Down-Regulating Causes of Fibrosis With Tamoxifen

A Possible Cellular/Molecular Approach to Treat Rhinophyma

Wyatt G. Payne, MD,† Francis Ko, BS,* Stephanie Anspaugh, BS,† Chad K. Wheeler, MD,† Terry E. Wright, MD,*† and Martin C. Robson, MD*†*

Abstract: Fibrosis and proliferative scarring are prominent features of the severe forms of rhinophyma. Up-regulation of growth and fibroblast kinetics are hallmarks of fibrosis. Persistent overexpression or dysregulated activation of the fibrogenic isoforms of transforming growth factor β (TGF-β) is associated with the increased fibroblast function leading to fibrotic conditions such as rhinophyma. Tamoxifen, a synthetic nonsteroidal antiestrogen, can neutralize or down-regulate TGF-β. Fibroblast-populated collagen lattices (FPCLs) were constructed from fibroblasts cultured from rhinophyma or normal nasal skin. One-half of each set of FPCLs was treated with Tamoxifen. Lattice contraction was serially measured over 5 days, and the supernatants of the cultures were analyzed for TGF-β-2 by immunoassay. Tamoxifen significantly decreased fibroblast activity by decreasing contraction of the treated lattices ($P < 0.05$) and significantly decreased the production/secretion of TGF-β-2 by rhinophyma fibroblasts ($P < 0.001$). These results suggest a possible new cellular/molecular approach to the treatment of the fibrotic varieties of rhinophyma.

Key Words: fibrosis, transforming growth factor β, rhinophyma

(*Ann Plast Surg* 2006;56: 301–305)

Although rhinophyma has classically been considered a form of end-stage acne rosacea, recent literature suggests that, at least in the severe varieties, fibrosis and proliferative scarring are important aspects of its pathobiology.[1–5] Up-regulation of growth and fibroblast kinetics have been demonstrated to occur in fibrotic conditions such as keloids, burn hypertrophic scars, and Dupuytren contracture.[6,7] The increased fibroblast activity in these and other fibrotic conditions such as lung fibrosis, cirrhosis, glomerulonephritis, and scleroderma is due to persistent overexpression or dysregulation of the isoforms of transforming growth factor β (TGF-β).[8–11] Of the isoforms of TGF-β, TGF-β-1 and TGF-β-2 have been associated with fibrogenic conditions, whereas TGF-β-3 tends to decrease fibrosis and scarring.[5,8,9,11–14] Both TGF-β-1 and TGF-β-2 have been shown to be elevated in rhinophyma tissue.[4,5] Data from our laboratory suggest that TGF-β-2 appears to be the most fibrogenic of the isoforms.[7,12,15–17]

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.[18–20] It has been demonstrated to have multiple effects, including altered RNA transcription, decreased cellular proliferation, delay or arrest of the cells in the G1 phase of the cell cycle, and interference with several growth factors such as TGF-β and insulin-like growth factor.[21–25] In vitro studies have shown that tamoxifen inhibits the proliferation of keloid fibroblasts, decreases the rate of collagen synthesis, decreases the production of TGF-β, and decreases the ability to contract fibroblast-populated collagen lattices (FPCLs).[11,23–25] Tamoxifen has also been demonstrated to decrease fibroblast function and down-regulate TGF-β-2 in Dupuytren affected palmar fascia.[7] Down-regulating fibroblast function and neutralizing TGF-β-2 resulted in a decrease in size and fibrosis in explanted samples of Dupuytren affected human palmar fascia.[26]

Since rhinophyma has a fibroproliferative component with increased TGF-β-2 production, the purpose of this study was to examine the role of tamoxifen at decreasing fibroblast function and down-regulating TGF-β-2 in collagen lattices populated with either fibroblasts extracted from rhinophyma specimens or normal nasal skin specimens.

