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GHAZI M. RAYAN* and JAMES J. TOMASEK†

GENERATION OF CONTRACTILE FORCE BY CULTURED DUPUYTREN'S DISEASE AND NORMAL PALMAR FIBROBLASTS

Keywords: Dupuytren's disease, myofibroblast, collagen lattice, fibroblast, tissue culture

ABSTRACT. Contractile fibroblasts are believed to be responsible for palmar fascia contracture in Dupuytren's Disease. An *in vitro* collagen lattice model was used to examine the contractile properties of Dupuytren's fibroblasts from 10 patients undergoing partial fasciectomy, and palmar fascia fibroblasts from 6 patients undergoing carpel tunnel release. Dupuytren's and palmar fascia fibroblasts cultured within a stabilized collagen lattice acquired morphological characteristics similar to those of 'myofibroblasts' in Dupuytren's diseased fascia. Both types of fibroblasts generated contractile forces that resulted in rapid collagen lattice contraction after release of the lattice from points of stabilization. Generation of contractile force by the fibroblasts was inhibited by disruption of the actin cytoskeleton, lack of cells, or serum removal. Afferent neuropeptides (substance P, galanin and neurokinin A) did not promote lattice contraction. These results demonstrate that normal palmar fascia fibroblasts can modulate into Dupuytren's-like fibroblasts and that cultured fibroblasts, from either Dupuytren's diseased or normal palmar fascia, can generate contractile forces that are transmitted to extracellular matrix. In addition, fibroblast contraction is an actin based process which requires specific factor(s) present in serum. It is suggested that in Dupuytren's disease extracellular cues trigger the modulation of fibroblasts to Dupuytren's fibroblasts and the promotion of contractile forces responsible for palmar fascia contracture.

Introduction

Dupuytren's disease is characterized by shortening of the palmar fascia leading to progressive, digital flexion deformity (review: McFarlane, McGrouther, and Flint, 1991). Studies have demonstrated that the disease has three histological stages; proliferative, involutional, and scar formation (Luck, 1959). Fibroblasts during the proliferative stage of the disease appear to modulate into specialized cells termed 'myofibroblasts' which by the involution stage of the disease appear to comprise most of the nodule (Gabbiani and Majno, 1972; Chiu and McFarlane, 1978; Tomasek, *et al.*, 1986). These specialized fibroblasts acquire specific morphological characteristics including bundles of intracellular actin microfilaments, a wrinkled nucleus, extracellular fibronectin fibrils at the cell surface, and a specialized transmembrane association linking intracellular actin filaments and extracellular fibronectin fibrils (Gabbiani and Majno, 1972; Tomasek, Schultz and Haaksma, 1987; Tomasek and Haaksma, 1991). Based on the presence of these specialized fibroblasts during active shortening of the palmar fascia and their histological characteristics, it has been proposed that contractile forces generated by these cells play an important role in the shortening of the palmar fascia (Schultz and Tomasek, 1990; Schurch, Skalli and Gabbiani, 1990).

Fibroblasts obtained from Dupuytren's diseased tissue can generate and transmit

* Hand Surgery Section, Orthopedic Surgery Department, University of Oklahoma Health Sciences Center, Baptist Medical Center, Oklahoma City, OK, USA.

† Department of Anatomical Sciences, University of Oklahoma Health Sciences Center, P.O.Box 26901, Oklahoma City, OK 73190, USA.
Correspondence and reprint requests to J. J. Tomasek.

