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RESEARCH ARTICLE (EXPERIMENTAL)

Characterisation of the inflammatory response in Dupuytren's disease

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Abstract

Background: Dupuytren's disease is characterised by fibrotic nodule and cord formation in the palmar aponeurosis. The pathophysiology of the disease is still unknown, although cell stress and subsequent activation of immune mechanisms seems to be crucial. *Materials and methods*: Surgically obtained tissue and blood samples of 100 Dupuytren patients were processed by immunohistochemistry, flow cytometry, as well as immunoscope analysis. Macroscopically normal aponeurotic tissue served as control. *Results*: Locally, microvascular alterations and massive infiltration by mononuclear cells (CD3+, CD4 > CD8, CD45RO > CD45RA, S100 protein, CD56, CD68, scarce CD19 and mast cells) forming perivascular clusters were found in DD tissue. Cytokine profiling of fibromatosis tissue-derived T-cells showed a Th1/TH17-weighted immune response. Immunoscope analysis revealed a restricted T-cell receptor α/β repertoire pointing to an (auto)antigen-driven process. *Conclusion*: The striking accumulation of immune cells, expression of leukocyte adhesion molecules, as well as pro-inflammatory and pro-fibrotic cytokines near markedly narrowed vessels supports the theory that the abnormal proliferation of fibroblasts and production of extracellular matrix proteins in DD seems to be related to immune-mediated microvascular damage. The restricted T-cell receptor repertoire of intra-lesional T-cells points to an antigen-driven process. T-cells seem to play an important role in the development of Dupuytren's disease.

Key Words: Palmar fibromatosis, immune cells, inflammation

Introduction

Palmar fibromatosis or Dupuytren's disease (DD) is characterised by fibrotic nodule and cord formation with progressive contraction of the palmar fascia resulting in finger flexion deformity. Currently, therapy consists of surgical excision of the abnormal fascia, collagenase injections, or percutaneous needle fasciotomy (PNF), neither of which helps to heal the deformity or prevent frequent recurrence [1,2]. Despite positive family histories, ~30% of cases are sporadic. Thus, a combination of environmental factors and genetic predisposition seems to underlie the pathogenesis of this condition [3,4]. In particular, damage to and occlusion of the palmar microvasculature are likely to be triggering events [5]. A range of predisposing ''stress factors'', such as smoking, and high alcohol consumption, on the one hand, and extreme and sustained vibration shock to the hand, on the other, are known to be associated with vascular damage and the development of DD, respectively [6,7]. T-cells play an important role in the induction and perpetuation of various fibrotic conditions [8]. In a recent review on the immunology of fibrosis, we set forth two paradigmatic axioms, namely (a) no fibrosis without prior and concomitant inflammation, and (b) irrespective of the primary disease underlying the development of fibrosis, the final profibrotic molecular pathway is always stereotypical [9]. So far, only a few reports have focused on the initial role of immune cells – especially T-cells – in the development of palmar fibromatosis [10,11]. Baird et al. [12] proposed that the onset of DD following injury in genetically susceptible individuals might be related to the large pool of activated T-cells and macrophages present in the affected palmar aponeurosis. In the context of capsular fibrosis around silicone breast implants, our group has shown that T regulatory and effector cells are main drivers in this fibrotic process [13].

In the present study, DD tissue-infiltrating immune cells were phenotypically characterised and analysed for their potential capability for cytokine production. The microvascular architecture with special focus on adhesion molecules was also investigated. In addition, fibromatosis-derived T-cells were isolated and analysed for signs of clonal expansion in order to support or exclude an antigen-driven process. Although microarray data are already available for DD tissue samples, extensive studies on the protein level are still needed [14]. We analysed the cellular and molecular composition of DD fibromatous tissue, with an extensive panel of antibodies in a great number of patients. Additionally, we tested the hypothesis that a combination of various ''stress factors'' leads to localised activation of the immune system in Dupuytren's patients.

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Materials and methods

Subjects and controls

One hundred patients undergoing fasciectomy for DD at the Department of Plastic and Reconstructive Surgery, Innsbruck Medical University, were included in the study (77 men, 23 women; age range = 21–83 years; mean = 60.3; median = 61). Preoperatively, all patients had confirmed diagnosis of DD. Tissue samples from Dupuytren's patients were obtained after partial or complete fasciectomy, these specimens consisted of excised cords and/or nodules, not otherwise needed for routine pathological diagnostics. Peripheral blood from DD patients, as well as macroscopically uncompromised aponeurotic tissue were used for control purposes. Table 1 displays sample numbers, which were included for final data generation. All study participants responded to a questionnaire about current health status and smoking habits. The Ethics Committee of the Medical University Innsbruck gave approval for the study (Study protocol #AN2218), and written informed consent was obtained from all participants. Blood was collected during general anaesthesia. Standardised pre- and postoperative pictures were taken from patients' hands (Figure 1).

Table 1. Overview of used methods and patient samples.

Techniques	Nodule, *n*	Cord, *n*	Normal fascia, *n*	Unspecified Dupuytren tissue*, *n*
Immunohistochemical analysis	31	37	22	63
Immunoscope analysis	15			
FACS analysis				33
Cytokine analysis				15

*These tissue samples were not classified from the surgeons into cord or nodule.

