HHS Public Access

Author manuscript

J Invest Dermatol. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

J Invest Dermatol. 2015 July ; 135(7): 1921–1924. doi:10.1038/jid.2015.50.

Keloid Pathogenesis: Potential Role of Cellular Fibronectin with the EDA Domain

Jonathan P. Andrews[1], **Jaana Marttala**[1], **Edward Macarak**[1,2], **Joel Rosenbloom**[1,2], and **Jouni Uitto**[1]

[1]Department of Dermatology and Cutaneous Biology, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, USA

[2]The Joan and Joel Rosenbloom Research Center for Fibrotic Diseases, Thomas Jefferson University, Philadelphia, PA, USA

Fibronectins (FNs) are high molecular weight glycoproteins present in extracellular connective tissue matrices (ECM) and extracellular fluids, including blood plasma. The human FN gene consists of 45 exons, and the primary mRNA transcripts are alternatively spliced to form up to 20 different mRNA variants (White *et al*., 2008). The FNs interact with other matrix proteins, such as collagens, glycosaminoglycans and fibrin, as well as cell surface receptors, including integrins α9β1, α5β1 and αvβ3, and Toll1like receptor 4 (TLR4) (Charo *et al*., 1990; Okamura*et al*., 2001). The precise role of individual isoforms of FN in ECM biology and pathology remains unclear. One of the alternatively spliced exons encodes the extra domain A (EDA), also known as extra type III repeat, that is regulated developmentally and is found exclusively in cellular fibronectin (cFN) but not in plasma fibronectin (pFN) (Muro *et al*., 1999). The latter form is synthesized by hepatocytes and secreted into the circulation, while cFN is produced by a variety of cells, including fibroblasts and epithelial cells, and is deposited as fibrils in the ECM.

The *in vivo* role of the EDA variant has been studied by constructing mouse strains either constitutively expressing (FN-EDA$^{+/+}$) or excluding it (FN-EDA$^{-/-}$) (Muro *et al*., 2003). No embryonic lethality or postnatal malformations were observed in the case of the homozygous mutant FN-EDA$^{-/-}$ mice, suggesting that EDA is not required for normal development. However, significant abnormalities were observed in adult FN-EDA$^{-/-}$ animals. Specifically, the EDA peptide segment is not found in the skin of wild-type animals, but FN-EDA was shown to be essential for normal wound healing, particularly with respect to re1epithelization. While wound healing in EDA$^{+/+}$ mice was indistinguishable from EDA$^{wt/wt}$ mice, the wounds in FN-EDA$^{-/-}$ mice showed ulcerations in the epidermis

Address for Correspondence: Jouni Uitto, M.D., Ph.D. Department of Dermatology and Cutaneous Biology Sidney Kimmel Medical College at Thomas Jefferson University 233 S. 10th Street, Suite 450 BLSB Philadelphia, PA 19107 Tel: 215-503-5785 Fax: 215-503-5788 jouni.uitto@jefferson.edu.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid.

resulting in delay of re1epithelialization. Furthermore, both FN-EDA$^{+/+}$ and FN-EDA$^{-/-}$ mice had shortened lifespan compared to wild1type animals.

Compelling recent evidence has accumulated implicating a crucial role for FN-EDA not only in normal wound healing but also in fibroproliferative disorders characterized by increased production and deposition of ECM. Treatment of normal fibroblasts with TGF-β, a critical cytokine in the pathogenesis of a variety of fibroproliferative disorders, results in a substantial increase in FN-EDA production (Balza *et al*., 1988; Chalmers, 2011). Interestingly, FN-EDA up1 regulation precedes that of collagen, and FN-EDA is required for the induction of the myofibroblastic phenotype by TGF-β. Furthermore, FN-EDA null mice failed to develop significant lung fibrosis after bleomycin administration (Muro *et al*., 2008). Recently, Varga and co-workers (Bhattacharyya *et al*., 2014) demonstrated increased FN-EDA in the skin and circulation of patients with scleroderma. Additionally, exogenous FN-EDA potently stimulated collagen production and myofibroblast differentiation *in vitro* which was mediated by TLR4. These observations stimulated our investigation of the role of FN-EDA in the development of keloids, an abnormal fibroproliferative response elicited by trauma to the skin of genetically susceptible individuals; the pathogenesis of this disorder remains obscure (Bran *et al*., 2009). Our results suggest that FN-EDA may play a critical role in the keloid disease process.

