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Dupuytren's disease

A MODEL FOR THE MECHANISM OF FIBROSIS AND ITS MODULATION BY STEROIDS

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Dupuytren's disease is a chronic inflammatory process which produces contractures of the fingers. The nodules present in Dupuytren's tissue contain inflammatory cells, mainly lymphocytes and macrophages. These express a common integrin known as VLA4. The corresponding binding ligands to VLA4 are vascular cell adhesion molecule-1 (VCAM-1) present on the endothelial cells and the CS1 sequence of the fibronectin present in the extracellular matrix. Transforming growth factor-beta (TGF-ß) is a peptide hormone which has a crucial role in the process of fibrosis.

We studied tissue from 20 patients with Dupuytren's disease, four samples of normal palmar fascia from patients undergoing carpal tunnel decompression and tissue from ten patients who had received perinodular injections of depomedrone into the palm five days before operation. The distribution of VLA4, VCAM-1, CS1 fibronectin and TGF-ß was shown by immunohistochemistry using an alkaline phosphorylase method for light microscopy.

In untreated Dupuytren's tissue CS1 fibronectin stained positively around the endothelial cells of blood vessels and also around the surrounding myofibroblasts, principally at the periphery of many of the active areas of the Dupuytren's nodule. VCAM-1 stained very positively for the endothelial cells of blood vessels surrounding and penetrating the areas of high nodular activity. VCAM-1 was more rarely expressed outside the blood vessels. VLA4 was expressed by inflammatory cells principally in and around the blood vessels expressing VCAM-1 and CS1 but also on some cells spreading into the nodule. TGF-ß stained positively around the inflammatory cells principally at the perivascular periphery of nodules. These cells often showed VLA4 expression and co-localised with areas of strong production of CS1 fibronectin.

Normal palmar fascia contained only scanty amounts of CS1 fibronectin, almost no VCAM-1 and only an occasional cell staining positively for VLA4 or TGF-ß.

In the steroid-treated group, VCAM-1 expression was downregulated in the endothelium of perinodular blood vessels and only occasional inflammatory cell expression remained. Expression of CS1 fibronectin was also much reduced but still occurred in the blood vessels and around the myofibroblast stroma. VLA4-expressing cells were also reduced in numbers. A similar but reduced distribution of production of TGF-ß was also noted.

Our findings show that adherence of inflammatory cells to the endothelial wall and the extravasation into the periphery of the nodule may be affected by steroids, which reduce expression of VCAM-1 in vivo. This indicates that therapeutic intervention to prevent the recommencement of the chronic inflammatory process and subsequent fibrosis necessitating further surgery may be possible.

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Dupuytren's disease is a condition in which the formation of nodules in the palm of the hand precedes eventual contracture of the fingers by fibrosis. The histopathological findings have been described.[1,2] In addition to myofibroblast cells from the nodule itself,[3-6] clusters of macrophages and T lymphocytes have been observed in Dupuytren's tissue.[7] These inflammatory cells have been shown to produce a spectrum of growth factors and cytokines such as basic fibroblast growth factor (bFGF), interleukin-1 (IL-1) alpha and beta, transforming growth factor beta (TGF-ß) 1 and 2, tumour necrosis factor alpha and IL-8[8,9] which regulate the migration, proliferation and contracture of Dupuytren's myofibroblasts.[10]

These macrophages and T lymphocytes express a common integrin, VLA4, which is important in controlling their adherence to activated endothelium at sites of inflammation and their subsequent transendothelial migration into the

tissue. VLA4 integrin is a cell-surface heterodimer expressed on most mononuclear leukocytes, eosinophils and basophils.[11,12] It has been shown to mediate cell adhesion to vascular cell adhesion molecule-1 (VCAM-1),[13-19] as well as to the HepII/IIICS region of fibronectin at which the CS1 site has the greatest binding activity for VLA4.[20-25]

There have been many studies on the extracellular matrix proteins in Dupuytren's tissue, in particular, the expression of fibronectin[26-28] and its co-expression with integrin expression on stromal cells.[29] It has been suggested that fibronectin acts to anchor the myofibroblast cell to the extracellular matrix and affects its contracture in the fibrotic process.[27] Just as the fibronectin of the extracellular matrix protein controls the properties of the myofibroblast, it also affects the activation and effect of inflammatory cells on the process of fibrosis. The CS1 sequence of fibronectin is the natural ligand for VLA4. Binding will influence the inflammatory cells carrying this integrin with regard to the activation and production of cytokines, including TGF-ß.