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force to a substratum as demonstrated by their ability to wrinkle a deformable rubber substratum (Hurst *et al.*, 1986). Recently, *in vitro* collagen lattice contraction models have been used to demonstrate that a variety of different fibroblasts can generate contractile force (Mochitate, Pawelek and Grinnell, 1991; Kolodney and Wysolmerski, 1992; Tomasek, *et al.*, 1992). Fibroblasts cultured within a stabilized collagen matrix will generate contractile force resulting in the development of isometric tension or stress (Mochitate, Pawelek and Grinnell, 1991; Kolodney and Wysolmerski, 1992; Tomasek *et al.*, 1992). If the stabilized collagen lattice is released from its points of attachment, thereby releasing the developed tension, the fibroblasts within the lattice will undergo isotonic contraction resulting in a rapid reduction in the diameter of the collagen lattice. We have previously used rapid reduction in the diameter of collagen lattices as a means to evaluate the amount of contractile force generated by fibroblasts (Tomasek *et al.*, 1992). In the present study an *in vitro* collagen lattice model system was utilized to compare fibroblasts isolated from Dupuytren's diseased tissue with palmar fascia fibroblasts from carpel tunnel release. We have examined: (1) the organization of the actin cytoskeleton and extracellular matrix attachments in these fibroblasts in a stabilized collagen lattice; (2) whether these fibroblasts can generate contractile force, as demonstrated by their ability to rapidly contract a collagen lattice once released from its points of attachment; (3) whether the generation of contractile force by these fibroblasts is an actin-mediated process; and (4) what factors may promote these fibroblasts to generate contractile forces. An understanding of the cellular mechanisms underlying the generation of contractile force by Dupuytren's diseased fibroblasts should provide information about their role in Dupuytren's contracture.

Materials and Methods

Cells

Dupuytren's fibroblast explant cultures from 10 patients undergoing surgery for Dupuytren's contracture and palmar fascia fibroblast explant cultures from 6 patients undergoing carpel tunnel release were used. Dupuytren's diseased tissue was placed in sterile Hank's balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, NY, USA) and the nodular tissue was dissected free from surrounding cord and normal appearing palmar fascia. Palmar fascia was also placed in HBSS and cut into pieces. Pieces of tissue were placed onto 60 mm culture dishes (Falcon, Oxnard, CA, USA), allowed to attach, and cultured in complete media containing M-199 media (GIBCO) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA), 2 mM glutamine, and 1% antibiotic-antimycotic solution. Dupuytren's and palmar fascia fibroblasts were harvested using trypsin-EDTA (GIBCO) and subcultured in 75 cm^2 tissue culture flasks (Falcon). For these experiments, 10 cell strains of Dupuytren's fibroblasts and 6 cell strains of palmar fascia fibroblasts were used. All cell strains used in these experiments were subcultured less than ten passages.

Preparation of collagen lattices

Fibroblasts were cultured within stabilized type I collagen lattices as previously described (Tomasek *et al.*, 1982; Tomasek *et al.*, 1992). The final collagen concentration was 0·65 mg/ml and the cell concentration was $1{\cdot}25 \times 10^5$ cells/ml. A 250 μl drop of the collagen/cell suspension was placed on a 35 mm plastic tissue culture dish (Falcon). The placement of a drop of collagen/cell suspension onto a dry plastic tissue culture dish ensured that the lattice would remain attached to the underlying plastic substratum, thereby stabilizing the collagen lattice. After 1 hr incubation at 37°C, to allow for gelation of the collagen, 1·5 ml of complete media was placed over the collagen lattice. Care was taken not to detach the lattices from the underlying plastic substratum. The stabilized lattices were then incubated for 5 days.

Collagen lattice contraction assay

After 5 days of incubation, the stabilized lattices were mechanically released from the underlying substratum by freeing the edge of the collagen lattice with metal forceps and releasing the rest of the area by gently pipetting media at the lattice-dish interface (Tomasek *et al.*, 1992). Culture dishes were then returned to the incubator. Rapid con-

traction was analyzed by measuring the diameter of the lattice before and at various times after release, using a Nikon SMZ-1 stereoscope. Lattice diameters were normalized due to variation in the initial diameter of the lattices which ranged from 14–16 mm. The relative lattice diameter was obtained by dividing the diameter of the collagen lattice at each time point by the initial diameter of the lattice. The percent contraction was obtained by subtracting the relative lattice diameter from 1. The percent contraction relative to that promoted by 10% fetal bovine serum was obtained by dividing the percent contraction by the percent contraction in the presence of 10% fetal bovine serum. All data are expressed as the mean ± standard deviation. For each individual cell strain the mean ± standard deviation is obtained by performing each experiment at least in duplicate with each experiment comprised of at least seven collagen lattices. Statistical analysis was done using an independent groups t-test (Crunch Software Corp., Oakland CA, USA).

