THE DIFFERENT CHARACTERISTICS OF DUPUYTREN’S DISEASE FIBROBLASTS DERIVED FROM EITHER NODULE OR CORD: EXPRESSION OF α-SMOOTH MUSCLE ACTIN AND THE RESPONSE TO STIMULATION BY TGF-β1

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Mechanisms behind the onset and progression of Dupuytren’s disease are poorly understood. Both myofibroblasts and transforming growth factor beta 1 (TGF-β1) have been implicated. We studied fibroblast cultures derived from nodules or cords of Dupuytren’s contracture tissue to determine the proportion of myofibroblasts present in comparison with flexor retinaculum fibroblast cultures. We identified myofibroblasts by immunohistochemical staining for α-SMA. We then investigated the effects of TGF-β1 stimulation on these fibroblasts.

Basal myofibroblast/fibroblast proportions were 9.7% in nodule cell cultures, 2.7% in cord cell cultures and only 1.3% in flexor retinaculum cell cultures. Nodule and cord myofibroblast proportions increased to 25.4% and 24.2%, respectively, in response to TGF-β1 treatment. Flexor retinaculum cell cultures showed no response to TGF-β1 stimulation.

Fibroblasts cultured from specific regions of Dupuytren’s tissue retain myofibroblast features in culture. TGF-β1 stimulation causes an increased myofibroblast phenotype to similar levels in both nodule and cord, suggesting that previously quiescent cord fibroblasts can be reactivated to become myofibroblasts by TGF-β1. This could be an underlying reason for high recurrence rates seen after surgery or progression following injury.


Keywords: Dupuytren’s contracture; myofibroblasts; transforming growth factor-β1; nodule cord

INTRODUCTION
Dupuytren’s disease of the hand is a common, sometimes disabling condition affecting up to 20% of men over the age of 65 (Mikkelsen, 1990). The underlying cause of the fascial fibrosis that characterizes the disease remains unclear despite significant advances in its cell and molecular biology, although several associations have been defined. These include diabetes, smoking, epilepsy and alcoholism (Leclercq, 2000).

Since the early 1970s a specialized cell, the myofibroblast, has been implicated in the causation and progression of Dupuytren’s disease (Gabbiani and Majno, 1972). This is currently believed to be a transformed fibroblast, which may pass through a proto-myofibroblast stage before becoming a differentiated myofibroblast (Tomasek et al., 2002). At present the most widely accepted method of identifying differentiated myofibroblasts is by their expression of intra-cellular α-smooth muscle actin (α-SMA) microfilaments. This can be demonstrated in both cell cultures and tissue sections using immunohistochemical techniques (Skalli et al., 1986).

Myofibroblasts are thought to be involved in excess collagen and extra-cellular matrix deposition in fibrotic conditions (Petrov et al., 2002; Serini and Gabbiani, 1999). Furthermore fibroblasts, when seeded into three-dimensional collagen lattices, produce a quantifiable contractile force (Eastwood et al., 1994) and the degree of contraction has been correlated with the myofibroblast content in cell cultures (Hinz et al., 2001; Tomasek and Rayan, 1995). Both of these effects are observed clinically in Dupuytren’s disease.

Luck (1959) described a clinicopathological staging system based upon the disease activity and histological appearances, dividing the affected tissue into proliferative, involutional and residual stage disease. This broadly encompasses the basic clinical picture of Dupuytren’s disease with nodules; fusiform swellings of involved tissue which display proliferative or involutional features; and cords which are fibrous, tendon-like and longitudinally arranged, which display residual characteristics. Very little work has been done regarding differences between the characteristics and cell phenotypes in these regions although many authors believe that the natural history of the disease is for active nodular tissue eventually to progress and “burn out” to become fibrous dormant cords (Luck, 1959; Rayan, 1999; Vande Berg et al., 1984).

The transforming growth factors are a family of ubiquitous polypeptide growth factors that are involved in the regulation of many cellular processes. Of these,
transforming growth factor beta 1 (TGF-β1) has been most widely studied and specifically implicated in wound healing, pathological scarring and fibrosis (Border and Noble, 1994). It has been shown to upregulate myofibroblast differentiation in several cell types and culture conditions (Desmoulière et al., 1993; Desmoulière and Gabbiani, 1994; Ignotz and Massague, 1986; Scrinia and Gabbiani, 1999), including Dupuytren’s fibroblasts (Dugina et al., 2001; Vaughan et al., 2000). Transforming growth factor-β1 also increases collagen production (Alioto et al., 1994; Petrov et al., 2002) and fibronectin synthesis (Grinnell et al., 1999). Fibroblast-mediated collagen gel contraction has also been shown to increase in response to TGF-β1 in several models (Brown et al., 2002; Montesano and Orci, 1988; Reed et al., 1994; Vaughan et al., 2000). No data are available on its comparative effects on specific nodule or cord cells.

