

FEEDBACK INHIBITION OF HIGH TGF- β 1 CONCENTRATIONS ON MYOFIBROBLAST INDUCTION AND CONTRACTION BY DUPUYTREN'S FIBROBLASTS

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Myofibroblasts and TGF- β 1 are implicated in Dupuytren's contracture. Transforming growth factor β 1 (TGF- β 1) (1–10 ng/ml) increases myofibroblast induction in Dupuytren's fibroblasts and contraction in a collagen model. However, higher doses (20–30 ng/ml) inhibit contraction in dermal fibroblasts. We hypothesized higher doses of TGF- β 1 would inhibit induction of myofibroblasts and contraction by Dupuytren's fibroblasts. Increasing doses of TGF- β 1 (0–30 ng/ml) were tested on Dupuytren's fibroblasts using immunofluorescence to determine myofibroblast upregulation and a 3D collagen model used to determine contractile forces. Flexor retinaculum fibroblasts were used as controls. TGF- β 1 induced myofibroblasts in Dupuytren's fibroblasts ($n = 3$) from 12% (0 ng/ml) to 23% (12.5 ng/ml) at 24 hours but dropped to 13% at 30 ng/ml ($P < 0.05$). This response was mirrored in the contraction profiles. These trends were similar for flexor retinaculum fibroblasts ($n = 3$), but contractile forces and myofibroblast induction were significantly less ($P < 0.001$). This is the first report of negative feedback inhibition of TGF- β 1 at higher concentrations in Dupuytren's fibroblasts.

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Dupuytren's disease is characterised by shortening of the palmar fascia leading to progressive flexion digital deformity (Lennox et al., 1993). The fascial contraction is believed by some to be due to the actions of the myofibroblast (Gabbiani and Majno, 1972), a type of cell which shares morphological features with both fibroblasts and smooth muscle cells (Gabbiani et al., 1979). It is currently believed to be a transformed fibroblast (Tomasek et al., 2002).

In mammals, three isoforms of Transforming growth factor beta (TGF- β) have been found (TGF- β 1, - β 2, - β 3) (Roberts, 1998), with the TGF- β 1 isoform the most widely studied, being specifically implicated in wound healing, pathological scarring and tissue fibrosis (Border and Noble, 1994). In earlier studies using immunohistochemical techniques to identify α -smooth muscle microfilaments, TGF- β 1 has been shown to increase the differentiation of the myofibroblast phenotype from Dupuytren's fibroblasts in both in vitro monolayer cell culture (Bisson et al., 2003) and in three-dimensional collagen matrices (Vaughan et al., 2000), in concentrations up to 2 ng/ml.

It is possible to quantify the contraction produced by fibroblasts by seeding them into collagen gels and measuring the reduction in area (Montesano and Orci, 1988) or attaching them to a highly sensitive force transducer and quantifying the forces generated (Eastwood et al., 1994). When Dupuytren's fibroblasts are seeded into collagen gels, the degree of contraction (as measured by a reduction in area) has been correlated to the myofibroblast content in cell culture (Tomasek and Rayan, 1995).

Previous studies on Dupuytren's fibroblast stimulation with TGF- β 1 have used lower concentrations (<2 ng/ml). However, higher concentrations have been tested only using dermal fibroblasts. TGF- β 1 increased the measurable contractile force generated by human dermal fibroblasts in tethered collagen gel in a friction-free floating environment up to 12.5 ng/ml. However, at concentrations above 15 ng/ml, it was noted that there was a significant reduction in measurable force generated (Brown et al., 2002).

In this study, we tested the hypothesis that higher doses of TGF- β 1 would inhibit the induction of myofibroblasts as well as the measurable contraction forces generated by Dupuytren's fibroblasts.

MATERIALS AND METHODS

Fibroblast culture

With local ethical committee approval, fibroblasts were cultured from Dupuytren's nodules or flexor retinaculum tissue obtained from patients undergoing surgery. Only the nodular part of the Dupuytren's diseased fascia was used as it has been reported previously that the natural history of the disease is for active nodular tissue to progress and "burn out" to become fibrous dormant cords (Vande Berg et al., 1984). As controls, flexor retinaculum fibroblasts were obtained from excised flexor retinaculum from patients undergoing

decompression of the carpal tunnel who did not have co-existing Dupuytren's disease.

The cells were cultured in T225 flasks (Corning Incorporated, New York, USA) in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Dorset, UK), supplemented with 10% foetal calf serum (Sigma-Aldrich, Dorset, UK), 100 U/ml penicillin/100- μ g/ml, streptomycin (Gibco BRL, Paisley, UK) and 2 mM L-glutamine (ICN Biochemicals Ltd, Thyne, UK) at 37 °C and 5% CO₂. At 80% confluence, the cells were enzymatically detached from the culture surface using trypsin (each time this is carried out, it is referred to as a passage) (Gibco BRL, Paisley, UK), as described previously by Brown et al. (2002). Cells for experimentation were used at passage 5, or less, and serum concentration of media dropped to 2%, as described previously by the same authors. Two per cent foetal calf serum was determined in earlier studies (Brown et al., 2002) to be the minimum serum concentration which will support a basal measurable contraction.

Immunofluorescent microscopy

Dupuytren's nodule 1×10^5 ($n = 3$) and flexor retinaculum fibroblasts 1×10^5 ($n = 3$) were counted using a haemocytometer and, then, seeded onto alcohol-sterilised, glass coverslips in six-well plates. The wells were flooded with 4 ml of supplemented Dulbecco's modified Eagle's medium, with 2% foetal calf serum and TGF- β 1 (PeproTech EC Ltd, London, UK). TGF- β 1 was diluted in phosphate-buffered saline to give final concentrations of 0 (negative control), 7, 12.5, 15 and 30 ng/ml. The cells were incubated at 37 °C and 5% CO₂, up to two time points of 24 and 72 hours.

