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Wound Healing-Associated Proteins Hsp47 and Fibronectin Are Elevated in Dupuytren's Contracture[1,2]

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Background. **Dupuytren's contracture or disease (DD) affects hand function by causing irreversible contraction of the palmar fascia. Histological analysis has shown that DD and wound granulation tissue share many cellular and biochemical characteristics, suggesting that DD may be an exaggerated wound-healing response. The goal of the present study was to examine the possible involvement of two important wound-healing-associated proteins—heat shock protein 47 (Hsp47), fibronectin (Fn), and its oncofetal isoforms—in DD, using clinical tissue samples and primary cell cultures.**

Materials and methods. **We examined the expression of Hsp47, Fn, and an oncofetal isoform of fibronectin (IIICS) in both normal and disease-matched surgical specimens and primary cell cultures using Western blot analysis, and immunocytochemistry (ICC).**

Results. **Our results indicate that Hsp47 and total fibronectin is elevated in DD lesional tissue. In addition, Western and ICC analysis of patient-matched (normal and disease) primary cultures show significantly elevated levels of oncofetal fibronectin (IIICS spliced variant) within disease primary cell cultures.**

Conclusions. **The high levels of expression of Hsp47 and adult and oncofetal fibronectin in DD suggests that cell-mediated alterations in the extracellular environment may play an important role in the disease process. Furthermore, the involvement of these wound healing-associated proteins in DD supports the notion that this disease may be an exaggerated form of wound healing.**

Key Words: **Dupuytren's disease (DD); wound healing; cellular contraction; Hsp47; fibronectin (Fn); oncofetal fibronectin; immunocytochemistry (ICC).**

INTRODUCTION

Dupuytren's contracture (DD) is a benign but debilitating fibroproliferative disease of unknown etiology [1]. The disease is characterized by a proliferation of fibroblasts and myofibroblasts in the palmar fascia resulting in a fixed, flexed position of the fingers. Although there is a considerable geographical variation in the incidence of the disease, its highest prevalence is within Caucasians of Northern European descent, with a reported incidence of 10–40% in males over the age of 65 [2, 3].

Clinically, DD progresses through distinct stages. The earliest stage is characterized by the appearance of small nodules of hyperproliferative cells within the palmar fascia that, over time, give rise to large bands of contracted, collagen-rich, fibrotic tissue (disease cords), which is a hallmark of the disease [4, 5]. A number of risk factors have been proposed for DD, including alcoholism [6], trauma [7], diabetes [8], smoking [9], and epilepsy [10]. However, their exact role in the disease is unclear and somewhat controver-

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sial [11]. In addition, despite cytogenetic and epidemiological evidence showing chromosomal abnormalities in diseased tissue [12–15], the identity of such genetic defect(s) has remained elusive [16].

Histopathology studies of DD have shown that it shares many biological features with wound granulation tissue [17]. Multiple structural proteins, including collagen Type 3 and signaling molecules such as transforming growth factor-β, are known to be differentially expressed both during wound closure and in DD [18], while cellular elements such as myofibroblasts are implicated in the contraction of both wound granulation tissue and the scar-like disease cords that define DD. These cells not only regulate the remodeling of the extracellular matrix (ECM), but also produce growth factors that can promote scar formation, in the case of DD, or further tissue repair events during normal wound healing [17]. Based on these and other studies, it has been suggested that DD might be akin to an exaggerated form of wound healing.

In this study, we set out to further delineate the similarities between DD and wound-healing tissue by investigating the involvement of two wound healing-associated proteins, heat shock protein 47 (Hsp47) and fibronectin (Fn) in clinical specimens and DD primary cultures. Hsp47 was chosen because it is known to act as a procollagen-specific chaperone [19] and may thus be involved in the regulation of fibrosis, which is a characteristic feature of DD and wound healing. Furthermore, we were specifically interested in identifying the specific isoforms of Fn that may be abberant in DD, as some of these have been shown previously to be differentially regulated during wound healing. As discussed below, our results support the notion that DD may serve as a useful model of wound-healing biology.

