The shed ectodomain of type XIII collagen associates with the fibrillar fibronectin matrix and may interfere with its assembly *in vitro*

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Type XIII collagen is a transmembrane collagen, which is known to exist also as a soluble variant due to ectodomain shedding. Earlier studies with the recombinant ectodomain have shown it to interact *in vitro* with a number of extracellular matrix proteins, e.g. Fn (fibronectin). In view of its strong binding to Fn, we examined in the present study whether the released soluble ectodomain can bind to the fibrillar Fn matrix under cell-culture conditions and, if so, influence its assembly. In this study, we demonstrate that the type XIII collagen ectodomain of mammalian cells can associate with Fn fibres and may eventually hamper incorporation of the

fibrillar Fn meshwork. The association between type XIII collagen and Fn was implicated to be mediated by the C-terminal end of type XIII collagen and the N-terminal end of Fn. The results presented here imply that the shedding of the type XIII collagen ectodomain results in a biologically active molecule capable of remodelling the structure of the pericellular matrix.

Key words: assembly, extracellular matrix, fibronectin, remodelling, shedding, type XIII collagen.

INTRODUCTION

ECM (extracellular matrix) remodelling is a dynamic and complex cell-mediated process that takes place during normal morphogenesis and tissue repair as well as under many pathological conditions. The correct cyclical deposition and degradation of its components and the balanced maintenance of its organization are pivotal for the functional integrity of ECM. It follows that deregulated remodelling and abnormal structure of ECM are associated with disruption of tissue architecture and impaired organ functions and are hence considered hallmarks of many pathological conditions [1–6]. Collagens and Fn (fibronectin) are major ECM components [7,8]. Ubiquitous and abundant in ECM, the insoluble fibrillar Fn meshwork makes a pliant and dynamic scaffold providing cues for a series of cellular processes, like cell adhesion, migration, proliferation and differentiation [9–12]. Furthermore, previous data have pointed to the critical role of Fn in regulating the deposition and stability of other ECM proteins and in maintaining the composition of the cell–matrix adhesion sites [13–17]. Owing to this central regulatory role, the assembly of the Fn matrix and the factors controlling Fn polymerization have been targets of intensive study.

The accumulated data imply reciprocity and interdependence between the assemblies of the Fn and collagen fibrillar networks. The correct incorporation of collagens is reflected in the structural and functional integrity of the Fn matrix. For instance, fibroblasts lacking type I collagen synthesis or expressing a mutant form of this collagen, or showing reduced synthesis and secretion of type VI collagen failed to incorporate the Fn matrix in an adequate amount or in its proper three-dimensional organization [18–20]. Dermal fibroblasts with mutations in *COL5A1* and *COL3A2* genes resulting in aberrant protein products showed reduced levels of Fn in the culture medium and a lack of the fibrillar Fn network [21].

Also, type IV collagen is required for correct Fn fibrillogenesis in Schwann cells [22]. On the other hand, polymerization of type I collagen and type III collagen into ECM was previously shown to be dependent on Fn, with a preformed Fn meshwork being a prerequisite for the formation of the collagen network [16,17]. Additionally, mutual binding sites have been described both in Fn and type I collagen [9,12,18,23–27].

Type XIII collagen is an integral membrane-bound protein localized in focal adhesions of cultured cells [28,29]. In addition, due to ectodomain shedding by proprotein convertases, it is also present as a cleaved soluble variant released from the cells [30,31]. We have recently found type XIII collagen to be up-regulated during malignant transformation, particularly in the dysplastic and tumour stromal compartments [32]. Our results imply that such an up-regulation of expression and the concomitantly increased amount of the cleaved ectodomain can result in an altered microenvironment non-supportive of cell adhesion, spreading and migration, particularly when present with vitronectin [31]. Given that the recombinant purified ectodomain was earlier found to interact *in vitro* with several ECM proteins, in particular with Fn [33], we examined in the present study whether the released type XIII collagen ectodomain could have Fn-specific effects on ECM, e.g. by associating and modifying the ongoing Fn matrix assembly, and whether it therefore could be described as being involved in the remodelling of ECM.