Discarded tissue specimens were obtained anonymously from patients undergoing cosmetic surgical excision of keloids and from patients undergoing panniculectomy (for patient information, see Supplementary Table S1). Use of discarded tissue was approved by the Institutional Review Board of Thomas Jefferson University. The tissues were dissected, sterilized with betadine solution, subcutaneous adipose tissue was removed, and the samples were prepared for gene expression analysis. Total RNA was extracted from all specimens using RNeasy Kit (Fibrous Tissue Kit, Qiagen, Alameda, CA) and reverse transcribed to cDNA. Primer pairs were designed to amplify FN-EDA gene sequences as well as the housekeeping gene, glyceraldehyde-3-phosphodehydrogenase (GAPDH) as a control (for primer sequence information, see Supplementary Table S2). Relative quantitative RT-PCR was performed to investigate the expression of total FN as well as FN-EDA in both keloid and control specimens. As demonstrated previously (Kischer and Hendrix, 1983; Sible *et al*., 1994), the total FN expression was increased in keloids by 10.7-fold over controls. However, the results indicated a dramatic, up to 70-fold ($p = 0.005851$) increase in FN1EDA mRNA in keloid tissues in comparison to controls ($n = 5$ both groups; Fig. 1a). Thus, the relative increase in cFN-EDA in comparison to total FN was ~7-fold higher in keloids.

To investigate whether the increased mRNA transcript levels result in similarly increased protein levels in keloid tissues, total protein was extracted from five keloids and control tissue specimens via bead-mill homogenization in the presence of protein lysis buffer containing RIPA buffer, PMSF, and EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) . Twenty μg of total protein from each sample was subjected to SDS/PAGE (4-20% gel) in the presence of reducing agent. Protein was electrotransferred to polyvinylidene fluoride membrane, and nonspecific binding sites on the membrane were blocked by incubation in 5% milk for 1 hour at room temperature. The blot was incubated with anti-fibronectin EDA antibody (IST-9) at 1:200 dilution overnight at 4°C and anti-β

actin primary antibody (ab8224, Abcam, Cambridge, MA), and then incubated with Licor 800cw infrared anti-mouse secondary antibody at 1:5,000 dilution for 1 hour at room temperature. After several washings, the signal was determined with Odyssey infrared image scanner (LiCor Biotechnology, Lincoln, NE).

Western blotting of the protein from keloids, as illustrated in Fig. 1b, revealed a significantly enhanced level of FN-EDA of the molecular weight of ~220 kDa, corresponding to a FN monomer, while very little, if any, protein was found in extracts of control specimens. Localization of FN-EDA in keloid tissues was determined by immunofluorescence staining with the same antibody (IST-9 monoclonal hybridoma supernatant, undiluted) as used for Western blotting, recognizing a 10-amino acid peptide sequence within the EDA segment, and an antibody to type III collagen (Rockland No. 600-401-105-0.1), overnight at 4°C followed by 3 washes in phosphate buffered saline (PBS). Sections were incubated for 1 hour at room temperature with 1:200 dilutions in PBS of Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit secondary antibodies (Invitrogen). After 3 washes in PBS, sections were then incubated at room temperature for 15 minutes with a 1:1,000 dilution of DAPI in PBS (Southern Biotech). After washing 3X with PBS, sections were mounted in Fluoromount G (Southern Biotech) and visualized. The results indicated intense staining of FN-EDA in association with broad collagen fibers within the keloids, as visualized by anti-type III collagen antibody, while little, if any, staining was noted in control skin (Fig. 1c, d). Thus, the keloidal lesions contain an abundance of FN-EDA.

While total FN expression has been shown to be higher in keloid fibroblasts compared to control dermal fibroblasts (Kischer and Hendrix, 1983; Sible *et al*., 1994), the high level of FN-EDA expression in keloid tissue has not been previously reported. Although the mechanisms of the high level of FN1EDA expression in keloid tissue and its virtual absence in normal tissue remains to be determined, its presence may explain in part the continuous excess matrix production. EDA acting through the TLR4 receptor may enhance the activation of fibroblasts resulting in increased production of TGF1β and subsequent production and accumulation of ECM molecules, particularly collagen, creating a positive feedback loop, thus establishing progressive fibrosis. This hypothesis suggests that blocking FN-EDA synthesis or its interactions with cell surface receptors may provide suitable strategies to interrupt the fibrotic reaction in keloids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The authors thank the clinical faculty and residents of the Department of Dermatology and Cutaneous Biology at Sidney Kimmel Medical College at Thomas Jefferson University for assistance in obtaining keloid samples. Dr. Patrick J. Greaney, Division of Plastic Surgery at Thomas Jefferson University, provided control skin tissue. Carol Kelly assisted in manuscript preparation. The authors wish to thank the National Institutes of Health (T32-AR060715) and the Orion1Farmos Research Foundation for their support for research and medical education.

Abbreviations

FN	fibronectin
EDA	extra domain A
ECM	extracellular matrix
TLR4	toll-like receptor 4

REFERENCES

Balza E, Borsi L, Allemanni G, et al. Transforming growth factor beta regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett. 1988; 228:42–4. [PubMed: 3422628]

Bhattacharyya S, Tamaki Z, Wang W, et al. Fibronectin EDA promotes chronic cutaneous fibosis through Toll1like receptor signaling. Sci Transl Med. 2014; 6:232ra50.