Immunohistochemistry and immunofluorescence

Serial paraffin sections (4 µm) of buffered formaldehyde fixed specimens were deparaffinised, subjected to antigen retrieval procedures adjusted for each single antibody-conjugate combination in pilot studies and stained immunohistochemically or by immunofluorescence (see Table 1). The number of slides and stainings, which we analysed per patient, varied due to the availability of different source material. The analysis of more than 3000 slides provided the basis for a semi-quantitative evaluation (Table 2). Appropriate negative controls consisting of either mouse isotype or rabbit immunoglobulin fractions were included in all assays.

Isolation and expansion of fibromatosis-derived T-cell lines

Isolation and expansion of lesion-derived T-cells was performed as detailed previously [13]. T-cell lines could be successfully cultured out of tissue samples from 33 patients. Briefly, the palmar fibromatosis cords and nodules were washed, cut into small pieces (ca. $2 \times 2 \times 2$ mm), and incubated without homogenisation (37°C, 5% CO_2) in a 6-well Petri dish plate in 1640 RPMI supplemented with IL-2 (20 U/ml, kindly provided by Dr E. Liehl, Novartis Research Institute, Vienna, Austria) on days 1 and 3. After 7 days, the T cell-containing supernatant was transferred to flat-bottom 24-well plates, stimulated with OKT3 (10 ng/ml Orthoclone OKT3, Janssen-Cilag, Saunderton, UK), IL-2 (20 U/ml) and autologous, irradiated (30 Gy) peripheral blood mononuclear cells (PBMCs) (5×10^5/ml) as feeder cells. Autologous PBMCs were cultivated in exactly the same way. In 15

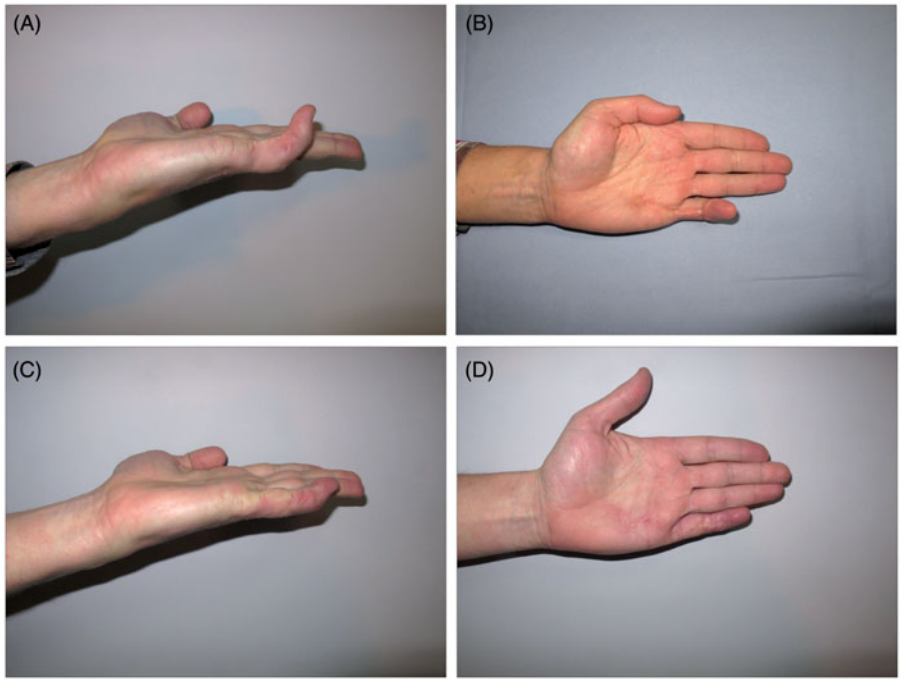

Figure 1. (A, B) Preoperative picture of Dupuytren's contracture involving the fifth ray of the left hand. Obvious extension deficit in the PIP joint. (C, D) Postoperative picture after partial fasciectomy.

Table 2. Overview on protein and cell expression in Dupuytren's tissue.

