SCIENTIFIC ARTICLE

Response of Dupuytren Fibroblasts to Different Oxygen Environments

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Purpose It is thought that local ischemia and oxygen radicals are responsible for fibroblast-to-myofibroblast cell transformation and proliferation. We hypothesized that hypoxia could differentially activate the contractility of fibroblasts from normal human palmar fascia and from fibroblasts-myofibroblasts of Dupuytren cords.

Methods Normal palmar fascia from 5 patients with carpal tunnel syndrome and Dupuytren cords from 5 patients were harvested. Cells were cultured from all tissue samples, and collagen lattices were prepared containing these cells. Oxygen treatment subgroups were created and incubated under hypoxic (1% O_2, 5% CO_2, and 94% N_2), normoxic (21% O_2, 5% CO_2, and 74% N_2), and hyperoxic (100% oxygen using 2.4 atm pressure twice a day for 7 d) conditions. After 7 days, each subgroup was photographed, and lattices were released from dishes. Postrelease photographs were taken immediately, 5 minutes after release, and after 1 hour. Areas of the lattices at each time point were calculated using MetaMorph software. Actin staining and live/dead cell analysis was performed. Linear repeated measures analysis of variance was used for data analysis given that contraction levels were measured over 3 distinct time points.

Results We found a statistically significant difference between normal samples and Dupuytren samples in mean contraction levels over time. There was no statistically significant difference between tissue groups over the 3 time periods based on the oxygen treatment received.

Conclusions Our results showed a greater degree of contractility in Dupuytren disease cells than normal fibroblasts. However, the contraction in either group was not affected by oxygen level. Future *in vivo* research is needed to better understand the nature of pathophysiology of Dupuytren disease. *(J Hand Surg 2013;■A:■−■. Copyright © 2013 by the American Society for Surgery of the Hand. All rights reserved.)*

Key words Dupuytren disease, fibroblast, hyperbaric oxygen treatment, hypoxia, myofibroblast.

DUPUYTREN DISEASE INVOLVES the palmar aponeurosis and causes progressive irreversible contracture of 1 or more digits.[1] Major factors increasing risk of disease onset are genetic predisposition, trauma, and age. Ischemic effects of smoking, diabetes, alcohol and/or barbiturate use, and epilepsy have been correlated with disease progression.[2,3]

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Despite a large body of clinical research on the contracture, the molecular pathology of Dupuytren disease is poorly understood.[4] Fibroblast cell proliferation as well as fibroblast to myofibroblast cell transformation are responsible for Dupuytren nodule and cord formation, leading to digit contracture.[5] Murrell et al[6] hypothesized that local ischemia and oxygen radicals are responsible for the pathophysiology of fibroblast to myofibroblast cell transformation and proliferation.

Both hypoxia and hyperoxia may cause formation of oxygen radicals such as hydrogen peroxide (H_2O_2).[7–9] Whereas H_2O_2 causes fatal injury to human fibroblast cell lines, low-dose H_2O_2 can in fact trigger proliferation of human fibroblasts.[10,11] We hypothesized that hypoxia could differentially activate the contractility of fibroblast cells from normal human palmar fascia and from fibroblast-myofibroblast cells of Dupuytren cords.

MATERIALS AND METHODS

Our institutional review board approved the study. Tissue samples from 10 patients were studied. The normal group tissue came from normal healthy palmar fascia tissue obtained from 5 male patients aged 57 ± 7 years, who underwent carpal tunnel surgery. Dupuytren group tissue came from Dupuytren cord samples obtained from 5 male patients aged 53 ± 8 years, who underwent Dupuytren disease fasciectomy.

Preparation of cell cultures

Dupuytren disease tissue and normal fascia samples were placed in sterile Hank's balanced salt solution. Each individual sample tissue was cut into small pieces and placed in a 60-mm culture dish. Careful attention was paid to maintaining maximum sterility and adherence of the cells to culture dishes. Tissues were cultured in 10-mL complete media containing M-199 media supplemented with 10% fetal bovine serum, 2 nmol/L glutamine, and 1% antibiotic-antimycotic solution and buffer. Cultures were incubated at 37°C in 21% O_2, 5% CO_2, and 74% N_2 for 10 days.

After removing sample tissue pieces from dishes, we harvested the first cell lines approximately 10 days later using 5 mL 0.25% trypsin–ethylenediaminetetra acetic acid solution for 10 minutes at 37°C. After application of trypsin–ethylenediaminetetra acetic solution, we micropipetted cells into 10% Dulbecco's Modified Eagle Medium F12 solution and centrifuged them for 10 minutes at 300*g*. The fibroblasts were then subcultured 3 to 6 times and placed in collagen lattices.

Preparation of collagen lattices

Fibroblasts were cultured within stabilized type I collagen lattices, as described by Tomasek et al.[12] The final collagen concentration was 0.65 mg/mL and cell concentration was 1.25×10^5 cell/mL. A 250-μL drop of collagen-cell suspension was placed on a 35-mm culture dish. After 1 hour of incubation at 37°C to allow gelatinization of the collagen, 2.5 mL of complete media was placed over the collagen lattice. Careful attention was paid to prevent lattice detachment from the underlying substratum. We prepared 15 dishes for each patient sample.