MATERIALS AND METHODS

Clinical Specimen and Primary Cultures

DD patient specimens (normal and disease palmar fascia) were collected in strict compliance with the Institute's chief pathologist and the research ethics committee for research involving human subjects at University of Western Ontario. Areas of diseased fascia and adjacent, uninvolved control palmar fascia were sampled during surgical resection of palmar fascia for Dupuytren's disease. Clinical specimens then were divided in two portions for protein analysis (immediately snap frozen) and primary cell cultures. DD explant cultures were initially cultured in starter media consisting of α-MEM (Gibco, Invitrogen Corporation) + 20% fetal bovine serum (FBS) (Clontech Laboratories, Palo Alto, CA) supplemented with antibiotics (Penicillin G + streptomycin sulfate, Gibco, Invitrogen Corporation). Established primary cultures were maintained in α-MEM +10% FBS + antibiotics. Culture flasks were incubated at 37°C in a humidified chamber with 5% CO_2. Medium was changed every 4–5 days and the cells were sub-cultured using 0.05% Trypsin-EDTA (Gibco, Invitrogen Corporation, Grand Island, NY) when confluent.

Preparation of Cell Extracts and Immunoblot Analysis of Clinical Specimen

Tissue protein extracts were prepared using a modified RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA) supplemented with a cocktail of protease inhibitors (1 mM PMSF, 1 μg/mL of aprotinin, leupeptin, pepstatin) and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF) (Sigma, St. Louis, MO). Tissue was homogenized at 4°C and the resulting homogenate centrifuged (15min, 4°C 12,000 $\times$ g) to remove cell debris. Sample protein concentrations were quantified using the BCA protein assay (Pierce, Rockford, IL). Equivalent levels of protein were analyzed by SDS-PAGE (12% or 10% gels) and subsequently transferred to polyvinylidene difuoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were blocked overnight (4°C) with a PBS solution containing 0.1% Tween 20 (PBST) and 5% fat-free skim milk, washed 2 times in PBST, and then probed with either an anti-human Fn antibody (1:500, clone IST-4, Sigma, St. Louis, MO), an anti-human oncofetal Fn antibody (1:250, FDC-6, Adeza Biomedical, Sunnyvale, CA), an anti-Hsp47 antibody (1:500, StressGen, Victoria BC, Canada), or an anti-actin antibody (1:500, C-11, Sigma, St. Louis, MO). After a series of brief PBS washes, the membranes were then incubated with the appropriate species-specific horseradish peroxidase-conjugated 2° antibodies (1:5000, Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature (RT). Antibody-specific bands were visualized using standard ECL chemiluminescence reagents and Kodak XLS film (Rochester, NY).

Fn Accumulation and Immunoblot Analysis of Primary Cell Cultures

Intact confluent monolayer cultures (*i.e.,* no enzymatic dissociation) were briefly washed in PBS and then lysed with the addition of 5 mL (per plate) of protein extraction buffer (25 mM Tris-HCl, pH 8.8, 2 mM EDTA, 2 mM iodoacetic acid, 2 mM *N*-ethylmaleimide, 2% deoxycholate, 1 mM PMSF) that was supplemented with a Sigma protease-inhibitor cocktail (1 μg/mL of aprotinin, leupeptin, and pepstatin). Briefly, cell extracts were centrifuged (15 min, 4°C, 12,000 $\times$ g) to remove insoluble debris. The large supernatant volumes of protein extract were then concentrated using Centricon Plus-20 centrifugal filer devices (PL-30 = 30 kDa molecular weight cutoff), according to the manufacturer's instructions (Millipore, USA). Equivalent protein was then subjected to SDS-PAGE and immunoblot analysis as described above.

Immunocytochemistry

Primary cell cultures (passages 3–6) were plated in 6- or 12-well plates (Costar, Corning, NY) containing glass coverslips (CS) and allowed to adhere overnight for immunoctochemistry (ICC) experiments. Cell-coated CS (50% or 90% cell confluency) were briefly washed in PBS and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 1 hour at RT, permeabilized in PBST (15 min, RT), and then blocked overnight at 4°C with PBS +2% donkey serum, (Jackson ImmunoResearch, West Grove, PA). PBS-washed CS were then incubated with either an anti-human Fn antibody (1:1000, clone IST-4, Sigma, St. Louis, MO) or an anti-human oncofetal Fn antibody (1:1000, FDC-6, Adeza Biomedical, Sunnyvale, CA) for 1 hour at 4°C. After 2 brief PBS washes, the cells were incubated with Cy3-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) for 45 min at RT, washed 2 times with PBS, incubated for 10 min at RT with the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR), and finally stained for 20 min at RT with Oregon Green Phalloidin (Molecular Probes, Eugene, OR). The coverslips then were washed briefly in PBS and mounted onto glass slides using DAKO faramount (DAKO Diagnostics, Carpinteria, CA). Dig-