TFG-ß is one of the fundamental groups of cytokines involved in stimulating the process of fibrosis. TGF-ß 1 and 2 are known to stimulate the synthesis of extracellular matrix and integrin expression in vitro[30-34] and are present in elevated amounts in sites of chronic inflammation. It has already been shown in vitro that cultured Dupuytren's cells produce TGF-ß, and that TGF-ß stimulates Dupuytren's fibroblasts to grow.[35] By augmenting integrin expression TGF-ß promotes leukocyte adhesion and stimulates release of further cytokines, refuelling the cycle. It also acts as an immunosuppressant, as has been shown by Kulkarni and Karlsson,[36] which suggests that while promoting fibrosis it can diminish inflammation. It may be that the balance between the TGF-ß 1, 2 and 3 isoforms and their receptors and the temporal sequence of release underlie this variable effect. These paradoxical functions of the TGF-ß molecule do not detract from its pivotal role in inflammation and fibrosis.

It has been noted that locally injected steroid may sometimes halt the progressive proliferation of Dupuytren's nodules macroscopically and indeed cause them to 'melt away'.[37] We have therefore studied the effect of local preoperative injections of hydrocortisone on the factors which control postoperative transendothelial migration and the subsequent re-initiation of the fibrotic process.

Patients and Methods

We studied 30 patients with Dupuytren's disease; 20 (17 men and 3 women) were in the group not treated with steroids and ten (8 men and 2 women) in the group which had an injection of steroid before operation. The mean age of the untreated group was 58 ± 14 years (40 to 77) and of the steroid group 54 ± 16 years (31 to 75). In the latter group 0.5 ml (20 mg) of depomedrone was injected into the perinodular area of the palm five days before operation.

All the patients were at a comparable stage of Dupuytren's contracture and activity. The operations were performed under regional anaesthesia and tourniquet control. A control group consisted of four patients undergoing routine carpal tunnel decompression who had a representative sample of palmar fascia excised. Samples of the excised Dupuytren's tissue and palmar fascia control tissue were placed in 10% neutral buffered formalin solution.

Immunohistochemistry. All procedures were carried out at room temperature unless otherwise stated. Tissue was dehydrated sequentially through graded alcohols and Histoclear solutions before embedding in paraffix wax. The tissue blocks were then cut and sections 7 µm thick were placed on slides coated with 3-aminopropyltriethoxy-silane (APES) and air-dried. Sections were then immunostained by the double-sandwich technique using the avidin-biotin alkaline phosphatase complex. They were dewaxed with Histoclear, rehydrated by graded alcohol solutions to Tris Buffer (0.05M) and then pretreated with trypsin (Sigma Chemical Company, Dorset, UK) for 15 minutes at 37°C. After three washes with 0.05M Tris Buffer Solution (TBS) at pH 7.2 the sections were incubated with the primary antibody for one hour. They were incubated with biotinylated secondary antibody (Sigma Immuno Chemicals; 1:400 solution) for 45 minutes and then washed three times with TBS at pH 7.2. Finally, the sections were incubated with extravidin alkaline phosphatase (Sigma Immuno Chemicals; 1:60 solution) for 45 minutes and finally washed three times with TBS at pH 7.2. Bound alkaline phosphatase was visualised using 5-bromo-4-chloro-indoyl phosphate (nitro blue tetrazolium) until visualisation was complete or background changes were becoming prominent. This gave a bluish/black/brown colour depending on the intensity of the reaction. The section was placed in distilled water to control this. The sections were then counterstained with Neutral Red (0.5%) and mounted in 50% glycerol solution with a coverslip. Control negative sections were run without primary antibody. The antibodies used were mouse anti-human CS1 fibronectin (Chemicon International Ltd, Harrow UK; 1:100 dilution), goat anti-human VCAM-1 (R&D Systems Europe Ltd, Abingdon, UK; 1:200 dilution), mouse anti-human VLA4 (alpha 4 beta 1 integrin dilution) (Histotec Range, Kidlington, UK; 1:100 dilution), and chicken anti-human TGF-ß 1 and 2 used as a cocktail together (R&D systems; 1:60 dilution solution). Positive-staining cell and tissue counts were made over six random ×25 magnification fields of comparable areas of three consecutive sections from every sample for VCAM-1, VLA4, CS1 fibronectin and TGF-ß.