MATERIALS AND METHODS

Cell culture

Primary human dermal fibroblasts, $\alpha 5$ integrin-positive CHO (Chinese-hamster ovary) cells and HT-1080 cells were grown in

Abbreviations used: CHO cells, Chinese-hamster ovary cells; COL3, collagenous domain 3 of type XIII collagen; delEC, a deletion variant of type XIII collagen lacking the extracellular domain; delIC, a deletion variant of type XIII collagen lacking the intracellular domain; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, foetal bovine serum; Fn, fibronectin; Fn120K, recombinant 120K fragment of Fn; FnIII₁-C, recombinant fragment of Fn consisting of the III₁ repeat unit; GAM, goat anti-mouse; GAR, goat anti-rabbit; IOD, integrated optical density; NC2, non-collagenous domain 2 of type XIII collagen; PFA, paraformaldehyde; RU, resonance unit; SAR, swine anti-rabbit; TEM, transmission electron microscopy; TRITC, tetramethylrhodamine β -isothiocyanate.

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DMEM (Dulbecco's modified Eagle's medium; Biochrom KG) with 10 % (v/v) FBS (foetal bovine serum; EuroClone). K562 cells were grown in Iscove's modified Dulbecco's medium (Gibco BRL) with 10 % FBS. Depletion of Fn from the serum was performed as described previously [23] with Gelatin Sepharose® 4B (Amersham Biosciences).

Antibodies

Synthetic peptides corresponding to the type XIII collagen NC2 (non-collagenous domain 2 of type XII collagen) and NC4 domains (residues 229–241 and 709–727 respectively) [34] were obtained from the peptide synthesis core facility at Biocenter Oulu (University of Oulu). The NC4 peptide was cyclized at cysteine residues and coupled with a carrier BSA (Imject® BSA; Pierce). The anti-NC4 domain antibody, named T22B, was produced in rabbits according to standard procedures [35] and affinity-purified as described earlier [28]. Its specificity was analysed by separating purified type I, III, IV, V and VI collagens, lysates of HT-1080 cells (positive control) and K562 cells expressing full-length type XIII collagen or the delNC4 variant on an SDS/8 % polyacrylamide gel, followed by immunoblotting. In a blocking experiment, T22B was blocked with a 4-fold molar excess of the peptide antigen before immunoblotting. Immunovisualization was done with ECL® (ECL Western Blotting Detection kit; Amersham Biosciences) or LumiLight detection (LumiLight Western Blotting Detection kit; Hoffman-La Roche). The production and purification of the anti-type XIII collagen NC3 domain antibody has been described earlier [28]. Anti-Fn antibody (IST-4) was purchased from Sigma and anti-Myc antibodies from NeoMarkers (clone 9E10.3) and Abcam (ab9106). The secondary antibodies GAM-Alexa 488 (where GAM stands for goat anti-mouse) was from Molecular Probes, SAR-TRITC (where SAR stands for swine anti-rabbit and TRITC for tetramethylrhodamine β -isothiocyanate) was from DAKO A/S and GAM-Cy2, GAM-Cy3 and GAR-Cy3 (where GAR stands for goat anti-rabbit) were from Jackson ImmunoResearch Laboratories.

Ectodomain association with the Fn matrix

The production and purification of the recombinant human type XIII collagen ectodomain has been described earlier [33]. CHO cells were cultured for 24 h with exogenously added 20 μ g/ml human Fn (Collaborative Biomedical Products) in serum-free DMEM to allow formation of a fibrillar Fn matrix. The cells were subsequently incubated with 20 μ g/ml recombinant type XIII collagen ectodomain in serum-free DMEM for 3 h. Primary fibroblasts were cultured in a serum-containing medium for 72 h to allow endogenous synthesis and deposition of the Fn matrix and a concomitant endogenous expression and cleavage of type XIII collagen ectodomain. Cells were fixed with 3 % (w/v) PFA (paraformaldehyde), stained with anti-Fn (IST-4) and anti-type XIII collagen NC3 domain antibodies and detected with GAM-Cy2 and GAR-Cy3 secondary antibodies. In the T22B blocking experiment, CHO cells were grown with exogenously added 20 μ g/ml Fn in serum-free DMEM for 24 h. Primary fibroblasts were grown in serum-containing medium for 48 h. The recombinant type XIII collagen ectodomain was preincubated with a 3-fold molar excess of T22B or rabbit non-immune IgG (Jackson ImmunoResearch Laboratories) before adding to cells for 3 h. In the peptide blocking experiment, primary fibroblasts, grown as in the T22B blocking experiment, were preincubated for 30 min with 20 μ g/ml of the type XIII collagen peptides