Antibody	Specificity	Quantification	Dupuytren's tissue expression
Adhesion molecules			
E-Selectin (CD62-E)	activated Ec	+ to ++	some vascular Ec
P-Selectin (CD62-P)	activated Ec	+++	many vascular Ec
VAP-1	Ec, SMC	+++	some vascular Ec
VCAM-1 (CD106)	Ec	+ to ++	sporadic positive cells; focal accumulation of positive cells adjacent to vessels
Cytokines and Enzymes			
CRP	acute phase protein	++ to +++	some positive vessels and proliferative nodules
IL-17R	cytoplasmic	+	
MIF	Ma in inflamed tissues	+ to +++	positive cells located adjacent to vessels in fibrocellular nodules
MRP-8	Mo, Ma in acute inflamed tissues	+ to ++	sporadic positive cells located adjacent to vessels
MRP-14	Mo, Ma in acute inflamed tissues	++ to +++	some positive cells located adjacent to vessels in fibrocellular and fibrotic stage
MMP-2	cytoplasmic	+ to ++	vascular SMC; cells located adjacent to vessels
TGFβ-1	cytoplasmic	++	many positive areas peripheral to nodules; vessel lumina
TNFα	Ma	+	weakly positive pericytes around vessels and myofibroblasts in proliferative nodules
Endothelin	Ec	+++	some positive vascular Ec
Synoviolin	cytoplasmic	+ to +++	interstitial tissue and Ec in small vessels
(Oxidative) Stress proteins			
Hsp60	HSP60, cytoplasmic	++	some positive cells in proliferative nodules and in MNC infiltrate; located near vessels
Fra-2	nuclear	+++	many positive cells in proliferative nodules and MNC clusters
Mononuclear cells			
CD1a	Dc; activ. T-cells	+ to ++	sporadic positive cells preferentially located adjacent to vessels
CD3	T-cells	+ to ++	cell clusters preferentially located adjacent to vessels
CD4	T helper cells	+ to ++	sporadic to view cells located adjacent to vessels; sporadic cell clusters
CD8	cytotoxic T-cells	+	sporadic positive cells located adjacent to vessels
CD11a	all Lc	++	sporadic positive cells located adjacent to vessels
CD15	mature granulocytes	+ to ++	some cells located adjacent to vessels and in proliferative nodules
CD18	most Lc	+ to ++	some cells located in proliferative nodules and adjacent to vessels
CD19	B-cells	+ to ++	cells preferentially located adjacent to vessels
CD25	activated T/B-cells	+ to ++	sporadic positive cells located in proliferative nodules and adjacent to vessels
CD29	most Lc	+++	many positive cells located in proliferative nodules
CD34	B lymphoid precursors	++ to +++	vascular Ecs
CD44	hyaluronan receptor	+ to +++	cells in proliferative nodules and located adjacent to vessels
CD45RA	naive T-cells	++	some cell clusters located adjacent to vessels
CD45RO	activated T-cells	++	some cell clusters located adjacent to vessels
CD47	Lc, Ec, fibroblasts	+ to ++	sporadic cells located adjacent to vessels
CD49d	all Ly	++ to +++	some cells located adjacent to vessels
CD54	Activated Ec, Mo, Ly	++ to +++	some positive vascular Ecs and cells located in proliferative nodules
CD56	natural killer cells	++	many positive cells in proliferative nodules; Ruffine bodies;
CD68	Ma	+ to ++	sporadic to some cells located adjacent to vessels
CD74	B-cells	+ to ++	some cells located adjacent to vessels and in proliferative nodules
CD79b	B-cells	+ to ++	sporadic positive cells located in proliferative nodules
foxp3	regulatory T-cells	+ to ++	sporadic to some positive cells located adjacent to vessels
HLA-DP, DQ, DR	antigen presenting cells	++ to +++	many positive cells located in proliferative nodules and HLA positive endothelial cells
Mastcell-Tryptase	mast cells	+++	sporadic cells preferentiallly located adjacent to vessels
S100	Dc	+++	some cells located between fat cells and vessels; many Ruffini bodies
TCRα	T cells	+	some positive cells located adjacent to vessels and focal accumulation
TCRγ/δ	T cells	+ to ++	sporadic cells located perivascular

Dc = dendritic cells; Ec = endothelial cells; SMC = smooth muscle cells; Ma = macrophages; Mo = monocytes; MNC = mononuclear cells; Lc = leucocytes; Ly = lymphocytes.

cases, T-cell lines and autologous PBMCs could be cultivated in completely parallel settings (data set for CD4/CD8 ratio calculations).

Flow cytometry analysis

The phenotype of the T-cell lines from 33 DD patients was determined by FACS analysis on a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ). Autologous PBMCs served as controls ($n = 21$). Briefly, fibromatosis-derived T-cell lines and autologous PBMCs were harvested after 21 days of culture and directly labelled using monoclonal antibodies (anti-CD3FITC (1F-202-T100), -CD4PE (1P-359-T100), -CD16FITC (1F-399-T100), -CD25FITC (1F-218-T100), -CD45RA/FITC (1F-223-T100), -CD56PE (1P-231-T100), Exbio, Prague, Czech Republic; -CD8Pe-Cy5.5 (341050), -CD28APC (559770), Becton Dickinson; -CD45RO/APC (17–0457-71), -TCR$\alpha\beta$/APC (17–9986-71), -TCR$\gamma\delta$/PE(12–9959-71), -Foxp3/APC (17–4776-71), eBioscience, San Diego, CA).

Cytokine analysis

Supernatant analysis for cytokine detection of T-cell lines was performed on samples of 15 different randomly selected Dupuytren's patients using a human Th1/Th2 11plex FlowCytomix Multiplex and a commercial ELISA kit (Bender MedSystems, Vienna, Austria).

RNA isolation and TCR CDR3 spectratyping

Total RNA was extracted directly from fibromatous nodules and autologous PBMCs from 15 randomly selected patients (age range = 41–75 years), using TRI reagent (Sigma Aldrich, St. Louis, MO). As previously described by Herndler-Brandstetter et al. [15], TCR Vβ transcripts were amplified by PCR using a Hot-Start Taq Master Mix kit (Qiagen, Vienna, Austria) and primers (MWG Biotech, Ebersberg, Germany) specific for each of the human Vβ families and a specific primer for the constant region of the β-chain (labelled with the fluorochrome 6-FAM). The distribution of clonality was calculated by a standard calculation of percentage.

Statistics

Statistical analysis was performed using Microsoft Excel and SPSS 15.0 software. Wilcoxon W, Kruskal–Wallis, Mann–Whitney *U*-test, and Fisher's exact test were used to estimate differences between groups and their respective *p*-values.

