Effects of Tamoxifen on Normal Human Dermal Fibroblasts

Mauro B. Ruffy, MD; Shaun S. Kunnavatana, BA; R. James Koch, MD

Objective: To evaluate the effects of tamoxifen on the growth and autocrine growth factor production of human dermal fibroblasts from the face.

Methods: In vitro study of normal adult dermal fibroblast cells developed from surgical specimens in a serum-free model. Cell cultures were exposed to 5-, 8-, 12-, 16-, and 50-µg/mL concentrations of tamoxifen solution. Cell counts were performed, and the cell-free supernatants were collected at 0, 1, 3, 5, and 7 days after the initial exposure. Population doubling times were calculated, and supernatants were quantitatively assayed for basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF-β1).

Results: Tamoxifen appears to delay cellular proliferation rates in a dose-dependent manner up to a concentration of 12 µg/mL. Higher concentrations, approaching 50 µg/mL, appear to have a toxic effect on cell growth. The analysis of growth factor production revealed decreased levels of bFGF and VEGF but no change in the levels of TGF-β1.

Conclusions: The in vitro findings of delayed cell proliferation and decreased production of VEGF and bFGF in cells exposed to tamoxifen are consistent with previous in vivo reports of delayed wound healing but improved scar formation. The in vitro findings of growth factor modulation by tamoxifen provide cellular and molecular evidence supporting the clinical use of tamoxifen to ultimately improve scar formation.

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It has been shown that postmenopausal women have improved scar formation compared with premenopausal women. Scars found in the postmenopausal group are paler and flatter than the more pigmented and everted scars found in the younger women. Shah and Maibach theorize that the difference in wound healing is in part due to the difference in estrogen levels between the 2 age groups. If this is true, the addition of an estrogen antagonist such as tamoxifen to a wound healing environment may augment wound healing and prevent poor scar formation.

Tamoxifen is a synthetic nonsteroidal antiestrogen agent that has been shown to inhibit keloid fibroblast proliferation and decrease collagen production. Effects of tamoxifen include altering transcriptional synthesis, decreasing cellular proliferation, and modulating production of multiple polypeptide growth factors. Mikulec et al reported that keloid and lethal fibroblasts cultured in serum-free media demonstrated a decrease in transforming growth factor (TGF-β1) levels in the presence of tamoxifen; however, there was no change in cell proliferation patterns in the presence or absence of tamoxifen.

METHODS

Normal dermal fibroblast cell lines were established from facial skin obtained from operative specimens. Approval to use operative specimens that would otherwise be discarded was obtained in advance from the human subjects committee of Stanford University. Using sterile technique under a laminar-flow hood, we minced the dermal specimens into approximately 1-mm³ fragments on a Petri dish with a sterile scalpel blade then washed in Dulbecco phosphate-buffered saline solution with 5% penicillin/streptomycin/amphotericin (PSA; Gibco, Grand Island, NY). The specimens were then placed in 75-cm² tissue culture flasks (T75;
read using an automated plate reader (E1X800; Bio-Tek Instruments Inc, Winooski, Vt). Optical densities were analyzed with KC4 software (Bio-Tek Instruments Inc), and cell counts were determined by comparison with a standard curve derived from known cell quantities calculated for each cell type and medium. Cell population doubling times were then calculated from the equation for exponential growth.

**AUTOCRINE PRODUCTION ASSAY**

In the second experiment, the cell-free supernatant for all groups was also collected from the wells at the 0-, 1-, 3-, 5-, and 7-day intervals. Samples were stored at −80°C in microcentrifuge tubes for use in growth factor assays. Expression of VEGF, bFGF, and TGF-β1 was evaluated by solid-phase enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn). Assays were performed in triplicate and read using an automated plate reader. Optical densities were analyzed with KC4 software, and growth factor quantities were determined by comparison with standard curves generated with each enzyme-linked immunosorbent assay performed.

Data analysis and statistics were performed using commercially available software (Microsoft Excel for Windows 2002; Microsoft Corp, Redmond, Wash; and Primer of Biostatistics, version 4.0; McGraw Hill, New York, NY). Statistical differences between groups were assessed using the paired t-test and considered significantly different at \( P < .05 \).