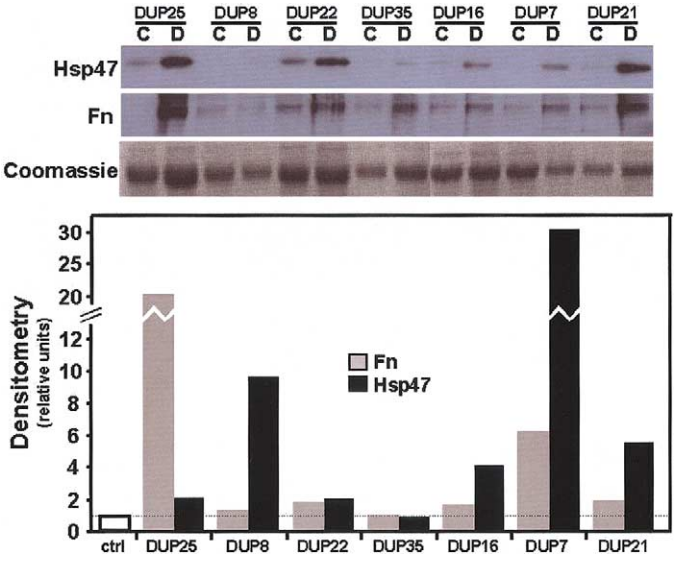

FIG. 1. Western blot analysis of Fn and Hsp47 in DD. Western blots of protein extracts from patient-matched control and disease tissues were probed with anti-Hsp47 antibodies (StressGen, Victoria BC, Canada) and anti-human Fn antibodies (clone IST-4, Sigma, St. Louis, MO). Six out of seven patients show elevated expression of both Fn and Hsp47. Due to the abundance of extracellular proteins in the tissue, equivalent protein loading was determined by coomassie staining. Bottom panel shows densitometric analysis. (Color version of figure is available online.)

ital images were acquired on a Nikon eclipse TE-200 inverted fluorescent microscope using a Photometrics series 300 cooled CCD camera, and deconvolved using *softWoRx* (v 2.5) software (Applied Precision Inc., Issaquah, WA). Oncofetal fibronectin fluorescence was quantified using the Data inspector tool of *softWoRx* v 2.5 Resolve 3D software. The data were presented as the total mean (Cy3) fluorescence ± the standard error of the mean for 5 low-power–10× fields, per coverslip, per group).

RESULTS

Hsp47 and Fn Protein Levels Are Elevated in Clinical Disease Samples

We examined the levels of expression of Hsp47 and Fn in clinical tissue specimens collected from seven patients. Hsp47 is a procollagen-specific molecular chaperone required for proper collagen biosynthesis and normal development [20, 21], while Fn, a glycoprotein of ~220 kDa, is secreted by various cell types in a variety of biological processes to form part of the extracellular matrix (ECM). The levels of Hsp47 expression are known to be elevated in a number of fibrotic conditions [22–24], while various alternatively spliced forms of Fn have been previously observed in DD palmar fascia [25, 26] and wound healing tissue [27, 28]. As shown in Fig. 1, Western blotting using an IST-4 antibody (identifies total Fn) showed elevated levels of total Fn (~4.9-fold) when normalized to total protein in the majority of the clinical disease specimens (6 of 7) examined. Probing for Hsp47 expression also showed a similar pattern of expression within the palmar fascia tissue, with the same (6 of 7) specimens showing ~8-fold higher levels of Hsp47 within the lesional tissue when normalized to total protein. Although previous work has documented elevated levels of total Fn expression in DD [25], this is the first report of elevated levels of Hsp47 in DD tissue. In addition, the increase in Hsp47 expression levels appears to exactly parallel the increases in total Fn expression seen in both normal and disease tissues. This finding, however, is in contrast to our previous work in primary disease cell cultures that did not show an elevated level of Hsp47 compared to control cultures [29, 30].

