached by trypsinization. Cells were centrifuged and suspended in the fresh culture medium. Cells were counted and reseeded on the culture dishes. Podocytes were then allowed for reattachment for 24h. Next day culture medium was changed. Cell death assay was performed by using Cyto Tox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) using manufacturer protocol.

Statistical analysis

Data handling, Statistical analysis and presentation were performed using Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism, v4.0 (GraphPad Software, Inc). Results were expressed as Mean ± SEM (standard error of the mean) of 3-6 independent experiments. Comparison between the means of 2 groups was made by Student's t-test. Comparison between the means of more than two groups was made by Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test. P value <0.05 was considered as significant. In our experiments we have considered the Collagen IV as a healthy component of extra cellular matrices and thus instead of plastic dishes as control we have used Collagen IV coated dishes as control.

Results

Characterization of alternatively spliced isoform EDA+Fn in CFn and PFn

To investigate the effect of EDA+Fn in human podocyte culture, I have used commercially available CFn isolated from human foreskin fibroblast and PFn from bovine plasma purchased from Sigma-Aldrich. In this experiment, I have characterised the presence and absence of EDA+Fn in CFn and PFn respectively by western blotting. I have subjected equal volume of CFn and PFn to western blotting. I have used antibodies targeted to EDA+Fn and Total Fn and looked for the presence of EDA+Fn (Figure 1, Panel B) and Total Fn (Figure 1, Panel A). The results from western blotting demonstrated that CFn contains the EDA, however, PFn lacks the EDA (Figure 1, Panel A and B).

Assessment of biological activity of CFn on TLR4 active HEK Blue cells

It has been shown that EDA+Fn can activate the TLR4 mediated signalling pathway. To test the biological activity of CFn (EDA+) isolated from human foreskin fibroblast, I have used HEK-Blue-hTLR4 cells, designed and commercially available by Invivogen which on activation with TLR4 ligand activates the NFkB signalling pathway which leads to the generation of blue colour product. The intensity of the blue colour represents the level of TLR4 activity. I have looked at TLR4 activation in HEK-Blue-hTLR4 cells by CFn (cell culture dishes coated with 5µg/cm^2 of cellular Fn) and compared with control by following the method described in Material and Method chapter section. I have used Lipopolysaccharide (LPS) 100ng/ml as a positive ligand for TLR4 activation in HEK-Blue-hTLR4 cells. The result from this studies demonstrated that CFn (EDA+) significantly induced the activation of the TLR4 pathway in HEK-Blue-hTLR4 cells. LPS 100ng/ml significantly induced the activation of the TLR4 pathway in HEK-Blue-hTLR4 cells (Figure 2).

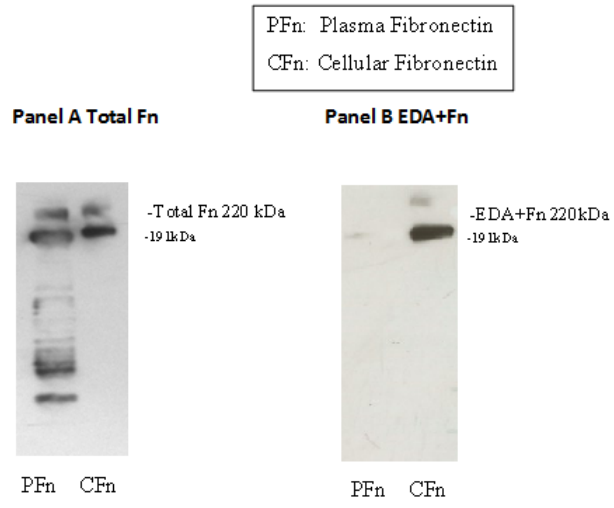

Figure 1. The expression of Total Fn (Panel A) and EDA+Fn (Panel B) protein in cellular Fn isolated from human foreskin fibroblast and plasma Fn from bovine plasma. An equal concentration (1µg/1µl) of cellular Fn from human foreskin fibroblast and plasma Fn from bovine plasma were subjected to western blotting and looked for expression of Total Fn and EDA+Fn. The results from western blotting demonstrate that cellular Fn contains the EDA+ however plasma Fn lacks the EDA+.