At 24 and 72 hours, the cells were rinsed three times in 4 ml of phosphate-buffered saline for 5 minutes each, followed by fixation with 100% methanol for 20 minutes at -20 °C and a further three washes of 5 minutes each in phosphate-buffered saline. The coverslips were incubated for 1 hour at room temperature with 100- μ l mouse monoclonal anti- α -smooth muscle actin primary antibody (Sigma-Aldrich, Dorset, UK), diluted 1:200 with phosphate-buffered saline. For negative controls, phosphate-buffered saline replaced the primary antibody. Human masseter myoblast cells were used as positive staining controls. After three washes of 5 minutes each in 4 ml phosphate-buffered saline, the samples were treated for 30 minutes with 100- μ l fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody (Dako A/S, Denmark), diluted 1:100 with the nuclear counter-stain propidium iodide (Sigma-Aldrich, Dorset, UK) diluted 1:25 with phosphate-buffered saline. Following further three washes of 5 minutes in 4 ml PBS, the coverslips were mounted onto slides using 1 drop of DABCO (Sigma-Aldrich, Dorset, UK), which prevents fading of fluorescence.

The slides were examined under ultraviolet illumination with excitation wavelengths for fluorescein isothiocyanate and propidium iodide of 490 and 535 nm, respectively, using an Olympus BH-2 Microscope. Three random fields per cover slip were chosen. The total number of nuclei stained red by propidium iodide per field was counted. Then, under UV illumination at the fluorescein isothiocyanate wavelength, the number of positively staining myofibroblasts (identified by their green actin filaments) were counted and the percentage of myofibroblasts was derived for each TGF- β 1 concentration. A minimum of 500 cells were counted.

Photographs were taken with an Olympus C35AD-4 camera at 200 \times magnification using Fujichrome Provia 400F film, which was processed at PDQ Photographic Ltd, Hertfordshire, UK. The photographs were scanned into a PC at 300 dpi. The two images at the different wavelengths were superimposed over each other using Adobe Photoshop v7 software (Adobe Corporation, San Jose, USA) to enable both the nuclei and the actin filaments to be displayed in the same picture.

The percentage of myofibroblasts in a field of fibroblasts was determined by first counting the total number of nuclei in the field and, then, counting the number of cells staining positive for intracellular α -smooth muscle actin filaments (myofibroblasts).

Measurement of contractile forces

The culture force monitor (Fig 1) is an instrument which measures forces generated by cells within a collagen matrix quantitatively (Eastwood et al., 1994). It comprises of a rectangular, three-dimensional, fibroblast-seeded collagen gel which is cast and floated in medium, tethered to two flotation bars on either of the short edges, in turn attached to an anchor point at one end and a force transducer at the other, via stainless-steel wire A-frames. Since very little deflection of the transducer was expected, the output signal was increased with a strain gauge amplifier and channelled into a digital voltmeter and then into an analog-to-digital converter installed onto a desktop computer, as previously described (Eastwood et al., 1998). Data was collected at a rate of one reading per second, using LabVIEW v6.1 software (National Instruments, Texas, USA) to produce graphical data points in real time of the continuous output of force generated, a contraction profile was generated over 24 hours. The apparatus was kept in a humidified incubator at a constant 37 °C and 5% CO₂.

The collagen gel was prepared by mixing 5 ml of 2.28 mg/ml solution of soluble, native acid type I rat tail collagen (First Link UK, West Midlands, UK) with 0.625 ml of 10 \times MEM (Gibco BRL, UK) to give a clear yellow solution. The gel was neutralised dropwise with 5 M NaOH, using the yellow to pink colour change to signify a neutral pH, before the addition of 0.5 ml of a suspension of 5 \times 10⁶ fibroblasts counted using a

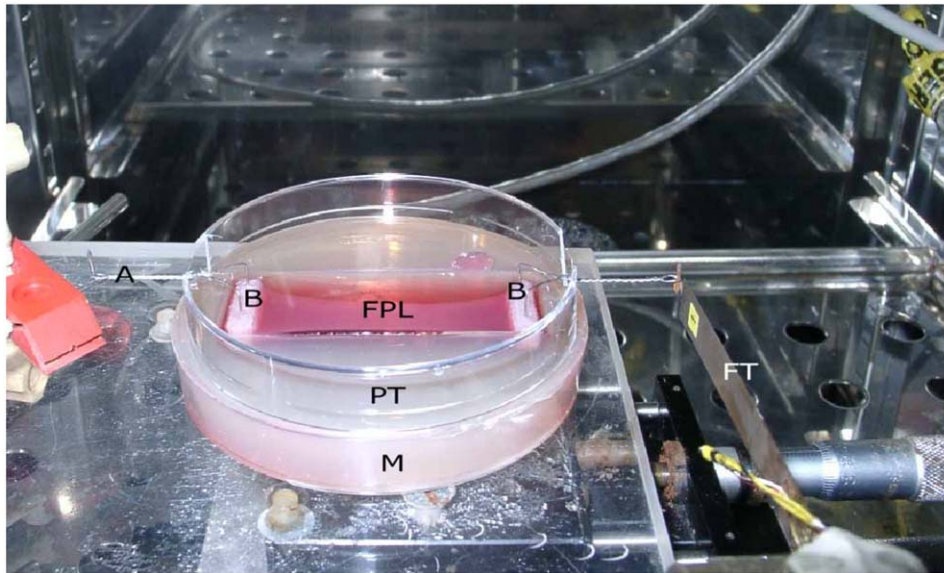


Fig 1 Culture force monitor apparatus. Floating in growth medium, within the Delron plastic mould (M), is a fibroblast-populated lattice (FPL) attached to flotation bars (B), one of which is anchored to a fixed point (A) and the other to a force transducer (FT) via stainless-steel wire A-frames. The setup is covered with a Petridish (PT).

