Tamoxifen Decreases Fibroblast Function and Downregulates TGFβ2 in Dupuytren's Affected Palmar Fascia

M. Ann Kuhn, M.D., X. Wang, Ph.D., Wyatt G. Payne, M.D., Francis Ko, B.S., and Martin C. Robson, M.D.

Institute of Tissue Regeneration, Repair, and Rehabilitation, Department of Veterans Affairs Medical Center, Bay Pines, Florida 33744; and Department of Surgery, University of South Florida, Tampa, Florida 33620

Submitted for publication June 19, 2001; published online February 13, 2002

INTRODUCTION

Dupuytren's contracture is a fibroproliferative disorder characterized by progressive, irreversible flexion of one or more digits that was initially described in 1832 [1-3]. While its etiology remains unclear, Dupuytren's contracture is known to result from changes produced in the dermis and palmar fascia [2]. These changes include proliferation of myofibroblasts, which were first defined as the dominant cell type associated with the formation of the pathognomonic nodule of the palmar fascia [4-6]. Dupuytren's contracture has also been associated with variable deposition of mature collagen fibers and enhanced synthesis of the extracellular matrix [2, 7].

Growth factors such as basic fibroblast growth factor and isoforms of transforming growth factor beta (TGFβ) are potent modulators of fibroblast and myofibroblast proliferation and differentiation. These cytokines have been shown to affect collagen synthesis as well and have been implicated in the pathobiology of proliferative scarring such as keloid formation and Dupuytren's disease [7-14].

Recent work produced in this laboratory suggests that TGFβ (especially TGFβ1) may be the key cytokine involved in the progressive fibrosis of Dupuytren's disease [15]. It was suggested that compounds that abrogate, neutralize, or downregulate the isoforms TGFβ1...
and/or TGF\(\beta\) may be useful in the treatment of the condition.

Tamoxifen, used mainly in the treatment of breast cancer, is a synthetic nonsteroidal antiestrogen that may also be effective in the treatment of abnormal proliferative healing disorders such as retroperitoneal fibrosis and desmoid tumors [16–19]. One of the demonstrated effects of tamoxifen is modulation of the production of growth factors such as TGF\(\alpha\), TGF\(\beta\), epidermal growth factor, and insulin-like growth factor (IGF) [16, 20–23]. In addition, tamoxifen has been demonstrated to decrease proliferation of both normal dermal and keloid fibroblasts as well as decrease the synthesis of collagen by keloid fibroblasts [16, 24].

Previous studies performed with human fibroblasts have demonstrated a decrease in fibroblast function in fibroblast-populated collagen lattices (FPCL) with tamoxifen treatment. Since Dupuytren's disease is a fibroproliferative process with increased TGF\(\beta\) production, we examined the role of tamoxifen at decreasing fibroblast function and downregulating TGF\(\beta\).

METHODS

Preparation of the fibroblast cultures. Primary cultures of fibroblasts were obtained from Dupuytren's affected fascia and carpal tunnel affected fascia [2, 8, 25]. Samples of palmar fascia were obtained at surgical release from six patients who had fasciectomy for treatment of Dupuytren's disease. Control palmar fascia was obtained from six patients who had hand surgery for carpal tunnel release. All patients were undergoing elective excision independent of this study. Primary cultures of fibroblasts from the surgical specimens were then established. Cells from passages 3 to 5 were used for experiments. The specimens were rinsed in 10 ml of calcium- and magnesium-free Dulbecco's phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO) supplemented with gentamicin (20 mg/ml) for 10 min at room temperature. A second antibiotic rinse using 10,000 u/ml penicillin G, 25 \(\mu\)g/ml amphotericin B, and 10,000 \(\mu\)g/ml streptomycin solution (Solute BRL, Grand Island, NY) was performed for 10 min. Each specimen was then cut into four pieces of equal dimensions and placed on the surface of a sterile 100-mm culture dish. The specimens were incubated with no additional culture medium for 15 min at 37°C. A 10-ml aliquot of Dulbecco's modified Eagle's medium (Gibco BRL) was carefully and slowly added to the culture dish which was then incubated at 37°C in 5% CO\(_2\). The cells were subcultured until 80% confluence was obtained by removing the medium and tissue fragments from the culture dish with calcium- and magnesium-free Dulbecco's phosphate-buffered saline solution (Gibco). Trypsin–ethylene diaminetetraacetic acid (0.25%) (Gibco) was added and the cultures were incubated at 37°C for 15 min. A 15-ml aliquot of soybean trypsin inhibitor (Sigma) was added. The cultures were centrifuged at 1000 \(g\) for 10 min. The supernatant was decanted and the cell pellets were resuspended in 5 ml Dulbecco's modified Eagle's medium. This rinse/wash and 10-min centrifuge was repeated three times. The cells were counted with a hemacytometer and trypan blue was used to determine cell viability. The cell density was adjusted to 5 \(\times\) 10\(^5\) cells/ml with Dulbecco's modified Eagle's medium.