described above, followed by incubation with 20 μ g/ml recombinant type XIII collagen ectodomain and the peptides for 3 h. Cells were fixed with 3 % PFA, stained with anti-Fn (IST-4) and anti-type XIII collagen NC3 domain antibodies and detected with GAM-Alexa 488 and SAR-TRITC-labelled secondary antibodies. Cells were mounted in Immu-Mount™ medium (Shandon), viewed with an Olympus BX50 microscope (Olympus) and photographed with a DP50 digital camera (Olympus). Double exposures were made digitally with the analysisIS software (Olympus).

TEM (transmission electron microscopy)

CHO cells were supplemented with 20 μ g/ml Fn in a serum-free medium for 24 h, after which the medium was changed to serum-free DMEM containing 15 μ g/ml recombinant type XIII collagen ectodomain for 3 h. The staining method has been described by others [36,37]. In short, the cells were fixed with 3 % PFA, blocked with 0.05 % glycine-PBS and 5 % (w/v) BSA with 0.1 % CWFS (cold water fish skin) gelatin-PBS (Aurion) and incubated with 20 μ g/ml anti-Fn antibody (IST-4), followed by rabbit anti-mouse antibody (Zymed Laboratories). Fn-bound antibody complexes were detected with Protein A-conjugated 5 nm gold particles (G. Posthuma, University of Utrecht, The Netherlands). After fixation with 1 % glutaraldehyde-PBS, the cells were incubated with anti-type XIII collagen antibody, which was detected with Protein A-conjugated 10 nm gold particles (G. Posthuma). To verify correct immunostaining of Fn and type XIII collagen ectodomain, control stainings with either of the primary antibodies omitted were performed. After immunolabelling, the cells were fixed in 2.5 % (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), post-fixed in 1 % osmium tetroxide (Electron Microscopy Sciences) and embedded in Epon Embed 812 (Electron Microscopy Sciences). Thin sections were cut with a Reichert Ultracut ultramicrotome and examined with a Philips CM100 transmission electron microscope. Images were captured by means of a CCD (charge-coupled-device) camera equipped with TCL-EM-Menu version 3 (Tietz Video and Image Processing Systems GmbH).

Expression constructs

The expression plasmid type XIII collagen was created in the mammalian expression vector pcDNA3.1(–)/Myc-HisTagB (Invitrogen) by ligating the NotI–EcoRI-digested full-length type XIII collagen cDNA lacking the stop codon, which was eliminated by site-directed mutagenesis (GeneEditor™ *in vitro* Site-Directed Mutagenesis system; Promega) to include an in-frame vector-derived Myc tag. The deletion variant delCOL2-NC4 (where COL2 stands for collagenous domain 2 of type XIII collagen) was created by ligating the NotI- and BamHI-digested full-length type XIII collagen cDNA into the pcDNA3.1(–)/Myc-HisC vector. The deletion variants delIC (a deletion variant of type XIII collagen lacking the intracellular domain), delEC (where EC stands for extracellular) and delCOL3-NC4 were made by site-directed mutagenesis, by creating new EcoRI restriction sites at either the 5'-end (delIC) or the 3'-end (delEC, delCOL3-NC4 and delNC4). The ensuing clones were digested with EcoRI (delIC) or with NotI and EcoRI (delEC, delCOL3-NC4 and delNC4), followed by insertion into the pcDNA3.1(–)/Myc-HisTagB vector. The primers (Sigma-Genosys) used in the mutagenesis were as follows: 5'-GACAGTGGCGCTGGCTGAGC-AGTGGCGCTAGGGAATTCATGCTGCCGAGCCCCGGGTG-CTGCCGACTGCTGGC-3' (for delIC), including an artificial

in-frame ATG codon, 5'-TTCGCTGGCGCTCAGCCTGCTCG-CCCACTTTGAATTCCGGACCGCGGAGCTGCAGGCCCCG-GGTGCTG-3' (for delEC), 5'-GCTTCAGGAGATCAGGACG-CTGGCCTTGATGGAATTCGGGCCTCCTGGTCTTCTTGG-ACAAACAGGCCC-3' (for delCOL3-NC4) and 5'-AAGGG-GGACCAAGGAGCACCTGGACTAGACGAATTCGCCCC-CTGCCCCGCTGGGAGAAGATGGC-3' (for delNC4). The 5'- and 3'-ends of the inserts were sequenced with an ABI Prism 377 DNA sequencer (Applied Biosystems). The plasmids were purified by an alkaline lysis method [38]. The enzymes were purchased from Amersham Biosciences.