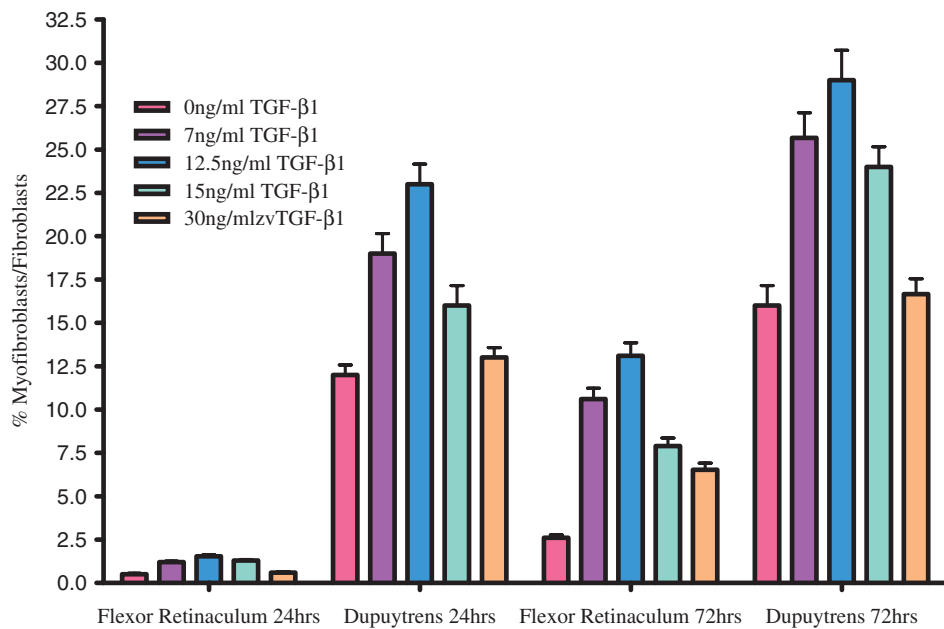


Fig 2 Histograms showing the per cent and standard error of the mean (SEM) of myofibroblast upregulation in flexor retinaculum and Dupuytren's fibroblasts at 24 and 72 hours. Note: Bell-shaped pattern with higher concentrations of TGF- β inhibiting myofibroblast upregulation.

haemocytometer. This gave 10^6 cells/ml of gel solution, in line with earlier studies (Brown et al., 2002). This was pipetted into a Delron plastic mould between the flotation bars. The gel was set for 30 minutes at 37°C, 5% CO₂, prior to being floated in 20 ml of supplemented Dulbecco's modified Eagle's medium with 2% foetal calf serum

and 100- μ l TGF- β 1 at concentrations 0 (i.e. 100- μ l phosphate-buffered saline added in place of TGF- β 1), 7, 12.5, 15 and 30 ng/ml. Data capture began immediately and was recorded over 24 hours. Two per cent foetal calf serum was determined in advance as the minimal serum-concentration to support a basal, measurable

contraction, minimising the contribution of serum-derived factors, such as lysophosphatidic acid (Tomasek et al., 1992). This ensured that the fibroblast populated collagen gel contracted under conditions limiting for TGF- β 1.

Power studies indicated a sample size of $n = 3$ assuming $\alpha = 0.05$, $\beta = 0.2$, power = 0.8, and a standard deviation of 20% from previous work carried out in this laboratory (Bisson et al., 2003, 2004). Rates of contraction and statistical analysis of data were carried out using standard computer software (Excel v2003, Microsoft Corporation, Redmond, USA and Prism v4, Graphpad, San Diego, USA). The Mann-Whitney statistical test was used to compare intragroup data and the repeated measures ANOVA was used to compare intergroup data. Linear regression and Spearman's rank correlation studies were used to ascertain the degree of correlation between the number of myofibroblasts and contractile force generated.

RESULTS

Myofibroblast upregulation

Fig 2 shows the percentage of myofibroblasts upregulated (i.e. increase in numbers and/or percentage from baseline levels tested) in a minimum of 500 cells of Dupuytren's nodule ($n = 3$) and flexor retinaculum ($n = 3$) fibroblasts at 24 and 72 hours upon stimulation with TGF- β 1. Figs 3 and 4 are representative fields from representative photomicrographs showing the expression of myofibroblasts in Dupuytren's nodule fibroblasts incubated for 24 and 72 hours, respectively.

The histogram in Fig 2 shows that the upregulation of myofibroblasts in Dupuytren's nodule fibroblasts is much greater, by a factor of almost 10, than the flexor retinaculum fibroblasts at all concentrations of TGF- β 1. This is statistically significant (ANOVA, $P < 0.001$). In addition, the percentage of myofibroblast upregulation