Microscopy

For actin and fibronectin localization, stabilized collagen lattices after 5 days of incubation were fixed with paraformaldehyde (Tomasek *et al.*, 1992). The actin cytoskeleton was visualized using bodipy phallacidin (Molecular Probes Inc., Eugene, OR; Barak *et al.*, 1980) as previously described (Tomasek *et al.*, 1992). To visualize fibronectin fibrils, fixed collagen lattices were incubated with an anti-human plasma fibronectin mouse monoclonal antibody (mAb) (Millis *et al.*, 1985) diluted 1:500 in phosphate buffered saline (PBS) followed by a biotinylated sheep anti-mouse IgG antibody diluted 1:200 in PBS (Amersham Corp., Arlington Heights, IL, USA) and subsequently a streptavidin peroxidase diluted 1:400 in PBS (Amersham Corp.). The peroxidase was visualized as previously described (Graham and Karnovsky, 1966). As a control, the primary mAb was replaced with PBS. All 10 cell strains of Dupuytren's fibroblasts and 6 cell strains of palmar fascia fibroblasts were examined for actin microfilaments and fibronectin fibrils.

For light and electron microscopy, stabilized collagen lattices after 5 days of incubation were fixed, dehydrated and embedded in Polybed 812 (Polysciences, Warrington, PA, USA) as previously described (Tomasek, *et al.*, 1992). One micrometer thick sections were stained with 1% toluidine blue and photographed with an Olympus Vanox photomicroscope. Thin sections were stained with uranyl acetate and lead citrate and photographed on a JEOL 100C transmission electron microscope. All 10 Dupuytren's and 6 palmar fascia fibroblasts strains were examined.

Treatment of collagen lattices

Cell concentration. Three concentrations were studied; collagen with no cells, $6{\cdot}25 \times 10^4$ cells/ml and $1{\cdot}25 \times 10^5$ cells/ml. The cell concentration used for all the rest of the studies was $1{\cdot}25 \times 10^5$ cells/ml.

Cytochalasin D treatment. Stabilized collagen lattices, after 5 days in culture, were treated for 30 min with cytochalasin D prior to release. The rate of lattice contraction was determined as described above. Cytochalasin D (Sigma, St Louis, MO, USA) was kept as a 2 mM stock solution in dimethyl sulfoxide (DMSO) at −20°C and added to the culture media for a final concentration of 6 μM. Control lattices received an equivalent concentration of DMSO (0·3%).

Serum removal. Stabilized collagen lattices, after 5 days in culture, were washed twice over a 5 min period with unsupplemented M-199 previously warmed to 37°C. After the final wash, some of the lattices received 10% fetal bovine serum immediately prior to release; other lattices were released and subsequently received 10% fetal bovine serum 45 min after release. The rate of lattice contraction was determined as described above.

Addition of neuropeptides. Stabilized collagen lattices, after 5 days in culture, were washed twice over a 5 min period with unsupplemented M-199 previously warmed to 37°C. Experimental lattices received one of the following immediately prior to release: 10^{-5} M substance P, 10^{-5} M galanin, or 10^{-5} M neurokinin A (Bachem California, Torrance, CA, USA). Control lattices received 10% fetal bovine serum immediately prior to release. The rate of lattice contraction was determined as described above.