The stabilized lattices were incubated 7 days after dish preparation. Three incubation subgroups of 5 dishes each were created among the 15 culture dishes prepared from each subject: the hypoxic, normoxic, and hyperoxic subgroups. The hypoxic subgroup was incubated in a low oxygen environment consisting of 1% O_2, 5% CO_2, and 94% N_2. The normoxic subgroup was incubated in normal 21% O_2, 5% CO_2, and 74% N_2. The hyperoxic subgroup was incubated in normal 21% O_2, 5% CO_2, and 74% N_2 and received daily hyperbaric oxygen treatments. The hyperbaric treatment was administered under 100% oxygen for 90 minutes twice daily at 2.4 atm pressure for 7 days using an experimental hyperbaric chamber.

Collagen lattice contraction assay

After 7 days of incubation, 3 35-mm dishes from each oxygen treatment subgroup were photographed. The camera (Nikon D40, Nikon, Tokyo, Japan) was placed 50 cm above a light table. Medium was removed from the dishes, and the dishes were placed on the light table. A ruler (for scale) was placed next to each culture dish and prerelease photographs were taken. After that, stabilized lattices were mechanically released from underlying substratum by freeing the edge of the collagen lattices with metal forceps and releasing the rest of the area by gently pipetting media at the lattice–dish interface using ×2.5 surgical loupes. Postrelease photographs were taken immediately (time 0) and 5 minutes after release (time 5). Dishes were returned to the incubator for 1 hour, when we again took photographs (time 60).

All photographs were downloaded to a computer using the MetaMorph (Microscopy Automation & Image Analysis) software program, Version 2.01 (Molecular Devices, LLC, Sunnyvale, CA); after calibration for each image individually, circumferential lengths and surface areas of lattices were calculated electronically.

Live/dead cell analysis

One 35-mm dish from each subgroup was analyzed using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies, Grand Island, NY). Healthy cells fluoresce green and dead cells fluoresce red. Live/dead viability/cytotoxicity was performed on the same sample in 5 different areas under ×40 magnification using a fluorescence inverted microscope.

Actin staining

One 35-mm dish from each subgroup underwent actin staining. Actin stain was applied to the cell collagen cultures, and the lattices were refrigerated at 4°C for 24 hours. Subsequently, actin properties of the lattices were determined using fluorescence.

Data analysis

We measured the surface areas of 3 samples from each subgroup in square millimeters at the following times: precontraction and postrelease times 0, 5, and 60. We computed summarized mean for each time point for the 3 samples (A, B, and C) and summary descriptive statistics of means, counts, and standard deviations, along with the coefficient of variation.

We analyzed data with linear repeated measures analysis of variance because contraction levels were measured repeatedly over 3 distinct time points. Our null hypothesis was that there would be no difference in mean contraction levels over time comparing oxygen treatment types and Dupuytren versus normal tissue. This hypothesis was tested with the omnibus analysis of variance F statistic. We used Mauchly W statistics to test the sphericity assumption that the variance of differences in contraction levels will be equal over time. However, because our sample size was small, we relied on the corrected F statistics from Greenhouse-Geisser, Huynh-Feldt, and Lower-bound to avoid excessive type II errors.[13,14]

RESULTS

Three lattices per oxygen treatment subgroup were released from culture dishes and photographed for the contraction assay. Table 1 lists descriptive statistics using geometric means for the 90 total samples (9 lattices per patient from 10 patient samples). Overall, we found a statistically significant difference in mean contraction levels over the time intervals (Table 1); normal samples decreased in size by 35%, and Dupuytren samples decreased by 41% (Greenhouse-Geisser: $F = 15.081$; $P < .001$). Our interaction term, to compare change in contraction over time between groups and treatment subgroups (time × group × treatments), was not statistically significant (Greenhouse-Geisser: $F = 0.027$; $P = .979$). In addition, the comparison of contraction between treatment subgroups within groups (time × treatment) was not statistically significant (Greenhouse-Geisser: Dupuytren $P = .981$, normal $P = .979$). Thus, there was no statistically significant difference between groups over the 3 time periods based on disease state and treatment received. The observed contractions for both normal and Dupuytren groups were not substantially modified by the type of oxygen treatments.

Live/dead analysis showed that over 90% cells were alive at the end of the study in all subgroups. Actin staining showed some elements of staining within all subgroups. We hypothesized that Dupuytren fibroblasts, which contracted more, would show increased actin staining; however, results from both Dupuytren groups and normal groups were not conclusive.

