The Effect of Copper Tripeptide and Tretinoin on Growth Factor Production in a Serum-Free Fibroblast Model

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Objective: To evaluate the effect of copper tripeptide and tretinoin on normal and keloid-producing dermal fibroblasts in a serum-free in vitro model. The cellular response was described in terms of viability and secretion of basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1).

Methods: Primary cell lines were established from patient facial skin samples obtained during surgery and plated in serum-free media. At 0 hour, copper tripeptide (1×10^{-9} mol/L), tretinoin (1×10^{-5} mol/L), or appropriate control vehicle was added. Cell counts and viability were established at 24, 72, and 120 hours. Supernatants were collected at the same intervals and were assessed for bFGF and TGF-β1 concentrations using the enzyme-linked immunosorbent assay technique.

Results: Cell lines showed viability between 86% and 96% (mean, 92%) throughout the experiment.

Tretinoin-treated normal fibroblasts secreted more bFGF than did controls at 24 hours ($P<.05$). Tretinoin-treated keloid-producing fibroblasts secreted more TGF-β1 than did controls at 120 hours ($P<.05$). Keloid-producing fibroblasts treated with copper tripeptide secreted less TGF-β1 than did controls at 24 hours ($P<.05$); a similar trend was observed in normal fibroblasts.

Conclusions: Normal fibroblasts treated with tretinoin produced more bFGF than did controls, and this might partially explain the clinically observed tightening effects of tretinoin. Normal and keloid-producing dermal fibroblasts treated with copper tripeptide secreted less TGF-β1 than did controls, suggesting a possible clinical use for decreasing excessive scar formation.

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ABERRANT WOUND healing is a significant problem for many surgical patients. Inadequate healing is often due to an underlying medical condition such as diabetes, previous radiation therapy, poor nutritional status, or malignancy. Other patients, at the opposite end of the spectrum, may form hypertrophic scars or keloid tissue. Keloids do not represent a more severe form of hypertrophic scars. There is no simple continuum from normal skin scar to hypertrophic scar to keloid tissue. Collagen bundles in hypertrophic scars remain parallel in orientation (as in normal skin), whereas keloids have randomly organized sheets of collagen. Also, whereas contractile myofibroblasts are common in hypertrophic scars, they are relatively absent in keloids.

Multiple treatment modalities have attempted to reduce such excess scarring, yet none have established long-standing results.[1] Keloid-producing dermal fibroblasts (KFs) have been shown[2,3] to produce substantially more procollagen and fibronectin than do normal dermal fibroblasts (NFs) in culture. The current research indicates that at the core of keloid etiology are wound-healing cytokines—growth factors that regulate production of extracellular matrix (ECM) components.

An imbalance in the levels of various cytokines generated in the wound-healing process may lead to keloid formation. In addition, it may be the proper balance of cytokines in the wound environment that allows for normal wound healing. Such growth factors, which promote cell growth, division, and migration in wounded tissue, are secreted by dermal fibroblasts. Two of these key growth factors were considered in the present study.

Transforming growth factor-β1 (TGF-β1) is a key cytokine in the initiation and termination of tissue repair. In relation to other known tissue repair cytokines, it strongly stimulates synthesis of the major ECM proteins, namely, collagen, proteoglycan, and fibronectin.[4,5] You-

MATERIALS AND METHODS

CELL CULTURES

Primary cultures of dermal fibroblasts were established from excisional biopsies of 3 different keloid and 3 different normal facial skin specimens using a standard explant technique. All cell lines were directly established from operative specimens. Keloid specimens were from the lobule and normal skin samples were from the preauricular and mental region from 5 different patients. The described method was approved by the institutional review board at Stanford University Medical Center.

The dermis was isolated from the specimens and minced. Antimicrobial treatment consisted of washing the specimens in Dulbecco phosphate-buffered saline solution (PBS) with 5% penicillin, streptomycin, and amphotericin (GIBCO, Grand Island, NY). The minced specimens were placed in scored 25-cm^2 tissue (T25) flasks (Falcon; Becton-Dickinson, Franklin Lakes, NJ) with a 2.5-mL solution of primary culture media (20% fetal bovine serum in Dulbecco modified Eagle medium; 1% penicillin, streptomycin, and amphotericin; and 1% L-glutamine) (GIBCO). The dermal specimens were stored and maintained at 37°C in a humidified 5% carbon dioxide atmosphere.