Preparation of collagen lattices. The collagen lattices were prepared from type I rat tail collagen (acetic acid extracted) as recommended by the manufacturer (Upstate Biotechnology, Lake Placid, NY). Undiluted collagen (3.85 mg/ml) was placed in 35-mm culture dishes (Falcon 1008) and 1 ml was evenly spread. The dishes were placed in an ammonia vapor chamber for 3 min to solidify. Sterile distilled water (5 ml) was added to the culture dishes, allowed to stand for 1 h, and then aspirated. This was repeated four times to remove excess ammonia and the collagen gel lattices were then incubated for 24 h at 4°C. Phosphate-buffered saline with 1.0% serum was added to replace the final aspirate. An 18-gauge needle was used to detach the collagen gel lattices from the surface of the culture dishes so that they were loose and suspended in the saline. A total of 72 collagen lattices were prepared to allow triplicate measurement from each specimen based on two treatment groups. To form the fibroblast-populated collagen lattices all saline was aspirated from the 35-mm culture dishes containing the collagen gel lattices. Two milliliters of 5 \(\times\) 10\(^4\) cells/ml was placed on the surface of each of the prefabricated collagen gel lattices. Tamoxifen (Sigma) was dissolved in PBS at a concentration of 8 \(\mu\)g/ml and was added on day 0 to 18 of the FPCLs containing Dupuytren's fibroblasts and 18 of the FPCLs containing carpal tunnel fibroblasts. The 8 \(\mu\)g/ml concentration was chosen because this dose has been shown not to be toxic to fibroblasts and to allow continued proliferation of fibroblasts [26]. This dose also allows fibroblast morphology to remain normal in collagen lattices [26].

Assay for gel contraction. The FPCLs were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide. The amount of gel contraction was measured every 24 h for 5 days. Acetate overlays were used to trace the area of the gels. Gels were performed in triplicate for each cell line established and measurements were then calculated using digital planimetry and Sigma Scan software (Jandel Scientific, Corte Madera, CA). Each collagen gel area measurement was converted to reflect the percentage of area remaining over time and subsequently the percentage of gel contraction. A one-way analysis of variance was used to determine significant differences among groups. When a difference was identified, Tukey's test (all pairwise multiple-comparison test) was used to delineate the differences. Sigma Stat statistical software (Jandel Scientific) was used for data analysis.

Immunoassay for TGF\(\beta\). The supernatant obtained from the culture medium following completion of the FPCL portion of the experiment was retained and used for a Quantikine human TGF\(\beta\) immunoassay (R&D Systems, Minneapolis, MN). The supernatant was initially stored at -85°C after being mixed with 1 ml DMEM, 1 ml 1% fetal bovine serum, and 1 ml 1% penicillin/streptomycin. The samples were activated with 1 N HCl and incubated 10 min. Then 1.2 N NaOH/0.5 M Hepes and 0.8 ml calibrator diluent RD51 were added. After 2 h, the samples were assayed. During this time TGF\(\beta\) standards were prepared. The assay procedure was as follows: 100 \(\mu\)l RD1-17 was added to each well then 100 \(\mu\)l standard or sample was added to each well. These were incubated for 2 h and then aspirated and washed three times. Next, 200 \(\mu\)l of conjugate was added to each well, incubated for 2 h, aspirated, and washed three times. A total of 200 \(\mu\)l of substrate solution was added to each well, the wells were incubated for 20 min, and then 50 \(\mu\)l of stop solution was added to each well. The wells were run upon completion of the immunoassay using a Microplate Manager 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA) at a wavelength of 450 nm with correction of 540 nm. Once the standard curve was complete the unknown concentrations were determined. The program calculated mean absorbance (OD), standard deviation, and concentration. Each unknown concentration was then multiplied by a factor of 7.8 to correct for the dilution factor. A one-way analysis of variance was used to determine significant differences among groups. When a difference was identified Tukey's test (all pairwise multiple-comparison test) was used to delineate the differences. Sigma Stat statistical software (Jandel Scientific) was used for data analysis.
RESULTS