Statistical analysis between counts was performed by the Mann-Whitney U test.

Results

Table I shows the results. In the Dupuytren's tissue, the endothelial cells of the blood vessels found at the periphery of active nodules and in the surrounding perinodular tissue

Table I. Mean (± SD) number of cells in areas of extracellular matrix which stained positively for VCAM, CS1 fibronectin (FN), VLA4 and TGF-β

Sample	VCAM	CS1 FN	VLA4	TGF-β
Dupuytren's tissue (n = 20)	2.87 ± 2.76	3.85 ± 8.06	2.22 ± 3.3	1.5 ± 2.72
Steroid-treated tissue (n = 10)	0.64 ± 0.97*	1.33 ± 1.80	1.02 ± 1.77	0.86 ± 1.69*
Palmar fascia tissue (n = 4)	0.28 ± 0.67†	0.18 ± 0.55†	0.14 ± 0.37†	0.28 ± 0.61†

* significant reduction of VCAM and TGF-β, $p < 0.02$
† significantly reduced presence in palmar fascia compared with Dupuytren's disease, $p < 0.02$

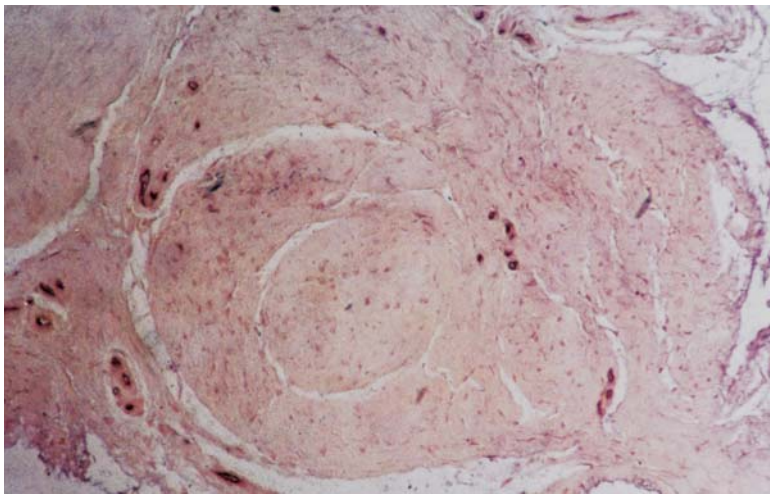

Fig. 1

Photomicrograph of Dupuytren's tissue in which antibody to VCAM-1 stained positively (dark purple) in the endothelium of peripheral blood vessels (counterstain with Neutral Red × 7).

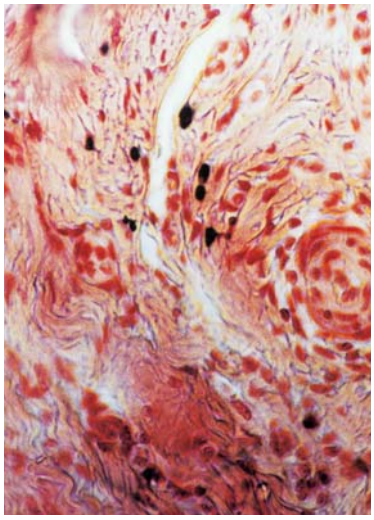

Fig. 2a

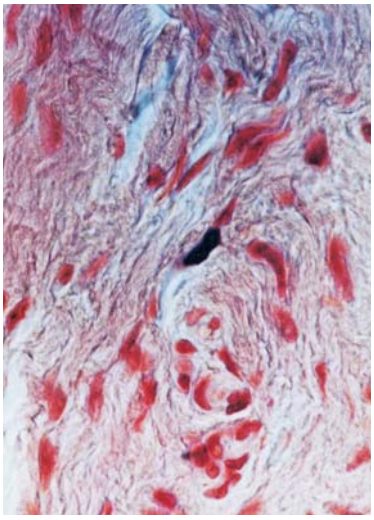

Fig. 2b

Photomicrograph of Dupuytren's tissue using antibody to VLA4. Figure 2a – Perivascular macrophages stained positive (dark purple). Figure 2b – A tissue section treated preoperatively with steroid. VLA4-positive cells (dark purple; macrophages) are still present around the blood vessels in the periphery of the nodule but in reduced numbers (counterstain with Neutral Red × 17).

showed considerable VCAM-1 staining (Fig. 1). By comparison the endothelium of the blood vessels in the palmar fascia and steroid-treated Dupuytren tissue did not express VCAM-1 strongly.