MATERIALS AND METHODS

Tissue Biopsy Collections

Rhinophyma biopsy specimens were obtained from discarded tissues from 5 white male patients undergoing elective excisions. Five additional white male patients who underwent simple excisions of nasal-skin benign lesions were chosen as control subjects. Normal nasal-skin biopsies adjacent to the lesions were obtained from these patients. These control subjects were chosen because it was not possible to obtain normal control tissue from the patients with rhinophyma lesions. The study was approved by the institutional review board at the Bay Pines VA Medical Center.

Received July 19, 2005, and accepted for publication, after revision, November 14, 2005.

From the *Institute for Tissue Regeneration, Repair, and Rehabilitation, Department of Veterans Administration Medical Center, Bay Pines, FL; and †Division of Plastic Surgery, University of South Florida, Tampa, FL.

Reprints: Martin C. Robson, MD, 2426 Waukazoo Trail, PO Box 345, Macatawa, MI 49434-0345. E-mail: robsonmd@earthlink.net.

ISSN: 0148-7043/06/5603-0301

DOI: 10.1097/01.sap.0000199155.73000.2f

Preparation of Fibroblast Cultures

Primary cultures of fibroblasts from the surgical specimens were then established. Cells from generations 2 to 3 were used for experiments. The specimens were rinsed in 10 mL of calcium- and magnesium-free Dulbecco phosphate-buffered saline solution (Sigma Chemical Co., St. Louis, MO) supplemented with gentamicin (20 mg/mL) for 30 minutes at room temperature. A second antibiotic rinse using 10,000 μg/mL penicillin G, 25 μg/mL amphotericin B, and 10,000 μg/mL streptomycin sulfate solution (Gibco BRL, Grand Island NY) was performed for 10 minutes. Each specimen was then cut into 4 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 minutes at 37°C. A 10-mL aliquot of Dulbecco modified Eagle 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 phosphate-buffered saline solution (Sigma). Trypsin-ethylenediaminetetraacetic acid (0.25%) (Gibco) was added and the cultures were incubated at 37°C for 15 minutes. A 15-mL aliquot of soybean trypsin inhibitor (Sigma) was added. The cultures were centrifuged at 1000*g* for 10 minutes. The supernatant was decanted and the cell pellets were resuspended in 5 mL Dulbecco modified Eagle medium. This rinse/wash and 10-minute centrifuge cycle was repeated 3 times. The cells were counted with a hemocytometer, and trypan blue was used to determine cell viability. The cell density was adjusted to 5×10^5 cells/mL with Dulbecco modified Eagle medium.

Preparation of the Collagen Lattices

The collagen lattices were prepared from type 1 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 minutes to solidify. Sterile distilled water (5 mL) was added to the culture dishes, allowed to stand for 1 hour, and then aspirated. This was repeated 4 times to remove excess ammonia, and the collagen lattices were then incubated for 24 hours 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 lattices from the surface of the culture dishes so that they were loose and suspended in the saline. A total of 60 (30 with tamoxifen, 30 without tamoxifen) collagen lattices were prepared to allow triplicate measurement from each specimen (based on 2 treatment groups). To form the FPCLs, all saline was aspirated from the 35-mm culture dishes containing the collagen gel lattices. Two milliliters of 5×10^5 cells/mL were placed on the surface of each of the prefabricated collagen gel lattices. Tamoxifen (Sigma) was dissolved in PBS at a concentration of 8 μmol/mL and was added on days 0 to 30 of the FPCLs containing nasal tissue fibroblasts (15 rhinophyma and 15 normal). The 8 μmol/mL concentration was chosen because this dose has been shown not to be toxic to fibroblasts and to allow continued proliferation of fibroblasts.[25] This dose also allows fibroblast morphology to remain normal in collagen lattices.[25] An equal amount of PBS was added to the remaining 30 FPCLs.