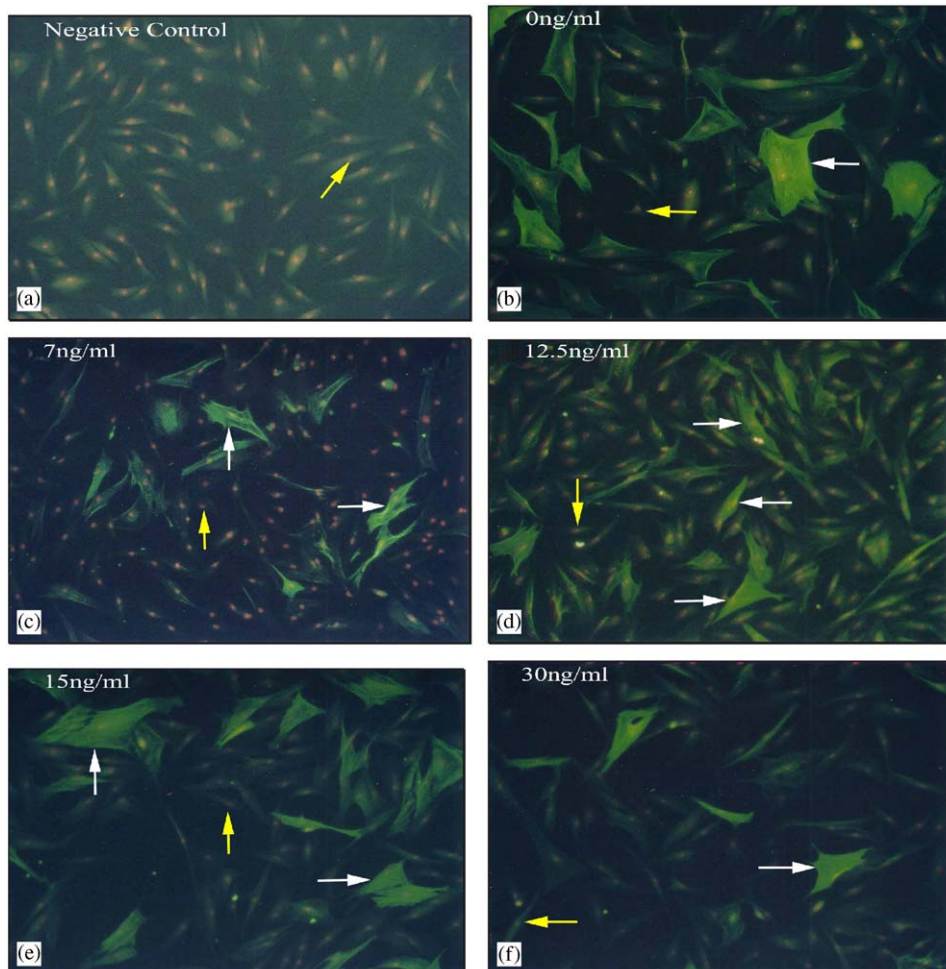


Fig 3 (a-f) Superimposed images ($\times 200$ magnification) of Dupuytren's fibroblasts cultured as a monolayer on glass coverslips at different concentrations of TGF- β 1 for 24 hours. Examples of fibroblasts are indicated with a yellow arrow whilst myofibroblasts are shown with a white arrow.

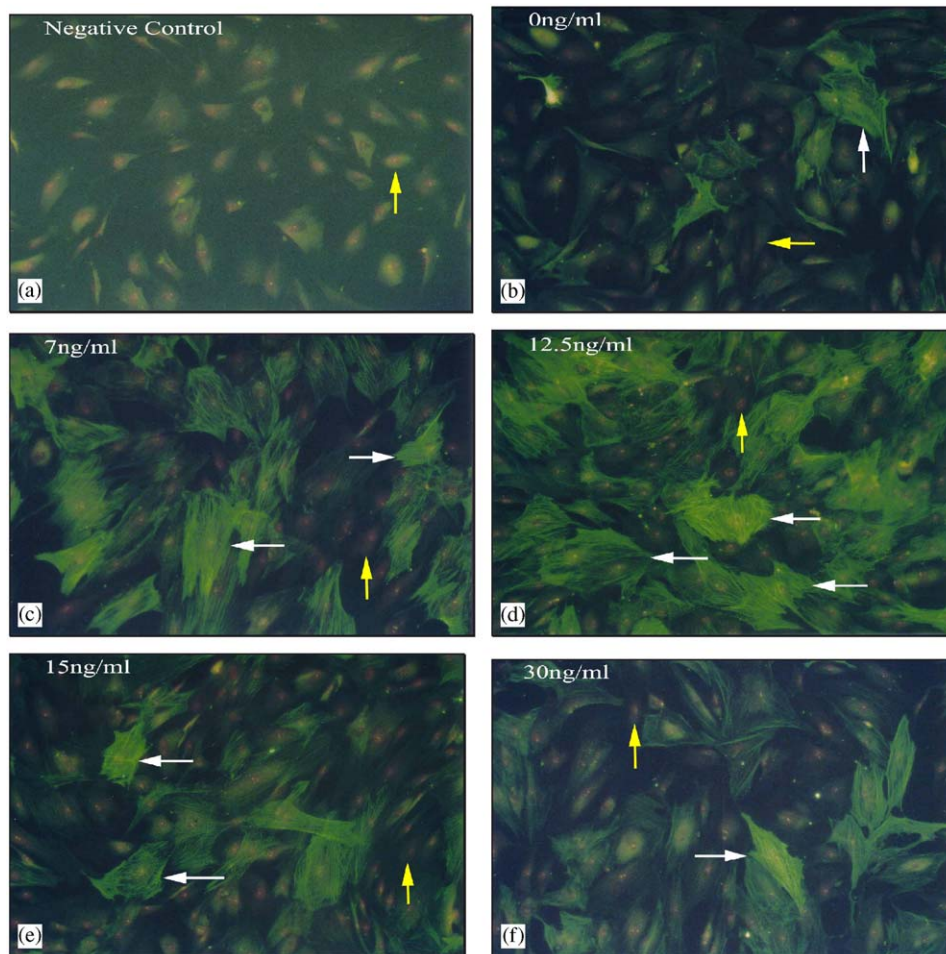


Fig 4 (a–f) Superimposed images ($\times 200$ magnification) of Dupuytren's fibroblasts cultured as a monolayer on glass coverslips at different concentrations of TGF- β 1 for 72 hours. Examples of fibroblasts are indicated with a yellow arrow whilst myofibroblasts are shown with a white arrow.

almost doubles from 0 to 12.5 ng/ml TGF- β 1 (Mann–Whitney, $P < 0.05$) but decreases to almost baseline levels, from 15 to 30 ng/ml TGF- β 1 (Mann–Whitney, $P > 0.05$) for both Dupuytren's nodule and flexor retinaculum fibroblasts.

When the Dupuytren's nodule and flexor retinaculum fibroblasts were cultured on glass coverslips for 72 hours, there was an increase in the expression of the myofibroblast phenotype compared to 24 hours incubation (Fig 2). For example, at 12 ng/ml of TGF- β 1, flexor retinaculum myofibroblast upregulation increased from 1.43% to 13.1% and Dupuytren's nodule fibroblasts increased from 23.0% to 29.0%. This increase in myofibroblast upregulation from 24 to 72 hours was significant for both types of fibroblasts (ANOVA, $P < 0.001$).

At 72 hours, there is a similar pattern as that with incubation at 24 hours, in that there is an increase from 0 to 12.5 ng/ml which, for flexor retinaculum fibroblasts,

was a threefold increase and, for Dupuytren's nodule fibroblasts, was a twofold increase (Mann–Whitney, $P < 0.05$ for both types of fibroblasts) and a decrease from 12.5 to 30 ng/ml which, for both types of fibroblasts, amounted to, approximately, a 50% reduction (Mann–Whitney, $P < 0.05$ for both types of fibroblasts). In addition, the upregulation of myofibroblasts in Dupuytren's nodule fibroblasts is significantly greater than flexor retinaculum fibroblasts (ANOVA, $P < 0.001$).

