The Effect of Shear Stress on Fibroblasts Derived From Dupuytren's Tissue and Normal Palmar Fascia

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This study examines the real-time intracellular calcium changes of palmar fascia from normal and Dupuytren's diseased fibroblasts in response to shear stress. The real-time cytosolic calcium changes were measured using fluorescence microscopy image processing. The preconfluent primary cultured cells were exposed to 1 minute of flow at 25 dyne/cm^2 after a 2-minute baseline of no flow. Additionally, the cells were exposed to an influx of Hank's buffered saline solution with 2% newborn bovine serum to examine the response to serum-born (chemical) agonists. Cytosolic calcium changes were measured as the percentage change over the 2-minute baseline of the mean $[Ca^{2+}]i$ peak. The mean change of the peak $[Ca^{2+}]i$ response of the normal palmar fascia was significantly greater than that of the cells from the Dupuytren's nodular and perinodular tissue. The response to the chemical agonist showed a robust but not statistically different response between the 3 cell types. Our work supports the hypothesis that palmar fascia responds to mechanical stress, specifically laminar fluid flow. These findings may help to explain that an underlying abnormality in the cells of the palmar fascia may be expressed by exposure to laminar fluid flow, a physical signal, rather than a chemical agonist. (J Hand Surg 1998;23A:945–950. Copyright © 1998 by the American Society for Surgery of the Hand.)

The fascia of the palm of the hand is a complex layer of tissue that serves to protect and provide structural integrity. Dupuytren's contracture or disease is characterized by an abnormal shortening of parts of the palmar fascia that results in a progressive

flexion deformity of the hand and fingers. The pathology in this disease is believed to be the result of uncontrollable tissue proliferation and the overproduction of extracellular matrix in the dermis and palmar fascia.[1] Early work focused on the study of the pathologic anatomy.[2,3] Other investigators followed with work relating collagen type and composition to disease activity.[4,5] Recent work has focused on the increased presence of various growth factors and their relationship to the disease.[1,6,7] Yet, the etiology and pathogenesis of this disease is still not entirely clear.

It is possible that the fibroblasts in patients with palmar fibromatosis are intrinsically different from fibroblasts in the normal population. As such, stresses that the hand is normally subjected to may produce an abnormal response in the diseased tissue.

Other investigators have reported early evidence that such is the case when studying the effect of mechanical stress on cellular proliferation and the production of growth factors. (Alman et al.: Trans Orthop Rel Res 1995;41;506, abstract). Using a well-developed model to deliver a consistent, reproducible physical stress,[8] we sought to further study the hypothesis that the fibroblasts of Dupuytren's contracture respond differently than the normal fibroblasts when subjected to mechanical stress. As calcium is a ubiquitous icon, cytosolic calcium flux was chosen as the secondary messenger to evaluate whether this physical signal was transduced into a biochemical response. This study examines the response of palmar fascia from normal and Dupuytren's diseased fibroblasts to shear stress from laminar fluid flow via real-time intracellular calcium changes.

Materials and Methods

Normal palmar fascia and Dupuytren's tissue were obtained from patients during open carpal tunnel release and routine excision of diseased tissue, respectively. Tissue was obtained from 8 patients to harvest the necessary specimens. Diseased tissue was obtained from 4 patients (3 men and 1 woman) with Dupuytren's diseased fascia, with an average age of 58 years (range, 43–77 age). All patients with Dupuytren's disease had nodular thickening of the palmar fascia with a progressive joint contracture and could be classified as stage II or active disease according to Chiu and McFarlane.[2] This stage of the disease was chosen for the following 2 reasons: first, as the fibroblasts in this stage are actively replicating, they would likely be most responsive to an agonist; and second, there would be less intervening collagen between fibroblasts in the specimens obtained during tissue harvest. Normal palmar fascia was obtained from 3 patients who had an open carpal tunnel release and 1 patient who had a trigger finger release. This group consisted of 3 women and 1 man with an average age of 66 years (range, 46–78 years).