Analysis of shedding of type XIII collagen deletion variants

Equal numbers of CHO cells were transfected with 3 µg of a given plasmid cDNA and 4.5 µl of FuGENE6 transfection reagent (Boehringer). The release of type XIII collagen deletion variants into the medium was verified by immunoblotting the methanol-precipitated culture medium with anti-Myc antibody (NeoMarkers). Densitometric scanning of the Western blots was performed with a GS-710 densitometer (Bio-Rad Laboratories) using Quantity One 4.4.1 software (Bio-Rad). For the immunofluorescent stainings, Fn matrix assembly was induced by adding exogenous 20 µg/ml Fn at the initiation of transfection. After 48 h, the cells were fixed with 3% PFA and stained with anti-Myc (Abcam) and anti-Fn (IST-4) antibodies, followed by SAR-TRITC and GAM-Alexa 488 antibodies.

Biacore™ interaction studies

The surface plasmon resonance technology (Biacore™) was used to determine the Fn domain(s) responsible for the interaction with type XIII collagen ectodomain. Ectodomain (20 µg/ml) in 10 mM sodium acetate (pH 5.5) was covalently immobilized by amine coupling with a carboxymethyl dextran CM5 sensor chip (Biacore™) activated with a mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide followed by ethanolamine. The experiments were run at +25°C using different concentrations of the full-length Fn or its recombinant fragments [Fn120K (recombinant 120K fragment of Fn), Fn70K, Fn45K, Fn40K, Fn30K and FnIII₁-C (recombinant fragment of Fn consisting of the III₁ repeat unit); Sigma or Chemicon]. The preliminary tests ($n=2$) with all the samples were run at concentrations of 25, 50 and 100 nM, whereas, with the Fn fragments that had initially shown binding to type XIII collagen ectodomain, the kinetic tests ($n=3$) were run at concentrations of 0, 20, 50, 100, 300 and 900 nM diluted in the running buffer of 50 mM Tris/HCl (pH 7.4) and 150 mM NaCl in order to reduce the bulk effect. The analytes were passed over the sensor chip at a constant flow-rate of 30 µl/min for 180 s. At the end of each cycle of the analysis, the sensor chip was regenerated with 2.5 M NaCl and 5 mM NaOH. The amount of analyte bound to the immobilized ligand was monitored by measuring the variation in the plasmon resonance angle as a function of time and expressed in terms of RU (resonance unit). The background signal was subtracted from the recorded values by a simultaneous injection of analytes over a blank surface (reference cell) composed of ethanolamine-substituted dextran and activated like the sample cells, but without the ligand. The fitting of the crude data, preparation of the overlay plots and determination of K_D (dissociation constant) values were performed using the Biaevaluation 3.0 software (Biacore™).

Analyses of Fn matrix assembly and morphology

For the immunofluorescent staining, equal numbers of primary fibroblasts were plated on coverslips in an Fn-depleted medium

Figure 1 Co-localization of type XIII collagen ectodomain with the Fn matrix

TEM analysis by the immunogold technique showed alignment of the recombinant type XIII collagen ectodomain with fibrillar Fn. Cells were labelled with anti-Fn antibody (shown by arrows) and anti-type XIII collagen NC3 domain antibody (shown by arrowheads) and detected with Protein A conjugated with 5 and 10 nm gold respectively. Scale bar, 200 nm.