The fact that myofibroblast expression in both types of fibroblasts increases significantly from 24 to 72 hours implies that upregulation is also a time-dependant process that takes days. In addition, these results imply that TGF- β 1 modulates this process in a dose-dependant manner. However, even at the highest dose tested (30 ng/ml), the myofibroblast de-differentiation did not fall below the levels of non-stimulated Dupuytren's nodule fibroblasts. This indicates that there is a threshold

level of differentiation, which cannot be inhibited at the levels of TGF- β 1 tested, viz 0 to 30 ng/ml.

Contractile forces

The contraction profiles for Dupuytren's nodule fibroblasts ($n = 3$) are shown in Fig 5. There is an increase in the contraction force generated as the concentration of TGF- β 1 increases. Maximum peak force generation (131 dynes) occurs when the fibroblasts are stimulated with 12.5 ng/ml TGF- β 1. However, as the concentration rises above 12.5 ng/ml, we see that the peak force starts to drop. The peak force at 24 hours generated by the fibroblasts when stimulated by 15 ng/ml is 88 dyn, compared to 131 dyn with 12.5 ng/ml. At 30 ng/ml, the peak force (41 dyn) at 24 hours drops below that of the baseline (0 ng/ml). ANOVA showed that the contraction profiles generated by the five different concentrations were significantly different from each other ($P < 0.001$). Testing specific concentrations against each other also showed a statistically significant difference (Mann-Whitney, $P < 0.01$) for the following pairs: 0 and 7, 7 and 12.5, 12.5 and 15 and 15 and 30 ng/ml.

The contraction profiles for flexor retinaculum fibroblasts ($n = 3$) are shown in Fig 6. As with Dupuytren's nodule fibroblasts, we see a similar pattern in that the force of contraction increases up to 12.5 ng/ml TGF- β 1 (63 dyn) but there is a fall in concentration

with 15 ng/ml (48 dyn) and 30 ng/ml (22 dyn). In addition, the forces generated under the influence of the five different concentrations were significantly different from each other (ANOVA, $P < 0.001$). Again, testing specific concentrations against each other also showed a statistically significant difference (Mann-Whitney, $P < 0.01$) for the following pairs: 0 and 7, 7 and 12.5, 12.5 and 15 and 15 and 30 ng/ml. The forces generated by the flexor retinaculum fibroblasts were significantly less than that generated by the Dupuytren's nodule fibroblasts. With 12.5 ng/ml, the peak force generated at 24 hours was 131 dyn with Dupuytren's nodule fibroblasts and 63 dyn with flexor retinaculum fibroblasts (Mann-Whitney, $P < 0.05$).

Fig 7 shows a dose-response plot comparing the response of Dupuytren's nodule and flexor retinaculum fibroblasts in terms of peak force generation at 24 hours to the different concentrations of TGF- β 1. ANOVA shows that the difference in force generation at the different TGF- β 1 concentrations between the two types of fibroblasts is significant ($P < 0.001$). As we have seen earlier with the contraction profiles (Figs 5 and 6), maximum force generation occurs under the influence of 12.5 ng/ml TGF- β 1 for both types of fibroblasts. One can see that the increase in force generation that Dupuytren's nodule fibroblasts exert compared to flexor retinaculum fibroblasts at the same concentrations of TGF- β 1 is not a linear one. Dupuytren's nodule fibroblasts show a 59% increase in force over flexor retinaculum fibroblasts at 0 ng/ml and a 106% increase

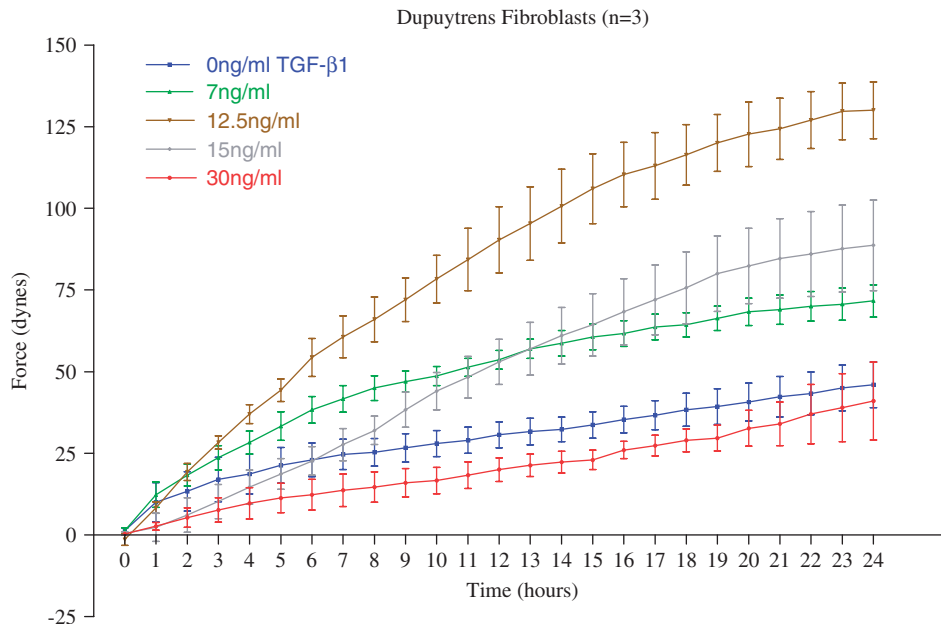


Fig 5 Time-force plots showing the mean and SEM of Dupuytren's fibroblasts contraction. TGF- β 1 increases peak contraction up to 12.5 ng/ml. Maximum force generation fell with concentrations 15 and 30 ng/ml. The five different concentrations varied significantly from each other ($P < 0.001$).

