Chapter 7

Involvement of pro-inflammatory cytokines and growth factors in the pathogenesis of the Dupuytren’s contracture: a novel target for a possible future therapeutic strategy?

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Abstract

Dupuytren’s contracture (DC) is a benign fibroproliferative disease of the hand causing fibrotic nodules and fascial cords which determine debilitating contracture and deformities of fingers and hands. The present study was designed to characterize pro-inflammatory cytokines and growth factors involved in the pathogenesis, progression and recurrence of this disease, in order to find novel targets for alternative therapies and strategies in controlling DC. The expression of pro-inflammatory cytokines and of growth factors was detected by immunohistochemistry in fibrotic nodules and normal palmar fascia resected respectively from patients affected by Dupuytren’s contracture and Carpal Tunnel Syndrome (as negative controls). RT-PCR analysis and immunofluorescence were performed to quantify the expression of TGF-β1, IL-1β and VEGFa by primary cultures of myofibroblasts and fibroblasts isolated from Dupuytren's nodules. Histological analysis showed high cellularity and high proliferation rate in Dupuytren’s tissue, together to the presence of myofibroblastic isotypes; immunohistochemical staining for macrophages was completely negative. In addition, a strong expression of TGF-β1, IL-1β and VEGFa was evident in the extracellular matrix and in the cytoplasm of fibroblasts and myofibroblasts in Dupuytren’s nodular tissues, as compared to control tissues. These results were confirmed by RT-PCR and by immunofluorescence in pathological and normal primary cell cultures. These preliminary observations suggest that TGF-β1, IL-1β and VEGFa may be considered potential therapeutic targets in the treatment of Dupuytren’s disease.

Introduction

Dupuytren’s disease (DD) has been defined as a benign progressive proliferative fibroplasia of the fascia palmaris of the hand, which results in contracture of the fingers leading to reduction in the movement and inability to
extend them [1]. The pathogenesis of DD still remains unclear; the stages of the fibrotic disease are classified as proliferative, involutional, and residual on the basis of the histological appearance of the affected fascia palmaris [2]. The proliferative phase is characterized by proliferation and differentiation of fibroblasts into myofibroblasts under the influence of several different factors, causing formation of the nodules [3, 4]. In the second phase, the involutional stage, myofibroblasts proliferate and align along the long axis of surrounding collagens bundles thus giving way to the formation of fibrotic cords [5]. Finally, in the residual phase, the myofibroblasts are replaced by fibrocytes that progressively decrease in number causing the formation of the avascular collagen cord [2]. Myofibroblasts seem to play a central role in the pathogenesis of the fibrotic disease. These cells show an intermediate phenotype between fibroblasts and smooth muscle cells and generate the forces responsible for palmar fascia contracture [6]. Myofibroblasts are responsible for matrix deposition and consequent contraction in Dupuytren’s disease. Many growth factors and cytokines seem to be implicated in the etiology of Dupuytren’s contracture, among all transforming growth factor beta (TGF-β) has been suggested to play a predominant role [7]. TGF-β is responsible for the up-regulation of collagens and also of other extracellular matrix components, all fundamental for connective tissue remodeling [8]. TGF-β transduces a signal through an heteromeric complex for formation of related type I and type II transmembrane serine/threonine kinase receptors [9]; the signal of the activated type I receptor induces SMAD signaling cascade and the heteromeric SMAD complexes (SMAD2/3-SMAD4) accumulated in the nucleus regulate the expression of a large array of target genes involved in myofibroblast proliferation, differentiation and extracellular matrix synthesis [10-11]. Several studies have identified pro-inflammatory cytokines in Dupuytren’s tissues, but the molecular mechanisms by which inflammatory mediators activate myofibroblast differentiation are still unknown [12]. II-6 is involved in the modulation of TGF-β and its receptor TGF-βRII, inducing then fibroblasts proliferation [13]. TNF-α is another central mediator of the fibrotic process, similar to TGF-β [14]: it has been identified as a mediator involved in
the differentiation of fibroblasts into myofibroblasts in the palm of patients affected by Dupuytren’s disease, via activation of Wnt signaling pathway [15]. TNF-α may directly regulate TGF-β1 expression, as shown in lung fibroblasts [16]. Yet, hypoxia and subsequent angiogenesis seem to play a role in the pathophysiology of this disease [17]. Angiogenesis is induced by several growth factors, but the most important molecule is vascular endothelial growth factor (VEGF), also known as vascular permeability factor [18]. Hypoxia activates the transcription of hypoxia-inducible factor alpha (HIF-1α), which itself positively regulates VEGF synthesis [19]. All these findings prompted us to investigate the structural alterations of the fibromatous palmar fascia in patients affected by Dupuytren’s contracture and to analyze expression and localization of the previously described growth factors in surgical samples of palmar aponeurosis. In parallel, immunofluorescence and RT-PCR analysis were conducted on primary cultures of fibroblasts and myofibroblasts explanted from Dupuytren’s nodules in the proliferative or involutional phases.