Elevated Levels of Oncofetal Fn in Primary Cell Cultures of DD

We also examined the levels of an oncofetal Fn (IIICS isoforms) in primary cell cultures (passages 2–6) derived from patient-matched lesional and normal fascia tissue. The appearance of various Fn isoforms is largely regulated by alternative splicing within three distinct regions (ED-A, ED-B, and IIICS) of the single Fn primary transcript [31, 32] and post-translational modifications [33] in a tissue-specific manner. Fn isoforms containing various combinations of the ED-A, ED-B and IIICS sequences are expressed preferentially during development [34, 35], transformation [36], wound healing [28], and tissues undergoing extensive extracellular matrix (ECM) remodeling [37, 38]. As shown in Fig. 2, ICC analysis of the primary cell cultures showed elevated levels of IIICS-positive Fn isoforms (IIICS$^+$ Fn) within the disease cells when compared to patient-matched control cultures. Quantitation of IIICS$^+$ Fn fluorescence was carried out using an anti-human oncofetal Fn antibody (FDC-6, Adeza Biomedical) that recognizes a specific *O*-linked *N*-acetylgalactosaminylated hexapeptide epitope within the Type 3 connecting segment (IIICS) of Fn [33]. These measurements showed an ~4-fold increase in the overall mean IIICS$^+$ Fn fluorescence within the disease cell cultures ($P < 0.007$, Student's t test). The only exception was a single disease cord culture (residual stage) derived from patient DUP46, which showed no significant differences in the levels of IIICS$^+$ Fn fluorescence compared to control cell cultures. The elevated level of IIICS$^+$ Fn within the disease cell cultures was also confirmed by Western analysis. As shown in Fig. 3, the disease cell extracts contained ~6.4-fold higher levels of IIICS$^+$ Fn relative to their patient-matched controls. A parallel increase in total Fn within the disease cell cultures was also observed by Western analysis (data not shown).

DISCUSSION

The aim of this study was to investigate the relationship between wound healing and DD by examining the