Assay for Gel Contractions

The FPCLs were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide. The amount of gel contraction was measured every 24 hours for 5 days. Acetate overlays were used to trace the area of the gels. Gels were performed in triplicate for the cell lines 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 1-way analysis of variance was used to determine significant differences among groups. When a difference was identified, Tukey 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-β-2

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-β-2 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 minutes. Then, 1.2-N NaOH/0.5-M HEPES and 0.8 mL calibrator diluent RD51 were added. After 2 hours, the samples were assayed. During this time, TGF-β-2 standards were prepared. The assay procedure was as follows: 100 μL RD1-17 was added to each well. These were incubated for 2 hours then aspirated and washed 3 times. A total of 200 μL of substrate solution was added to each well, the wells were incubated for 20 minutes, and then 50 μL of stop solution was added to each well. The wells were read upon completion of the immunoassay using a Microplate Manager 4.0 (Bio-Rad Laboratories, Inc, Hercules, CA) at a wavelength of 450 nm with a 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 1-way analysis of variance was used to determine significant differences among groups. When a difference was identified, Tukey test (all pairwise multiple-comparison test) was used to delineate the differences. When 2 groups were compared directly, Student *t* test was used. Sigma Stat statistical software (Jandel Scientific) was used for data analysis.

RESULTS

FPCL Contraction

Fibroblasts from normal nasal skin and from rhinophymas contracted the collagen lattices over time. Compared

with the normal nasal skin fibroblasts, rhinophyma fibroblasts contracted the lattice significantly more by the fifth day ($P < 0.05$) (Fig. 1). Tamoxifen significantly inhibited the FPCL contraction in both the rhinophyma fibroblast-populated lattices and the normal nasal skin fibroblast-populated lattices ($P < 0.05$) (Fig. 2). The inhibition of contraction began immediately and was noted to a greater degree each successive day. Because the degree of lattice contraction was greater with the rhinophyma fibroblasts, the degree of contraction inhibition with Tamoxifen was also greater with the rhinophyma fibroblasts.

TGF-β-2 Immunoassay

There was a significant increase in TGF-β-2 expression in the supernatants obtained from rhinophymas compared with the supernatants obtained from FPCLs populated with fibroblasts obtained from normal nasal skin ($P < 0.001$) (Fig. 3). Tamoxifen treatment resulted in a down-regulation of TGF-β-2 in both rhinophyma and normal nasal skin fibroblasts, although to a significantly greater degree in the rhinophyma fibroblasts ($P < 0.001$).