Materials and Methods

Tissue samples were obtained from 26 patients (22 males and 4 females, mean age 58 years, mean duration of clinical history 2.5 years) undergoing surgical dermo-fasciectomy for Dupuytren’s contracture (n=18) and Carpal Tunnel Syndrome (n=8, as negative controls). Pathological tissues were sampled from areas of Dupuytren's nodules (4 specimens for each nodule). Control samples (2 specimens for each tissue fragment), characterized by normal palmar fascia tissues, were collected from patients undergoing hand surgery for Carpal Tunnel Syndrome (CTS). During excision, apart from anesthesia, no other chemical products or pharmaceutical drugs have been administered. All samples were collected with the informed consent of the patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Experiments were performed in compliance with the Italian laws and guidelines concerning the patients’ written informed consent. The Ethics Committee of the Policlinico Umberto I Hospital approved our study according to European
Community and Italian laws.
Samples (72 from DD and 16 from control patients) were fixed in formalin and embedded in paraffin to be processed for histological staining and immunohistochemistry. The sections were subjected to Hematoxylin & Eosin and Masson’s Trichrome staining. In parallel, other tissue samples were used to obtain primary cultures of pathological and normal fibroblasts.

**Immunohistochemistry**

The immunohistochemical analysis was conducted using the ABC/HRP technique (avidin complexed with biotinylated peroxidase) on 4 µm thick paraffin sections that were cut using a rotative microtome. These sections were deparaffinized and hydrated through decreasing ethanol series to distilled water, then subjected to microwave irradiation and immersed in citrate buffer (pH = 6) twice for 5 minutes each time. Subsequently, endogenous peroxidase activity was quenched using 0.3% hydrogenous peroxide in methanol for 30 minutes. To evaluate the immunolocalization of pro-inflammatory factors, VEGF, marker of myofibroblasts and proliferating cell nuclear antigen (PNCA), the following antibodies were employed: mouse anti-αSMA monoclonal antibody (Leica, USA 1:100); rabbit anti-TGF-β1 polyclonal antibody (Santa Cruz, CA, USA 1:200); rabbit anti-IL1β polyclonal antibody (Santa Cruz, CA, USA 1:100); rabbit anti-IL6 polyclonal antibody (Santa Cruz, CA, USA 1:50); mouse anti-VEGFα monoclonal antibody (Santa Cruz, CA, USA 1:100); mouse anti-TNF-alpha monoclonal antibody (Santa Cruz, CA, USA 1:200); mouse anti-ICAM-1 monoclonal antibody (Santa Cruz, CA, USA 1:25) and mouse anti-PNCA monoclonal antibody (Santa Cruz, CA. USA 1:100).