and were allowed to stay for 2 h to ensure proper cell adherence. The culture medium was then changed to serum-free DMEM with exogenous 10 µg/ml Fn and 50 µg/ml recombinant type XIII collagen ectodomain or type I collagen for 3 h, followed by fixation with 1% PFA and staining with anti-Fn (IST-4) and GAM-Cy3 antibodies. The experiment was repeated with 100 µg/ml recombinant collagens. Capturing of the fluorescence images with identical settings and the quantitative evaluation of Fn matrix-associated fluorescence were done as described by others [21], with minor modifications. The analyses were done on ten randomly selected viewing fields per sample with MetaMorph 6.1 image analysis software (Universal Imaging) and results were expressed in terms of IOD (integrated optical densities) related to the area containing the fluorescent signal. For the quantification of the endogenous Fn assembly, equal numbers of primary fibroblasts were plated in a serum-containing medium. After adhering for 2 h, the cells were thoroughly washed with PBS and supplemented with 50 µg/ml recombinant type XIII collagen ectodomain, type I or type VI collagen in serum-free DMEM for 24 h. The Fn matrix isolation procedure has been described by others [39]. In short, the cells were disrupted with 25 mM NH₄OH, washed with PBS, followed by isolation of the Fn matrices with 10 mM Tris/HCl (pH 8.0), 8 M urea, 1% SDS and 15% (w/v) 2-mercaptoethanol. Samples were boiled for 5 min and equal volumes of samples were separated on an SDS/5% polyacrylamide gel under reducing conditions, followed by Western blotting with anti-Fn antibody (IST-4). Densitometric scanning of the Western blots was performed with a GS-710 densitometer (Bio-Rad).

RESULTS

Association of type XIII collagen ectodomain with the fibrillar Fn matrix

The hypothesis that type XIII collagen ectodomain associates with the fibrillar Fn matrix *in vitro* was tested with CHO cells and primary fibroblasts. Double immunofluorescent staining with anti-type XIII collagen and anti-Fn antibodies showed that, in

Figure 2 Analysis of shedding of type XIII collagen deletion variants

(**A**) Schematic representation of the full-length type XIII collagen and the deletion variants covering both the ectodomain and the intracellular domains. White boxes denote collagenous sequences, grey boxes non-collagenous sequences and black boxes transmembrane domains. Abbreviations: NC, non-collagenous domain; COL, collagenous domain; IC, intracellular domain; EC, ectodomain. (**B**) CHO cells were transiently transfected with a given plasmid cDNA for 48 h. The release of type XIII collagen deletion variants into the cell-culture medium was detected by immunoblotting the methanol-precipitated medium with anti-Myc antibody. Sample 1, type XIII collagen ectodomain; sample 2, delIC; sample 3, delEC; sample 4, delCOL2-NC4; sample 5, delCOL3-NC4; sample 6, delNC4. Arrowheads on the right point to the masses of the detected type XIII collagen deletion variants. The 50 and 30 kDa bands in the blot result from unspecific binding of the antibody to unrelated Myc epitopes. The blot shown is representative of three independent assays. (**C**) Densitometric scanning of the Western blots ($n=3$) shows that the type XIII collagen ectodomain variants are released from the plasma membranes in comparable amounts with negligible differences. Results are expressed as means with the bars indicating the S.D.

CHO cell culture with the matrix formed from exogenously added Fn, the recombinant ectodomain fully associated with the Fn fibres (results not shown). In line with this, with primary fibroblasts, the endogenously synthesized and cleaved type XIII collagen ectodomain organized into an overlapping pattern with the endogenously deposited fibrillar Fn matrix (results not shown). Ultrastructural analysis by the immunogold technique and TEM verified the impeccable linear alignment of type XIII collagen ectodomain along the Fn fibres (Figure 1). The specificity of the double immunostaining was controlled by excluding either anti-type XIII collagen NC3 domain or anti-Fn antibodies from the staining method. If anti-type XIII collagen antibody was left out, only Fn fibres labelled with 5 nm gold particles were correctly detected in the sections. If anti-Fn antibody was omitted, only Fn-associated type XIII collagen ectodomain labelled with 10 nm particles was detected (results not shown). These excluded unspecific cross-reactions in the immuno-TEM double staining.

Figure 3 Association of the type XIII collagen ectodomain variants with the fibrillar Fn matrix

CHO cells were transfected with a given plasmid cDNA, with Fn matrix assembly induced by exogenous Fn supplementation at the initiation of transfection. After 48 h, the cells were fixed and stained with anti-Myc and anti-Fn antibodies. Immunofluorescent double stainings showed co-localization of the shed ectodomains of (**A**) type XIII collagen and (**B**) delIC with Fn, as shown by the yellow colour in the merged images. (**C**) delNC4 showed a grossly diminished ectodomain association with Fn, resulting in very faint type XIII collagen and overlay images. In some images, the merged signal at the cell surfaces of the transfected cells is likely to stem from the pericellular Fn fibres overlapping the unshed type XIII collagen present on the plasma membrane. Scale bar, 10 μ m.