FPCL Contraction

When comparing the percentage of contraction over the 5-day study period, FPCLs populated with fibroblasts obtained from fascia affected by Dupuytren’s disease contracted significantly more at days 1, 2, 3, 4, and 5 compared to the FPCLs populated with fibroblasts from the carpal tunnel control \((n = 18, P < 0.05, \text{ANOVA})\) (Fig. 1). FPCLs populated with fibroblasts obtained from Dupuytren’s disease affected fascia that were treated with tamoxifen had significantly decreased contraction rates on days 1, 2, 3, 4, and 5 compared to untreated FPCLs \((n = 18, P < 0.05, \text{ANOVA})\) (Fig. 2). Fibroblasts obtained from patients with carpal tunnel (normal palmar fibroblasts) that were treated with tamoxifen did not affect FPCL contraction on days 3, 4, and 5 when compared to untreated fibroblasts \((n = 18, P < 0.05, \text{ANOVA})\) (Fig. 3). However, the FPCL contraction was to a lesser degree than that of the FPCL containing fibroblasts obtained from Dupuytren’s affected fascia.

TGF\(_{\beta2}\) Immunoassay

There was a significant increase in TGF\(_{\beta2}\) expression in the supernatant obtained from FPCLs populated with fibroblasts obtained from Dupuytren’s affected fascia compared to the supernatant obtained from FPCLs populated with fibroblasts obtained from carpal tunnel affected fascia \((n = 18, P < 0.05, \text{ANOVA})\) (Fig. 4). Tamoxifen treatment of fibroblasts obtained from Dupuytren’s affected fascia resulted in a significant downregulation of TGF\(_{\beta2}\) expression compared to untreated fibroblasts \((n = 18, P < 0.05, \text{ANOVA})\) (Fig. 5). Supernatant from FPCLs populated with fibroblasts obtained from carpal tunnel fascia (normal) that were treated with tamoxifen did not show any significant downregulation of TGF\(_{\beta2}\) compared to untreated fibroblasts \((n = 18)\) (Fig. 6).

DISCUSSION

Dupuytren’s disease is a proliferative fibrotic disorder and while its etiology remains unknown, we theorize that it is pathobiologically related to other progressive fibrosing human physiologic disorders such as keloid and proliferative scar formation. All recent evidence suggests that TGF\(_{\beta1}\) and TGF\(_{\beta2}\) are the key cytokines in scarring and fibrotic conditions [8, 9, 27–34]. It has been demonstrated that TGF\(_{\beta2}\) plays an important role in the fibrogenic nature of Dupuytren’s disease [2, 12]. Fibroblasts are abundant within the affected palmar fascia and TGF\(_{\beta2}\) has been demonstrated to be a stimulus for both collagen and noncollagen production by these cells [7, 14, 35].

Tamoxifen, a synthetic nonsteroidal anti-estrogen, has been demonstrated to have multiple effects includ-
FIG. 2. Comparison of fibroblasts derived from Dupuytren's affected fascia treated with tamoxifen. Fibroblasts treated with tamoxifen had significantly less contraction of a collagen lattice compared to untreated fibroblasts.

ing altered RNA transcription, decreased cellular proliferation, delay or arrest of the cells in the G1 phase of the cell cycle, and interference with multiple growth factors such as TGFβ and IGF [24, 26]. It is the interference with TGFβ that has created interest in the area of manipulating proliferative scarring and the treatment of fibroproliferative disorders. TGFβ is known to contribute to the excessive production of collagen, which in turn leads to fibrosis that is characteristic of fibroproliferative disorders. Inhibition of TGFβ can decrease collagen production [24]. In vitro studies have demonstrated that tamoxifen inhibits the proliferation of keloid fibroblasts and their rate of collagen synthesis and decreases their ability to contract a collagen lattice [24, 26]. It has also been reported that benign tumors such as desmoids and retroperitoneal fibrosis have been treated successfully with tamoxifen [17, 26]. Mancell et al. have demonstrated a decreased production of TGFα in keloid fibroblasts that were treated with tamoxifen [36].