Incubation with the primary antibodies was performed overnight at 4ºC. Optimal antibody dilution and incubation times were assessed in preliminary experiments. As negative control, the primary antibodies were omitted. After exposure to the primary antibodies all slides were rinsed twice in phosphate buffer (pH=7.4) and incubated for 1 hour with the appropriate secondary biotinylated antibody at the final dilution of 1:200. The secondary biotinylated antibodies against rabbit and mouse immunoglobulins were purchased from
Abcam (biotinylated goat anti-mouse antibody and biotinylated goat anti-rabbit antibody). The slides were then incubated with peroxidase-conjugated avidin (Vector laboratories, Burlingame, CA, USA, Vectastain Elite ABC kit Standard*PK 6-100) for 30 min. Slides were washed in phosphate buffer (pH=7.4) and treated with 0.05% 3,3-diaminobenzidine (DAB) and 0.1% H₂O₂. Finally, sections were counterstained with Mayer’s hematoxylin and dehydrated rapidly. The staining assessment was made by three experts. The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. Twelve 100 µm² areas were delineated in each section by measuring the diaphragm. The system was calibrated taking the background obtained in sections exposed to non-immune serum as zero. Quantitative data of the intensity of immune staining were analyzed statistically by analysis of variance (ANOVA) followed by Dunca’s multiple range test as a post hoc test. The comparison of the expression levels of each antigen between the palmar fascia from Dupuytren’s disease and CTS patients was carried out by Student’s t-test. Statistical analysis was performed using the GraphPad Prism (La Jolla, CA). The results were considered statistically significant with p-value <0.05.

**Cells Cultures**

Dupuytren’s nodule tissues and normal palmar fascia tissues from CTS patients were obtained by surgery. All samples were minced using a sterile technique and placed in sterile 30 mm single well culture dishes. The wells were then flooded with 2.0 ml of Dulbecco’s essential medium (DMEM, Gibco, Grand Island, NY) containing 4% penicillin/streptomycin (PS, Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS, Gibco, Grand island, NY). The media was renewed three times weekly. Cells were observed adhering to the bottom of the wells and were allowed to grow to confluence. The cells were lifted from the wells using Trypsin/EDTA, pelleted, washed and re-suspended in DMEM with 10% FBS and 4% penicillin/streptomycin. Four cell strains were used in these experiments (4 from DD patients and 4 from control patients) and sub-cultured by less than 10 passages. These primary cultures of pathological and normal
cells were used for establishing protein and mRNA expression of specific pro-
inflammatory factors and VEGF by immunofluorescence and RT-PCR.

**Immunofluorescence**

For immunofluorescence fibroblast primary cultures were grown directly on Labteck chamber slides (Nunc) for at least 24 h, the cells were then washed with PBS with Ca/Mg, and fixed with 4 % buffered paraformaldehyde for 20 min at 4 °C. Fixed cells were incubated overnight at 4°C with the primary antibody for α-SMA, TGFβ1, IL1β, IL6, TNF-α and VEGFa. After three washes in 0.1% Tween in PBS for 10 min each, the cells were incubated with the secondary antibody, anti-mouse-fluorescein antibody (Abcam USA (MA) 1:200) or anti-rabbit– rhodamine antibody (Abcam USA (MA), 1:200) for 2 hours at room temperature. The nuclei were stained with DAPI (Vectashield Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA, USA). The immunofluorescence was examined by confocal laser microscope (Nikon TE2000). Student’s t-test was used to evaluate the expression of each analyzed antigen in DD and control cells. The data were considered statistically significant with p-value <0.05.