After 24 hours, the media were changed with 5.0 mL of primary culture media. The media were then changed every 2 days until fibroblasts were visualized under light microscope to be growing outward from the explanted tissue. At this time the tissue was removed. With sufficient outgrowth of fibroblasts, cells were passed into 75-cm^2 tissue (T75) flasks using 0.05% trypsin (GIBCO) in PBS. Primary culture media were changed every third day, and successive cultures were passed at confluence. Cells from passages 4 and 5 were used for experimentation. All work was performed under a laminar flow hood using a sterile technique.

MODULATORS

A concentrate was prepared by dissolving the GHK-Cu (Procyte Corp, Kirkland, Wash) in PBS just before experimentation, which was then diluted in serum-free media to a concentration of 1.0×10^{-9} mol/L. Previous studies[9,10] have shown this concentration to be effective in stimulating collagen and glycosaminoglycan production by fibroblasts.

Tretinoin (Sigma-Aldrich Corp, St Louis, Mo) was dissolved in ethanol immediately before use, then diluted in commercially available serum-free media (UltraCulture; Biowhittaker, Walkersville, Md) to achieve a final concentration of 1.0×10^{-5} mol/L. This concentration has proven to be most effective for in vitro studies of procollagen inhibition.[21,24] Total concentration of solvent was less than 0.1%. This concentration has been shown to be nontoxic to fibroblasts and has no effect on collagen metabolism by these cells.[21] The corresponding amount of ethanol was added to the serum-free media in control samples.

CELL PLATING IN SERUM-FREE MEDIA

At the time of experimentation, fibroblasts were released from flask walls using 0.05% trypsin solution. The trypsin was inactivated using trypsin soybean inhibitor (GIBCO) in a 1:1 ratio. Cells were suspended in UltraCulture and then transferred into 24-well culture plates (Falcon; Becton-Dickinson) at a concentration of 6×10^4 cells/well. UltraCulture was selected for its ability to sustain dermal fibroblast growth to at least 7 days with greater than 90% viability.[25] Cells were counted in duplicate using phase-contrast microscopy and a hemocytometer. Viable cells were determined using trypan blue exclusion. The plates were incubated for 48 hours to allow for adequate settling. After this time, cells were washed in PBS and fresh UltraCulture was added, this time with the appropriate modulator or vehicle included (0 hour).

MEASUREMENT OF GROWTH FACTOR CONCENTRATIONS

The supernatant was drawn from the culture wells at 24, 72, and 120 hours and stored at –70°C until the time of assay. Each sample was assayed using the enzyme-linked immunosorbent assay technique. Growth factors bFGF and TGF-β1 were assayed using Quantikine High Sensitivity and Quantikine assay kits (R&D Systems, Minneapolis, Minn), respectively. Assays were read using an automated plate reader (Elx800; Bio-Tek Instruments Inc, Winooski, Vt). Optical densities were analyzed with KC4 software (Bio-Tek Instruments Inc). Assays were read with the specified filter for each assay with application of a reference filter to correct for optical imperfections in the plate.

STATISTICAL ANALYSIS

Each data point represents duplicate cell counts with assays performed in duplicate. Statistical differences were assessed using 2-sample and paired *t* tests. Differences at the 5% level were considered statistically significant.

nai et al[6] investigated the in vitro effects of TGF-β1 on fibroblasts and found that KFs produce many times more collagen than do NFs when stimulated by TGF-β1. Cultured KFs also secrete more TGF-β1 than do NFs—further evidence that excessive amounts of this cytokine in the wound environment may be central to keloid formation and growth.[7]

The second growth factor assayed in our study, basic fibroblast growth factor (bFGF), has been shown to inhibit hydroxyproline biosynthesis, an index of collagen production, in cultured KFs.[8] Methods that increase or stabilize bFGF secretion might therefore decrease aberrant scar or keloid formation by reducing the amount of collagen deposited during wound healing.