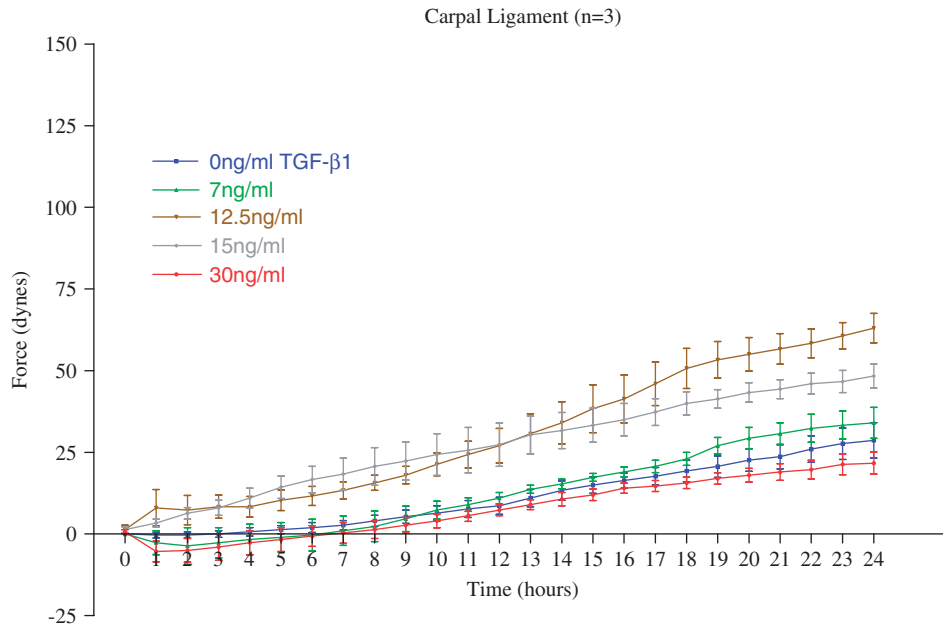


Fig 6 Time–force plots showing the mean and SEM of flexor retinaculum fibroblasts contraction. TGF-β1 increases peak contraction up to 12.5 ng/ml. Maximum force generation fell with concentrations 15 and 30 ng/ml. The five different concentrations varied significantly from each other ($P < 0.001$).

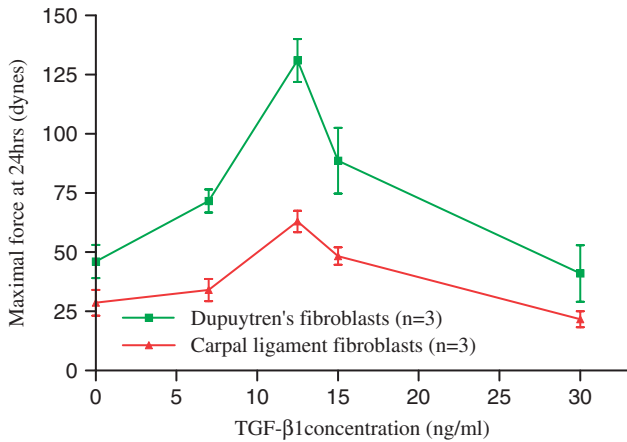


Fig 7 Dose–response plots derived from data in Figs 5 and 6 showing the mean and SEM of the maximal response at 24 hours. Peak force generation to TGF-β1 occurs at 12.5 ng/ml. Contraction of Dupuytren's fibroblasts for each different TGF-β1 concentration at 24 hours was significantly greater than flexor retinaculum fibroblasts ($P < 0.001$). There was a 59% increase at 0 ng/ml and a 106% increase at 12.5 ng/ml.

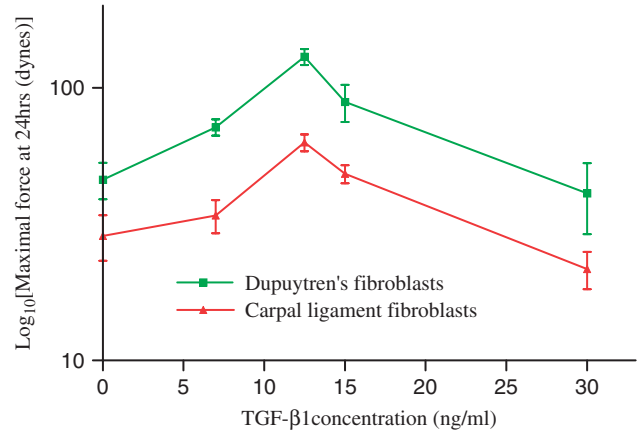


Fig 8 Dose–response plot derived from data in Fig 7 with the contractile force generated plotted as a logarithmic scale. At any given TGF-β1 concentration, the force generated by Dupuytren's fibroblasts is twice that of flexor retinaculum fibroblasts.

at 12.5 ng/ml. Plotting the maximal force generated at 24 hours on a logarithmic scale shows a more constant increase in the force produced by Dupuytren's nodule fibroblasts over that of carpal ligament fibroblasts (Fig 8). At any given TGF-β1 concentration, the force generated by Dupuytren's nodule fibroblasts is twice that of flexor retinaculum fibroblasts.

Correlation

Plotting the percentage change over baseline (0 ng/ml) of peak contractile force and myofibroblast upregulation and applying linear regression studies in Fig 9 reveals a relationship between contraction and myofibroblast upregulation in Dupuytren's nodule fibroblasts