**RT-PCR**

Cultured cells were suspended in TRIzol reagent (Invitrogen Corporation, CA) and total RNA was isolated using RNeasy Micro Kit (Qiagen, CA, USA). Real time PCR was conducted to determine the differences in mRNA expression levels of TGF-β1, IL-1β and VEGFa between normal and pathological fibroblasts in culture. The purity of the RNA was assessed using a UV/visible spectrophotomer (SmartSpec 3000, Bio-Rad Laboratories, CA, USA). 1 µg total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription (RT) kit (Applied Byosystems, CA, USA) according to the manufacturer’s instructions. RNA samples, RT buffer, dNTP mix, RT random primers, multiscrime reverse transcriptase, RNase inhibitor and DEPC-treated distilled water were added in RNase-free tubes on ice at the final volume of 20 µl. The thermal cycler was programmed as follows: 25°C for 10 min, 37°C for 120 min and the reaction
was stopped at 85ºC for 5 min. For the RNA reactions we used the follow primer pairs (Bio Basic In, NY, USA): for TGF-β1 primer forward 5’-GCTGGACAGGAAGCTGGG-3’ and primer reverse 5’-GGACACAACACGAGGAGA-3’, for IL-1β primer forward 5’-GCTTCTGGTGATTCCGC-3’ and primer reverse 5’-GGGCTGTGAGGTTCTTG-3’, for VEGFa primer forward 5’-AACCCCTAGGCCAGGTTGA-3’ and primer reverse 5’-CGGGATATGGAAGGGAAGCC-3’. For glyceraldehyde 3 phosphate dehydrogenase (GAPDH) we used primer forward 5’-GAGCAGTCCGGTGTCACTAC-3’ and primer reverse 5’-TAGTAGCCGGCCCTACTTT-3’. The specificity of the primers was verified by searching in the NCBI database for possible homology to the cDNA of unrelated proteins. Each PCR tube contained the following reagents at the final volume of 50 µl: 0.2 µM of forward and reverse primers, 1 µg template cDNA, 0.2 mM dNTP mix, 2.5 U RedTaq Genomic DNA polymerase (Sigma-Aldrich), MgCl2 and reaction buffer 1X. The amplification was started with an initial denaturation step at 94°C (2 minutes) and was followed by 35 cycles consisting of denaturation (30 seconds) at 94°C, annealing (30 seconds) at the appropriate temperature for each primer pair and extension at 72 ºC (1 minute). Using the comparative critical cycle methods, the expression levels of the target genes were normalized to the GAPDH endogenous control. Data were analyzed using the 7900 HT SDS software version 2.1 provided by applied Biosystems. Statistical analysis was performed using Student’s t-test (GraphPad Prism). The results were considered statistically significant when the p-value was <0.05.

On the basis of these experiments we observed that TGF-β1 mRNA expression was the most significant among the examined molecules, so we performed Real Time PCR on paraffinized tissue in order to confirm mRNA expression data obtained from cell cultures. For this last procedure we used the same number of specimens as before (4 from DD patients and 4 from control patients). Real Time PCR was performed with the same protocol employed in previously described PCR.
Results

The rate of cell proliferation was first evaluated in Dupuytren’s tissues, by analyzing the cellular proliferation marker known as Proliferating Cell Nuclear Antigen (PCNA). Fibroblasts and myofibroblasts of Dupuytren’s nodules had a strong nuclear staining of PCNA (Fig. 1A), in contrast with the normal palmar fascia tissue, in which cell staining was completely absent (Fig. 1B). We quantified the number of positive cells on the total number of cells in 10 different areas for each experimental group and we expressed the ratio between PCNA-positive cells and total cells as percentages (Fig 1C). The incidence of PCNA-positive nuclei in the fibromatosis nodules (87.65 % ± 4.98 %) was significantly (P<0.0001) higher than that in the normal palmar fascia tissues (18.43 % ± 7.15 %). Our data demonstrate that these pathological samples are in the same proliferative stage of the disease that causes thickening and contracture of the palmar fascia. CD68 immunostaining was performed and it was completely negative in Dupuytren's nodules (Fig 2A) similarly to normal tissues (Fig. 2B), thus confirming the absence of macrophages and monocytes in the proliferative phase of the disease. Spindle-shaped cells in Dupuytren's nodule resulted alpha-SMA-positive (Fig. 2C), this is a reliable marker of the myofibroblastic phenotype; α-SMA staining was weak or absent in the control tissues (Fig. 2D). The α-SMA high expression in Dupuytren’s tissues confirmed the trans-differentiation from fibroblasts to contractile myofibroblastic phenotype.