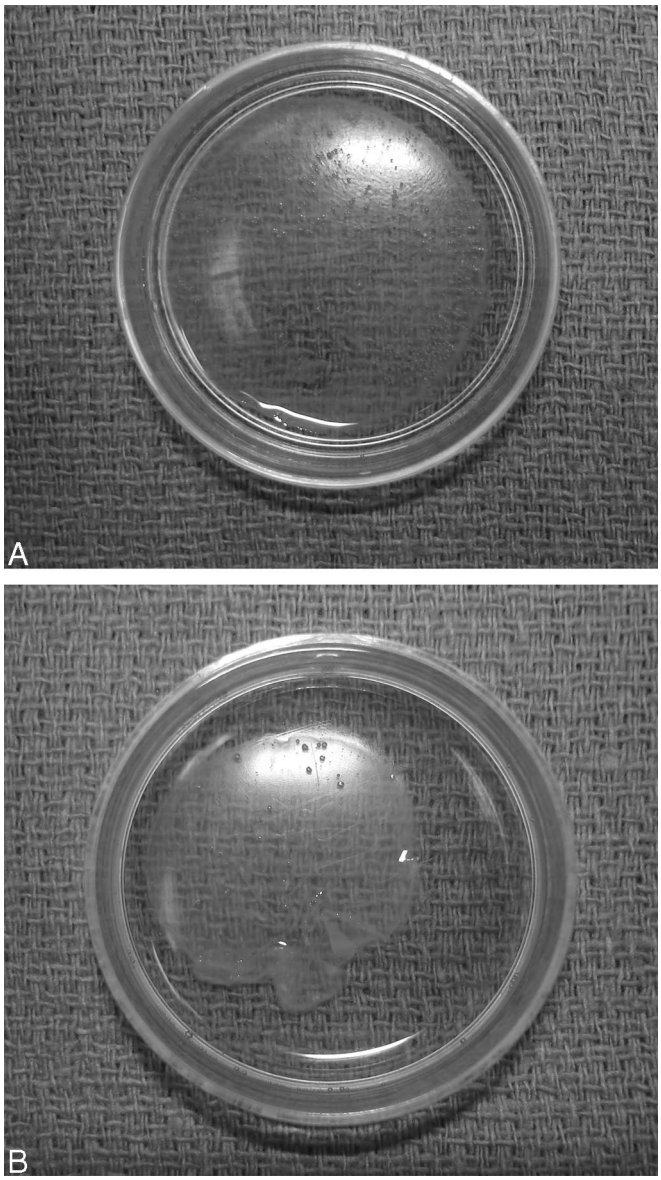

FIGURE 1. A, Fibroblast-populated collagen lattice (FPCL) in the uncontracted state on day 0 and in the (B) contracted state on day 5.

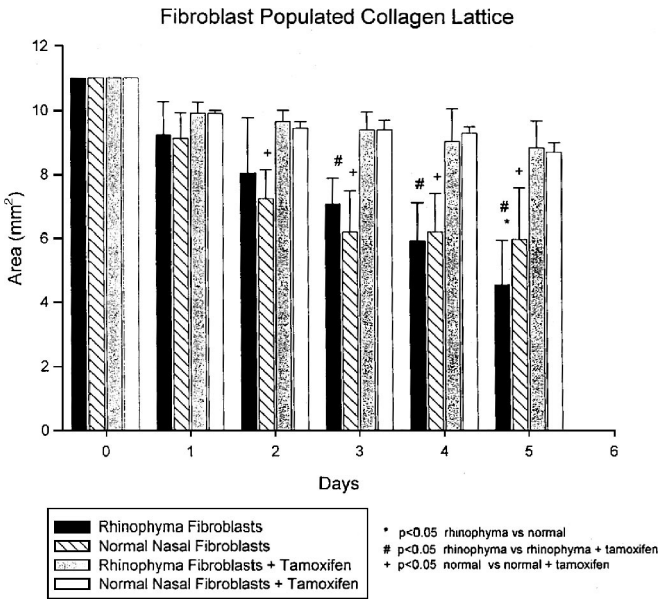

FIGURE 2. Fibroblasts from both rhinophyma-affected skin and normal nasal skin contracted the collagen lattices. Rhinophyma fibroblasts were more active by day 4 and significantly so by day 5 ($P < 0.05$). When fibroblasts derived from rhinophyma tissue were treated with Tamoxifen, they had significantly less contraction of a collagen lattice compared with untreated rhinophyma fibroblasts ($P = 0.05$). Similarly, normal nasal skin fibroblasts treated with Tamoxifen had significantly less ability to contract a collagen lattice than untreated normal nasal skin fibroblasts ($P < 0.05$).

DISCUSSION

Rhinophyma has a significant proliferative fibrotic component that has been suggested to be pathobiologically related to other progressive fibrosing dermal disorders such as keloids, burn hypertrophic scars, and Dupuytren contracture.[4,5] All recent evidence suggests that TGF-β-1 and TGF-β-2 are the key cytokines in scarring and fibrotic conditions.[8–10,17,23,27–31] TGF-β-1, TGF-β-2, and the TGF-β type-II have all been shown to be up-regulated in rhinophyma tissue.[4,5] The excess amounts of fibrogenic isoforms of TGF-β are directly related to fibroblast growth and kinetics, excessive production of collagens, and contraction of FPCLs.[7,11]