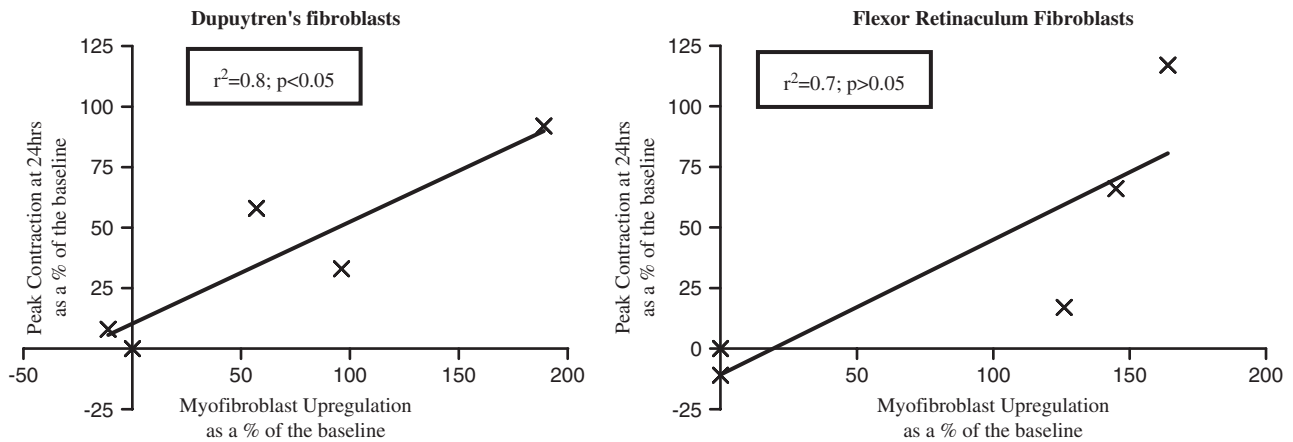


Fig 9 Graphs showing myofibroblast upregulation against peak contractile force at 24 hours as % change over baseline for Dupuytren's and flexor retinaculum fibroblasts. Linear regression analysis show that the linear relationship between myofibroblast upregulation and peak contractile force is significant in Dupuytren's fibroblasts but not in flexor retinaculum fibroblasts.

($r^2 = 0.8$) and flexor retinaculum fibroblasts ($r^2 = 0.7$), which was statistically significant for Dupuytren's nodule ($P < 0.05$) but not for flexor retinaculum fibroblasts ($P > 0.05$).

DISCUSSION

Myofibroblast upregulation

The myofibroblast, a specialised fibroblast phenotype expressing α -smooth muscle actin plays a key role in the pathogenesis of Dupuytren's contracture (Gabbiani and Majno, 1972). There was some variation between our results and other studies using immunofluorescent techniques to quantify the number of myofibroblasts in a fibroblast monolayer cell culture. Tomasek and Rayan (1995) found 14% (SD \pm 8.1) myofibroblasts in Dupuytren's fibroblasts and 5% (SD \pm 2.8) in flexor retinaculum fibroblasts after 48 hours incubation, whilst Bisson et al. (2003) found 9.7% (SD \pm 4.5) in Dupuytren's fibroblasts and 1.3% (SD \pm 1.9) in flexor retinaculum fibroblasts after 4 days incubation. However, these workers not only used different incubation lengths, they also cultured their fibroblasts in 10% foetal calf serum, whereas, in this study, cells were cultured initially in 10% foetal calf serum, before switching them over to 2% foetal calf serum prior to fixation, as explained earlier in the method (2% foetal calf serum was determined in earlier studies (Brown et al., 2002) to be the minimum serum concentration which will support a basal measurable contraction). This would, obviously, mean a reduction in the availability of growth factors for fibroblasts. In addition, Tomasek and Rayan (1995) cultured their fibroblasts in the presence of an antimycotic agent. Patient factors, such as age and gender, may also have contributed to the differences.

Despite the variation when compared to other studies, which could be attributable to slight methodological differences, the trend remains the same, in that there is a significantly greater proportion of myofibroblasts upregulated in cultured Dupuytren's fibroblasts than flexor retinaculum fibroblasts. This implies that Dupuytren's fibroblasts are altered in some way phenotypically to increase the number of myofibroblasts when compared to flexor retinaculum fibroblasts.

TGF- β 1 plays a key role in amplifying the transformation of the myofibroblast from the fibroblast (Badalamente et al., 1996). Vaughan et al. (2000) found that 5 days incubation with 1 ng/ml TGF- β 1 increased myofibroblast expression from approximately 2% to 26% in Dupuytren's fibroblast-populated collagen lattices. Bisson et al. (2003) found that 4 days incubation with 2 ng/ml TGF- β 1 increased myofibroblast expression from 9.7% (SD \pm 4.5) to 25.4% (SD \pm 5.5) in monolayer cell cultures of Dupuytren's fibroblasts. However, none of these authors tested the dose response of increasing TGF- β 1 concentrations on myofibroblast upregulation.

Our results show that TGF- β 1 up to 12.5 ng/ml increases the expression of the myofibroblast phenotype in Dupuytren's fibroblasts. At 15 ng/ml, the level of expression started to fall by approximately 30% (at 24 hours) and, with 30 ng/ml, the levels fell to near baseline levels. This phenomenon of high concentrations of TGF- β 1 reducing the levels of myofibroblast expression in Dupuytren's fibroblasts has not been previously documented in the literature.

Flexor retinaculum fibroblasts also showed a statistically significant increase in myofibroblast expression when treated with TGF- β 1. Bisson et al. (2003) found that when flexor retinaculum fibroblasts are treated with 2 ng/ml for 4 days the levels rose from 1.3% (SD \pm 1.9)

to 2.6% (SD \pm 1.9). However, they did not find the increase to be statistically significant. As with Dupuytren's fibroblasts, the level of upregulation peaked at 12.5 ng/ml (1.5% at 24 hours) then downregulated (decrease in numbers and/or percentage when compared to normal or pre-treatment group) by about 13% and 60% at 15 ng/ml (1.3% at 24 hours) and 30 ng/ml (0.6% at 24 hours), respectively. This increase in myofibroblast upregulation from 0 to 12.5 ng/ml and the decrease from 12.5 to 30 ng/ml was significant (Mann-Whitney, $P < 0.05$). Why TGF- β 1 would display this negative feedback inhibition at higher concentrations is unclear at present but one could speculate that it is part of a regulatory mechanism to prevent excessive myofibroblast upregulation in fibroblasts with the consequences of excessive contraction.