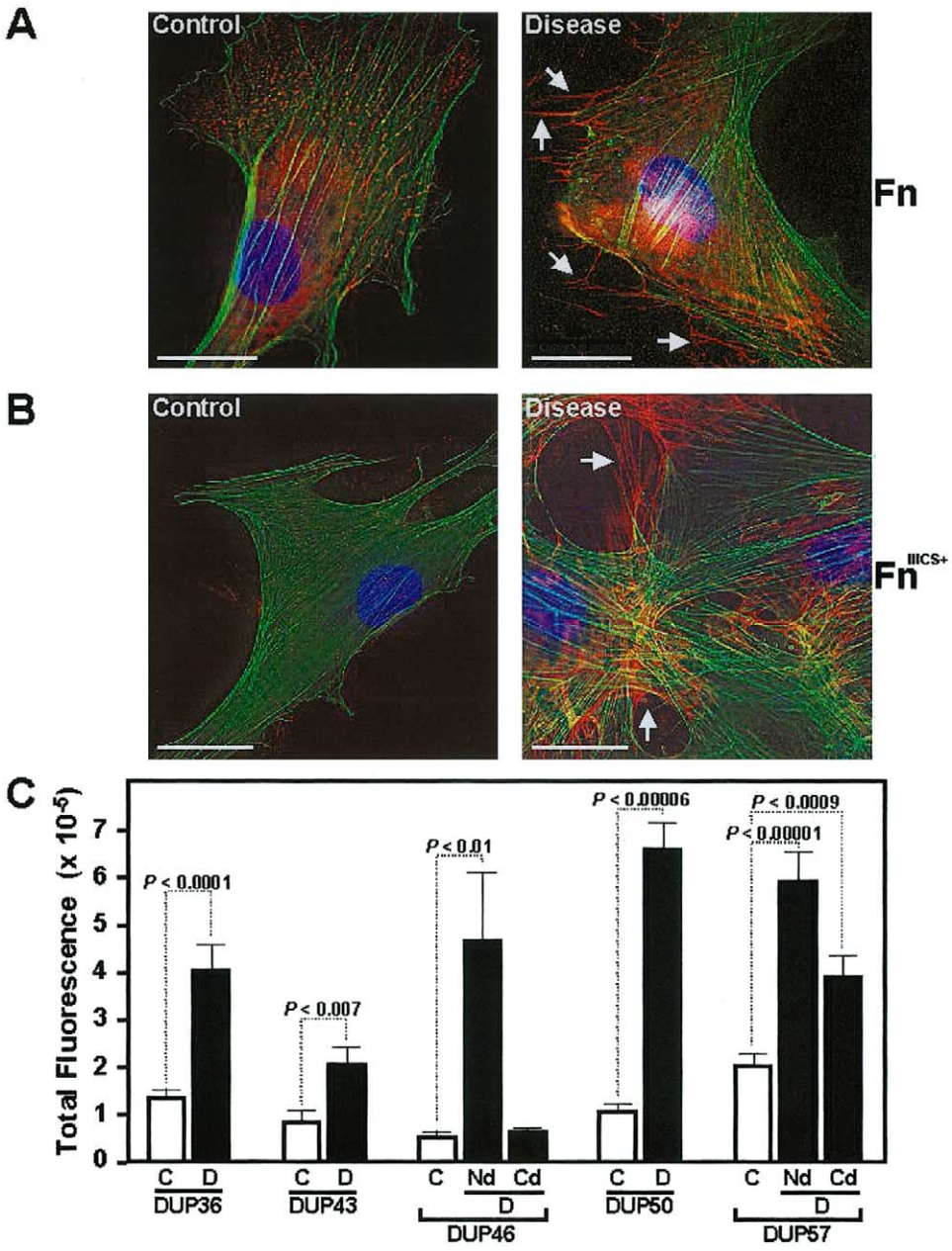

FIG. 2. Immunocytochemistry analysis of Fn in Dupuytren's disease primary cultures. (A) Representative photomicrographs of primary cell cultures derived from patient-matched control and disease palmar fascia tissue were plated on glass coverslips (CS), fixed, and stained with anti-human Fn antibodies (clone IST-4, Sigma, St. Louis, MO). Cells were also co-stained for DNA and filamentous actin using Alexa 488 Phalloidin (green) and DAPI (blue), respectively (Molecular Probes, Eugene, ON). Cy3-labeled secondary antibodies (Jackson Immuno-Research, West Grove, PA) were used to visualize the anti-human Fn antibodies (red). Images were deconvolved using DeltaVision *softWoRx* (v 2.5) software (Applied Precision Inc., Issaquah, WA). Arrows highlight the extensive Fn matrix deposits produced by the disease cell cultures. (B) Glass CS containing primary cell cultures (control and disease) were also fixed and stained with anti-human oncofetal (IIICS$^+$-specific) Fn antibodies (FDC-6, Adeza Biomedical, Sunnyvale, CA). Cells were also co-stained for filamentous actin and DNA using Alexa 488 Phalloidin and DAPI, respectively (Molecular Probes, Eugene, ON). Cy3 labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used to visualize the anti-human oncofetal Fn antibodies (red). Images were deconvolved using DeltaVision s*oftWoRx* (v 2.5) software (Applied Precision Inc., Issaquah, WA). Arrows highlight the extensive oncofetal Fn matrix produced by the disease cell cultures. (C) The immunofluorescence of oncofetal (IIICS$^+$) Fn was quantified using the Data Inspector tool of DeltaVision *softWoRx* (v 2.5) software (Applied Precision Inc., Issaquah, WA). The data are plotted as the total Cy3 fluorescence ± SEM (standard error of the mean) for each of the indicated primary cell lines. A total of 5 random selected low power (10×) fields per coverslip ($n = 3$) per patient ($n = 5$) were used to calculate these measurements. DUP, Dupuytren; N, normal; D, disease; ED, early disease; LD, late disease.

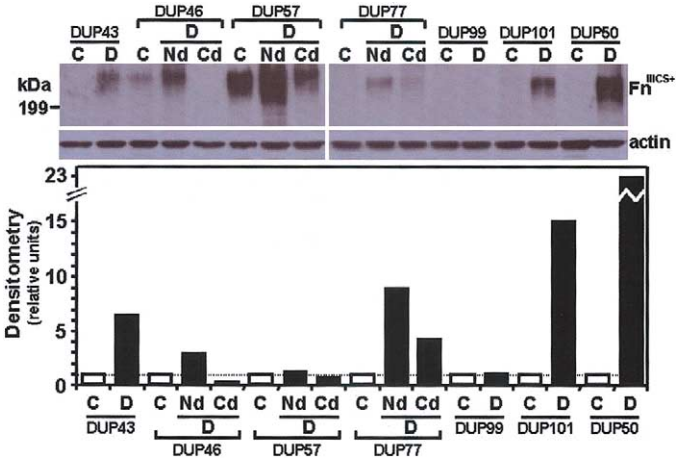

FIG. 3. Western analysis of oncofetal Fn expression in Dupuytren's disease primary cultures. Western analysis was carried out on protein extracts prepared from intact primary monolayer cultures derived from the patient-matched control (C) and disease (D) tissue. Where indicated early or active disease corresponds to nodular disease tissue, whereas late or residual stage disease corresponds to disease cord tissue. Membranes were probed with an affinity purified anti-actin antibody (C11, Santa Cruz, CA) to confirm equivalent protein loading, and an affinity purified anti-human oncofetal (IIICS$^+$-specific) Fn antibody (FDC-6, Adeza Biomedical, Sunnyvale, CA). (Color version of figure is available online.)

expression of two important wound healing-associated proteins, an oncofetal isoform of Fn and Hsp47, in patient-matched palmar fascia tissue (lesions and normal) and primary cell cultures.