Results

Evaluation of questionnaires

No significant difference was found between DD patients and healthy controls when compared for the possible presence of autoimmune diseases, allergies, and vascular diseases. A statistically significant number (32.9%) of DD patients in this cohort were smokers, compared to only 18.6% of the control group ($p = 0.047$). However, no correlation was found between smoking habits and stage or recurrence of disease, number of affected finger strings, or number of affected hands.

Fibromatosis-derived T-cells have a predominantly CD4 + phenotype

Most of the fibromatosis patients had a mixed phenotype, with CD4 + T-cells (median = 80%) prevailing over CD8 + T-cells (median = 22%). The CD4/CD8 ratio, calculated for 15 patients for whom data from fibromatosis T-cells and autologous PBMCs were available, was increased compared to peripheral autologous T-cells ($p = 0.07$) (Figure 2). Foxp3 + regulatory T-cells were detected in higher numbers in the fibrotic tissue (median = 7%) as compared to peripheral PBMCs (median = 2%), and TCR$\alpha\beta$+ T-cells prevailed over TCR$\gamma\delta$ T-cells.

Fibromatosis-derived T-cells produce mainly IFNγ, IL-6, IL-8, and IL-17

DD tissue-derived T-cells from 15 different patients and T-cells expanded from autologous PBMCs were analysed for their cytokine secretion profile (Table 3). Fibromatosis tissue-derived T-cells produced similar amounts of the Th1 cytokine IFNγ (mean = 3139 pg/ml) as autologous peripheral T-cells (mean = 2908 pg/ml). A significant difference ($p < 0.001$) in the production of the profibrotic cytokine IL-6 was found between fibromatosis-derived T-cells (mean = 5649 pg/ml) and autologous peripheral mononuclear cells (mean = 105 pg/ml). IL-8, which is mainly produced by macrophages and ECs, but also by T-cells, was secreted to a large extent by T-cells from both sources (T-cells mean = 6859 pg/ml; PBMCs mean = 5956 pg/ml). Moreover, IL-17 secretion turned out to be significantly different ($p = 0.031$) between fibromatosis-derived T-cells (mean = 639 pg/ml) and autologous PBMCs (mean = 261 pg/ml).

Activated endothelium and focal accumulation of mononuclear immune cells

A semiquantitative overview of the immunostaining of sections of Dupuytren's tissue is given in Table 2. Several proinflammatory cytokines were detected *in situ*, e.g. macrophage migration inhibitory factor (MIF, Figure 3(A); Figure 3(B) represents the negative control rabbit Ig fraction on an adjacent section to 3(A)) and vascular adhesion protein (VAP-1, Figure 3(C)). The profibrotic cytokine TGFβ1 was abundantly expressed in the areas containing excessive

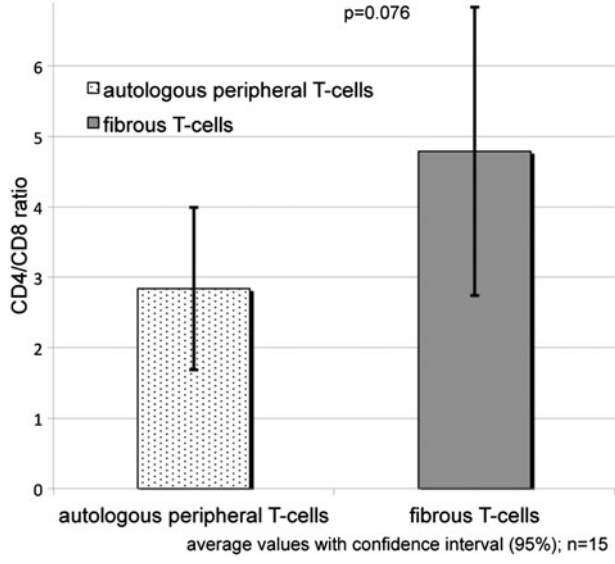

Figure 2. T-cells isolated from palmar fibromatosis tissue show an increased CD4/CD8 ratio compared to autologous peripheral blood-derived T-cells ($p = 0.076$).

Table 3. Cytokine expression profile of fibromatosis tissue-derived ($n = 15$) and autologous peripheral T-cells ($n = 13$).

	Conc. pg/ml	IL-12p70	IFNg	IL-2	IL-10	IL-8	IL-6	IL-4	IL-5	IL-1b	TNF-a	TNF-b	IL-17	TGFb-1
PBMCs	Mean value	1	2 908	417	152	5 956	105	23	402	18	269	63	261	565
	Median	0	1 772	61	59	6 145	82	21	69	1	167	6	74	588
	Min	0	256	0	0	706	0	0	0	0	16	0	9	328
	Max	10	12 633	1657	591	10 629	380	90	3419	82	1255	410	1242	772
Fibromatous	Mean value	2	3 139	90	36	6 859	5 649	21	261	100	118	6	639	640
T-cell	Median	0	1 945	0	17	7 119	5 115	15	14	50	91	0	467	643
	Min	0	0	0	0	4 374	355	0	0	0	4	0	19	347
	Max	32	7 406	156	138	9 290	17 063	75	2581	234	436	47	1242	863
							$p > 0.001$						$p = 0.031$	

Note: dupuytren's tissue-derived T-cells secrete mainly IFNγ, IL-8, IL-6, IL-17.

extracellular matrix (ECM) molecules (Figure 3(D)), exceeding TNFα (Figure 3(E) and (F)).