Tamoxifen has previously been demonstrated to inhibit FPCL contraction containing normal dermal fibroblasts, keloid fibroblasts, and Dupuytren affected palmar fascia fibroblasts.[23,25,26] This effect was due to Tamoxifen's inhibitory effects on TGF-β. Estrogen has been demonstrated to increase TGF-β production, so it is reasonable that an antiestrogen such as Tamoxifen would decrease levels of TGF-β-1 and TGF-β-2.[32] A decrease in TGF-β-2 occurred in supernatants

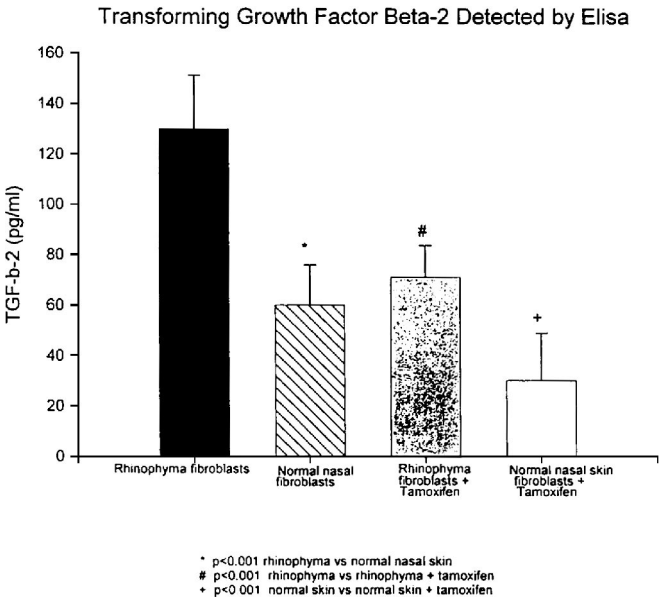

FIGURE 3. When TGF-β-2 detection (ELISA) of fibroblasts from rhinophyma tissue was compared with normal nasal skin fibroblasts, there was a significantly greater amount of TGF-β-2 detected in the rhinophyma tissue ($P < 0.001$). Tamoxifen significantly decreased the amount of TGF-β-2 detected in the rhinophyma tissue ($P < 0.001$). TGF-β-2 in normal nasal skin fibroblasts was also down-regulated by treatment with Tamoxifen ($P < 0.05$). However, the amount of down-regulation was to a much lesser degree than in rhinophyma fibroblasts.

from collagen lattices populated with Dupuytren affected palmar fascia fibroblasts when treated with Tamoxifen.[7]

In the present study, we have been able to demonstrate a marked decreased function of fibroblasts derived from rhinophymas when treated with Tamoxifen (Fig. 2). We have also shown a down-regulation of TGF-β-2 in these same fibroblasts (Fig. 3). Normal nasal skin shows less active fibroblasts and significantly less TGF-β-2 than the rhinophyma fibroblasts. Both fibroblast function and TGF-β-2 are decreased in normal nasal fibroblasts by Tamoxifen treatment, but to a much lesser extent.

It is possible that some of the effect on fibroblast function may be due to a direct effect on the fibroblasts by the Tamoxifen such as altered RNA transcription, decreased cellular proliferation, and/or delay or arrest of the cells in the G1 phase of the cell cycle.[21] However, the 8 μmol/mL concentrations of Tamoxifen used have been shown not to significantly inhibit fibroblast proliferation in collagen lattices.[25] Therefore, it appears that the effect is due to Tamoxifen's ability to down-regulate TGF-β-2 and, consequently, fibroblast activity.