The purpose of this study was to establish whether fibroblasts derived from Dupuytren’s nodules and cords expressed similar levels of α-smooth muscle actin and to determine if this was affected by stimulation with TGF-β1.

MATERIALS AND METHODS

Cell culture

Following local ethical committee approval, samples of Dupuytren’s diseased tissue and control carpal ligament tissue were obtained from patients undergoing routine fasciectomy, or carpal tunnel decompression. Each Dupuytren’s specimen was classified into regions of nodule or cord depending on the macroscopic appearances and texture (Fig 1). Nodules were defined as firm fusiform swellings of affected tissue whilst cords were fibrous longitudinally orientated tendon-like structures. Specimens that did not have clearly different nodule and cord regions were excluded. Primary cell cultures were then obtained from specific areas of nodule or cord using an explant technique. The same method was used to culture flexor retinaculum fibroblasts from patients unaffected by Dupuytren’s disease. Cell cultures were bathed in Normal Growth Media (NGM) which was composed of Dulbecco’s Modified Eagles Media (Gibco, Paisley, Scotland) supplemented with 10% foetal calf serum (Gibco), penicillin/streptomycin (100 U/ml and 100 μg/ml, Gibco) and L-glutamine (2mM, Gibco), and buffered with 1 M Hepes solution (3.5 ml). Cells were passaged at confluence and for experimental purposes were used at or below passage 5. Ten Dupuytren’s nodule and 11 cord cell lines, and four flexor retinaculum cell lines were used. In the TGF-β1-stimulated group seven Dupuytren’s nodule, seven Dupuytren’s cord and four flexor retinaculum cell lines were used, with each cell line of each type (cord, nodule) originating from a different patient. Only tissue from primary Dupuytren’s procedures was used, excluding any cases of recurrent disease, and nodule and cord cultures were established from each Dupuytren’s specimen. The mean patient age was 63 for the Dupuytren’s patients and 65 for the flexor retinaculum patients.

Immunohistochemistry

Fibroblasts were prepared for α-smooth muscle actin staining as previously described (Skalli et al., 1986; Desmoulière et al., 1993). Briefly 1 × 10⁶ cells were seeded onto alcohol-sterilized glass coverslips in six-well plates. They were incubated at 37°C in 5% CO₂ in NGM for 4 days. The cells were then fixed in ice-cold methanol for 20 min. Mounted cover slips were stored at −20°C until ready for staining.

Cell lines from nodule, cord and carpal ligament were used and the experiment was repeated having incubated the cells in NGM containing 2ng/ml TGF-β1 (R&D Systems) for 3 days prior to fixation.

A mouse monoclonal anti-α-smooth muscle actin primary antibody (Sigma Chemicals, Dorset) was used at a dilution of one in 800 and a FITC-conjugated rabbit anti-mouse (Dako A/S, Denmark, one in 400 dilution) secondary antibody was then added. This secondary antibody was combined with propidium iodide (Sigma) as a nuclear counter stain.

Digital images of the cover slips were captured under UV illumination using a Zeiss Axioscope 20 microscope with a Leica DC200 mounted camera and software (Leica DC Viewer, Leica Microsystems Ltd.). Three random fields per cover slip were captured with each cell line repeated on triplicate cover slips. The total number of cells per field and the number of positively staining myofibroblasts was counted and a percentage of myofibroblasts was derived for each cell line with and without TGF-β1.

Statistical analysis was performed using the student’s t-test (SigmaStat, Jandel Corps.).

RESULTS

Cells staining positively for α-smooth muscle actin demonstrated green intra-cellular microfilaments with
the nucleus counterstained red. Typical fields from nodule, cord and carpal ligament cultured fibroblasts are illustrated in Fig 2.