We have been able to demonstrate decreased function of fibroblasts derived from Dupuytren's affected fascia in FPCL. We have also shown a downregulation of TGFβ in these same fibroblasts. In carpal tunnel cases (normal), the fascia shows less active fibroblasts in FPCL when treated with tamoxifen but not significantly lower levels of TGFβ. Although FPCL contraction in the carpal tunnel derived fibroblasts is slightly decreased by tamoxifen, it does not appear to be a result of TGFβ downregulation. It is possible that there may be a small direct effect on the fibroblasts such as altered RNA transcription, decreased cellular proliferation, and/or delay or arrest of the cells in the G1 phase of the cell cycle. However, the 8 μmol/ml concentration of tamoxifen used has been shown not to significantly inhibit fibroblast proliferation in collagen lattices [26]. Because the normal fascia control did not respond to tamoxifen with downregulation of TGFβ, it can then be inferred that the fibrotic nature of Dupuytren's disease is related to activated fibroblasts and secretion of fibroblastic cytokines like TGFα, TGFβ, and TGFβ.

If stimulation of fibrosis by TGFβ plays a role in the pathogenesis of Dupuytren's disease, recent data support other forms of treatment as well as tamoxifen. Neutralizing antibodies to TGFα and TGFβ have been demonstrated to reduce dermal scarring in rat dermal wounds [37, 38]. TGFβ antibody has been demonstrated to decrease contraction of keloid or burn hypertrophic scar fibroblast-populated collagen lattices [39]. These observations have been extended to in vivo demonstrations that explanted human proliferative scar collagen production can be downregulated by TGFβ antibody [40]. There are other ways to attack overproduction of TGFα and TGFβ. McCallion and Ferguson have used exogenous mannose 6-phosphate to reduce scarring in rodent, porcine, and human wounds [41]. Mannose 6-phosphate prevents activation of TGFα by
FIG. 3. Carpal tunnel fibroblasts (normal) treated with tamoxifen had significantly less ability to contract a collagen lattice than untreated fibroblasts.

Competitive inhibition of binding of the critical latency-associated peptide [27]. Decorin is a leucine-rich proteoglycan that is important in collagen fiber formation [42]. Fibroblasts from burn hypertrophic scars produce less decorin than normal dermal fibroblasts [43]. Decorin binds and neutralizes all three TGFβ isoforms and the protein has also been used in the central nervous system to decrease fibrotic scarring known to be secondary to TGFβ [44, 45]. Finally, Tredget et al. have been able to decrease the volume of hypertrophic scars with the use of systemic interferon α-2b [46]. The action appears to be secondary to the ability of interferon α-2b to normalize elevated levels of TGFβ1 and TGFβ3.

Another area of interest that has not been extensively explored is the topical application of tamoxifen. It is known that oral doses of tamoxifen similar to those used in breast cancer have been used successfully for the treatment of desmoid tumors [47, 48]. There has been a comparison of the distribution of tamoxifen metabolites in the plasma and in normal

FIG. 4. TGFβ detection ELISA of fibroblasts derived from Dupuytren's affected palmar fascia compared to carpal tunnel derived fibroblasts (normal). There was a significantly greater amount of TGFβ detected in the Dupuytren's contracture group.

FIG. 5. TGFβ detection ELISA comparing fibroblasts derived from Dupuytren's affect palmar fascia, untreated and treated with tamoxifen. Tamoxifen significantly decreased the amount of TGFβ detected.
and tumor breast tissue following oral versus topical application [49]. Comparing topical and oral doses has been difficult and it is believed that various factors influence the rate of hydroxylation in the liver and, subsequently, the proportion of tamoxifen metabolites [26]. It may be possible, with further clinical investigation, to use the topical application of tamoxifen for the treatment of fibroproliferative disorders such as Dupuytren's disease.

Tamoxifen, by neutralizing or downregulating TGF-β, may prove to be a method to manipulate and control Dupuytren's contracture in the clinical setting. Even though the treatment of fibroproliferative disorders such as Dupuytren's contracture may prove to be a method to manipulate and control Dupuytren's contracture in the clinical setting, it is believed that various factors influence the rate of hydroxylation in the liver and, subsequently, the proportion of tamoxifen metabolites [26]. It may be possible, with further clinical investigation, to use the topical application of tamoxifen for the treatment of fibroproliferative disorders such as Dupuytren's disease.

REFERENCES