Many HLA-DR-positive and S100 protein-positive dendritic cells were found mainly adjoining blood vessels (Figure 3(G) and (H)). Endothelial cells of venules in Dupuytren's tissue also showed a positive staining pattern for HLA-DR.

B-cells and Foxp3-positive regulatory T-cells were scattered within the mononuclear cell infiltrates (Figure 4(A) and (B)). Foxp3 cells reside mostly adjacent to vessels and were found in nodular as well as cord tissue. In sequential sections, CD45RA-positive naïve T-cells were markedly outnumbered by CD45RO-positive activated T-cells (Figure 4(C) and (D)). Numerous partially occluded vessels were dispersed throughout the fibromatosis tissue, accompanied by clusters of naïve and activated T-cells (Figure 4(E) and (F)). Massive expression of the stress protein, heat shock protein 60 (HSP60), was observed too – especially in hyperproliferative nodular areas (Figure 4(H)).

Adjacent autologous aponeurosis tissue, macroscopically unaffected, also turned out to be slightly positive with several of the antibodies applied, e.g. CD8, CD44, CD45RO (Figure 4(G)), CD68, and HLA-DR. Thus, macroscopically "normal" aponeurotic tissue already contained typical cellular inflammatory infiltrations, albeit to a much lower degree.

T-cells from Dupuytren's nodules have a restricted T-cell repertoire

Figure 5 shows the predominantly oligo-/monoclonally restricted T-cell repertoire of α/β T-cells accumulating in fibromatosis tissue (43%) compared to only 12% of peripheral T-cells ($p < 0.001$). In contrast, peripheral T-cells showed a predominantly polyclonal TCR repertoire (55%), whereas almost no polyclonality was found in fibromatosis nodules-derived T-cells (1%, $p < 0.001$).

Discussion

In spite of recent attempts to elucidate the pathogenesis of DD via genome-wide association studies, the precise role of immune mechanisms involved in the development of this disease is not yet understood [16]. DD is characterized by three histological phases: (a) the proliferative phase, characterised by local fibroplasia and the development of nodular lesions with high density of myofibroblasts, followed by (b) the involutional phase, where cells within the nodule realign themselves with the lines of stress in the tissue, and, finally, (c) the residual phase during which nodules disappear leaving relatively acellular, scar-like tissue with an absence of myofibroblasts. Based on our long-standing experience in the study of fibrotic diseases including DD, we believe, that (a) in all forms of fibrosis, inflammatory-immunologic reactions take place in the earliest stages, promoting subsequent profibrotic processes, and (b) elements of both the innate and adaptive immune system are involved in this sequence of events [9].

We detected perivascular clusters of activated T-cells (Figure 4(E) and (F)), mostly positive for TCR receptor $\alpha\beta$ (TCR$\alpha\beta$). Foxp3-positive regulatory T-cells (Figure 4(B)) and B-cells were also present. Meek et al. [17] showed in this context that the inflammatory cells in the nodules – mainly macrophages and lymphocytes – express a common integrin