Tissue harvest of all specimens began less than 90 minutes after surgical excision from the patient. Using ×3.5 magnifying loupes, Dupuytren's nodular and perinodular tissue was cut into 1-mm^3 cubes. Palmar fascia was also prepared in a similar manner. These cubes were placed in the center of a 60 × 15 mm Petri dish with Dulbecco's modified Eagle's medium and 50% fetal bovine serum. The cells were incubated at 37°C with 5% CO_2. After 3 days, the tissue was adherent to the dishes. The media was
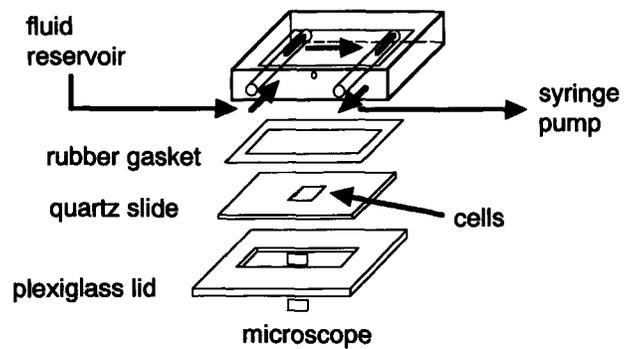

Figure 1. The flow chamber used to stimulate the cells provided a consistent, reproducible agonist to the cells.

replaced with Dulbecco's modified Eagle's medium with 10% fetal bovine serum, oxalicetic/sodium pyruvate/insulin solution, and penicillin/streptomycin/amphotericin B solution and was changed every 2 to 3 days. Once the cells were near confluence, they were passaged with cold trypsin onto Quartz culture slides to fit in specially designed laminar fluid flow chambers. The parallel plate flow chamber has a uniform-thickness rubber gasket that separates the 2 parallel plates (Fig. 1). One plate is the quartz slide with the cultured cells; the other plate is the plexiglass block with a flow channel milled in it. A mechanical syringe draws the fluid from a fluid reservoir through the assembled chamber, thereby exposing the cells to a fluid-induced wall shear stress. The fluid-induced wall shear stress is proportional to the applied flow rate for a constant chamber geometry. All experiments were performed using preconfluent, primary fibroblast cultures. We used a previously described model to subject the cultured cells to shear stress associated with laminar fluid flow.

The cellular response to this physical signal was measured via cytosolic calcium shifts. The results are demonstrated via fluorescence microscopy image processing. The cells were topically loaded with a FURA-2 dye (Molecular Probes, Eugene, OR) in its ester form, in which it penetrates the cell membrane and establishes itself in the cytoplasm. This dye has an affinity to bind to the ionic calcium. We measure the calcium concentration by exciting the dye with 2 different wavelengths of light. The first wavelength 340 nm maximally excites the bound Ca^{+2}–dye complex. The 360 nm excites all the fura complexes in the cell. A ratio is established to determine the cytosolic ionic calcium concentration. A dose-response curve is established with standard, known
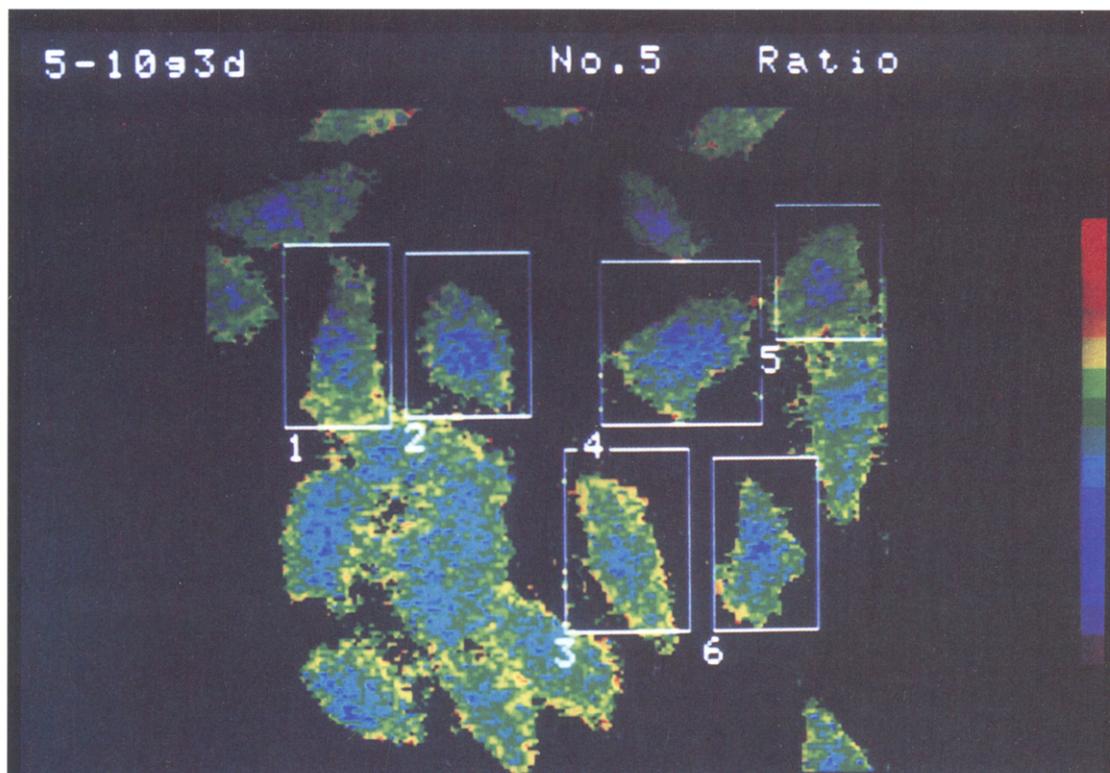
Figure 2. Six individual cells of normal palmar fascia were selected for examination before stimulation.