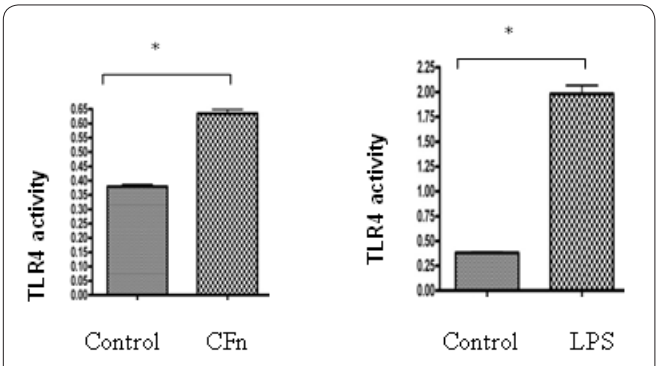

Figure 2. The TLR4 activity of cellular Fn (EDA+) and LPS (Lipopolysaccharide) as compared to control in HEK-Blue-hTLR4 cells. HEK-Blue-hTLR4 cells were treated with cellular Fn (cell culture dishes coated with 5µg/cm2 of cellular Fn), LPS 100 ng/ml and control by following the method described in Material and Method chapter section. Student's t-test is performed and the graph represents TLR4 activity expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

Effect of CFn, PFn and collagen IV on human podocytes cellular morphology

Podocytes were grown on 35mm cell culture dishes (plastic) or dishes coated with CFn (EDA+), PFn (EDA-) and COL IV with the concentration of 5µg/cm^2 by following the method as described in Material and Method section. Podocytes were allowed to differentiate for 14 days. Changes in cellular morphology were observed by taking a picture with phase contrast microscope (40x magnification). Phase contrast light microscope pictures demonstrated that differentiated human podocytes in culture exhibited visible changes in their morphology in response to CFn (EDA+), PFn (EDA-) and COL IV as compared to plastic as routine cell culture dishes (Figure 3).

Effect of CFn, PFn and COL IV on TGFβ1-mediated alternative splicing of EDA+Fn in human podocytes culture

Podocytes were grown on 35mm cell culture dishes (plastic) or dishes coated with CFn (EDA+), PFn (EDA-) and COL IV with the concentration of 5µg/cm^2 by following the method as described in Material and Method section. Podocytes were allowed to differentiate for 14 days. Serum-starved overnight and treated with TGFβ1

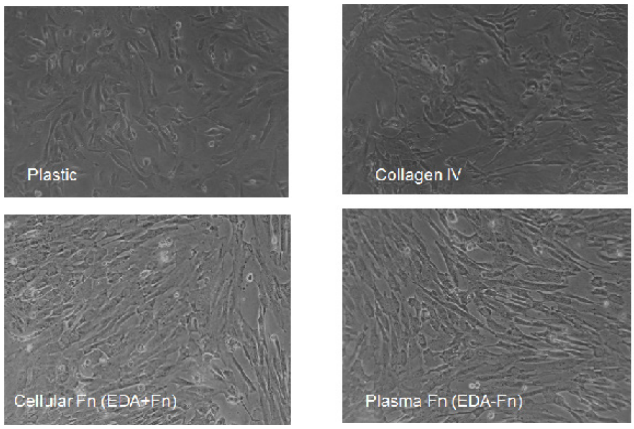

Figure 3. The phase contrast light microscope (40x magnification) pictures of differentiated human podocytes. Human podocytes culture with 80-90% confluence was grown on 35mm cell culture dishes (plastic) or dishes coated with cellular Fn (EDA+), Plasma Fn (EDA-) and collagen IV with the concentration of 5µg/cm^2. Differentiated human podocytes in culture exhibit visible changes in their morphology in response to EDA+Fn, EDA-Fn and collagen IV as compared to plastic as routine cell culture dishes. n=4 (independent experiments).