The proportion of myofibroblasts in cell cultures from nodule was 9.7% (SD ± 4.5) compared with 2.7% in cord (SD ± 1.5), (P < 0.001). Control flexor retinaculum cell cultures contained only 1.3% myofibroblasts (SD ± 1.9) which was significantly different from nodule (P < 0.001), but not from cord. These differences reflect the in vivo cell phenotype where nodules are rich in myofibroblasts. Relatively inactive or quiescent tissues such as cord or flexor retinaculum display a very low level of basal myofibroblast phenotype cell presence.

After addition of TGF-β1 there was a significant (P < 0.001) up-regulation of the positively staining myofibroblast phenotype in the Dupuytren’s cell lines (Figs 3a and b) to 25.4% (±5.5) in nodule and to 25.8% (±5.8) in cord. This was not the case in flexor retinaculum control fibroblasts (2.6% ± 0.6; Fig 3c).

Dupuytren’s fibroblasts therefore, seem to be inherently more susceptible to stimulation by TGF-β1 irrespective of whether from nodule or cord. Overall, however, there was a greater percentage increase in the myofibroblasts seen in cord-derived cultures (from 2.7% to 25.8%) when stimulated with TGF-β1 than nodules (from 9.65% to 25.4%), and this difference just reached statistical significance (P < 0.05 Mann-Whitney Rank Sum test). It is not clear from our data as to whether this difference represents a greater susceptibility of the cord cells to TGF-β1 or a lesser basal stimulation leading to less myofibroblast differentiation within the non-stimulated cord cultures.

**DISCUSSION**

The myofibroblast plays a key role in the pathogenesis of Dupuytren’s contracture of the hand but the natural history of the condition and relative contribution of cells from either the nodule or the cord remains unclear. Vande Berg et al. (1984) studied nodule and cord characteristics in tissue specimens using electron microscopy and went on to culture cells from the two regions. They found ultrastructural features of myofibroblastic type cells to be prevalent in nodule tissue but absent in cord, with these findings maintained in cultured fibroblasts. Nodule myofibroblasts in addition proliferated at a slower rate in culture. We did not formally examine this aspect of behaviour but it did not appear to be the case in our cell lines. Badalamente et al. (1983), using ultrastructural electron microscopic studies of diseased tissue, have also found myofibroblasts in nodule tissue but not in cords whilst others have found myofibroblasts in only a single cord specimen (Pasquali-Ronchetti et al., 1993) or stated that α-SMA positive cells were rarely found in cord tissue (Qureshi et al., 2001). We have cultured cells specifically from nodule and cord regions of resected Dupuytren’s tissue and

**Fig 2** Representative fields of (a) Dupuytren’s nodule, (b) Dupuytren’s cord and (c) flexor retinaculum cell cultures viewed under UV illumination after immunofluorescence staining for α-smooth muscle actin (green intra-cellular microfilaments) × 200 magnification.
shown, using immunohistochemical staining for α-SMA, that they retain similar basal differences in the myofibroblast phenotype distribution in vitro as shown by Vande Berg et al. (1984). Contrary to their work however, our cord cell cultures did contain a few (2%) positively staining myofibroblasts. They used electron microscopic examination of the cell ultrastructure and in fact did identify 40 to 80 Å microfilaments within some cells from cords, although they did not term these myofibroblasts, interpreting them as a random morphological feature. Only when they observed consistent prominent 40 to 80 Å microfilament bundles, as seen in their nodules, did they define them as myofibroblasts. With our technique we have been able to calculate the percentage of cells expressing the myofibroblastic phenotype in both nodule and cord cultures, as well as flexor retinaculum cultures as a comparison. Tomasek and Rayan using similar methods found a much greater variability in the percentage of α-SMA positive myofibroblasts than we did in both cultured nodule (1–26%) and flexor retinaculum cells (1–9%) (Tomasek and Rayan, 1995). Their cells were only incubated for 2 days before fixation whilst we used a 4 day time point: this may account for some of the differences observed although the trend was similar. They did not investigate myofibroblasts in cord cultures.

There are several potential reasons for the differences between the virtual absence of myofibroblasts in vivo in cords and the in vitro findings of a small percentage present in all cell cultures. Explant culture of fibroblasts inherently produces some degree of cellular heterogeneity and this may be additionally exacerbated by the nature of the tissue categorized as cord. It is occasionally observed on standard histology sections of cord that there are foci of much more cellular diseased tissue within the sparsely fibroblast populated, thick parallel collagen bundles. If these cell “nests” become incorporated into the explanted tissue an increase in the myofibroblast phenotype may be found in culture. Secondly, culture of fibroblasts in two dimensions on the base of a tissue flask or glass coverslip provides a stiff environment for the cells to attach to and will stimulate stress fibre formation (Tomasek et al., 2002).