### RESULTS

#### CELL PROLIFERATION

Population doubling times were calculated for each of the experimental cell cultures exposed to varying concentrations of tamoxifen. At lower concentrations, tamoxifen appeared to have a dose-dependent negative effect on cell proliferation up to 12 µg/mL. Each treatment well received a different concentration of tamoxifen to provide the cells with continuous exposure to the modulator.

Cell counts were performed in triplicate using the WST-1 assay (Boehringer Mannheim, Indianapolis, Ind) at 0, 1, 3, 5, and 7 days after initial exposure to tamoxifen and a growth curve was generated with the data. The WST-1 assay is a colorimetric assay used in the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Assays were performed with cells in their first or second passage. Confluent fibroblasts were washed with phosphate-buffered saline and released from the flask wall using 0.03% trypsin. The trypsin was then inactivated using Trypsin Soybean Inhibitor (Gibco) in a 1:1 ratio. Cell viability was determined by comparison with a standard curve derived from known cell quantities calculated for each cell type and medium. Cell population doubling times were then calculated from the equation for exponential growth.

### CELL PROLIFERATION ASSAY

In the first experiment, varying concentrations of tamoxifen were dissolved in UltraCULTURE with 1% L-glutamine yielding different concentrations of tamoxifen (1, 8, 12, 16, and 50 µg/mL). The commercially available tamoxifen was initially dissolved in dimethyl sulfoxide (DMSO), and the negative control consisted of the equivalent DMSO solution without the tamoxifen. The positive control contained UltraCULTURE with 10% fetal bovine serum. Each treatment well received a different concentration of tamoxifen to provide the cells with continuous exposure to the modulator.

Cell counts were performed in triplicate using the WST-1 assay (Boehringer Mannheim, Indianapolis, Ind) at 0, 1, 3, 5, and 7 days after initial exposure to tamoxifen and a growth curve was generated with the data. The WST-1 assay is a colorimetric assay used in the quantification of cell proliferation and cell viability based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Assays were performed with cells in their first or second passage. Confluent fibroblasts were washed with phosphate-buffered saline and released from the flask wall using 0.03% trypsin. The trypsin was then inactivated using Trypsin Soybean Inhibitor (Gibco) in a 1:1 ratio. Cell viability was determined by comparison with a standard curve derived from known cell quantities calculated for each cell type and medium. Cell population doubling times were then calculated from the equation for exponential growth.

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<th>Tamoxifen Concentration, µg/mL</th>
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<tr>
<td>Positive control</td>
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*The 5-, 8-, and 12-µg/mL concentrations of tamoxifen appeared to have a dose-dependent inhibitory effect (longer population doubling time) on fibroblast cell growth. At 16-µg/mL concentrations, the population doubling time paralleled that of the positive control. The 50-µg/mL exposure (not shown in the Table) demonstrated no cell growth, which suggests a toxic dose to the cells. The differences in population doubling times between the positive control group and the 8- and 12-µg/mL groups were statistically significant (\( P < .05 \)).

Falcon, Becton-Dickinson, Franklin Lakes, NJ) with 10 mL of primary culture medium made up of 10% fetal bovine serum in Dulbecco modified Eagle medium with 1% L-glutamine and 1% PSA. These flasks were stored in an incubator at 37°C in a humidified 5% carbon dioxide atmosphere.

After 48 hours, the medium was changed with 10 mL of primary culture medium and then changed every 3 days until fibroblasts were visualized under light microscopy to be growing outward from the explanted tissue. At that time, the tissue fragments were removed, and the cells were subcultured into T75 culture flasks. The primary culture medium was changed every 2 to 3 days, and successive cultures were passed at confluence.

Experiments were performed with cells in their first or second passage. Confluent fibroblasts were washed with phosphate-buffered saline and released from the flask wall using 0.03% trypsin. The trypsin was then inactivated using Trypsin Soybean Inhibitor (Gibco) in a 1:1 ratio. Cell viability was determined by comparison with a standard curve derived from known cell quantities calculated for each cell type and medium. Cell population doubling times were then calculated from the equation for exponential growth.

