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Type III and V collagens modulate the expression and assembly of EDA$^+$ fibronectin in the extracellular matrix of defective Ehlers–Danlos syndrome fibroblasts

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ABSTRACT

Background: Alternative splicing of EDA fibronectin (FN) region is a cell type- and development-regulated mechanism controlled by pathological processes, growth factors and extracellular matrix (ECM). Classic and vascular Ehlers–Danlos syndrome (cEDS and vEDS) are connective tissue disorders caused by *COL5A1*/*COL5A2* and *COL3A1* gene mutations, leading to an *in vivo* abnormal collagen fibrillogenesis and to an *in vitro* defective organisation in the ECM of type V (COLLV) and type III collagen (COLLIII). These defects induce the FN-ECM disarray and the decrease of COLLs and FN receptors, the $\alpha2\beta1$ and $\alpha5\beta1$ integrins. Purified COLLV and COLLIII restore the COLL-FN-ECMs in both EDS cell strains.

Methods: Real-time PCR, immunofluorescence microscopy, and Western blotting were used to investigate the effects of COLLs on *FN1* gene expression, EDA region alternative splicing, EDA$^+$-FN-ECM assembly, $\alpha5\beta1$ integrin and EDA$^+$-FN-specific $\alpha9$ integrin subunit organisation, $\alpha5\beta1$ integrin and FAK co-regulation in EDS fibroblasts.

Results: COLLV-treated cEDS and COLLIII-treated vEDS fibroblasts up-regulate the *FN1* gene expression, modulate the EDA$^+$ mRNA maturation and increase the EDA$^+$-FN levels, thus restoring a control-like FN-ECM, which elicits the EDA$^+$-FN-specific $\alpha9\beta1$ integrin organisation, recruits the $\alpha5\beta1$ integrin and switches on the FAK binding and phosphorylation.

Conclusion: COLLs regulate the EDA$^+$-FN-ECM organisation at transcriptional and post-transcriptional level and activate the $\alpha5\beta1$–FAK complexes. COLLs also recruit the $\alpha9\beta1$ integrin involved in the assembly of the EDA$^+$-FN-ECM in EDS cells.

General significance: The knowledge of the COLLs-ECM role in FN isotype expression and in EDA$^+$-FN-ECM-mediated signal transduction adds insights in the ECM remodelling mechanisms in EDS cells.

1. Introduction

Ehlers–Danlos syndrome (EDS) refers to a heterogeneous group of rare autosomal dominant and recessive connective tissue disorders, characterised by joint hypermobility, hyperextensible, elastic and fragile skin with impaired wound healing. The involved genes, encoding type V, type I, and type III collagens (COLLs) or enzymes entering into the COLLs triple helices processing, differ between the six major EDS types [1,2]. Among them, the classical, or type I EDS (cEDS, or EDSI) and the vascular, or type IV EDS (vEDS, or EDSIV) are caused by mutations in *COL5A1* or *COL5A2* and in *COL3A1* genes, respectively. Ultrastructural studies of *in vivo* cEDS skin biopsies showed a decrease of COLL fibrils density and the presence of large irregular COLL fibrils (cauliflower shape), whereas in vEDS biopsies a thinner dermis than control ones and a reduced number of COLL bundles with small diameter fibrils were reported [3]. The abnormal COLL fibrillogenesis might be due either to primary defect in COLL genes and to alteration of non-collagenous extracellular matrix (ECM) components, known to influence the COLL fibrils assembly. *In vitro* cultured skin fibroblasts derived from cEDS and vEDS patients do not organise fibronectin (FN) in the ECM [4–8], consequently to an altered COLL expression and deposition into the ECM, caused by COLL gene mutations [6,8]. In particular, the defective synthesis and organisation of type V COLL (COLLV) or type III COLL (COLLIII) are associated to a decreased organisation of the specific COLL receptor, the $\alpha2\beta1$ integrin [6]; this event leads to the disorganisation of the FN-specific cell surface receptor, the $\alpha5\beta1$ integrin, triggering an FN-ECM disarray [6].