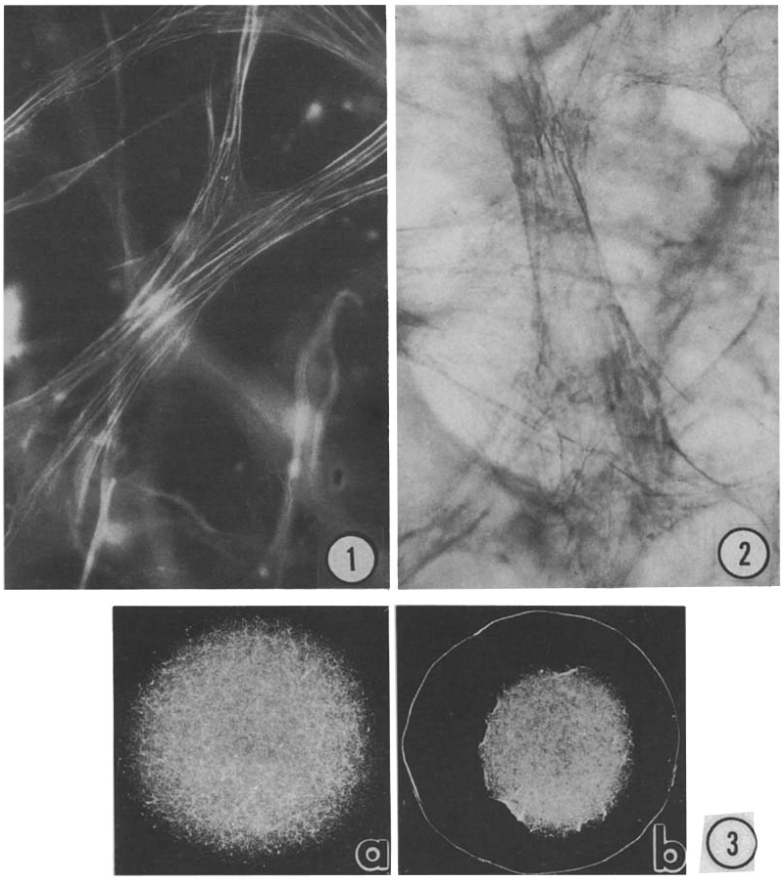

Fig. 1. Fluorescence micrograph of Dupuytren's fibroblasts cultured within an attached collagen lattice for 5 days and stained with bodipy phallacidin. Fibroblasts illustrated are present within the collagen lattice. Large bundles of actin microfilaments traverse the length of these cells. ×500.

Fig. 2. Light micrograph of Dupuytren's fibroblasts cultured in an attached collagen lattice for 5 days stained with an anti-fibronectin antibody using the immunoperoxidase technique. Stained fibronectin fibrils are present at the cell surface. ×500.

Fig. 3. Dark field photomicrographs illustrating the rapid contraction of a collagen lattice upon release from the substratum. (a) An attached collagen lattice containing Dupuytren's fibroblasts cultured for 5 days has a circular outline. (b) 10 min after release the diameter of the same collagen lattice as in (a) is dramatically reduced. The circumference of the lattice prior to release is visible as a white line due to the scrapping of the plastic substratum with metal forceps to mechanically free the attached collagen lattice. ×2·25.

In each of the above experiments, all 10 cell strains of Dupuytren's fibroblasts and all 6 cell strains of palmar fascia fibroblasts were examined. Each cell strain was examined in at least duplicate experiments with each experiment comprised of at least four collagen lattices.

Results

Actin cytoskeleton and fibronectin fibrils in Dupuytren's and palmar fascia fibroblasts

Dupuytren's fibroblasts and palmar fascia fibroblasts cultured within stabilized collagen lattices for 5 days were examined for the

acquisition of morphological characteristics which define the 'myofibroblast' in Dupuytren's diseased palmar fascia. The most dominant characteristic is the presence of large bundles of actin microfilaments. Fibroblasts, from all cell strains, contained numerous bundles of actin microfilaments, which stained brightly with the f-actin probe bodipy-phallacidin. Figure 1 illustrates a fibroblast from a Dupuytren's cell strain cultured for 5 days in an attached collagen lattice and stained with bodipy phallacidin. The presence of bundles of actin microfilaments was confirmed by electron microscopy (not illustrated). No differences in the organization of actin microfilaments in Dupuytren's fibroblasts and palmar fascia fibroblasts cultured for 5 days in a stabilized collagen lattice were observed in the cell strains examined.