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DMSO indicates dimethyl sulfoxide.
centration of 16 µg/mL, the population doubling time of 21.9 hours decreased to a level similar to that of the positive control, which suggests an upper limit to inhibitory effect on cell growth. No growth was seen in the plates exposed to 50 µg/mL of tamoxifen, which suggests a toxic effect on the fibroblast cells.

**AUTOCRINE GROWTH FACTOR PRODUCTION**

Levels of VEGF decreased in the presence of tamoxifen. Compared with the negative control (DMSO only), the cells exposed to tamoxifen appeared to produce lower levels of VEGF beyond day 3. The only exception was the 16-µg/mL group, which appeared to produce levels that paralleled the VEGF levels of the negative control. The VEGF levels found in each experimental group when compared with the DMSO negative control were found to be statistically significant (P<.05) (Figure 1).

Levels of TGF-β1 were increased in the presence of tamoxifen. Compared with the negative and positive controls, the cells exposed to all levels of tamoxifen appeared to produce higher levels of TGF-β1, though these increases were not statistically significant (Figure 2).

Tamoxifen appeared to have no effect on bFGF production. Notably, the 16- and 50-µg/mL concentrations of bFGF appeared elevated at day 0 and were thought to be related to toxic cell lysis releasing intracellular stores into the supernatant (Figure 3).

This study explored the modulation of tamoxifen, a known estrogen antagonist, on human fibroblast cells. In vitro, tamoxifen appears to negatively affect cell proliferation rates in a dose-dependent fashion. Our group’s previous research has demonstrated that tamoxifen has no effect on the cellular proliferation rates of keloid fibroblast cells. The present study demonstrates that tamoxifen does indeed slow proliferation of normal fibroblasts by increasing the population doubling times, thereby delaying the time it takes for cells to double its population size. This occurred in a dose-dependent fashion at lower concentrations (up to 12 µg/mL), but at higher concentrations, tamoxifen appeared to lose its effect on cell growth. There was a return to normal population doubling times at a tamoxifen concentration of 16 µg/mL, which suggests an upper limit whereby tamoxifen delays proliferation rates.

The negative control contained the same concentration of DMSO used to dissolve the tamoxifen in all of the experiments and was used to distinguish any effects of the tamoxifen from DMSO alone on population doubling times. Interestingly, the DMSO alone appears to demonstrate a positive effect on cell proliferation. The population doubling time of the negative control was 12.2 hours compared with 25.4 hours for the positive control. If tamoxifen truly exhibits a negative effect on cell proliferation and DMSO truly exhibits a positive one, the data suggest that the negative effects of tamoxifen outweigh the positive effects of the DMSO. The fact that we find statistically significant population doubling times at 8- and 12-µg/mL concentrations strengthens the argument that tamoxifen delays cellular proliferation because these delays had to overcome the positive effects of DMSO. Ideally, a negative control should not exhibit any effect on cell growth. A study of solvents for tamoxifen other than DMSO is warranted to confirm and further evaluate its effect on cell proliferation.

The serum-free experimental model used to conduct these experiments proved to be a reliable assay for determining autocrine growth factor production by eliminating the presence of confounding growth factor levels found in serum. The in vitro studies suggest that tamoxifen decreases levels of VEGF and bFGF and increases levels of TGF-β1. The delay in population doubling times noted in the first experiment may be explained by the decrease in bFGF production given its role as an important mitogenic growth factor for fibroblasts.
It is interesting to find that VEGF levels were decreased in all cells exposed to tamoxifen. Vascular endothelial growth factor is implicated in angiogenesis and the recruitment of new vessels at the site of injury. Ashcroft et al. have demonstrated that ovariectomized rats had delayed wound healing but improved scarring. The scars appeared pale and thin compared with the thicker, more everted scars on the rats with intact ovaries. Shah and Maibach theorize that the decreased levels of estrogen contribute to the clinical effects found in the improved scar formation. Exposure to tamoxifen decreases estrogen levels to approximately those found in ovariectomized rats. Our in vitro findings of decreased levels of VEGF in response to tamoxifen may help explain the paler appearance of the scars in ovariectomized rats. It is possible that the clinical findings of paler, thinner scars and delayed wound healing can be explained by decreased levels of VEGF production by the fibroblasts, resulting in less vascularity at the wound site.