2.5ng/ml and vehicle (0.1% w/v BSA) for 24h. RT-PCR was performed to look for EDA+Fn to EDA-Fn mRNA ratio. TGFβ1 significantly increased EDA+/-Fn mRNA ratio as compared to vehicle on plastic in human podocytes. In human podocyte grown on CFn (EDA+) and PFn (EDA-) and COL IV display a distinct level of alternative splicing of EDA+Fn with or without TGFβ1 (Figure 4).

Effect of CFn, PFn and COL IV on TGFβ1-mediated signalling and synaptopodin gene expression in human podocytes culture

Podocytes were grown on 35mm cell culture dishes coated with CFn (EDA+), PFn (EDA-) and COL IV with the concentration of 5µg/cm^2 by following the method as described in Material and Method section. Podocytes were allowed to differentiate for 14 days. Serum-starved overnight and treated with TGFβ1 2.5ng/ml and vehicle (0.1% w/v BSA) for 1h. Cells were lysed and protein extraction was done. Western blot analysis was performed

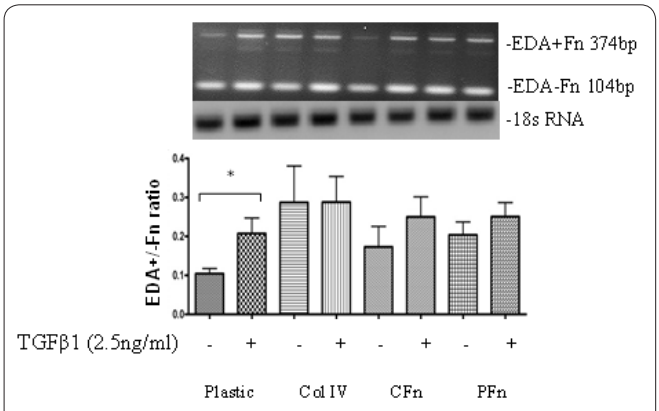

Figure 4. EDA+/-Fn mRNA expression in human podocytes culture grown on coated dishes. Serum-starved podocytes were treated with TGFβ1 2.5ng/ml and vehicle (0.1 % w/v BSA) for 24h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

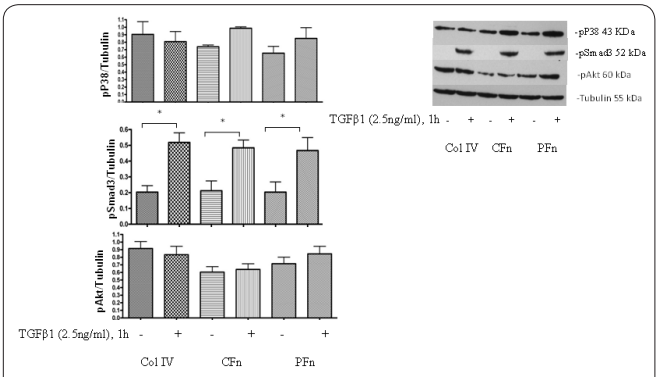

Figure 5. The phosphorylation of pSmad3, pP38 and pAkt indexed to tubulin as housekeeping protein corresponds to western blot. Podocytes were grown on 35mm cell culture dishes coated with 5µg/cm^2 of cellular Fn (EDA+), plasma Fn (EDA-) and collagen IV serum starved overnight and treated with TGFβ1 2.5ng/ml and vehicle (0.1 % w/v BSA) for 1h. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

to look for pSmad3, pP38, pAkt phosphorylation and tubulin as housekeeping protein. TGFβ1 significantly induced the phosphorylation of pSmad3 however it did not alter the phosphorylation of pP38 and pAkt as compared to vehicle in human podocytes grown on dishes coated with CFn, PFn and COL IV (Figure 5).