The modulators selected for evaluation were the copper tripeptide complex glycyl-L-histidyl-L-lysine-Cu^{2+} (GHK-Cu) and all-*trans* retinoic acid (tretinoin). A naturally occurring tripeptide, GHK-Cu has been shown to have significant clinical application in the field of wound healing and tissue repair. It has been demonstrated that GHK-Cu stimulates cultured NFs to synthesize collagen and induces a dose-dependent increase in the synthesis of glycosaminoglycans.[9-11] It also has been shown that ECM accumulation increases in the rat wound model
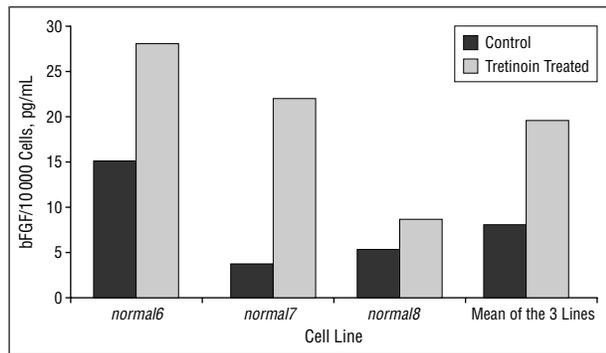
Figure 1. *Secretion of basic fibroblast growth factor (bFGF) by normal dermal fibroblasts 24 hours after application of tretinoin (1×10^{-5} mol/L). In each cell line, treated cells secreted more bFGF than did controls (mean comparison, $P<.05$).*
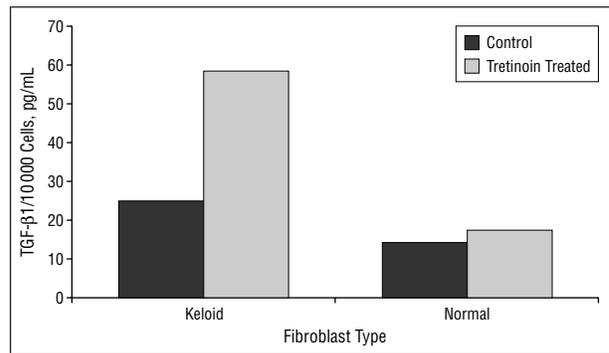

Figure 2. *Comparison of normal and keloid-producing dermal fibroblast mean secretion of transforming growth factor-β1 (TGF-β1) 120 hours after application of tretinoin (1×10^{-5} mol/L). Keloid fibroblasts show sensitivity to tretinoin treatment ($P<.05$), whereas normal dermal fibroblasts do not.*

as a result of GHK-Cu application.[12] Clinical studies[13,14] have demonstrated the efficacy of copper tripeptide preparations in facilitating an increased rate of wound healing in diabetic ulcers and in patients who have undergone Mohs surgery. Additional cosmetic uses of copper tripeptide complex are currently being researched.

Tretinoin is frequently prescribed for its collagen-tightening effects and is useful in the treatment of a variety of skin conditions, including wrinkling, acne vulgaris, photoaging, early stretch marks, and hyperkeratosis.[15,16] Several studies[17-19] have also demonstrated the possible antitumor effects of tretinoin in the treatment of basal cell carcinoma, dysplastic nevi, and cutaneous malignant melanoma. The dermal tightening effect of tretinoin in the treatment of photodamaged skin has been well studied and its histologic effects described.[20] Central to the clinical effects of reduced wrinkles and skin roughness is the partial restoration of the facility of normal skin cells to produce collagen. Retinoids, however, also have been shown to decrease the amount of collagen produced in KF cultures, and results of clinical trials[21,22] indicate that topical treatment with retinoic acid (0.05%) reduces keloid size in most cases.

Cell culture–based research is an effective means of studying wound healing at the cellular level because it offers a controlled environment. The presence of serum in the culture medium has long been a necessary component of this model because it allows for sustained cell growth. The presence of serum components, however, hinders any experiment seeking to accurately measure growth factor production by the cells themselves. Use of a serum-free cell culture model addresses this shortcoming because it allows for a controlled environment in which the growth factor–secreting effects of potential wound-healing modulators can be evaluated without confounding effects from serum. The Wound Healing and Tissue Engineering Laboratory of Stanford University Medical Center, Stanford, Calif, has developed a fibroblast in vitro model that uses serum-free growth medium.[23]

The purpose of this study was to grow NFs and KFs in a serum-free model, treat them with GHK-Cu or tretinoin, and assess the cellular response in terms of cell viability and autocrine growth factor production. An attempt is made to characterize the growth factor profiles of KFs and NFs based on these 2 treatment modalities.