The C-terminal end mediates binding of type XIII collagen to Fn

A series of expression plasmids encoding Myc-tagged full-length type XIII collagen and deletion variants covering both the ectodomain and intracellular domains were constructed for the characterization of the domain(s) of type XIII collagen involved in mediating the association with Fn (Figure 2A). As analysed by Western blotting of the methanol-precipitated culture media, the transiently transfected CHO cells showed the release of the ectodomain deletion variants into the culture media in correct masses corresponding to the calculated masses based on amino acid sequences (Figure 2B). The densitometric quantifications of the precipitated ectodomain variants ensured that the shedding of these variants had occurred at a sufficiently similar efficiency to allow the presence of comparable amounts of the soluble ectodomain variants in the media (Figure 2C). Subsequently, immunofluorescent double stainings with anti-Myc and anti-Fn antibodies revealed that the cleaved ectodomains of the full-length type XIII collagen (Figure 3A) and delIC variant (Figure 3B) had associated with the Fn fibres. The overlapping alignments were visualized by the yellow overlay colour in the merged images (Figures 3A and 3B, Merge). Interestingly, delNC4 showed a markedly reduced association with the Fn matrix (Figure 3C). The truncated ectodomain variants of delCOL2-NC4 and delCOL3-NC4 did not associate with the Fn matrix at all (results not shown), and as expected, no ectodomain staining was observed with the delEC variant (results not shown).

The data implied that the conserved C-terminus of type XIII collagen is involved in mediating the association between type XIII collagen ectodomain and Fn. This was studied further by producing a specific polyclonal antibody T22B against the NC4 domain. In the tests for its specificity, the affinity-purified T22B detected a protein with identical mass with that recognized by the previously described anti-NC3 domain antibody [28]. Binding to

Figure 4 T22B antibody blocks the type XIII collagen ectodomain association with Fn

(A) T22B detected a protein with identical mass with that recognized by the previously described anti-NC3 domain antibody. Binding was blocked by a peptide corresponding to the epitope of T22B. No cross-reactivity against type I, III, IV, V or VI collagens was observed. HT-1080 cell lysate was included as a positive control. The domain specificity of T22B was verified by the lack of binding to delNC4 variant expressed in K562 cells. In the T22B blocking experiment (B, C) CHO cells with an Fn matrix made of exogenous Fn and (D, E) primary fibroblasts with an endogenously deposited matrix were treated with type XIII collagen ectodomain preincubated with T22B or rabbit non-immune IgG. Immunofluorescent staining with anti-type XIII collagen NC3 domain antibody revealed that the type XIII collagen ectodomain association with the Fn matrix was almost completely blocked with T22B (B, D), but not with non-immune rabbit IgG (C, E). Fn matrices stained with anti-Fn antibody are shown in the insets. Scale bar, 20 μ m. Abbreviations: COLI, type I collagen; COLIII, type III collagen; COLIV, type IV collagen; COLV, type V collagen; COLVI, type VI collagen; COLXIII, type XIII collagen.

type XIII collagen was blocked by a peptide corresponding to the epitope of T22B, and no cross-reactivity against type I, III, IV, V or VI collagens was observed (Figure 4A). The domain specificity of T22B was ascertained by the lack of binding of T22B to delNC4 variant expressed in K562 cells (Figure 4A). Type XIII collagen ectodomain was preincubated with T22B and then applied to either CHO (Figure 4B) or primary fibroblast (Figure 4D) cell cultures with preformed Fn matrices (Figures 4B and 4E, insets). This blocked almost completely the association between type XIII collagen ectodomain and Fn fibres of both cell cultures. As a control, pretreatment of type XIII collagen ectodomain with non-immune rabbit IgG did not accomplish the same (Figures 4C and 4E). The epitope peptide corresponding to the NC4 domain did not show a similar blocking effect, nor did a control peptide against the NC2 domain (results not shown).