From the above experiment, I have looked at the basal expression of pAkt indexed to tubulin as housekeeping protein in human podocytes grown on dishes coated with CFn (EDA+), PFn (EDA-) and COL IV. This result shows that in human podocyte grown on CFn (EDA+Fn) shows significantly downregulated pAkt phosphorylation as compared to human podocytes grown on COL IV (Figure 6).

I have looked at the basal gene expressions of synaptopodin and GAPDH as a housekeeping gene in hu-

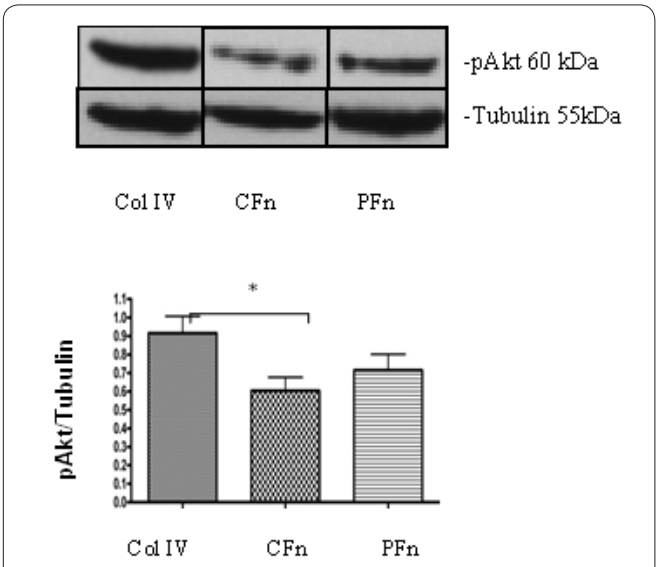

Figure 6. The basal pAkt expression indexed to tubulin as housekeeping protein. Podocytes were grown on 35mm cell culture dishes coated with 5µg/cm^2 of cellular Fn (EDA+), plasma Fn (EDA-) and collagen IV. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

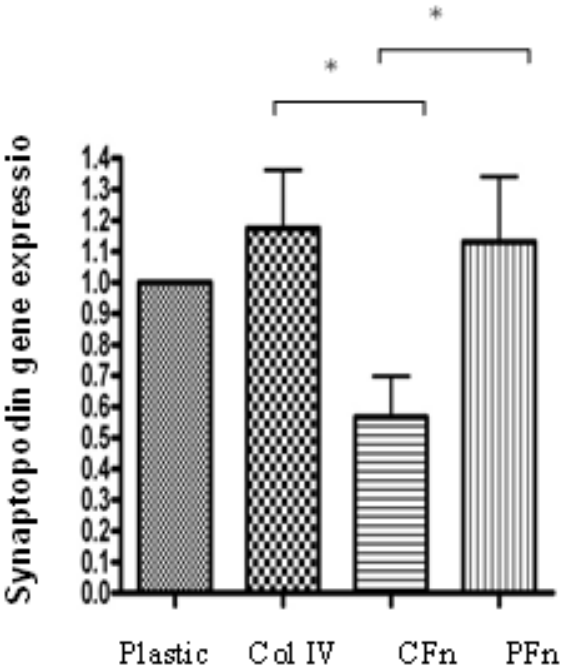

Figure 7. The basal gene expression of synaptopodin normalised to GAPDH gene expression. Podocytes were grown on 35mm cell culture dishes (plastic) or dishes coated with 5µg/cm^2 of cellular Fn (EDA+), plasma Fn (EDA-) and collagen IV. Analysis of Variance (ANOVA) with Bonferroni multiple comparisons post hoc test was performed. The graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

man podocytes grown on cell culture dishes (plastic) or dishes coated with CFn (EDA+), PFn (EDA-) and COL IV. In human podocytes grown on CFn (EDA+) shows significantly downregulated gene expression of synaptopodin normalised to GAPDH gene expression as compared to human podocytes grown on COL IV and PFn (EDA-) (Figure 7).