The FN-ECM regulates a variety of biological processes, including cell adhesion, migration, proliferation, survival and gene expression, *via* signal transduction pathways which can be differentially activated by several ECM ligands interacting with specific integrin settings [9–12]. At least ten different FN specific integrins are reported; between them,

Abbreviations: CE, cell extract; CM, culture medium; COLLIII, type III collagen; COLLV, type V collagen; COLLs, collagens; DOC-IS, deoxycholate-insoluble; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; FN1, fibronectin gene

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the α5β1 integrin is the main FN receptor in dermal skin fibroblasts, recognising the FN RGD binding site and regulating outside-in and inside-out signalling for cell proliferation, survival and migration [9].

In EDS cells the α5β1 integrin is replaced by another FN receptor, the αvβ3 integrin [6,8], allowing cell survival in the absence of an organised COLL-FN-ECM [8]. Indeed, in these cells the αvβ3 integrin transduces signals through the FAK-independent paxillin. In this signalling the src family proteins are involved in the activation of paxillin, which maintains a pre-apoptotic cell behaviour: EDS cells proliferate in the absence of ECM, actin cytoskeleton and anti-apoptotic factors [8]. Purified exogenous COLLV and COLLIII restore the COLLs-ECM in cEDS and vEDS cells, respectively, through the recruitment of the α2β1 integrin and the αvβ3 substitution with the α5β1 integrin, finally supporting the FN-ECM re-organisation, through a yet unknown α2β1 integrin-mediated signal transduction pathway [6].

The defective FN-ECM assembly reported in different EDS fibroblasts' types has been associated to a decrease of EDA^+ (or EDI or EIIIA) FN mRNA level [13], one of the alternative spliced regions of the *FN1* gene [14–17]. FN alternative splicing, also acting at the EDB domain (or EDII or EIIIB) and at the type III connecting segment (IIICS), is regulated in a cell type-, development- and age-dependent manner and in pathological processes. Skin fibroblasts preferentially synthesise the EDA^+-FN mRNA [18–20]; the EDA^+-FN enters in the cellular FN (cFN) isotype, organising in the fibrillar ECM [13]. Plasma FN (pFN) mainly contains the EDA^- isotype. *In vivo*, the EDA^+-FN is poorly represented in the ECM of adult tissues and normal adult skin fibroblasts *in vivo* mainly produce EDA^--FN [21,22]. EDA^+-FN is overexpressed in fibroblasts and epithelial cells during tissue remodelling and cell migration occurring in developing embryos [23–26], during wound healing [21,22,27], liver fibrosis, myofibroblast differentiation [28,29] and in some tumours [20,30,31]. Although in EDS patients the *in vivo* FN-ECM organisation was not investigated, the clinical defects and the impaired wound healing observed suggest a role of FN in the connective tissue haemostasis. Growth factors, cytokines, hormones and stress stimuli regulate FN alternative splicing either *in vivo* or *in vitro* [32–38]. Furthermore, different ECM proteins can control and modulate this process, *i.e.*, laminin and type IV COLL [39].

Here we report the effect of purified human COLLIII and COLLV on the modulation of EDA^+-FN mRNA synthesis and on the EDA^+-FN organisation in FN-ECM defective EDS cells, leading to the α9β1 and α5β1 integrin recruitment and signalling to FAK protein.

2. Materials and methods

2.1. Cell strains

Human control skin fibroblasts were established in our lab from three skin biopsies of age- and sex-matched healthy donors. cEDS and vEDS fibroblasts carried dominant missense mutations in the genes encoding for the α1 chains of COLLV (*COL5A1*, G1181C) and COLLIII (*COL3A1*, G769S), respectively [6,8]. Five cEDS cell strains (P1–P5) carrying different mutations in *COL5A1* and *COL5A2* genes [7 and unpublished results] and three vEDS cell strains (P1–P3) carrying different *COL3A1* gene mutations [40] were also analysed in some experiments. The cells at the 8th *in vitro* passages were grown in Earle's Modified Eagle's Medium (MEM) (Invitrogen/Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technology), 100 IU/ml penicillin and 100 μg/ml streptomycin (complete MEM) at 37 °C in a 5% CO_2 atmosphere. Alternatively, control and EDS cells were seeded in complete MEM supplemented with 1.0, 2.5, 5 and 10 μg/ml purified human COLLV or COLLIII.