Tamoxifen may mimic the wound environment found in postmenopausal women and in ovariectomized rats by decreasing the amount of estrogen present in the wound milieu. Ashcroft et al. have shown that the delayed wound healing in ovariectomized rats was reversed with the introduction of hormone replacement therapy containing estrogen. It was theorized that the effects were in part due to increased levels of TGF-β1.

We have shown that TGF-β1 levels were not significantly increased in the presence of tamoxifen when compared with the negative control. This is not consistent with what Ashcroft et al. have shown in the rat model. Various theories to explain this discrepancy may include the possibility that the rat and human models are so disparate at a cellular level that comparisons between the two species are not strong and conclusions cannot be extrapolated from one to the other.

Another possibility is the duality of tamoxifen as an estrogen agonist on certain tissues such as the endometrium as opposed to an estrogen antagonist on other tissues such as breast tissue. This duality may be due to different estrogen receptors expressed on the different tissue types. Further studies to explore which type of estrogen receptors expressed on fibroblasts is affected by tamoxifen can help elucidate the mechanism by which tamoxifen affects fibroblasts.

Mikulec et al. have previously shown that tamoxifen decreased levels of TGF-β1 in keloid cells. It appears that keloids have deranged wound healing caused by excessive amounts of TGF-β1. In a sense, keloids that have increased levels of TGF-β1 are seen as the opposite type of scar compared with the estrogen-deficient scars that do not have elevated levels of the growth factor. The fact that TGF-β1 levels did not increase in response to tamoxifen may be beneficial in that this modulator should not promote the formation of a keloid scar by increasing TGF-β1 production.

Improved scar formation in the presence of tamoxifen has been further demonstrated by a collagen lattice study performed by Hu et al. They showed that at 10µM to 20µM concentrations, tamoxifen exhibited a dose- and time-dependent inhibition of scar contraction that was reversible with removal of the modulator. Our studies echo the dose dependency of tamoxifen between 5 and 12 µg/mL in terms of its inhibitory effects on cellular proliferation. We have shown that concentrations greater than 16 µg/mL appear to reverse its inhibitory effects, and at concentrations approaching 50 µg/mL, tamoxifen appears to be toxic. Hu et al. showed that complete inhibition of scar contraction was present at 50µM and 100µM concentrations, which may be explained by the decreased, almost toxic effect on cell proliferation demonstrated by our in vitro studies.

It is hard to extrapolate these in vitro findings to an in vivo model or to human clinical trials. Our data suggest that tamoxifen decreases fibroblast proliferation and may decrease angiogenesis, which may result in a cosmetically pleasing scar, but it may result in a weaker wound. In the animal studies published by Ashcroft et al., ovariectomized young rats had delayed and impaired wound healing by the measures of reepithelialization time, wound width, and collagen deposition. Animal models testing the tensile strength of the wound treated with tamoxifen may help elucidate whether a cosmetically pleasing scar is worth the price of a weaker wound-healing response.

Future in vitro studies should explore the mechanisms by which estrogen modulates wound healing. Thus far, to our knowledge, there are no growth factor studies demonstrating the effect estrogen has on VEGF production or fibroblast proliferation. The serum-free model would be ideal to assess the effects of exogenous estrogen on fibroblast proliferation and production of VEGF.

In conclusion, tamoxifen may play a role in improving wound healing by inhibiting cell proliferation rates of fibroblasts. Growth factor production may be directly or indirectly affected by tamoxifen. The delayed wound healing reported in other studies in postmenopausal women and ovariectomized rats may be explained by decreased levels of bFGF resulting in decreased cellular proliferation rates in the presence of tamoxifen. Furthermore, VEGF levels were found to be decreased, which may help explain the paler, thinner scars reported in other studies. The use of tamoxifen as a potential modulator of wound healing and scar improvement may have a cellular basis for its effects.

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REFERENCES