Effect of CFn and COL IV on gene expression of ECM proteins in human podocyte culture

Podocytes are grown on 35mm cell culture dishes coated with CFn (EDA+) and COL IV with the concentration of 5µg/cm^2 by following the method as described in Material and Method section. Podocytes were allowed to differentiate for 14 days. Cells were lysed and RNA was extracted. Real Time-PCR was performed to look for the gene expressions of Fn, collagen I and GAPDH as housekeeping gene. In human podocytes grown on CFn (EDA+) shows significantly induced gene expression of Fn and collagen I normalised to GAPDH gene expression as compared to human podocytes grown on COL IV (Figure 8).

Effect of CFn and COL IV on detached differentiated human podocytes

Podocytes were grown on 35mm cell culture dishes (Plastic) and allowed to differentiate for 14 days. Completely differentiated human podocytes were detached and reattached on 35mm cell culture dishes (plastic) or dishes coated with CFn (EDA+), PFn (EDA-) and COL IV with the concentration of 5µg/cm^2 by following the method as described in Material and Method section. Cell death was determined by cell death assay after 72h

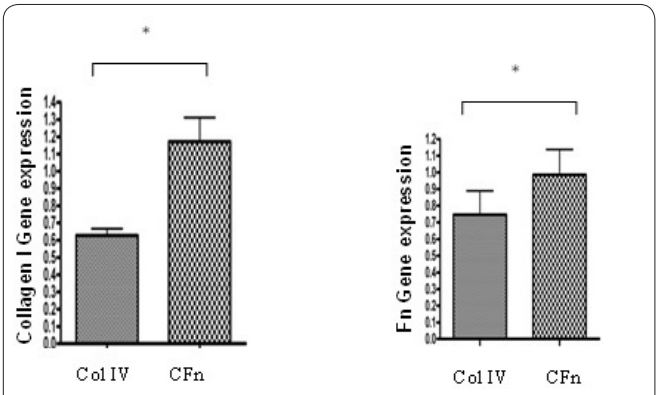

Figure 8. The gene expression of Fn and Collagen I normalised to GAPDH gene expression. Podocytes were grown on 35mm cell culture dishes (plastic) or dishes coated with 5µg/cm^2 of cellular Fn (EDA+Fn) and collagen IV. Student's t-test was performed and the graph represents band density expressed as SEM with n=4 (independent experiments). P value <0.05 was considered as significant.

of reattachment. In detached human podocytes grown on CFn (EDA+) shows significantly induced cell death as compared to human podocytes grown on COL IV (Figure 9).

Discussion

In these studies, I have investigated the effect of EDA+Fn on podocyte responses in culture. These studies describe the responses of human podocytes grown on CFn (EDA+), PFn (EDA-) and COL IV. I have investigated the human podocyte morphology, TGFβ1 mediated alternative splicing of EDA+Fn, TGFβ1 mediated signalling, gene expressions of podocyte differentiation marker synaptopodin, ECM protein Fn gene expression and scar protein collagen I gene expression and detached human podocytes cell death.

The CFn is synthesised by many cell types such as fi-

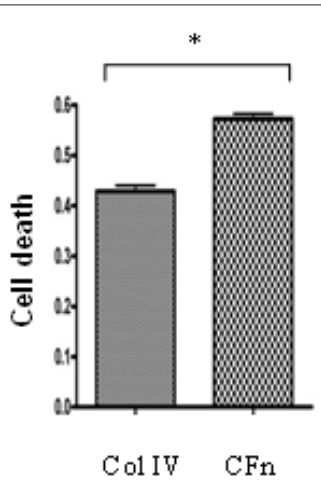

Figure 9. The cell death in detached human podocytes grown on cellular Fn (EDA+) and collagen IV. Podocytes were grown on 35mm cell culture dishes (Plastic) and allowed to differentiate for 14 days. Completely differentiated human podocytes were detached and reattached on 35mm cell culture dishes (plastic) or dishes coated with cellular Fn (EDA+) and collagen IV with the concentration of 5µg/cm^2. Cell death was determined by cell death assay. Student's t-test was performed and the graph represents band density expressed as SEM with n=4. P value <0.05 was considered as significant.