The statistically significant increase in myofibroblast expression from 24 to 72 hours in both Dupuytren's and flexor retinaculum fibroblasts implies that the upregulation of the myofibroblast phenotype increases with time. When we compare the level of myofibroblast expression in the absence of TGF- β 1 from 24 to 72 hours, there is a significant increase for both types of fibroblasts (Mann-Whitney, $P < 0.05$). This has been observed before and has been attributed to the glass coverslip providing a stiff environment for the cells to attach to and stimulate actin microfilament stress fibres (Tomasek et al., 2002). However, as the culture conditions were the same for all groups tested and the only variation was the concentration of TGF- β 1, the significant upregulation of myofibroblast phenotype can only be attributed to TGF- β 1 stimulation.

Contractile forces

Using the Culture Force Monitor model (Eastwood et al., 1994), we were able to determine the effects of TGF- β 1 on collagen lattice contraction of Dupuytren's and flexor retinaculum fibroblasts. TGF- β 1 increased the peak contraction generated at 24 hours in Dupuytren's fibroblasts up to 12.5 ng/ml but there was a fall in peak contraction with 15 and 30 ng/ml. This pattern was also seen with flexor retinaculum fibroblasts. Others have found that treatment with TGF- β 1 increases the force of contraction generated. Vaughan et al. (2000) found that TGF- β 1 up to 1 ng/ml would increase the contraction of Dupuytren's fibroblasts in stressed-relaxed collagen lattices in a dose-dependent manner. Bisson et al. (2003) found that 2 ng/ml TGF- β 1 significantly increased the force generated by both Dupuytren's and flexor retinaculum fibroblasts in a culture force monitor. This TGF- β 1 augmentation of contraction has also been seen with other types of fibroblasts (Hinz et al., 2001; Montesano and Orci, 1988).

However, this phenomenon of negative feedback inhibition by TGF- β 1 at higher concentrations on the contractile force generated by fibroblasts in a three-

dimensional collagen lattice has only been previously described before in dermal fibroblasts (Brown et al., 2002), but never in Dupuytren's or flexor retinaculum fibroblasts. An important event during wound healing is the contraction of newly formed connective tissue by fibroblasts and TGF- β 1, which has been shown to enhance the ability of fibroblasts to contract collagen gels in vitro markedly (Montesano and Orci, 1988). We propose that it would, therefore, be prudent to have a fail-safe mechanism to prevent excessive contraction if the levels of TGF- β 1 were to rise.

Dupuytren's fibroblasts generated greater contractile forces than flexor retinaculum fibroblasts. This is in agreement with current literature (Bisson et al., 2004). However, our data on contractile forces using the same measuring device to quantitate forces are lower than that reported by Bisson et al. (2004). Bisson et al. (2003) reported Dupuytren's fibroblasts produced peak contraction forces of 145 ± 7.9 dyn and carpal ligament fibroblasts 39.9 ± 13.1 dyn at 20 hours. In that study, force measurements of fibroblast-populated collagen matrices were recorded in 10% foetal calf serum, with cells being preincubated in 2% foetal calf serum and 2 ng/ml TGF- β 1 for 4 days. In the present study, as stated earlier, cells were culture expanded in 10% foetal calf serum initially before switching to 2% foetal calf serum over the 24 hours of force measurements of fibroblast-populated collagen lattices using TGF- β 1 stimulation at various concentrations, which were added at time 0, at the start of force measurements. This was similar to the setup used and reported by Brown et al. (2000). The aim of this work was to test the feedback inhibition of increasing concentrations of TGF- β 1 on Dupuytren's nodule-derived fibroblasts. Reduction of the foetal calf serum level is known to reduce contraction of the fibroblast-populated collagen lattice, in part by limiting the availability of lysophosphatidic acid, which acts through myosin light chain kinase and phosphatase (Grinnell, 1994; Tomasek et al., 1992).

Correlation

Tomasek and Rayan (1995) have described a significant positive correlation between the expression of α -smooth muscle actin and the generation of contractile force in cell strains of Dupuytren's fibroblasts ($r^2 = 0.68$, $P < 0.05$) and in flexor retinaculum fibroblasts ($r^2 = 0.92$, $P < 0.05$). Hinz et al. (2001) also found a significant positive correlation ($r^2 = 0.88$) in the presence of TGF- β 1, but no P -value was given. Our results showed a significant positive correlation between myofibroblast upregulation and peak contractile force generated at 24 hours as percentage increases over the baseline with Dupuytren's fibroblasts. There was also a positive correlation with flexor retinaculum fibroblasts, but this was not statistically significant. We can,

therefore, conclude that, in the case of Dupuytren's fibroblasts the contractile force generated was as a direct result of the action of the myofibroblasts contracting.

Clinical implications

The mainstay of treatment remains surgical excision of the affected fascia \pm overlying skin, but disease recurrence can be as high as 77% in those followed up over 30 years (Leclerq, 2000). Non-operative treatments targeting the abnormal biochemical processes of Dupuytren's disease are currently being investigated. For example, 5-fluorouracil has been shown to inhibit both proliferation and myofibroblast differentiation in Dupuytren's cell cultures (Jemec et al., 2000). Subsequently, a double-blind randomised clinical trial in 15 patients, assessing whether intraoperative topical treatment with 5-fluorouracil reduces the recurrence rate after limited excision of Dupuytren's tissue compared to placebo, found no significant difference between control and 5-fluorouracil-treated digits with respect to flexion deformity after 18 months of follow-up (Bulstrode et al., 2004). Abnormal collagen metabolism has been targeted through the use of collagenase. In a series of controlled phase-2 clinical trials, excessive collagen deposition in Dupuytren's disease has been targeted by using enzyme (Clostridial collagenase) injection therapy to lyse and rupture finger cords causing metacarpophalangeal and/or proximal interphalangeal joint contractures. Results so far have been encouraging (Badalamente et al., 2002).

Can we extrapolate our laboratory findings to the clinical situation? For example, could humanised TGF- β 1 antibodies, currently available and used in clinical trials as anti-scarring agents in ophthalmology, be utilized? Our findings indicate that there is a band of TGF- β 1 concentrations (0–12.5 ng/ml) where such antibodies may have an inhibitory effect on myofibroblasts. However, after a certain concentration (> 15 ng/ml), there is a feedback inhibitory effect where, perhaps, the use of such antibodies might actually cause greater myofibroblast differentiation and contraction.

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