concentrations of calcium. During each experiment, individual cells are selected and the calcium concentration, $[Ca^{+2}]i$, is calculated based on this ratiometric analysis of fluorescent intensities (Figs. 2 and 3).

After the cells were loaded with fura2-AM dye, the cells were exposed to modified Hank's buffered saline solution as the perfusion media. The cells were exposed to 1 minute of flow at 25 dyn/cm^2 after a 2-minute baseline of no flow. Data were collected for an additional minute after the signal was stopped. This flow rate was based on results from previous experiments using other cell types. This flow rate consistently elicited a response from the other cell lines.[8] In addition, the cells were exposed to an influx of Hank's buffered saline solution with 2% newborn bovine serum as a flow rate corresponding to 3 dyne/cm^2 to examine the response to serum-born (chemical) agonists. Cytosolic calcium changes were measured as the percentage change over the 2-minute baseline of the mean $[Ca^{2+}]i$ peak for cells from both Dupuytren's tissue and the palmar fascia. ANOVA testing with Scheffe's test was performed to analyze the data.

Results

The experiments were performed on 2 separate days. Each value for percent change in the peak

$[Ca^{+2}]i$ was calculated by evaluating the peak value compared with the average value for the 2-minute baseline (Fig. 4). There was no statistically significant difference between the responses obtained on separate days. Experiments were performed using cells obtained from all 8 patients. Approximately an equal number of cells were analyzed from each of the 4 normal palmar fascia cultures (n = 82). Each of the 4 Dupuytren's disease samples provided 2 cultures (nodular and perinodular tissues). Similarly, an approximately equal number of cells from each of the cultures were analyzed.