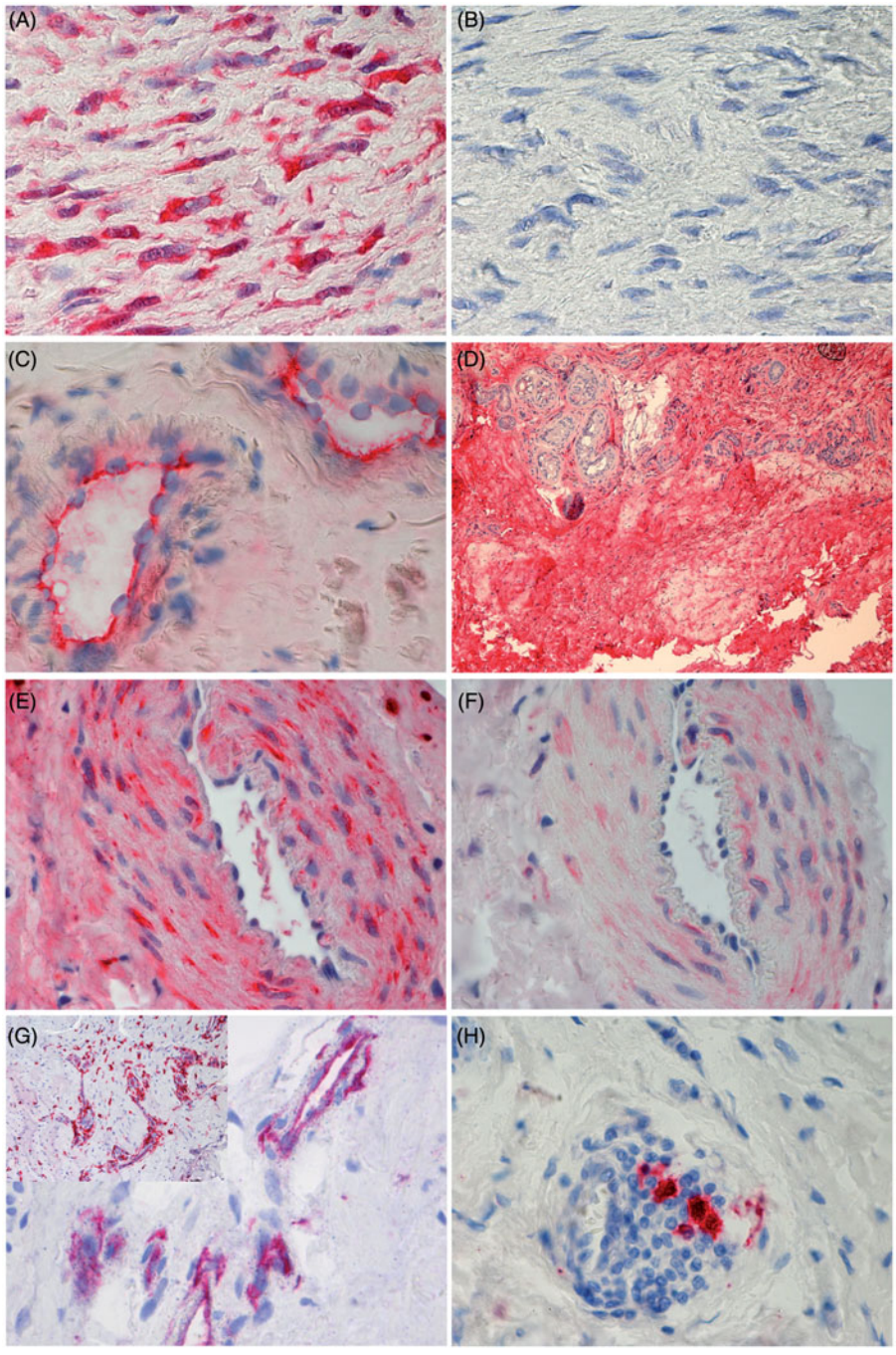

Figure 3. (A) MIF (macrophage migration inhibitory factor = proinflammatory cytokine) positive fibromatosis tissue (74 years, male; original magnification 600×); (B) Negative control rabbit Ig fraction on an adjacent section to 3(A) (74 years, male; original magnification 600×); (C) VAP-1 (vascular adhesion protein-1) positive vascular endothelium in Dupuytren's tissue (75 years, male; original magnification 600×); (D) Abundant TGFβ-1 expression in Dupuytren's tissue (72 years, male; original magnification 100×); (E) Blood vessel with exceptionally thick walls showing intense TGFβ-1 staining in the cytoplasm of DD cord (53 years, male; original magnification 600×); (F) Serial section to area depicted in (E) shows moderate TNFα immunoreactivity in DD cord (53 years, male; original magnification 600×); (G) HLA-DR-positive cells in DD nodule (75 years, male; original magnification 200×); HLA-DR-positive endothelial cells on venules in DD cord (55 years, male; original magnification 400×); (H) S100-positive dendritic cells in DD cord (58 year old, male; original magnification 600×); for Fgures 2(A–H) immunohistochemical stainings using alkaline-phosphatase and Fast Red for detection were applied.

known as VLA4, which functions as ligand to VCAM-1 present on endothelial cells and the CS1 sequence of fibronectin as well. In contrast to Qureshi et al. [18], we found S100 protein-positive dendritic cells also in DD cord tissue, which provide the local prerequisite for antigenic stimulation of T-cells (Figure 3(H)). Several proinflammatory cytokines, e.g. MIF (Figure 3(A) and (B) – negative control rabbit Ig fraction), and the key pro-fibrotic cytokine TGFβ1 (Figure 3(D)), which lead to recruitment and local proliferation of macrophages, were also detected. Interestingly, immunohistochemistry showed extensive expression of TGFβ1 throughout all DD specimens, while cytokine profiling