RESULTS

All cell lines grew in the modulated, serum-free environment, with cell viability ranging from 86% to 96% (mean, 92%). Differential growth factor secretion patterns were observed and are described in the following 2 subsections.

bFGF SECRETION

Levels of bFGF measured in supernatant samples peaked at 24 hours and progressively declined throughout the experiment for KFs and NFs, with no significant difference between the 2 cell types. Greater concentrations of bFGF were detected in samples of NFs treated with tretinoin than in controls at 24 hours (mean, 19.6 and 8.0 pg/mL, respectively; $P<.05$) (**Figure 1**). No differential secretion pattern was observed in NFs treated with GHK-Cu. Similarly, KFs treated with either modulator did not demonstrate any trend in bFGF secretion compared with controls.

TGF-β1 SECRETION

Samples obtained from KFs had higher levels of TGF-β1 than did those from NFs in control and tretinoin-treated groups at 120 hours (not statistically significant) (**Figure 2**). Tretinoin-treated KFs also secreted more TGF-β1 than did controls at 120 hours (mean, 58.5 and 24.6 pg/mL, respectively; $P<.05$), whereas NFs showed no such sensitivity to tretinoin in terms of TGF-β1 concentrations (Figure 2). This pattern was consistent for each keloid specimen (**Figure 3**). Both fibroblast types treated with GHK-Cu secreted less TGF-β1 than did controls, although this was significant only for KFs at 24 hours (mean, 29.9 and 17.7 pg/mL, respectively; $P<.05$) (**Figure 4**). This pattern was consistent for each keloid specimen (**Figure 5**).

COMMENT

Cell culture has long been a primary means toward understanding the activity of human dermal fibroblasts—normal or keloid. It has often proved an especially useful way of differentiating the behavior of these 2 cell types
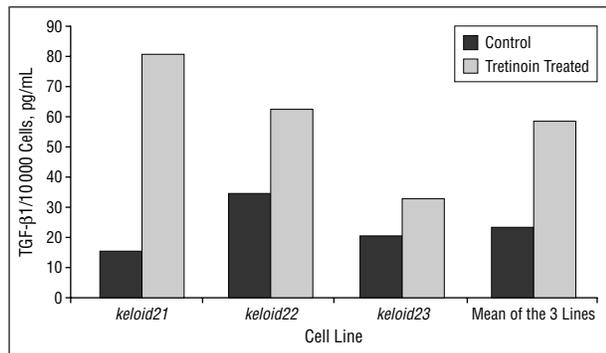
Figure 3. *Secretion of transforming growth factor-β1 (TGF-β1) by keloid-producing fibroblasts 120 hours after application of tretinoin (1×10^{-5} mol/L). In each cell line, treated cells secreted more TGF-β1 than did controls (mean comparison, $P<.05$).*

Figure 4. *Comparison of normal and keloid-producing dermal fibroblast mean secretion of transforming growth factor-β1 (TGF-β1) 24 hours after application of glycyl-L-histidyl-L-lysine-Cu²⁺ (GHK-Cu) (1×10^{-9} mol/L). Although both cell types show sensitivity to GHK-Cu treatment, only keloid fibroblasts demonstrated statistically significant reductions ($P<.05$).*

Figure 5. *Secretion of transforming growth factor-β1 (TGF-β1) by keloid-producing fibroblasts 24 hours after application of glycyl-L-histidyl-L-lysine-Cu²⁺ (GHK-Cu) (1×10^{-9} mol/L). In each cell line, treated cells secreted less TGF-β1 than did controls (mean comparison, $P<.05$).*

as they relate to the wound-healing environment. Keloid fibroblasts, eg, have been shown in vitro to produce more collagen than their normal dermal counterparts. Although many studies have demonstrated differences by measuring specific cellular proteins such as collagen, rarely has a model specifically focused on the growth factors that trigger their production. This is largely because of the difficulty in controlling for growth factor levels in a serum-containing cell culture model, traditionally used to maintain cell growth. Our study demonstrated that NFs treated with tretinoin produce more bFGF than do controls, whereas NFs and KFs treated with copper tripeptide secrete less TGF-β1 than do controls. Each result suggests a correlation between growth factor production and known clinical effects.