The N-terminal end mediates binding of Fn to type XIII collagen

The surface plasmon resonance method was used to pinpoint which domain(s) of Fn mediate its binding to type XIII collagen ectodomain. Our group has previously characterized the interaction of the full-length Fn with type XIII collagen ectodomain *in vitro* [33], and in the preliminary tests, we also confirmed this binding in our present experimental set-up (results not shown). We then used a series of recombinant Fn fragments covering

Figure 5 Binding of Fn70K and Fn45K fragments to type XIII collagen ectodomain

(A) In the preliminary tests with all the Fn fragments, only Fn70K and Fn45K showed binding to the immobilized type XIII collagen ectodomain. (B) Kinetic tests with the Fn70K and Fn45K. The amount of bound analyte was monitored by measuring the variation in the plasmon resonance angle as a function of time and expressed in terms of RU. The recorded RU values, from which the background signal has been subtracted, are shown with various symbols depending on the concentration used. The fitting of the crude data and preparation of the overlay plots were performed using the Biaevaluation 3.0 software. The continuous lines in the graphs indicate the fitted curve. The graphs presented are representative of three individual runs.

almost the entire length of the full-length Fn molecule to study which of these fragments, i.e. corresponding Fn domains, bind to type XIII collagen ectodomain. Only the N-terminal Fn fragments Fn70K and Fn45K containing the collagen/gelatin-binding domain [1,8,9] associated with the immobilized ectodomain (Figure 5A). These interactions were subsequently characterized in more detail in the kinetic tests ($n = 3$). The associations were analysed in a Langmuir 1:1 model and the mean K_D values were estimated to be approx. 0.5 μ M for the Fn70K and 15.6 μ M for the Fn45K, with χ^2 values for the K_D fittings in the range 2.1–3.8 and 0.38–0.47 respectively (Figure 5B).

Type XIII collagen interfered with the Fn matrix assembly

The effect of type XIII collagen ectodomain on the matrix incorporation and morphology was studied by immunofluorescent staining. Recombinant type XIII collagen ectodomain or type I collagen, the latter used as a collagen control, was added to fibroblast cultures with exogenous Fn. Staining with anti-Fn antibody showed that when compared with the untreated sample (Figure 6A), the recombinant 50 μ g/ml type XIII collagen ectodomain (Figure 6B) had interfered with the assembly of the matrix from the exogenous Fn ($n = 2$). This reduction was estimated by measuring the matrix-associated fluorescence intensity from ten randomly selected viewing fields, and was approx. 35 % (Figure 6E).

Figure 6 Type XIII collagen ectodomain hampers the Fn matrix assembly

Equal numbers of primary fibroblasts were plated and supplemented with exogenous Fn. In (A), the control culture was without additional supplementations, whereas in the others, (B) 50 $\mu\text{g/ml}$ type XIII collagen ectodomain, (C) 100 $\mu\text{g/ml}$ type XIII collagen ectodomain or (D) 50 $\mu\text{g/ml}$ type I collagen was added to the cultures for 3 h. The matrices formed were stained with anti-Fn antibody. (E) The graph shows the quantitative evaluation by image analysis of fluorescence associated with the fibrillar Fn matrices formed, as measured from ten randomly selected viewing fields per sample and expressed in terms of IOD. The sample in (C) was not evaluated quantitatively due to the strong fluorescence associated with the precipitations. Scale bar, 20 μm . (F) Equal numbers of primary fibroblasts were plated in serum-free DMEM and supplemented with type XIII collagen ectodomain, type I or VI collagen for 24 h. The cells were disrupted with 25 mM NH_4OH , followed by isolation of the Fn matrices. Equal volumes of samples were separated on an SDS/5% polyacrylamide gel under reducing conditions, followed by Western blotting with anti-Fn antibody. The graph shows results of densitometric scanning of the Western blots, expressed as means with the bars indicating the S.D. The statistical differences were evaluated with Student's *t* test. * $P < 0.05$, ** $P < 0.01$. N.S., not statistical. The blot shown is representative of five independent experiments. Abbreviations: COLXIII EC, type XIII collagen ectodomain; COL I, type I collagen; COL VI, type VI collagen.