Experiments were performed to established that our cell cultures would respond with this model. Two percent newborn bovine serum was used as a biologic signal with the flow rate of 3 dyne/cm^2. Seventy-two cells were evaluated for percent change in the peak $[Ca^{+2}]i$ for 4 minutes without any agonist. The average change for the 4 minutes was 6.38 ± 1.33. The 3 cell populations we studied were the normal palmar fascia (n = 82), the Dupuytren's nodular tissue (n = 60), and the Dupuytren's perinodular tissue (n = 52). All cell populations responded with a cytosolic calcium flux. The mean percent change over baseline was different for each of the 3 cell populations. The values were 281.57 ±
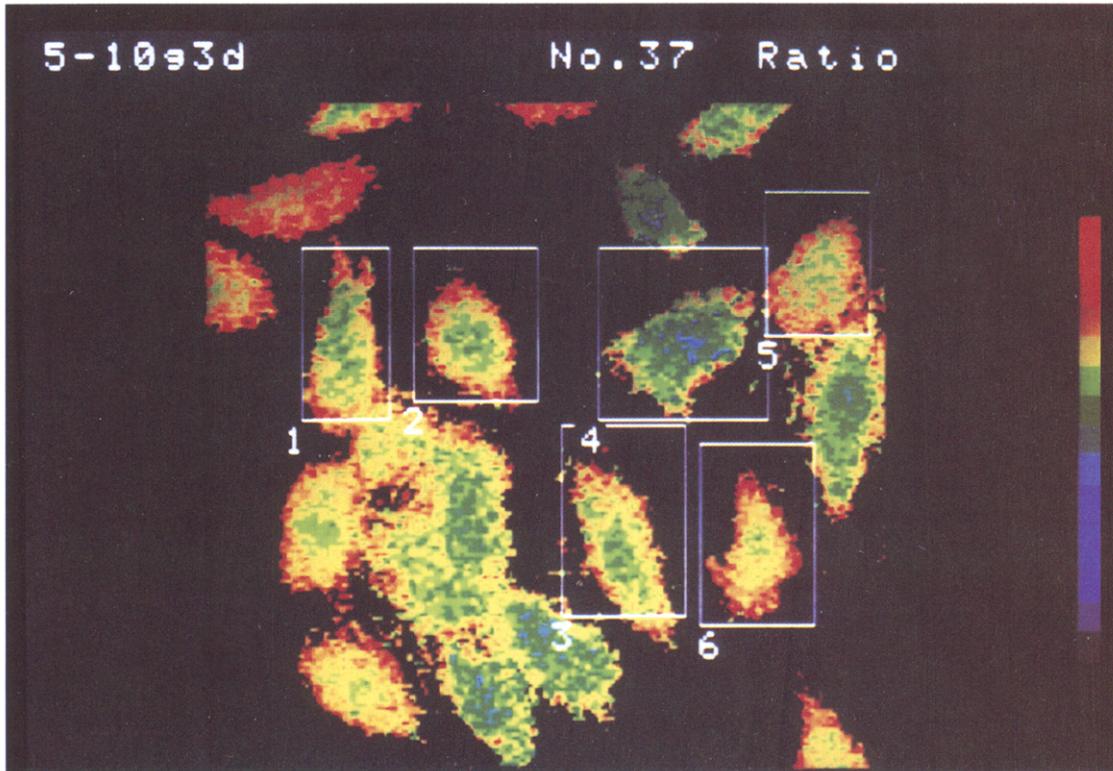
Figure 3. The 6 cells shown in Fig. 2 after they had been stimulated with an agonist, the laminar fluid flow.

9.26, 236.87 ± 7.13, and 293.49 ± 18.90, respectively. These responses were not statistically significant (Fig. 5).

The thrust of the experiments was directed at examining the response that the 3 cell types had to a specific mechanical agonist: laminar fluid flow (25 dyne/cm^2). The mean percent change of peak $[Ca^{2+}]i$ response of the normal palmar fascia (n = 105) was 142.5 ± 12.2, while the Dupuytren's nodular issue (n = 161) and the Dupuytren's perinodular tissue

(n = 167) were 60.3 ± 4.1 and 68.1 ± 5.2, respectively. The response of the cells from the normal palmar fascia was significantly greater than that of the cells from the Dupuytren's nodular and perinodular tissues (p < .0001). All responses were statistically different from the no-flow controls (Fig. 6).
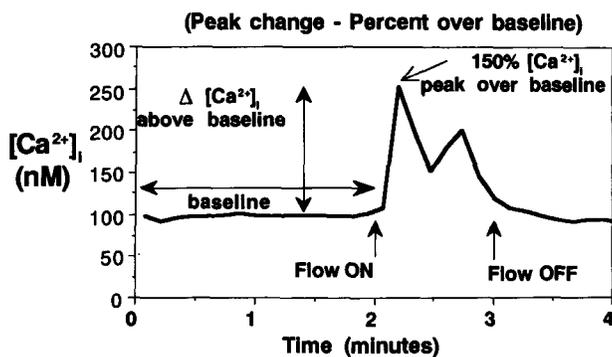

Figure 4. The typical data obtained from 1 cell (normal palmar fascia) using fluorescent image processing and cytosolic calcium flux as the secondary messenger system.
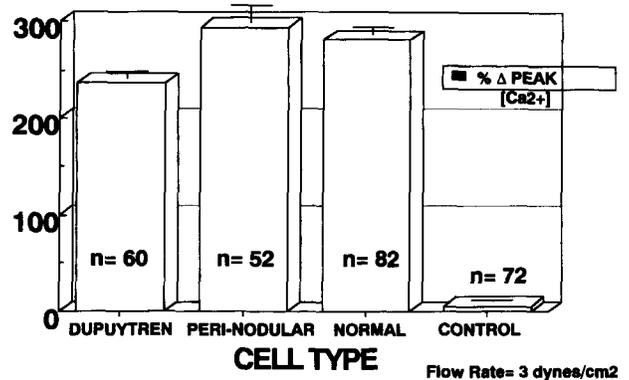