broblasts, epithelial cells, endothelial cells and shown to contains the various isoforms of Fn including EDA+Fn. PFn is soluble form synthesised by hepatocytes and shown to lack EDA+Fn. The CFn can exist as a mixture of both excluded and retained EDA and EDB segments whereas PFn does not contain any EDA or EDB domain (12, 24). In our studies, we have established the presence of EDA in CFn (purchased from Sigma-Aldrich) by western blotting. The results of this experiment suggest that CFn isolated from human foreskin fibroblast contains the EDA, however, plasma Fn from bovine plasma does not contain the EDA.

I have assessed the biological activity of CFn (EDA+) using HEK-Blue-hTLR4 cells. It has been shown that EDA+Fn can activate the TLR4 mediated signalling pathway similar to lipopolysaccharide (LPS) in HEK-Blue-hTLR4 cells (25). HEK-Blue-hTLR4 cells on activation with TLR4 ligand activates the NFkB signalling which can be quantified by spectrophotometric method. The result from this experiment suggests that similar to LPS, CFn (EDA+) isolated from human foreskin fibroblast was biologically active and activated the TLR4 receptor-mediated NF/kβ signalling in HEK-Blue-hTLR4 cells.

Maintenance of podocytes differentiated structure is key for active functioning of glomerular filtration barrier. Any alteration in podocyte specialised differentiated phenotype could disturb the podocyte SD structure and thus the active functioning of glomerular filtration barrier (4,23). The studies in mammary epithelial cells demonstrated that the switch in alternative splicing of structural protein 4.1 modulates the morphological state of epithelial cells. The human mammary epithelial cells grown on complex ECM show a marker of differentiation, an alternatively spliced isoform of structural protein 4.1. The switch in alternative splicing changes the pattern of expression of different alternative spliced isoform of structural protein 4.1 and thus alters the morphological state of mammary epithelial cells when grown on complex ECM (26). Similarly, in our studies, we have investigated the morphological changes in human podocytes grown on CFn (EDA+), PFn (EDA-) and COL IV with a phase contrast light microscope. Results from these studies show that on COL IV human podocytes display morphology as arborized, distinct cell bodies with interlinked processes similar to differentiated human podocytes grown on cell culture dishes (plastic) however cells appeared to be compressed in size. Podocytes grew on CFn and PFn display marked differences in their morphology from the morphology of podocytes grown on collagen IV and plastic. Human podocytes grown on CFn and PFn appeared to be more elongated and densely packed. Human podocytes grown on CFn and PFn appeared to be similar but not exactly same. These results suggest that ECM proteins can induce the alteration in the morphology of human podocytes culture. And more specifically the CFn (EDA+) could alter the podocyte morphology. Subsequently, I have investigated the podocyte differentiation marker synaptopodin gene expression. Podocytes grown on CFn (EDA+Fn) show a significant loss in gene expression of synaptopodin. Above mentioned results suggest that ECM proteins; CFn, PFn and COL IV could alter the podocyte differentiation morphology and change in morphology were associated with loss of podocyte differentiation marker synaptopodin.