In this study, we have found that Hsp47 and Fn are expressed at high levels in lesional palmar fascia tissue derived from DD patients when compared to adjacent patient-matched uninvolved palmar fascia tissue. While this pattern of expression in DD has been previously reported for various Fn isoforms, including oncofetal isoforms of Fn, this is the first report describing the elevated expression of Hsp47, a procollagen-specific molecular chaperone, in DD. Moreover, previous DD studies of oncofetal Fn have been largely confined to immunohistochemical or RNA expression analysis, and to our knowledge never performed on primary cell cultures derived from patient-matched disease and normal palmar fascia tissue.

From our Western and ICC analysis, it is clear that the primary disease cell cultures produce significantly more oncofetal (IIICS$^+$) Fn matrix relative to their control cell cultures (~4-fold, $P < 0.007$, Student's t test), suggesting that the disease cells are actively modifying their extracellular matrix (ECM) environment. Although ECM remodeling ultimately facilitates the mechanical process of disease cord-contraction, the exact mechanisms underlying the cell-ECM interactions that are important to this process are largely ill defined for DD. Nevertheless, Fn does appear to play an important role in this disease process. Bundles of pericellular Fn are major components of the "fibronexus" complex, which physically links the intracellular actin cytoskeleton (contractile machinery) to the collagen-rich ECM of the disease cords [39]. Besides providing structural support, Fn is also known to bind to specific integrins and trigger cell signaling events that are important to cell proliferation, survival, migration, and cell-mediated collagen contraction [40, 41]. This latter activity is particularly noteworthy given the elevated levels of Fn expression seen within disease tissue, and the enhanced spreading and collagen contraction of disease cells when placed within three-dimensional FPCL cultures [29]. The potential role that oncofetal (IIICS$^+$) Fn has to play in DD is worth noting, particularly in light of the purported function of IIICS containing Fn isoforms in regulating cell-ECM adhesion dynamics [42], and the subsequent regulation of these events by fibrogenic cytokines such as TGB-β1 [43]. The IIICS region encodes two cell-specific binding sites (CS1 and CS5) that mediate adhesion exclusively via $\alpha4\beta1$ [44], an integrin that is believed to confer increased cell motility [45], as well as increased invasiveness and metastatic potential in tumor cells [46]. In addition, the CS-1 integrin binding site of IIICS Fn has been shown to mediate Fn matrix assembly via $\alpha4\beta1$ in an RGD-independent manner, suggesting that inappropriate activation of $\alpha4\beta1$-positive cells at sites of Fn expression could lead to fibrosis through excessive Fn matrix formation [47]. Although the exact function of this oncofetal form of Fn in DD is unclear, the increased deposition of IIICS Fn matrix by the disease cells may provide these cells with an important mechano-transduction advantage, since: 1) fibroblast-mediated collagen contraction is Fn-dependent; and 2) Fn-induced collagen contraction can be blocked by agents that inhibit Fn polymerization [41]. Based on these findings it seems reasonable to suggestion that the excessive Fn matrix deposition, which is characteristic of these disease cells, may be a key factor contributing to the excessive ECM remodelling events associated with this disease, most notably disease cord contraction during the active stages of the disease. Although the cell signaling factors that regulate these events (Fn production and matrix assembly) are not entirely clear, recent evidence from our lab suggests that the Wnt/β-catenin pathway may be involved [29, 30].

The elevated level of the Hsp47 in DD tissue is certainly consistent with the role that this protein plays in collagen biosynthesis and other fibrogenic conditions [23], such as keloids [19] and intimal hyperplasia [48]. Although it is not surprising to see elevated levels of Hsp47 in DD, given the production of collagen-rich disease cords are a hallmark of the disease, it has been suggested that Hsp47 may constitute a potential therapeutic target for several fibrotic disorders [19, 49, 50]. It would, thus, clearly be of further interest to

determine exactly how Hsp47 and oncofetal forms of Fn are regulated during DD.

In summary, the data presented here provide further support for the notion that DD is an exaggerated or uncontrolled form of wound healing. Future studies of Hsp47 and Fn may therefore provide new insight into the underlying molecular mechanisms of cell-ECM remodelling events that are characteristic of DD and other fibrosing conditions.

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