Figure 5. The normal and control groups represent the cells obtained from normal palmar fascia. The perfusate for the control group was only Hank's buffered saline solution and did not include 2% newborn bovine serum. The perfusate for the 3 remaining groups did include the 2% newborn bovine serum.
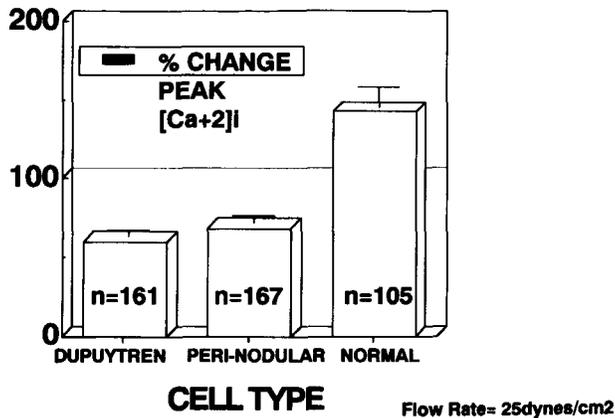
Figure 6. The data for the 3 cell types with only Hank's buffered saline solution as the perfusate. The difference in $[Ca^{+2}]i$ between Dupuytren's disease tissue and normal palmar fascia was statistically significant ($p < .0001$).

Discussion

Much work has been done in an attempt to elucidate the etiology and pathogenesis of Dupuytren's disease. The early understanding of Dupuytren's disease stemmed from McFarlane's[3] classic description of the anatomic pathology of the palmar fascia. After Gabbiani and Majno[9] described the concept of fibroblast contraction, Chiu and McFarlane[2] brought forth the idea that the cell of the active disease was the myofibroblast. Gelberman et al.[5] reported that the chemical composition of the palmar fascia was similar for different patients with Dupuytren's disease and that the fascia contained increased levels of collagen type III and reducible cross-links of collagen. Brickley-Parsons et al.[4] reported that all the palmar fascia in patients with Dupuytren's disease was abnormal. Although the gross and histologic findings of the fascia and nodular tissue may be different, similar biochemical changes are found throughout the fascia. Terek et al.[7] reported the expression of the platelet-derived growth factor gene in patients with Dupuytren's contracture. Badalamante et al.'s[10] work showed that the intracellular contractile mechanism of myofibroblasts is on the myofilaments.

At present, there is no clear understanding of the etiology of Dupuytren's contracture. Furthermore, there is little available data about the response of these cells to various agonists. In response to 12 hours of mechanical stretch via a vacuum pump, there was an increase in cellular proliferation and PDGF-A expression in cultured cells. (Alman et al.:

Trans Orthop Rel Res 1995;41:506, abstract). Tomasek et al.[11] have recently shown that both the actin cytoskeleton and the extracellular matrix in fibroblasts can transduce cell signalling as gelatinase A activation may be regulated by mechanical stress. Our work supports the hypothesis that palmar fascia responds to mechanical stress, specifically laminar fluid flow. As we wanted to study real-time cytosolic calcium flux as the secondary messenger for a physical signal, fluorescence microscopy image processing was the tool used to provide these data. If we had used the same mechanical agonist that Alman et al. (Trans Orthop Rel Res 1995;41:506, abstract), had used, the mechanical stretch of the membrane on which the cultured cells grew would have changed the focal length for the microscope. This would have limited our ability to obtain real-time data. Our conclusions are not to state that this particular type of mechanical stress (shear stress secondary to laminar fluid flow) is physiologic or the only type of mechanical stress that the palmar fascial cells respond to. Rather, we present the notion that these cells respond differently to a mechanical agonist compared with a chemical agonist. All 3 cell populations responded in a similar fashion to the chemical agonist, yet there was a statistically significant different response ($p < .0001$) between the cells from diseased Dupuytren's tissue and normal cells from the palmar fascia when subjected to the laminar fluid flow. It is possible that an underlying abnormality in the different cell types is expressed when exposed to a mechanical agonist.