As for the morphology, in the type XIII collagen ectodomain-treated culture, the Fn fibre structure seemed unaffected. However, increasing the type XIII collagen ectodomain concentration up to 100 $\mu\text{g/ml}$ did not add to the observed inhibitory effect. The assembly of the Fn meshwork was again clearly hampered, but the higher ectodomain concentration caused the Fn to aggregate (Figure 6C) ($n = 2$). As opposed to type XIII collagen ectodomain, type I collagen did not interfere with the Fn matrix assembly (Figure 6D).

The effect of type XIII collagen ectodomain on the endogenous FN matrix polymerization was also analysed ($n = 5$). The amounts of the isolated Fn matrices were semi-quantitatively evaluated by Western blotting. Again, the presence of the soluble recombinant type XIII collagen ectodomain had diminished the amount of the endogenously formed Fn matrix, this time approx. 25%, when compared with the untreated control. Also here, the influence of type XIII collagen ectodomain was distinctly different from that of the other collagens (Figure 6F).

DISCUSSION

We have recently discovered that, during malignant transformation, the expression of type XIII collagen is up-regulated in the tumour stroma [32], which is known to be strikingly different from a normal stroma [5,6,40–43]. Although the exact function of the stromal type XIII collagen up-regulation is still obscure at the moment, we assume that it may have distinct repercussions for the composition and dynamics of the altered tumour stroma. Namely, up-regulation of type XIII collagen expression is concomitantly accompanied by an enhanced shedding of its ectodomain and we know the type XIII collagen ectodomain to be capable of altering the vitronectin-rich environment that is non-supportive of many adhesion-dependent cell functions [31,32]. In the present study, we propose an extension to the hypothesis of type XIII collagen ectodomain participating in the remodelling of the stroma by reporting that type XIII collagen ectodomain, whether exogenously supplemented or endogenously released by the cells, can associate fully with the fibrillar Fn matrix under cell-culture conditions, in line with our group's earlier *in vitro* interaction findings between type XIII collagen ectodomain and Fn [33].

The C-terminal end of type XIII collagen and the N-terminal end of Fn were implied to mediate the association. The involvement of the C-terminal amino acids of type XIII collagen encompassing the NC4 domain and part of the COL3 domain is significant, because the last 100 amino acids at the C-terminus of type XIII collagen are highly conserved, e.g. they are identical between mouse and human. As shown, the NC4 domain alone is of substantial importance in mediating the fibre alignment. Since the COL3 domain contributes to the effect, it seems that both domains are needed for the association. As for Fn, the identification of its N-terminus as a collagen-binding unit is consistent with what is already known about Fn–collagen associations [9,12,23,26,44,45]. The precise mechanism of Fn–collagen interaction is, however, still incompletely understood [45], as is the case here with type XIII collagen and Fn, and clearly, this topic remains to be elucidated.

The results also suggested that type XIII collagen ectodomain may interfere with the assembly of the Fn matrix, as opposed to other collagens tested. The N-terminus of Fn contains the matrix assembly domain necessary for the efficient elongation of the growing Fn fibres [1,8,9], and only this end of Fn showed binding to type XIII collagen ectodomain. We thus propose a model whereby the observed type XIII collagen ectodomain-derived reduction in the Fn matrix assembly may result from either steric hindrance of the free joining Fn dimers incoming to the assembly site and/or reduced availability of the juxtaposed assembly site due to preoccupation of the N-terminal end of Fn by the large type XIII collagen ectodomain molecule. Such a masking effect would explain why type XIII collagen ectodomain did not totally block, yet did hamper, the assembly. We feel that the influence of type XIII collagen ectodomain may, nevertheless, be of biological significance when (i) contributing to a multitude of other changes of the altered tumour stroma and/or (ii) occurring in the pericellular space of the stromal cells with an induced type XIII collagen expression and type XIII collagen ectodomain shedding.

In summary, shedding of type XIII collagen ectodomain by mammalian cells results in the release of a biologically active soluble molecule with distinct matrix-specific effects on cell behaviour [31,32]. These novel observations on the Fn matrix assembly broaden our present knowledge of the biology of type XIII collagen and may be of relevance when considering the central involvement of the Fn matrix in a wide variety of physiological

and pathological conditions. By modulating the expression of the transmembrane type XIII collagen and the shedding of its ectodomain, cells may change the structure and function of the surrounding ECM, with distinct ramifications in terms of cell behaviour.

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