By maintaining some of the in vivo characteristics when cultured such differential culture techniques provide a model to attempt to elucidate the role of nodules and cords in disease progression.

The basal differences in myofibroblast content that we have shown, taken in context with other known differences in nodule and cord properties such as greater
total collagen in nodule tissue (Bazin et al., 1980; Brickley-Parsons et al., 1981), suggest that the nodule is indeed active compared to cord. Cord tissue is much more like the flexor retinaculum and it is quite possible therefore that any potential non-surgical therapy or surgical adjuvant treatment may need to target nodular active disease to be effective.

Transforming growth factor-β1 has been shown to induce myofibroblast transformation from fibroblasts in several studies (Desmoulière et al., 1993; Ignatoff and Massague, 1986; Vaughan et al., 2000) and currently it is believed that fibroblasts are stimulated to differentiate into proto-myofibroblasts and then myofibroblasts expressing z-SMA by TGF-β1 in addition to a range of other factors (Tomasek et al., 2002). Desmoulière et al. (1993) demonstrated an upregulation of myofibroblasts in a rat in vivo model after subcutaneous injection of TGF-β1 to granulating wounds. They subsequently found a corresponding increase in z-SMA synthesis in cultured fibroblasts from both rat and humans using Western blot analysis. This increase occurred across a whole range of TGF-β1 concentrations from 1 ng/ml to 10 ng/ml. In our study we have used a concentration of 2 ng/ml which is the standard concentration used in our laboratory for stimulation of myofibroblast differentiation and falls within this range.

Dugina et al. used Dupuytren’s nodule fibroblasts to study the formation of focal adhesions in myofibroblasts in response to TGF-β treatment (Dugina et al., 2001). In untreated cultures they found only 3% myofibroblasts, although this was in serum-free conditions whereas our cultures were incubated with 10% serum, which is likely to account for our higher basal level of expression. After TGF-β stimulation for 5 days they found myofibroblast numbers increased to 71%, significantly more than we encountered. Our cultures were stimulated for 3 days, although preliminary data (not shown) indicated there was little difference between levels seen at 3 and 5 days. Additionally Dugina et al. used TGF-β2 rather than TGF-β1 but did state that similar changes were observed with both growth factors and this has been found in other studies (Serini and Gabbiani, 1999). They also used a concentration of 5 ng/ml, more than double the amount used here.

Other authors (Vaughan et al., 2000) have determined myofibroblast percentages in stressed fibroblast-populated collagen lattices by staining for z-SMA. They used a maximum concentration of 1 ng/ml TGF-β1 and showed an increase from 7.9% to 23.4% after stimulation of Dupuytren’s-derived fibroblasts for 5 days.

TGF-β1 has been demonstrated in Dupuytren’s disease using various techniques (Badalamente et al., 1996; Baird et al., 1993; Zamora et al., 1994), as have its receptors (Kloen et al., 1995). Berndt et al. (1995) showed a greater intensity of staining for TGF-β1 protein in proliferative nodules and colocalization of TGF-β1 synthesis and the myofibroblast phenotype to these regions. It is likely that this growth factor plays a central function in the development and progression of the disease.

Fibroblasts resident within cords of Dupuytren’s disease may be quiescent, as indicated from previous histochemical studies and our basal, unstimulated results. However, when exposed to a stimulus they appear capable of becoming as differentiated as the traditionally active fibroblasts within nodules. This has important implications for clinical practice and could explain the high recurrence rate following treatment for Dupuytren’s disease. The local trauma of surgical excision and the natural wound healing response will lead to release of large amounts of TGF-β1 and any residual tissue of nodule or cord origin will be susceptible to stimulation, myofibroblast transformation, cell proliferation, collagen deposition and the formation of recurrent disease. Other authors have correlated recurrence with presence of myofibroblasts (Gelberman et al., 1980).

The evidence presented in this paper is yet another indication that surgical intervention for Dupuytren’s disease should be contemplated only when the degree of contracture reaches the point where the benefit of correction is outweighed by the risk of recurrence.

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References


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