Brickley-Parsons et al.[4] stated that all the palmar fascia in patients afflicted with Dupuytren's disease is pathologic. Electron microscopic examination of perinodular tissue did not give evidence of any pathology. The number of reducible cross-links after reduction with tritiated sodium borohydride in perinodular tissue was similar to that found in the nodular tissue. Our work supports this hypothesis. The nodular and perinodular cells from the same patients were dissected with the aide of microscopic magnification to better define any gross morphologic changes. When the cultured cells were subjected to shear stress, the cells from the Dupuytren's perinodular tissue responded most similarly, not to the normal palmar fascial cells, but rather to the cells from the Dupuytren's nodular tissue. Fibroblasts in the grossly unaffected areas of the palmar fascia of Dupuytren's tissue were not examined. Therefore, no conclusion can be made about the nature of those fibroblasts from our experiments.

Rayan et al.[12] have shown that nifedipine and

verapamil actually block the lysophosphatidic acid-induced contraction of the cultured cells on a collagen lattice. These agents block the flux of extracellular calcium. Our study examines the response of intracellular calcium flux. Further work using agents that block intracellular calcium flux, such as neomycin and thapsigarin, need to be conducted.

There are limitations to our study. Although a defined, reproducible model was used to deliver the physical agonist, there was heterogeneity in the fluid-induced shear stress. The wall shear stress was calculated as a uniform macroscopic shear stress and not as the actual shear stress on each cell membrane.[8] Thus, the differences in clustering may have had an effect on the cytosolic flux. Although unlikely with this method of examining the cells, the possibility that fibronectin anchoring strands may be present cannot be ruled out. Furthermore, additional attempts need to be made to deliver a more physiologic mechanical stress to the cell. As these were preconfluent cultured cells, the in vivo response of the palmar fascia may be different. Further work needs to be completed to define how this change in calcium flux is translated into various cellular pathways.

Our findings may help to explain that an underlying abnormality in the cells of the palmar fascia may be expressed by exposure to laminar fluid flow, a physical signal, rather than a chemical agonist. Further investigation is necessary to understand how this difference translates into changes in cellular proliferation and the manifestation of the disease.

References

1. Kloen P, Jennings CL, Gebhardt MC, Springfield DS, Mankin HJ. Transforming growth factor-β: possible roles in Dupuytren's contracture. J Hand Surg 1995;20A:101–108.
2. Chiu HF, McFarlane RM. Pathogenesis of Dupuytren's contracture: a correlative clinical-pathological study. J Hand Surg 1978;3:1–10.
3. McFarlane RM. Patterns of the diseased fascia in the fingers in Dupuytren's contracture. Plast Reconstr Surg 1974;54:31–44.
4. Brickley-Parsons D, Glimcher MJ, Smith RJ, Albin R, Adams JP. Biochemical changes in the collagen of the palmar fascia in patients with Dupuytren's disease. J Bone Joint Surg 1981;63A:787–797.
5. Gelberman RH, Amiel D, Rudolph RM, Vance RM. Dupuytren's contracture. J Bone Joint Surg 1980;62A:425–432.
6. Ian BA, Naber SP, Terek RM, et al. Platelet-derived growth factor in fibrous musculoskeletal disorders: a study of pathologic tissue sections and in vitro primary cell cultures. J Orthop Res 1995;13:67–77.
7. Terek RM, Jiranek WA, Goldberg MJ, Wolfe HJ, Alman BA. The expression of platelet-derived growth-factor gene in Dupuytren contracture. J Bone Joint Surg 1995;77A:1–9.
8. Hung CT, Pollack SR, Reilly TM, Brighton CT. Real-time calcium response of cultured bone cells to fluid flow. Clin Orthop 1995;313:256–269.
9. Gabbiani G, Majno G. Dupuytren's contracture: fibroblast contraction? An intrastructural study. Am J Pathol 1972; 66:131–146.
10. Badalamente MA, Stern L, Hurst LC. The pathogenesis of Dupuytren's contracture: contractile mechanisms of the myofibroblasts. J Hand Surg 1983;8:235–243.
11. Tomasek JJ, Halliday NL, Updike DL, et al. Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. J Biol Chem 1997;272: 7482–7487.
12. Rayan GM, Parizi M, Tomasek JJ. Pharmacologic regulation of Dupuytren's fibroblast contraction in vitro. J Hand Surg 1996;21A:1065–1070.