VLA4-integrin-expressing cells were abundant in the endothelium and perivascular areas corresponding to areas of high VCAM-1 expression in the Dupuytren tissue (Fig. 2). In the steroid-treated samples VLA4-expressing cells were still present in similar areas but in reduced amounts (Fig. 2b). In both groups, these cells were predominantly macrophages and lymphocytes. The sections of palmar fascia contained only a very few cells with VLA4 expression and these were randomly distributed.

The CS1 binding site of fibronectin was present around the endothelial cells of blood vessels in the perinodular tissue, but also around active myofibroblasts, principally at the periphery of many of the active areas of the Dupuytren's nodule (Fig. 3). In steroid-treated Dupuytren tissue there was a generalised reduction in expression of the CS1 sequence. The palmar fascia showed only limited and random positive staining for CS1.

TGF-ß 1 and 2 stained positively principally around the inflammatory cells and some myofibroblasts at the periphery of nodules, often in a perivascular relationship which co-localised with areas of expression of the CS1 sequence of fibronectin (Figs 4 and 5). Dupuytren tissue treated with

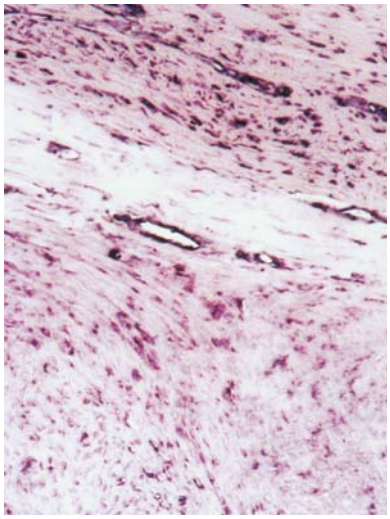

Fig. 3a

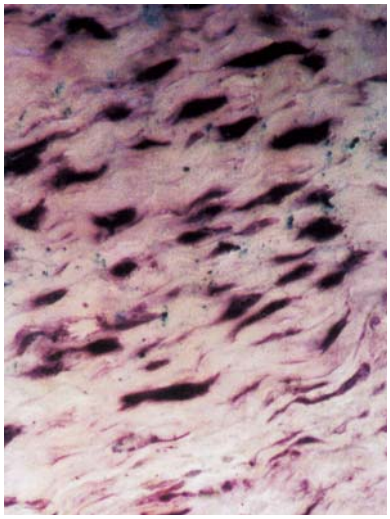

Fig. 3b

Photomicrograph of Dupuytren's tissue using antibody to the CS1 sequence of fibronectin. Figure 3a – Positive staining (dark purple) of blood vessels and the myofibroblast (×7). Figure 3b – Positively-stained myofibroblasts (counterstain with Neutral Red×28).

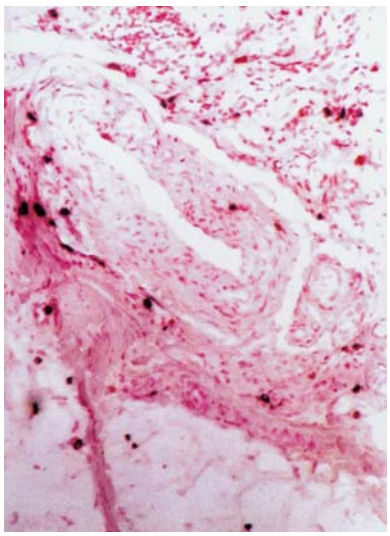

Fig. 4a

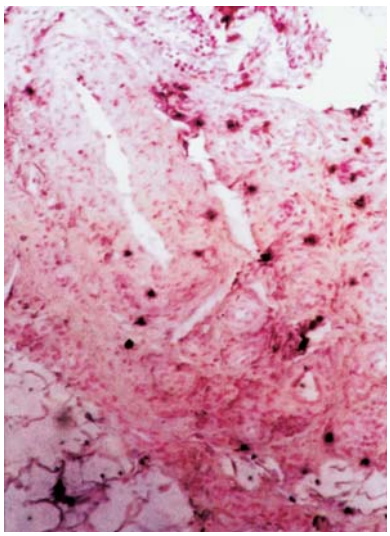

Fig. 4b

Photomicrographs of Dupuytren's tissue using antibody to a) TGF-ß 1 and 2 and b) to the CS1 sequence of fibronectin. There is co-localisation of positively stained cells and extracellular matrix (dark purple) in both regions (counterstain with Neutral Red×7).