Then TGF-β1 was highly expressed in the extracellular matrix and by fibroblasts and myofibroblasts of Dupuytren’s proliferative nodules (Fig. 3A); at the same time, a weak expression of TGF-β1 was evidenced in the fibroblasts and vascular endothelial cells of normal tissues, while it was completely negative in the extracellular matrix of normal palmar fascia (Fig. 3B). IL-1β was then evaluated, showing positive immunoreaction in the cytoplasm of fibroblasts and myofibroblasts, extracellular matrix and vascular endothelial cells of the fibro-proliferative nodules (Fig. 3C). This pro-inflammatory cytokine was completely
absent in the extracellular matrix of control samples, but weakly present in the
cytoplasm of capillary endothelial cells and fibroblasts (Fig. 3D).
IL-6 was strongly expressed in the extracellular matrix in its soluble form, and
moderately present in the proliferative myofibroblasts and fibroblasts of the
Dupuytren’s nodules (Fig. 3E). In the controls, IL-6 was completely absent at
level of the loose connective tissue but moderately expressed in the vascular
endothelial cells and in the fibroblasts (Fig. 3F), confirming that this cytokine is
involved in the inflammatory process that activate the fibrotic process of
Dupuytren’s disease.
TNF-α was then analyzed and it resulted moderately positive in the extracellular
matrix, fibroblasts and vascular endothelial cells of the Dupuytren’s proliferative
site (Fig. 4A) similarly to the control tissue (Fig. 4B). Finally, VEGFa was
strongly positive in vascular endothelium, fibroblasts and myofibroblasts of
Dupuytren’s nodules (Fig. 4C); differently, VEGF-a was completely absent in the
control tissues (Fig. 4D). All these data were supported by immunofluorescence
of the cultured fibroblasts and myofibroblasts isolated from Dupuytren’s
nodules, in which α-SMA was expressed by 50% of the cells, with uniform
cytoplasmic distribution (Fig. 5A), confirming the presence of myofibroblasts;
while α-SMA was completely absent in the cells isolated from normal palmar
fascia (Fig. 5B). Pathological cultured fibroblasts and myofibroblasts were also
strongly positive for TGF-β1 expression, as compared to normal fibroblasts,
showing a granular cytoplasmic distribution (Fig. 5 C-D). Also IL-1beta was
strongly present in Dupuytren’s cell cultures (Fig. 5E), but weakly present in
normal fibroblasts. Finally, IL-6 was weak and moderately present in
pathological (Fig. 5G) and normal (Fig. 5H) cultured cells. TNF alpha was
moderately expressed by pathological cells (fig 6A), similarly to control
fibroblasts (fig 6B). VEGFa was found to be strongly expressed in the cytoplasm
of fibroblasts and myofibroblasts isolated from Dupuytren’s tissue (fig 6C),
while was totally absent in normal fibroblasts (Fig 6D). Our study confirmed
that there was no difference in the intensity of the expression of this pro-
inflammatory cytokine and in the percentage of TNF-α-positive cells between
control cells and pathological fibroblasts (Fig. 7). The difference in the number
of IL-6-positive cells between the pathological and the normal cell lines of fibroblasts was significant for p<0.05 (Fig. 7). On the basis of the immunohistochemical and immunofluorescence results that showed a stronger expression of TGF-β, IL-1β and VEGFa in pathological tissue, respect to normal tissues, we then quantified their differences in mRNA expression between normal and pathological tissues by RT-PCR (Fig. 8). An upregulation of TGF-β1 (2.30 ± 0.05), IL-1β (2.02 ± 0.07) and VEGFa (1.97 ± 0.04) was demonstrated in fibroblasts from Dupuytren’s nodules compared to normal fibroblasts (Fig. 9A). RT-PCR data were demonstrated either on cultured cells or in de-paraffinized tissue samples with p-value <0.05.