Another characteristic of 'myofibroblasts' in Dupuytren's diseased palmar fascia is the presence of fibronectin fibrils at the cell surface (Tomasek *et al.*, 1986; Tomasek and Haaksma, 1991). Fibroblasts, cultured within attached collagen lattices, assembled fibronectin into fibrils at the cell surface (Fig. 2); similar to 'myofibroblasts' in Dupuytren's diseased palmar fascia. No differences in the formation of fibronectin fibrils were observed in all of the cell strains examined.

Generation of contractile force

Dupuytren's fibroblasts and palmar fascia fibroblasts were examined for their ability to generate contractile force using an *in vitro* collagen lattice contraction assay. Fibroblasts, from all cell strains, could generate contractile force as determined by their ability to rapidly contract a collagen lattice after release from its points of stabilization. Figure 3 illustrates a 5-day collagen lattice containing fibroblasts from a Dupuytren's cell strain immediately prior to release and 10 min after release. Within 10 min after release of the stabilized collagen lattice from the underlying substratum, there is a visible reduction in its diameter from 15 mm to 9 mm (Fig. 3). Collagen lattice contraction was found to be a cell-mediated process dependent on the number of fibroblasts cultured within the stabilized collagen lattice (Fig. 4). Most of the rapid contraction occurred within the first 10 min after release.

The amount of contractile force generated by Dupuytren's fibroblasts and palmar fascia fibroblasts was compared using the *in vitro* collagen lattice contraction assay. For this experiment each cell strain was examined in at least duplicate experiments with each experiment comprised of at least seven collagen lattices. Mean percent contraction of collagen lattices by Dupuytren's fibroblasts

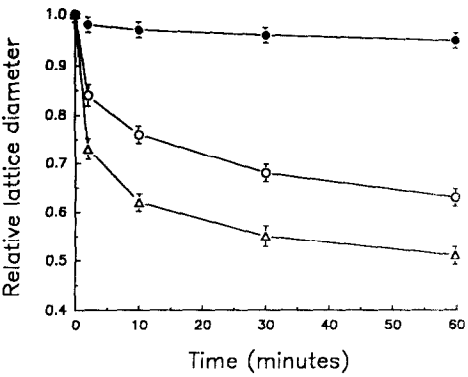

Fig. 4. Collagen lattice contraction is dependent on cell concentration. The concentration of Dupuytren's fibroblasts incorporated into the lattice at the initiation of the culture were none (●), $6{\cdot}25 \times 10^4$ (○), or $1{\cdot}25 \times 10^5$ (△) cells/ml. A large amount of the contraction occurs in the first 2 min after release. The data are averages of quintuplicate cultures of a strain of Dupuytren's fibroblasts. Standard deviations are shown.

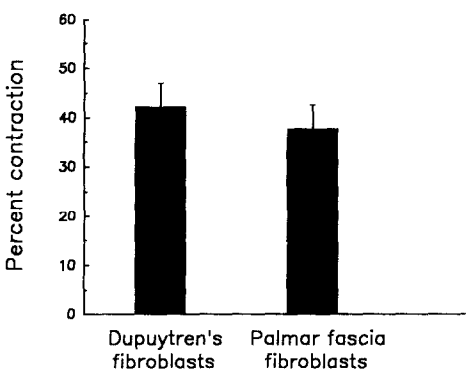

Fig. 5. Dupuytren's fibroblasts and palmar fascia fibroblasts generate equivalent amounts of contractile force. The mean percent contraction of collagen lattices 10 min after release from the substratum is illustrated. 10 strains of Dupuytren's fibroblasts and 6 strains of palmar fascia fibroblasts were used to determine mean percent contraction. There was no statistical difference in percent contraction between these two groups of cells at $p \leq 0{\cdot}05$. Standard deviations are shown.