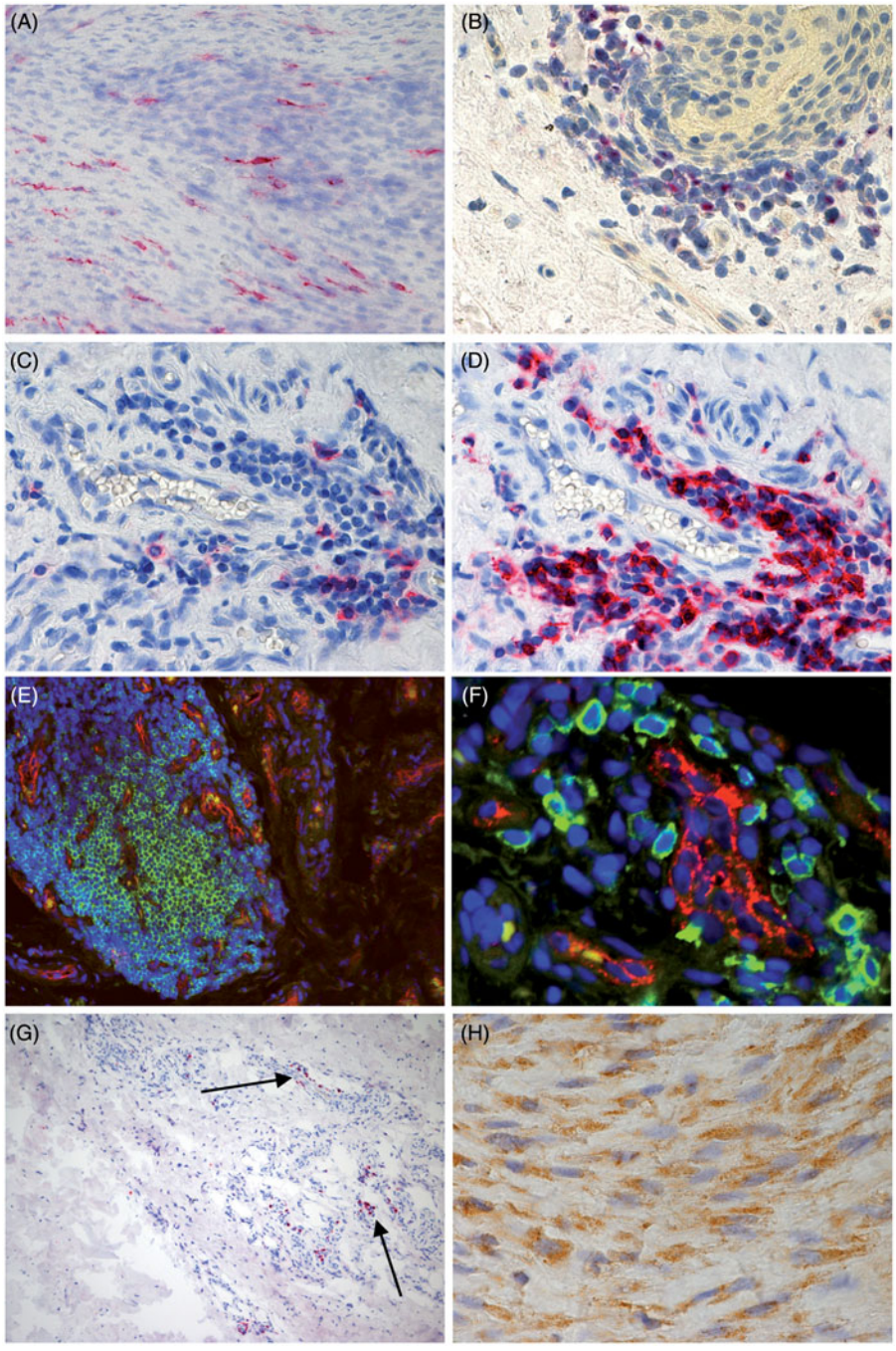

Figure 4. (A) CD74-positive B cells in cell-dense, actively proliferating DD nodule (62 years, female; original magnification 200×); (B) Foxp3-positive regulatory T-cells in palmar fibromatosis tissue (73 years, male; original magnification 600×); (C) CD45RA-positive naive T-cells (50 years, male; original magnification 600×); (D) Section adjacent to 4(C) stained for CD45RO displays more activated T-cells supporting our FACS data; original magnification 600×); (E) Immunofluorescence triple staining of naive CD45+ T cell cluster (Alexa488, green), von Willebrand factor-positive endothelial cells (TRITC, red) and nuclei (DAPI, blue) in Dupuytren's tissue, original magnification 200×; (F) Immunofluorescence triple staining of perivascularly CD45RO + activated accumulation of T-cells (Alexa488, green), von Willebrand factor-positive endothelial cells (TRITC, red), and DAPI- (blue) positive nuclei, in DD tissue, original magnification 600×; (G) CD45RO + activated T-cells in ''normal'' palmar aponeurosis adjacent to Dupuytren tissue (45 years, male; original magnification 100×); (H) HSP60 expression in recurrent Dupuytren's cord (53 years, male; original magnification 600×). Peroxidase anti-peroxidas and DAB (diaminobenzidine) were used for detection of HSP60. For (A–D) and (G), immunohistochemical stainings using alkaline-phosphatase and Fast Red for detection were applied.

of fibromatosis tissue-derived T-cells showed, compared to PBMCs, almost identical TGFβ1 levels. A possible explanation for this discrepancy is the fact that cytokine analysis was not performed *ex vivo*, but after a series of clonal T-cell expansion lasting 14 days. Th1 cells in DD tissue expressed IFNγ, which locally activates macrophages and induces the expression of MHC class II antigens and acute phase proteins, such as IL-6, which is known to exert profibrotic effects [19]. O'Reilly et al. [20] have shown recently that the profibrotic effect of IL-6 is dependent on STAT3; however, the effect is

indirect and mediated by enhanced TGF-β signalling and the classic downstream cellular mediator Smad3. Endothelial cells in Dupuytren's tissue also showed a positive staining for HLA-DR. MHC class II is constitutively expressed in a very restricted number of cell types, such as B-cells and dendritic cells, which are specialised in antigen presentation. However, MHC class II expression can be induced by T-cell derived IFNγ in a large variety of other cell types, including endothelial cells [21]. Wick et al. [22] showed HLA-DR positive stainings in atherosclerotic lesions, but not in normal intima, and they hypothesised that this aberrant MHC II expression may participate in the perpetuation of the atherogenic autoimmune reaction. In the present study, endothelial cells of venules with perivascular mononuclear cell infiltration were HLA-DR-positive (Figure 3(G)). Hence, the question arises if T-cells residing in palmar fibromatosis tissue react to pathogenetically relevant antigens. Immunoscope analysis showed that T-cells in DD tissue preferentially display a mono- and oligoclonally-restricted α/β T-cell receptor repertoire compared to autologous peripheral blood (Figure 5). This finding suggests that, in palmar fibromatosis tissue, an expansion of specific T-cell clones takes place, pointing to an antigen-driven mechanism. This is in analogy with the initiation of another fibrotic condition, namely atherosclerosis, where T-cells with a mono- and oligoclonally restricted α/β T-cell receptor repertoire play a crucial role. In the latter case, the target antigen for these specific T-cell clones is heat shock protein 60 (HSP60). Several studies provided evidence for increased expression of heat shock proteins, e.g. HSP47 and HSP70, in DD tissue [23]. We additionally found that the stress protein HSP60, a phylogenetically conserved and highly immunogenic molecule, was expressed in hyperproliferative areas of DD nodules at the earliest stage of the disease. Moreover, mononuclear cell infiltrates and ECs were positive for this stress protein, which in our experience represents an important possible candidate for the antigen(s) triggering the local immune response in the palmar tissue of DD patients in response to various stress factors.