DISCUSSION

Murrell et al[6] proposed that local ischemia and oxygen radicals are responsible for the pathophysiology of Dupuytren disease. They suggested that local ischemia in palmar fascia leads to adenosine triphosphate breakdown and increased conversion of hypoxanthine to xanthine and xanthine to uric acid in endothelial cytoplasm. Xanthine dehydrogenase, responsible for the conversion, is converted to xanthine oxidase, which releases free oxygen radicals that cause microvessel dysfunction and, perhaps more relevantly, conversion of fibroblasts into myofibroblasts, which create the changes characteristic of Dupuytren disease.[6,11,15] Drawing from this theory, we hypothesized that hypoxia would differentially activate the contractility of fibroblasts, particularly those from patients with Dupuytren disease. The data in our study did not support our hypothesis that oxygen conditions influenced fibroblast contractility.

The electron microscopy study of Gabbiani and Majno[5] suggested that only the cells of the Dupuytren nodules are responsible for contraction in the disease. They were not able to show pathologic conditions in collagen fibers. Their work inspired the design of our study, and we therefore used only the fibroblasts from patient samples in combination with type I collagen. According to Murrell et al,[16] the ratio of type III:type I collagen changes in Dupuytren disease, showing a greater proportion of type III collagen owing to diminished type I production. Because type I collagen was not implicated as a contributor to Dupuytren

TABLE 1. Data and Statistical Analysis of Dupuytren and Normal Samples

Group	Time 0 (Mean [SD] Area)	Time 5 (Mean [SD] Area)	Time 60 (Mean [SD] Area)	% Contraction	*P* Value
Dupuytren	391 (126)	249 (74)	229 (69)	41	< .001
Hypoxic	368 (155)	233 (77)	214 (78)	42	Treatment subgroup contrast (time × treatment) = .981
Normoxic	376 (135)	243 (101)	217 (84)	42	
Hyperbaric	428 (102)	272 (44)	257 (47)	40	
Normal	453 (98)	322 (58)	295 (66)	35	< .001
Hypoxic	465 (118)	327 (88)	302 (88)	35	Treatment subgroup contrast (time × treatment) = .979
Normoxic	456 (84)	326 (47)	289 (47)	37	
Hyperbaric	437 (110)	314 (68)	295 (72)	33	

There was a statistically significant change over the 3 time periods within each group in the mean area of contraction. There was a statistically significant difference in mean contraction comparing groups: $F = 15.081$, $P < .001$. Overall contrast of mean contraction between groups and subgroup treatments (time × group × treatment) was insignificant; $F = 0.80$, $P = .976$.

contracture, we believe that using type I collagen would be the appropriate approach to mimic palmar fascia in the laboratory environment. It has also been suggested that stromal cells, via α5β1 integrins acting on fibronectin in the surrounding extracellular matrix, have greater contractile power, which implies that fibronectin and not collagen is a key component and that this cellular receptor may be involved in Dupuytren disease–related contraction.[17] Because our interest was in cell dynamics and not matrix alterations, we used type I collagen. With this in mind, our findings suggest that the fibroblasts in Dupuytren disease lesions are not fundamentally or irreversibly altered, at least with respect to oxygen level sensitivities.

There are several possible explanations for the lack of an observed response to oxygen treatment. It is possible that the absence of a significant oxygen effect would have resulted from a lack of cell survival and not cell dysfunction. That being said, live/dead analysis revealed over 90% living cells in every gel lattice tested. Another possibility is that the 3-dimensional *in vitro* conditions of our study did not sufficiently replicate the complex *in vivo* environment. Thus, absence of the underlying cell–matrix interactions specific to Dupuytren disease might be responsible for lack of contraction in this study. It may be also speculated that if the fibroblast cells were exposed to hypoxia for a longer period of time, they might have exhibited more contraction. This speculation could be supported by the study of Madden et al,[18] in which interosseous muscle biopsies were taken from 3 patients who experienced hand ischemia and subsequent intrinsic contracture. They showed a large number of modified fibroblast-myofibroblast cells, which resembled the cells in Dupuytren disease, and concluded that ischemia lasting 8 to 16 weeks resulted in intrinsic muscle contracture. In the *in vitro* environment of our study, we increased the time of hypoxia exposure by 40% beyond the original 5-day model, yet we saw no difference in contraction. It is unclear whether a further increase of hypoxia exposure *in vitro* would be possible or exhibit any changes. Of course, in the diseased hand, hypoxia leads to other phenomena such as inflammation, which could secondarily influence cell behavior, perhaps via cytokine activity, leading to dysfunctional contractions.

It is possible that hypoxia does not have a role in the Dupuytren disease process. Our results showed that Dupuytren-diseased cells provided more contractility than normal palmar fascia; however, this contractility was not affected by oxygen level variations. Furthermore, Schrader et al[19] showed that, contrary to previously described hypovascularization of the palm, even in Dupuytren patients, the palm does have adequate blood supply, which suggests that Dupuytren contracture may not be related to hypoxia at all. Future *in vivo* research is needed to better articulate the nature of pathophysiology of Dupuytren disease. We encourage future investigation to address the current uncertainty in the underlying Dupuytren disease pathophysiology.

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