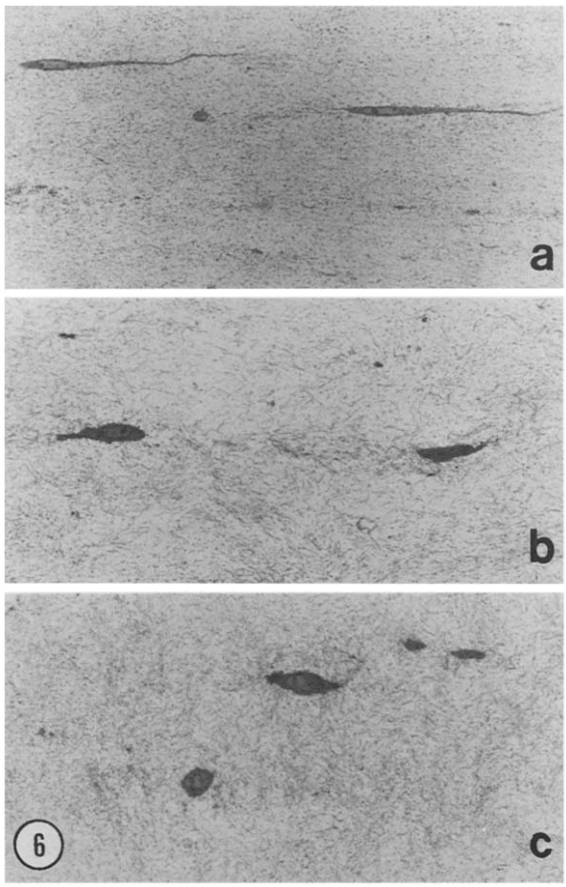

Fig. 6. Light micrographs of 1 μm thick epoxy sections cut perpendicular to the underlying substratum after 5 days in culture (a), 2 min after release from the substratum (b), or 10 min after release from the substratum (c). (a) Dupuytren's fibroblasts are elongate, bipolar cells which are aligned parallel to the underlying plastic substratum. (b) Dupuytren's fibroblasts have become rounded with numerous surface protrusions. (c) The fibroblasts appear even more contracted than in (b). ×450.

and palmar fascia fibroblasts 10 min after release were compared and analyzed using an independent groups T-test (Fig. 5). There was no statistical difference in percent contraction between these two groups of cells at a p value of less than or equal to 0·05. Other time points from 2–60 min after release were compared and showed a similar result with respect to amount of contraction.

The contraction of the collagen lattice is the result of contraction of the fibroblasts within the collagen lattice. Figure 6 illustrates Dupuytren's fibroblasts cultured within collagen lattices and examined prior to release, 2 min, and 10 min after release. Prior to release, the fibroblasts had an elongate, bipolar shape and the surrounding collagen was oriented parallel with the long axis of the cell (Fig. 6a). 2 min after release, the fibroblasts had dramatically changed their shape, becoming shortened and rounded with numerous cell protrusions (Fig. 6b). 10 min after release, the fibroblasts appeared to have contracted further (Fig. 6c). Similar morphological changes were observed in all cell strains examined.

Fibroblast contraction is an actin based process

To determine whether the generation of contractile force by Dupuytren's and palmar fascia fibroblasts is an actin based process, fibroblasts were treated with cytochalasin D. Cytochalasin D disorganizes the actin cytoskeleton and inhibits actin based processes (Schliwa, 1982). Treatment of stabilized collagen lattices with cytochalasin D, 30 min prior to release, disrupted the actin cytoskeleton (not illustrated) and inhibited collagen lattice contraction by both Dupuytren's fibroblasts and palmar fibroblasts (Fig. 8). Each cell strain was examined in at least duplicate experiments with each experiment comprised of at least four collagen lattices.

Contractile force generation is promoted by serum factor(s)

The following set of experiments were done to determine if serum factors play a role in promoting the generation of contractile force by Dupuytren's and palmar fascia fibroblasts. Fetal bovine serum, which is present in the culture media and contains a variety of hormones and growth factors, was removed for 5 min prior to release of the collagen lattice from the substratum. This resulted in inhibition of collagen lattice contraction, which could be reversed by adding 10% fetal bovine serum either immediately prior to release, or 30 min after release (Fig. 7). Similar results were observed in all 10 cell strains of Dupuytren's fibroblasts and all 6 cell strains of palmar fascia fibroblasts (Figure 8). Each cell strain was examined in at least duplicate experiments with each experiment comprised of at least four collagen lattices.