Brandes et al. [24] found ECs responding to mechanical stress applied from the outside by producing a remarkable and highly ordered microfilament system, the so-called ''stress fibres''. Murrell et al. [25] hypothesised that narrowing of microvessels may be initiated by genetic factors, ageing, or environmental exposures, leading to localised ischaemia and the generation of free oxygen radicals. A positive feedback mechanism has been proposed: free radicals derived from stressed ECs damage the surrounding tissue and stimulate the proliferation of fibroblasts which excessively synthesise ECM molecules, mainly collagen, leading to further microvascular narrowing, local ischaemia, and oxidative stress. We have evidence in support of Murrell's hypothesis [25], but our focus has been on the role of T-cells as initially triggering factor(s). Thus, we hypothesise that microvascular ECs are functionally impaired by a combination of exogenous and endogenous ''stress factors''. Functional impairment leads to the attraction and accumulation of T-cells, which react against stress-induced molecules. Subsequently, ''innocent by-stander'' T-cells and macrophages are attracted, producing further pro-inflammatory and pro-fibrotic cytokines. The latter mediate the myofibroblast transdifferentiation and uncontrolled production of ECM, leading to the development of full-blown Dupuytren's contracture.

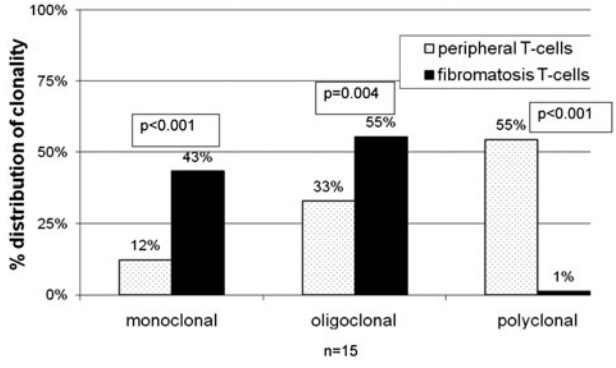

Figure 5. Immunoscope analysis shows distribution of TCR Vβ transcripts in intrafibromatosis and autologous peripheral T-cells. A significant monoclonal or oligoclonal restriction of the TCR$\alpha\beta$ repertoire was detected in fibromatosis-derived T-cells, while autologous PBMCs displayed a predominantly polyclonal pattern.

Macroscopically uncompromised aponeurotic tissue, adjacent to Dupuytren tissue, was used for control purposes. Interestingly, we found strong indicators (e.g. activated T-cells, HLA-DR + cells, CD4, CD8, DCs) for a local cellular inflammatory process in this ''normal'' palmar aponeurotic tissue, most probably representing the earliest steps of the disease process (Figure 4(G)). These sporadically appearing cells were not comparable with DD tissue-infiltrating cells in number, phenotype, or in distribution pattern. However, the presence of inflammatory hallmarks in adjacent ''normal'' palmar tissue might precede the development of Dupuytren's disease.

The striking accumulation of immune cells, leukocyte adhesion molecules, as well as pro-inflammatory and pro-fibrotic cytokines near markedly narrowed vascular luminae with thickened EC layers strongly supports the theory that the abnormal proliferation of cells in DD is related to immune-mediated microvasculature damage. The analysis of fibromatosis-derived T-cells shows a Th1/TH17-weighted immune response. Our findings show a restricted α/βTCR repertoire of intralesional T-cells, which points to an antigen-driven process, although the involved (auto)-antigen(s) have not yet been identified. An (auto)-immune mechanism, similar to that in the development of earliest atherosclerotic vascular changes triggering an immune reaction to (a) altered self molecules and (b) HSPs, might contribute to the development of DD. Thus, it would appear that DD does not occur ''spontaneously'', but rather is triggered by various endothelial stress factors. The next step in our investigations would be the determination of the antigenic specificity of palmar fibrosis-infiltrating T-cells. HSP60 is an important and plausible candidate in this context.

Mechanical stress, possibly combined with other stress factors (alcohol, smoking, etc) leads to local immune reactions against autoantigens, reflected by the restriced T-cell-receptor repertoire. These stressors induce simultaneous expression of adhesion molecules and HSP 60 or other stress proteins in the palmar aponeurosis. Mechanically induced vascular damage

represents an additional stress factor, leading to increased permeability, extravasation of inflammatory cells, and hypoxic condition.

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