The studies in hepatocytes demonstrated that hepatic cells grown on the basement membrane rich in laminin show downregulated inclusion of EDA+ in Fn as compared to hepatic cells grown on plastic. The laminin and collagen IV inhibit the inclusion of EDA+ in Fn. This study demonstrates the basement membrane components can regulate the alternative splicing of Fn (27). Similarly, in our study, I have investigated the effect of CFn, PFn and COL IV on the alternative splicing of EDA+Fn in human podocytes culture. Results from this experiment show that TGFβ1 had tended to induce the alternative splicing of EDA+Fn in human podocyte grown on CFn and PFn similar to human podocytes grown on cell culture dishes (plastic) however the induction was not statistically significant. In human podocytes grown on COL IV basal alternative splicing of EDA+Fn was comparatively higher than the human podocytes grown on cell culture dishes (plastic). However, TGFβ1 did not further induce the basal alternative splicing in human podocytes grown on COL IV. These results suggest that basement membrane components can alter the alternative splicing of EDA+Fn in human podocytes culture.

Further, we have investigated the TGFβ1 mediated signalling in human podocytes grown on CFn, PFn and COL IV. In human podocyte grown on COL IV shows up-regulated phosphorylation of pAkt. High basal phosphorylation of pAkt could have induced the high basal alternative splicing of EDA+Fn in human podocytes grown on COL IV. TGFβ1, however, did not induce pAkt phosphorylation in human podocytes grown on COL IV, CFn and PFn. This finding raised the question whether TGFβ1 was active on different cellular matrices. To answer this, we have investigated the TGFβ1 mediated pSmad3 phosphorylation in human podocytes grown on all the cellular matrices and results from this experiment suggest that TGFβ1 was active and induced the phosphorylation of pSmad3 in human podocytes grown on all the cellular matrices. In human podocytes grown on CFn and PFn, TGFβ1 tends to increase the pP38 phosphorylation. These results support the previous conclusion that PI3K/Akt pathway was involved in basal alternative splicing of EDA+Fn and p38 MAP kinase pathway was specifically involved in TGFβ1 mediated alternative splicing of EDA+Fn in human podocytes culture (results not shown).

Further, in human podocytes grown on CFn (EDA+) shows significantly induced gene expression of Fn and collagen I as compared to human podocytes grown on collagen IV. These results suggest that loss of differentiation of podocytes leads to podocytes to be more actively involved in developing the pathologies associated with ECM and scar protein up-regulation.

To establish that once human podocytes are differentiated and detached whether they could be able to reattach and survived on different cellular matrices, I have investigated the effect of the different cellular component on cell death of detached human podocytes. Detached human podocytes grown on CFn (EDA+) shows significantly induced cell death as compared to human podocytes grown on COL IV. These results demonstrated that detached differentiated human podocytes can

reattach however after the reattachment CFn (EDA+) increased cell death in detached human podocytes. This result suggests that the detached podocytes can reattach, but the survival of reattached podocytes depends on the composition of the ECM proteins.

Podocytes have been shown to express α3β1 integrins which have been described to bind with GBM proteins including Fn. Selective deletion of the α3 and β1 subunit in mice podocytes has shown to induce the podocyte abnormalities and proteinuria (13,6). Integrins are shown to mediate outside-in signalling and inside-out signalling. Outside-in signalling involves the clustering of various kinases such as focal adhesion kinases (FAK) and integrin-linked kinases (ILK). Selective deletion of ILK in podocytes is demonstrated to cause the aberrant distribution of SD protein nephrin which leads to early foot process effacement and morphological abnormalities (10). Another study suggests that urokinase interaction with αvβ3 integrin could lead to podocyte foot process effacement and proteinuria. Blockade of αvβ3 integrins reduces the podocyte motility and proteinuria in mice (28). Thus the possible mechanism of alteration of podocyte responses could involve the interaction of CFn (EDA+), PFn (EDA-Fn) and COL IV with podocyte integrins.

Altogether these studies describe that different components of cellular matrices specifically CFn (EDA+Fn) could alter the podocyte responses in health and diseases.

Alteration of the constituents of the GBM is likely to significantly alter podocyte cellular responses to growth factors involved in podocytopathies, such as TGFβ.

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