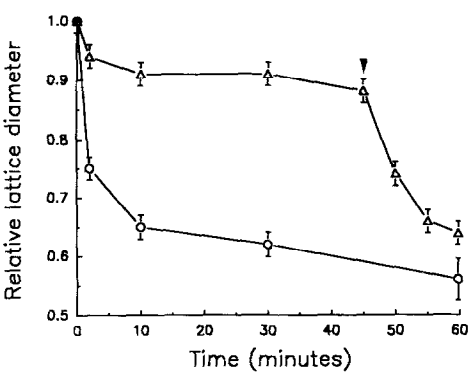

Fig. 7. Contraction of collagen lattices by Dupuytren's fibroblasts is promoted by fetal bovine serum (FBS). Dupuytren's fibroblasts were cultured within attached collagen lattices for 5 days. Collagen lattices were washed with M-199 media lacking FBS and then received either M-199 (△) or M-199 + 10% FBS (○) immediately prior to release. 45 min after release of collagen lattice containing only M-199, 10% FBS was added to the culture media (arrow). The lattices immediately contracted on addition of FBS. The data are averages of quadruplicate cultures. Standard deviations are shown.

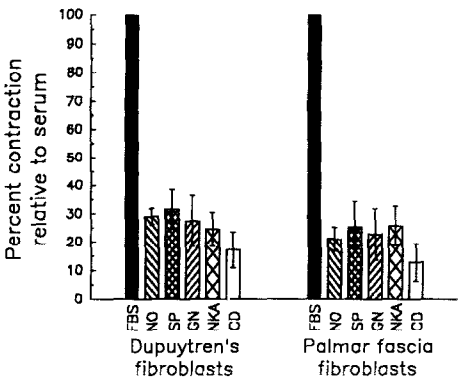

Fig. 8. Contraction of collagen lattices by Dupuytren's fibroblasts or palmar fascia fibroblasts. Collagen lattices, after 5 days in culture, were washed with M-199 media lacking fetal bovine serum and then received immediately prior to release either: 10% fetal bovine serum (FBS); no fetal bovine serum (NO); 10 μM substance P (SP); 10 μM galanin (GN); or 10 μM neurokinin A (NKA). Some of the lattices were treated with 6 μM cytochalasin D for 30 min prior to release (CD). The mean percent contraction relative to serum of collagen lattices 10 min after release from the substratum is illustrated. 10 strains of Dupuytren's fibroblasts and 6 strains of palmar fascia fibroblasts were used to determine mean percent contraction. Standard deviations are shown.

Contractile force generation is not promoted by neuropeptides

The afferent neuropeptides substance P, galanin, and neurokinin A, which have efferent functions on smooth muscle cells (Shew, Papka and McNeill, 1991, 1992), were added in place of fetal bovine serum to collagen lattices just prior to release. None of these neuropeptides promoted collagen lattice contraction of Dupuytren's or palmar fascia fibroblast strains in the absence of fetal bovine serum (Fig. 8). Each cell strain was examined in at least duplicate experiments with each experiment comprised of at least four collagen lattices.

Discussion

This study has demonstrated that fibroblasts from both Dupuytren's diseased fascia and normal appearing palmar fascia, when cultured within a stabilized collagen lattice, will acquire similar morphologies and resemble the 'myofibroblast' observed in Dupuytren's diseased palmar fascia. Using an *in vitro* collagen lattice contraction assay it was demonstrated that these fibroblasts can generate contractile force and that the amount of forces generated are similar in both cell types. In addition, the generation of contractile force was determined to be an actin-based process requiring an organized actin cytoskeleton. The generation of contractile force was dependent upon the continuous presence of a specific factor or factors present in fetal bovine serum and contractile force could not be promoted by afferent neuropeptides previously demonstrated to generate contractile force in smooth muscle cells.