Although Tamoxifen is usually administered systemically and is fairly well tolerated when given orally, Hu et al[25] have suggested its use topically. Pujol et al[33] reported a percutaneous gel administration of Tamoxifen in a small randomized study. A chlorinated Tamoxifen analogue, Toremifene, has been investigated experimentally in a topical methylcellulose formulation.[34] Since Tamoxifen was successful in this study at decreasing fibroblast function and down-regulating TGF-β-2, a cellular/molecular approach may be feasible for the treatment of rhinophyma. Not only Tamoxifen may be successful, but other compounds known to down-regulate or neutralize the fibrogenic isoforms of TGF-β may also be successful. As suggested by Pu et al,[4] these compounds would include neutralizing antibodies,[8,35] mannose-6-phosphate,[9,36] decorin,[37,38] and TGF-β-3.[8,14]

REFERENCES

1. Tope WD, Sangueza OP. Rhinophyma's fibrous variant: histopathology and immunohistochemistry. *Am J Dermatopathol*. 1994;16:307–310.
2. Aloi F, Tomasini C, Soro E, et al. The clinicopathologic spectrum of rhinophyma. *J Am Acad Dermatol*. 2000;42:468–472.
3. Hoasjoe DK, Stucker FJ. Rhinophyma: review of pathophysiology and treatment. *J Otolaryngol*. 1995;24:51–56.
4. Pu LLQ, Smith PD, Payne WG, et al. Overexpression of transforming growth factor-beta-2 and its receptor in rhinophyma: an alternative mechanism of pathophysiology. *Ann Plast Surg*. 2000;45:515–519.
5. Payne WG, Wang X, Walusimbi M, et al. Further evidence for the role of fibrosis in the pathobiology of rhinophyma. *Ann Plast Surg*. 2002; 48:641–645.
6. Polo M, Smith PD, Kim YJ, et al. Effect of TGF-beta-2 on proliferative scar fibroblast cell kinetics. *Ann Plast Surg*. 1999;43:185–190.
7. Kuhn MA, Wang X, Payne WG, et al. Tamoxifen decreases fibroblast function and downregulates TGF-beta-2 in Dupuytren's affected palmar fascia. *J Surg Res*. 2002;103:146–152.
8. Shah M, Foreman DM, Ferguson MWJ, et al. Neutralization of TGF-beta-1 and TGF-beta-2 or exogenous addition of TGF-beta-3 to cutaneous wounds reduces scarring. *J Cell Sci*. 1995;108:985–1002.
9. O'Kare S, Ferguson MWJ. Transforming growth factor-betas and wound healing. *Int J Biochem Cell Biol*. 1997;29:63–78.
10. Border WA, Noble NA. Transforming growth factor-beta in tissue fibrosis. *N Engl J Med*. 1994;321:1286–1292.
11. Robson MC. Proliferative scarring. *Surg Clin North Am*. 2003;83:557–569.
12. Wang X, Smith PD, Pu LLQ, et al. Exogenous TGF-beta-2 modulates collagen I and collagen III synthesis in proliferative scar xenografts in nude rats. *J Surg Res*. 1999;87:194–200.
13. Polo M, Ko F, Busillo F, et al. The Moyer award: cytokine production in patients with hypertrophic burn scars. *J Burn Care Rehabil*. 1997;18: 477–482.
14. Cox DA. Transforming growth factor-beta-3. *Cell Biol Int*. 1995;19: 357–370.
15. Polo M, Kim YJ, Kucukcelebi A, et al. Special article: an in vivo model of human proliferative scar. *J Surg Res*. 1998;74:187–195.
16. Smith PD, Mosiello G, DeLuca L, et al. TGF-beta-2 activates proliferative scar fibroblasts. *J Surg Res*. 1999;82:319–323.
17. Kuhn MA, Singh S, Smith PD, et al. Periprosthetic breast capsules contain the fibrogenic cytokines TGF-beta-1 and TGF-beta-2, suggesting possible new treatment approaches. *Ann Plast Surg*. 2000;44:387–391.
18. Owens LV, Cance WG, Huth JF. Retroperitoneal fibrosis treated with tamoxifen. *Ann Surg*. 1995;61:842–844.
19. Clark CP, Vanderpool D, Preskill JT. The response of retroperitoneal fibrosis to tamoxifen. *Surgery*. 1991;109:502–506.
20. Timmons MJ. Fibromatosis, desmoids, fibroblasts, and tamoxifen. *Br J Plast Surg*. 1994;47:378–380.
21. Dickens TA, Colletta AA. The pharmacological manipulation of members of the transforming growth factor-beta family in the chemoprevention of breast cancer. *Bioessays*. 1993;15:71–74.
22. Pollock M, Constantino J, Polychronakos C, et al. Effect of tamoxifen on serum insulin-like growth factor I levels in stage 1 breast cancer patients. *J Natl Cancer Inst*. 1990;82:1693–1697.
23. Mancoll JS, Zhao J, McCauley RL, et al. The inhibitory effect of tamoxifen on keloid fibroblasts. *Surg Forum*. 1996;47:718–720.
24. Chau D, Mancoll JS, Lee S, et al. Tamoxifen downregulates TGF-beta production in keloid fibroblasts. *Ann Plast Surg*. 1998;40:490–493.
25. Hu D, Hughes MA, Cherry GW. Topical tamoxifen: a potential thera-