Bran GM, Goessler UR, Hormann K, et al. Keloids: current concepts of pathogenesis (review). Int J Mol Med. 2009; 24:283–93. [PubMed: 19639219]

Chalmers RL. The evidence for the role of transforming growth factor-beta in the formation of abnormal scarring. Int Wound J. 2011; 8:218–23. [PubMed: 21449929]

Charo IF, Nannizzi L, Smith JW, et al. The vitronectin receptor alpha v beta 3 binds fibronectin and acts in concert with alpha 5 beta 1 in promoting cellular attachment and spreading on fibronectin. J Cell Biol. 1990; 111:2795–800. [PubMed: 1703545]

Kischer CW, Hendrix MJ. Fibronectin (FN) in hypertrophic scars and keloids. Cell Tissue Res. 1983; 231:29–37. [PubMed: 6342808]

Muro AF, Caputi M, Pariyarath R, et al. Regulation of fibronectin EDA exon alternative splicing: possible role of RNA secondary structure for enhancer display. Mol Cell Biol. 1999; 19:2657–71. [PubMed: 10082532]

Muro AF, Chauhan AK, Gajovic S, et al. Regulated splicing of the fibronectin EDA exon is essential for proper skin wound healing and normal lifespan. J Cell Biol. 2003; 162:149–60. [PubMed: 12847088]

Okamura Y, Watari M, Jerud ES, et al. The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem. 2001; 276:10229–33. [PubMed: 11150311]

Sible JC, Eriksson E, Smith SP, et al. Fibronectin gene expression differs in normal and abnormal human wound healing. Wound Repair Regen. 1994; 2:3–19. [PubMed: 17168907]

White ES, Baralle FE, Muro AF. New insights into form and function of fibronectin splice variants. J Pathol. 2008; 216:1–14. [PubMed: 18680111]

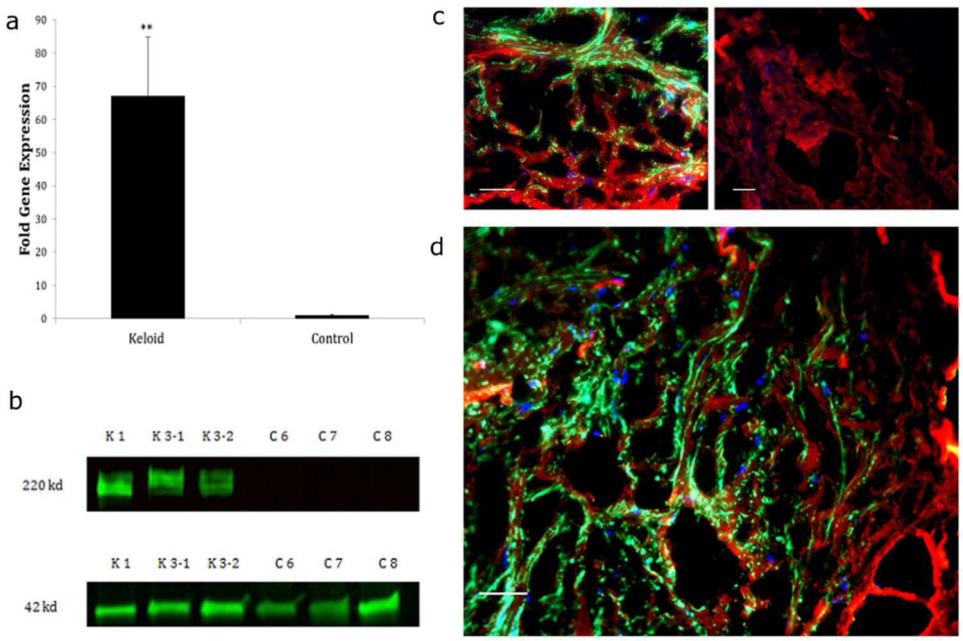

Figure 1.

Expression of FN-EDA in keloid tissues (a). Note 70-fold increase in mRNA levels in keloid tissue versus control (n = 5 for both groups; mean ± SE; p = 0.005851). Western blot results for tissue protein isolates from three keloid samples and three control samples (b). A total of 20μg protein was loaded into each well; presence of fibronectin1EDA (220 kD) is demonstrated in keloid extracts versus near total absence in controls. Positive control (β-actin) is present in each protein isolate (42 kD). Immunofluorescence of frozen sections of keloid (1c, left and 1d; scale bar = 30 μm) and control skin (1c, right). Note the heavy decoration of the surfaces of the type III collagen fibers (shown in red) with FN-EDA antibody (shown in green), in keloids in contrast to complete absence of FN-EDA in control skin (1c, right).