**Discussion**

Dupuytren’s disease is a condition in which the formation of nodules in the palm of the hand precedes eventual contracture of the fingers due to fibrosis [20]. Clusters of macrophages and T-lymphocytes have been observed, in addition to myofibroblasts, in the pathological tissue [6, 21-24]. Several growth factors and inflammatory cytokines have been reported in the literature as molecules probably involved in the modulation of the pathogenesis of Dupuytren’s disease [25-27]. TGF-β is undoubtedly one of the cytokines most involved in the process of fibrosis and is present at high amounts in sites of chronic inflammation [28-32]. Moreover, some studies have also demonstrated in vitro that cultured Dupuytren’s cells produce TGF-β and that TGF-β stimulates the growth of Dupuytren’s fibroblasts [33]. As demonstrated by Kulkarni and Karlsson [34] TGF-β plays other important roles in the modulation of fibrosis and inflammation. Our data confirm that TGF beta is more strongly present in DD extracellular matrix and cells, than in the correspondent normal tissue and cells. A new finding, not described in the previous available literature on this topic, is the presence of a cytoplasmic granular fluorescent staining for TGF-β1, whose characteristic appearance represents an interesting typical finding observed in DD myofibroblasts. A recent study performed by Iqbal et al. [35] confirmed the importance of myofibroblasts in the pathogenesis of DD also
introducing the possibility of alternative sources of DD myofibroblasts originating from skin overlying nodule (SON) and perinodular fat (PNF). Verjee et al. [15] have recently identified another possible therapeutic target in the TNF, whose role may be hopefully relevant in the better future knowledge of pathogenesis and therapy of DD. Nowadays, there are different therapeutic options for the treatment of DD [36], but the real question remains still: what is the primary cause in its pathogenesis? An important recent study [37] postulates diverse origins of the myofibroblasts responsible for kidney fibrosis. Notwithstanding the differences in comparison with DD a possible general “common control” of the mechanisms of fibrosis may be activated even in different areas of the body in response to appropriate stimuli. This “common control” has to be elucidated in further studies, but it probably constitutes the central problem in DD and in other fibrosis-related pathologies. Some authors recently underline the possible role of Wnt signaling [38] in the pathogenesis of DD, but this study implicates even nine different loci involved in genetic susceptibility to Dupuytren's disease. The fact that six of these nine loci harbor genes encoding proteins in the Wnt-signaling pathway suggests that aberrations in this pathway may be key to the process of fibromatosis in Dupuytren's disease. However further genome studies should be performed in order to elucidate this interesting hypothesis.

Our results demonstrated that TGF-β, IL-1β and VEGFa are markedly expressed in Dupuytren’s tissue and cultured myofibroblasts. This finding led us to postulate a pivotal role for these molecules in the development of Dupuytren’s contracture. The obtained informations may provide a basis for the research of non-surgical treatment regimens to reduce the recurrence and the progression of this disease, ultimately attenuating hospitalization and post-surgical rehabilitation for patients.