peutic regimen in treating excessive dermal scarring? *Br J Plast Surg*. 1998;51:462–469.
26. Kuhn MA, Payne WG, Kierney PC, et al. Cytokine manipulation of explanted Dupuytren's affected palmar fascia. *Int J Surg Invest*. 2001; 2:443–456.
27. Border WA, Rouslahti E. Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest*. 1992;90:1–7.
28. Younai S, Nichter LS, Wellisz T, et al. Modulation of collagen synthesis by transforming growth factor-beta in keloid and hypertrophic scar fibroblasts. *Ann Plast Surg*. 1994;33:148–151.
29. Younai S, Venters G, Vu S, et al. Role of growth factors in scar contraction: an in vitro analysis. *Ann Plast Surg*. 1996;36:495–501.
30. Lee TY, Chin GS, Kim WJH, et al. Expression of transforming growth factor beta-1, -2, and -3 proteins in keloids. *Ann Plast Surg*. 1999;43: 179–184.
31. Igarashi A, Nashiro K, Kikuchi K, et al. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. *J Invest Dermatol*. 1996;106:729–733.
32. Ashcroft GS, Dodsworth J, VanBoxtel E, et al. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-beta-1 levels. *Nat Med*. 1997;3:1209–1215.
33. Pujol H, Girault J, Rouanet P, et al. Phase I study of percutaneous 4-hydroxytamoxifen with analysis of 4-hydroxytamoxifen concentrations in breast cancer and normal breast tissue. *Cancer Chemother Pharmacol*. 1995;36:493–498.
34. Maenpaa J, Dooley T, Wurz G, et al. Topical toremifene: a new approach for cutaneous melanoma? *Cancer Chemother Pharmacol*. 1993;32:392–395.
35. Shah M, Foreman DM, Ferguson MWJ. Control of scarring in adult wounds by neutralizing antibody to transforming growth factor-beta. *Lancet*. 1992;339:213–214.
36. McCallion RL, Ferguson MWJ. Fetal wound healing and the development of antiscarring therapies for adult wound healing. In: Clark RAF, ed. *The Molecular and Cellular Biology of Wound Repair*. New York, NY: Plenum Press; 1996:561–600.
37. Logan A, Berry M, Gonzales AM, et al. Effect of transforming growth factor-beta-1 on scar production in the injured central nervous system of the rat. *Eur J Neurosci*. 1994;6:355–363.
38. Hildebrand A, Romaris M, Rasmussen LM, et al. Introduction of the small interstitial proteoglycans biglycan, decorin, and fibromodulin with transforming growth factor-beta. *Biochem J*. 1994;303:527–534.