Palmar fascia fibroblasts cultured in stabilized collagen lattices acquire bundles of actin microfilaments, fibronexus associations, and the ability to generate large amounts of contractile force; similar to Dupuytren's fibroblasts. Previous studies have demonstrated that fibroblasts, from a wide variety of sources, when cultured on a planar substratum acquire bundles of actin microfilaments characteristic of 'myofibroblasts' (Byers, White and Fujiwara, 1983). These results are consistent with previous studies on fibroblasts demonstrating that strong adhesion to a stabile substratum will promote the acquisition of bundles of actin microfilaments and the development of isometric tension (Burridge, 1981; Ingber, 1991). Although Dupuytren's and palmar fascia fibroblasts are phenotypically different *in vivo*, they respond similarly when cultured within stabilized collagen lattices under isometric tension. These results suggest that aspects of the environment in which fibroblasts reside, such as the stability and adhesivity of the extracellular matrix and/or the presence of certain growth factors, can regulate their behavior.

The ability of fibroblasts to rapidly contract a collagen lattice is indicative of the generation of contractile force. The generation of contractile force was abolished by the addition of cytochalasin D, demonstrating the critical importance of actin filaments in this process. Large bundles of actin microfilaments are present in fibroblasts generating contractile forces within the collagen lattices. Similar bundles of actin microfilaments have been observed in 'myofibroblasts' present in Dupuytren's diseased palmar fascia (Gabbiani and Majno, 1972; Chiu and McFarlane, 1978; Tomasek *et al.*, 1986; Tomasek and Haaksma, 1991). Previous studies have demonstrated that isolated actin microfilament bundles from fibroblasts, termed stress fibers, are highly contractile (Isenberg *et al.*, 1976; Kreis and Birchmeier, 1980). Whether these bundles of actin microfilaments are responsible for the generation of contractile force in fibroblasts in collagen lattices and in Dupuytren's disease palmar fascia remains to be determined.

The generation of contractile force by fibroblasts is dramatically reduced on removal of fetal bovine serum. This is not an irreversible process since generation of contractile force can be immediately restored upon re-addition of fetal bovine serum to the culture media. These results demonstrate that the continuous presence of extracellular factor(s) in fetal bovine serum are necessary for active fibroblast contraction. In this study, we examined a variety of afferent neuropeptides known to have an efferent role on smooth muscle (Shew, Papka, and McNeill, 1991, 1992). These neuropeptides have no effect on the generation of contractile force by either Dupuytren's or palmar fascia fibroblasts when added at concentrations known to elicit contraction of uterine smooth muscle. The extracellular factor(s) present in fetal bovine serum which promote contraction are not known. Recently, we have demonstrated that thrombin can promote fibroblast contraction by proteolytic activation of its cell surface receptor (Pilcher *et al.*, 1994). However, thrombin does not appear to be the primary agent in fetal bovine serum responsible for promoting contraction. It is expected that other extracellular factors will be identified that can promote or inhibit fibroblast contraction.

The rapid contraction observed in the *in vitro* collagen lattice contraction model is the result of its design. The stabilization of the collagen fibrils results in development of isometric tension (Delvoye *et al.*, 1991; Kolodney and Wysolmerski, 1992), which upon

release of the lattice from its points of attachment results in its rapid isotonic contraction. The granulation tissue of healing wounds, if attached to a force transducer, can generate measurable amounts of isometric force (Higton and James, 1964). Similar to the release of an attached collagen lattice, granulation tissue of a wound splinted open for 10 days will dramatically reduce its size within 30 min after release from the splint (Abercrombie, James and Newcombe, 1960). Although rapid contraction does not normally occur during the shortening of the palmar fascia in Dupuytren's contracture or of granulation tissue during wound healing, the rapid contraction observed on release of an attached collagen lattice most likely represents an exaggeration of certain aspects of the normal tissue contraction.

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