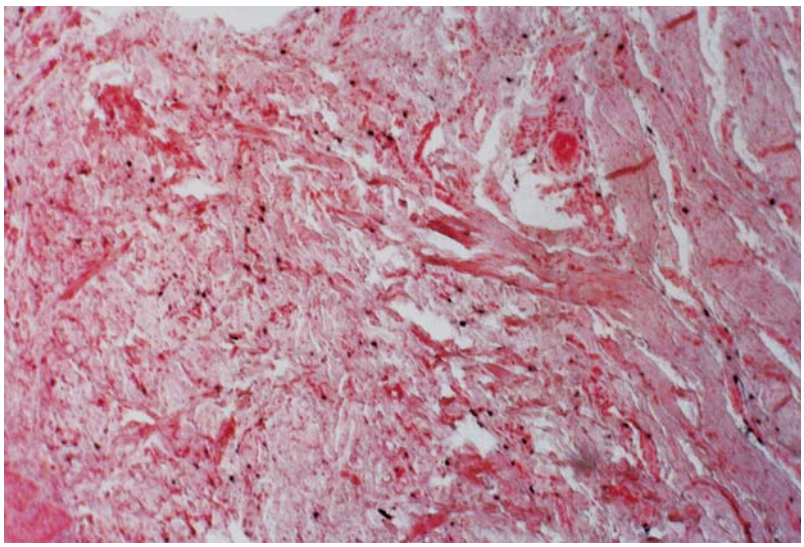

Fig. 5

Photomicrograph of Dupuytren's tissue using antibody to TGF-ß 1 and 2 (dark purple). There are many areas expressing production in the periphery of the nodule (counterstain with Neutral Red×7).

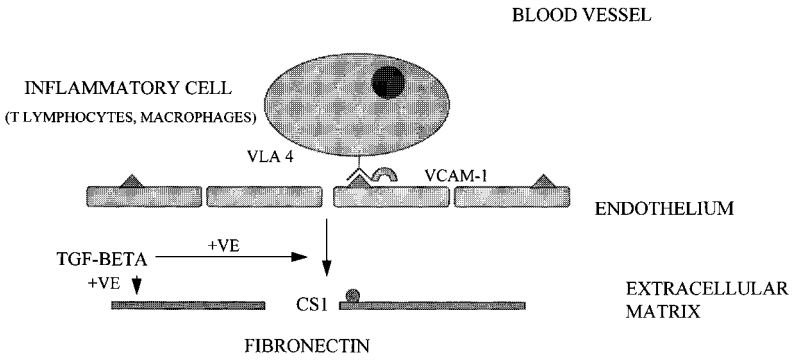

Fig. 6a

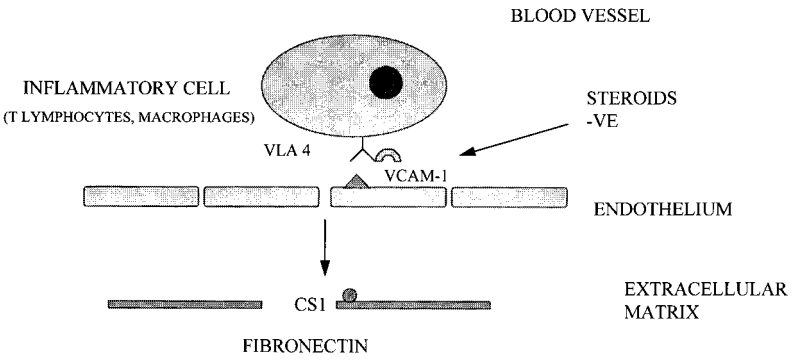

Fig. 6b

Figure 6a – The transendothelial migration of inflammatory cells expressing VLA4 integrin into tissues necessitates binding to the VCAM-1 adhesion molecule or CS1 sequence of fibronectin. The inflammatory cells in areas of high CS1 fibronectin presence stimulate TFG-ß 1 and 2. Figure 6b – This process may be influenced by the downregulation of VCAM-1 expression by steroids.

steroid showed a decrease in expression of TGF-ß 1 and 2 in these same areas. The palmar fascia had relatively little TGF-ß 1 or 2.