2.2. Antibodies and reagents

Mouse anti-α5β1 (clone JBS5), anti-αvβ3 (clone LM609) integrins monoclonal antibodies (mAbs) and purified human COLLV and COLLIII were from Millipore, Chemicon (Billerica, MA). The goat anti-COLLV and anti-COLLIII polyclonal antibodies (Abs) were from LifeSpan BioSciences, Inc. (Seattle, WA). The anti-FAK and anti-integrin α9 Abs, the anti-phospho-tyrosine (anti-p-Tyr) (PY20) and the anti-GAPDH (clone 0411) mAbs were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-integrin α4 (EPR1355Y) Ab and the anti-α5 integrin subunit mAb (clone NKI-SAM1) were from Abcam (Cambridge, UK). Fluorescein isothiocyanate (FITC)- and rhodamine-conjugated goat anti-rabbit and goat anti-mouse secondary Abs were from Calbiochem-Novabiochem INTL. Horseradish-peroxidase (HRP)-conjugated anti-rabbit, anti-goat and anti-mouse IgGs, BSA, rhodamine-conjugated rabbit anti-goat IgGs, the affinity purified rabbit anti-human FN Ab (F3648) and anti-tenascin mAb (clone BC-24), recognising all of the human FNs and tenascins, respectively, were from Sigma Chemicals (St. Louis, MO). The anti-human EDA FN (clone DH1) mAb was from BIOHIT (Celbio, Italy). The anti-FN Ab and the anti-EDA FN mAb did not recognise the bovine FN (SM 1B to Fig. 1). The rabbit anti-u-PA Ab was from Technoclone GmbH (Alifax, Italy).

2.3. RNA extraction and retro-transcription reaction

Total RNA was isolated from 1.5×10^6 control and EDS fibroblasts grown for 48 h to confluence, treated or not with 5.0 μg/ml purified human COLLV and COLLIII, using TRIzol reagent according to the manufacturer's instructions (Invitrogen/Life Technologies, Carlsbad, CA). The RNA was recovered by precipitation with isopropyl alcohol, washed by 75% ethanol solution and dissolved in RNase free water. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer and quality was checked using the AGILENT Bioanalyzer 2100 lab-on-a-chip technology. Retro-transcription (RT) was performed using the Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) provided by Invitrogen. Total RNA (3 μg) from each cell strain were mixed with 2.2 μl of 0.2 ng/μl random hexamer, 10 μl of 5× buffer, 10 μl of 2 mM dNTPs, 1 μl of 1 mM DTT, 0.4 μl of 33 U/μl RNasin, 2 μl MMLV-RT (200 U/μl), in a final volume of 50 μl. The reaction mix was incubated at 37 °C for 2 h and the enzyme was heat-inactivated at 95 °C for 10 min. Samples were stored at −20 °C and cDNA was used for Real-time PCR.

2.4. Real-time PCR by TaqMan probes

The expression levels of EDA^+- and EDA^--FN isoforms were analysed by means of an Applied Biosystem 7500 Real-time PCR system based on a 5′ nuclease assay (TaqMan). For the quantitative measurement, the comparative threshold cycle (ΔΔCt) method was used according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Porphobilinogen deaminase gene (*PBGD*) was used for normalisation of possible fluctuations in quantitative values of the target transcripts. PCR was carried out with TaqMan Universal PCR Master Mix (Applied Biosystem), which contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference and optimised buffer components. AmpErase UNG treatment was used to prevent the possible reamplification of carryover PCR products. Thermal cycling was initiated with incubation at 50 °C for 2 min and 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase, respectively. After this step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95 °C for 15 s for melting and 60 °C for 1 min for annealing and extension. 20 ng of cDNA, 7.5 pmol of each primer and 5 pmol of labeled probe were used in each Real-time PCR, in a final volume of 25 μl.

Primers and probes of the housekeeping gene *PBGD* and of the EDA^+- and EDA^--FN mRNA isoforms were previously described [41]. In brief, the forward primer of EDA^+-FN lies within the EDA region, whereas the forward primer of the EDA^--FN isoform does not include the EDA region. The reverse primers and probes are similar for EDA^+ and EDA^-. The specificity and efficiency of both primer sets were