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References

**Figure 1.** Immunohistochemical analysis of PCNA, CD68 and α-SMA markers in Dupuytren’s nodules and control tissues. The photomicrographs of Dupuytren’s nodular tissues showed an increasing number of PCNA (A) and α-SMA-positive cells (C) compared to the control tissues in which these markers resulted completely negative (D, F). Immunohistochemistry for CD-68 showed that the macrophages and monocytes were completely absent in the pathological nodule (B) similarly to the normal tissue (E). (magnification 40X).
Figure 2. Cell proliferation index in Dupuytren’s nodules and normal fascia palmar tissues. PCNA proliferative index is represented as a percentage of PCNA-positive nuclei on the total number of cells in Dupuytren’s nodules (87.65% ± 4.98) and normal fascia palmar tissues (18% ± 7.15). The bar graph indicates the mean percentage of PCNA positive cells ± SD. Statistical analysis is performed using Student’s t-test. *p-value < 0.05.
**Figure 3.** Immunohistochemical analysis of pro-inflammatory markers TGF-β, IL-1β and IL-6 in Dupuytren’s nodules and controls. TGF-β was strongly expressed in the extracellular matrix and cytoplasm of proliferative myofibroblasts and fibroblasts in the pathological tissue (A). TGF-β was moderately present in the cytoplasm of fibroblasts scattered in the loose connective tissue of normal fascia palmar (D). IL-1β was strongly positive in Dupuytren’s myofibroblasts - rich nodules showing cytoplasmic localization (B). IL-1beta was completely absent in the extracellular matrix and weakly present in the vascular endothelium and fibroblasts of normal palmar fascia (E). IL-6 was moderately expressed in the extracellular matrix and fibroblasts of Dupuytren’s tissues (C) similarly to the control tissue (F). (magnification 40X).
Figure 4. Immunohistochemical analysis of TNF-α and VEGF in Dupuytren’s nodules and control tissues. A moderate expression of TNF-α is seen in the extracellular matrix and fibroblasts of Dupuytren’s nodules (A) and control tissues (C) (magnification 40X). VEGF immunoreactivity appears to be strongly positive in the extracellular matrix, fibroblasts and myofibroblasts of Dupuytren’s nodules (B) (magnification 40X). compared to controls in which the staining was completely absent in the amorphous substance and weakly expressed in the cytoplasm of normal fibroblasts (D) (magnification 20X).
Figure 5. Immunofluorescence for α-SMA and pro-inflammatory factors TGF-β, IL-β, IL-6 in fibroblast cultures isolated from Dupuytren’s nodules and normal palmar fascia. The pathological fibroblasts isolated from Dupuytren’s nodules (A) showed a strong expression of α-SMA demonstrating that 50% of fibroblasts differ in myofibroblasts. In the normal fibroblasts, α-SMA expression was completely absent (E). TGF-β and IL-β were strongly expressed in the pathological fibroblasts isolated from Dupuytren’s tissues (B, C,) compared to the normal fibroblasts (F, G,) that showed a weak expression of these pro-inflammatory factors. IL-6 was moderately expressed in the cytoplasm of pathological (D) and normal fibroblasts (H) (magnification 20X).
**Figure 6.** Immunofluorescence for TNF-α and VEGF in fibroblast cells isolated from Dupuytren’s nodules and normal palmar fascia. TNF-α was moderately expressed in the pathological fibroblast cultures (A) similarly to the control fibroblasts (C). A strong VEGF expression was found in the cytoplasm of Dupuytren’s fibroblasts (B) differently from fibroblasts isolated from normal palmar fascia in which this factor was completely absent (D). (magnification 20X).
Figure 7. Pro-inflammatory cytokines and VEGF positive cell index in the fibroblasts isolated from Dupuytren's contracture and normal palmar fascia tissue. The bar graph indicates the mean % growth factors-positive cells ± SD. Statistical analysis is performed using Student's t-test. * p<0.0001 or ** p<0.05.
Figure 8. Quantitative PCR analysis in Dupuytren’s nodules-derived cells, in control tissue and in paraffinized tissue samples. A (upper): PCR analysis of mRNA expression for TGF-β₁, IL-1β and VEGF-A in nodules-derived cells and in control normal tissue. Data are presented as mean ± SEM. Statistical analysis is performed using Student’s t-test. * p-value <0.05.

B (down): PCR analysis of mRNA expression for TGF-β₁ in paraffinized tissue samples from DD patients and control ones.