Discussion

Fibrosis is a process which involves the regulation of mononuclear and lymphocyte cells. Clinical examples of fibrosis include systemic sclerosis,[38] renal interstitial fibrosis,[39,40] sarcoidosis,[41] idiopathic pulmonary fibrosis,[42,43] Reidel's thyroiditis,[44] retroperitoneal fibrosis[45,46] and Dupuytren's disease.[7,47] The presence of macrophages, eosinophils or lymphocytes has been initially demonstrated in all of these conditions. Many have shown impressive improvement after treatment with steroids.

The inflammatory cells infiltrate the site after triggering by chemotactic and activating mediators. This is followed by an escalation of cytokines which induce fibroblast and endothelial proliferation with the subsequent deposition of extracellular matrix. In the absence of inhibitory signals the continued production of these mediators sustains the accumulation of connective tissue resulting in permanent alteration in the structure and function of the tissue.

Dupuytren's disease is a chronic inflammatory disorder characterised by palmar nodules of proliferating fibroblasts, which eventually become incorporated into cords of mature scar tissue, producing contracture of the fingers. Inflammatory cells have been found at the periphery of Dupuytren nodules.[7,47] Our findings have confirmed this. These appear to produce a number of potent growth factors and cytokines[8,48,49] which are thought to stimulate Dupuytren's myofibroblasts to express their contractile fibrosing phenotype.[10] To prevent the accumulation of these initiating inflammatory cells it is necessary to discover the pathway for their migration.

Many cell types migrate transendothelially into sites of inflammation. In Dupuytren's disease lymphocytes and macrophages are the predominant inflammatory cells and express the integrin VLA4. This binds with the inducible adhesion molecule VCAM-1 and the CS1 domain of fibronectin. The latter has recently been shown to be involved in cellular migration and to be present on the endothelium as well as in the extracellular matrix. Our results have shown that it would be possible for VLA4-expressing inflammatory cells to adhere to blood vessels in the periphery of Dupuytren's nodules and the peripheral nodular tissues as they express VCAM-1 molecules and the CS1 site of fibronectin.

Inflammatory cells undergo transendothelial migration and initiate effects on the extracellular matrix to produce fibrosis. Of critical importance among the other cytokines and growth factors is the TGF-ß group. We have shown that TGF-ß co-localises with CS1 fibronectin in Dupuytren's tissue, suggesting that it is implicated in the fibrosis of Dupuytren's disease.

Steroids affect the initial stages of transendothelial migration in a number of ways. For example, they may reduce the inducible adhesion molecules expressed by activated endothelium.[50] We have now shown that after treatment with steroids there is a massive reduction in VCAM-1

expression by Dupuytren's endothelial cells. Nevertheless, some cells expressing VLA4 continued to accumulate in the extracellular matrix of steroid-treated Dupuytren's tissue possibly because the steroids did not totally block expression of CS1 fibronectin in either the blood vessels or in the extracellular matrix. Some of the cells expressing VLA4 appeared to be elongated like fibroblasts and were found to express Mac387 and CD68 markers. This suggested that they were macrophages behaving like fibroblasts rather than VLA4-expressing myofibroblasts.

Steroids can also modify the subsequent inflammatory process by altering the production of growth factors and cytokines, for example, that of TGF-ß which is reduced but not totally blocked in Dupuytren tissue. This is probably due to the continued presence of VLA4 inflammatory cells which co-localised with TGF-ß, suggesting that they were a source of this cytokine.

Thus it is likely that the steroids reduce the presence of TGF-ß 1 and 2 in Dupuytren's tissue by a combination of decreasing transendothelial migration of inflammatory cells (Fig. 6) and local production by cells already present in the inflammatory site.

Dupuytren's contracture is a useful model of chronic inflammation and fibrosis because it displays the entire temporal and histological architecture of cells, cytokines and extracellular matrix. Given the critical role of TGF-ß in fibrosis further work is required to allow modulation of its production and more precise control of the transendothelial migration into the tissues of inflammatory cell populations and their subsequent synthesis of growth factors. This can be achieved by modifying their binding properties to the activated endothelium and matrix molecules. There are already drugs which block CS1 fibronectin of VLA4 integrin itself and when they are in clinical use they may allow further immunomodulation of Dupuytren's disease.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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