Only recently have studies[23] established KF and NF cell lines in serum-free media. The present study demonstrates the viability of such cells in a serum-free model and assayed for growth factors known to figure prominently in the wound-healing process. Such methods have been adopted previously, although serum-free media have not been used past the incubation phase of cell culture.[7] A potential disadvantage of serum-free media is that fibroblast proliferative characteristics and viability are generally not as good as with serum-based models. In short-term culture, however, the medium used in our experiments supported similar growth characteristics and comparable cell viability to that of serum-based models of similar experiment duration.[25]

Altering the wound environment through chemical modulators, as demonstrated in the present study, might provide insight as to the link between proven clinical applications and the induced cellular response. The mechanism by which tretinoin exerts its cellular effects is linked to the retinoinc acid receptors, discovered in 1987.[26] Tretinoin binds these intracellular receptors—similar in makeup and function to steroid/thyroid hormone receptors—which in turn bind regulatory regions of cellular DNA, causing activation of gene transcription. Several of these target sequences are contained by genes that have been shown to be markers of tretinoin stimulation, including cellular retinoic acid binding protein. By directly stimulating the transcriptional machinery of the cell, tretinoin is able to modulate the production of proteins central to cell growth and differentiation.
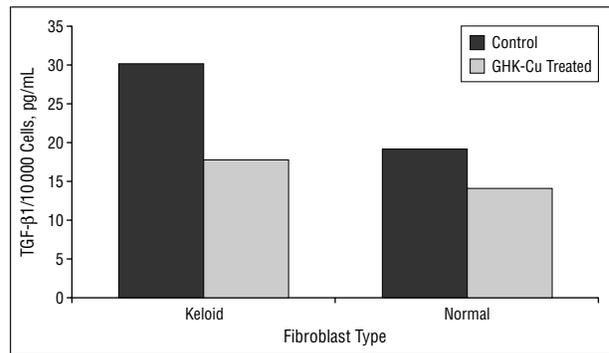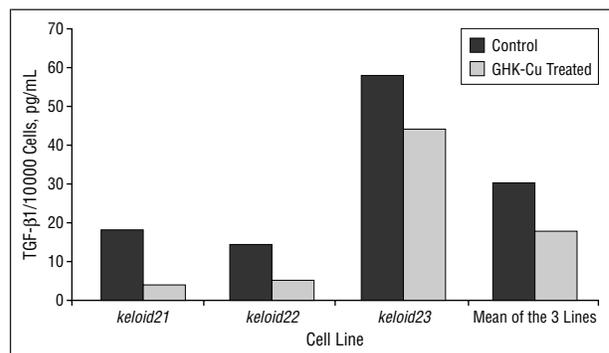
These proteins might then initiate a cascade effect whereby other DNA segments are transcribed, including those coding for ECM proteins, and thus account for the ultimate clinical effect of tretinoin.

In the present study, retinoic acid seems to stimulate secretion of "collagen tightening" growth factor (bFGF) by NFs. This may partially explain its known clinical utility. That maximal levels of bFGF were observed at 24 hours in all cell lines is consistent with the half-life of bFGF (25 hours).[27] The application of tretinoin initially stimulated fibroblasts to produce bFGF. Levels then gradually declined over the course of the experiment as the bFGF degraded. Although some clinical studies have shown topical tretinoin use to reduce the size of keloid scars, our data suggest a mechanism other than modulation of TGF-β1, as keloid cell lines secreted more of this growth factor than did controls.

Treatment with GHK-Cu stimulates glycosaminoglycan and collagen production in human fibroblasts, critical to the postinflammatory phase of wound healing. More recently it has been postulated that GHK-Cu stimulates specific matrix metalloproteinases.[28] Other studies[29,30] have demonstrated that in addition to its direct wound-healing effects, GHK-Cu enables angiogenesis and leukocyte chemoattraction. The exact mechanism whereby copper tripeptides alter cellular activities has yet to be worked out, despite such observed phenomenon. Modu-

lation of local growth factor production at the site of active wound healing might be involved in these processes.

As already described,[7] KFs produce more TGF-β1 than do NFs in culture, and our data reinforce this property. In the present study, copper tripeptide therapy seems to suppress secretion of "fibrogenic" growth factor (TGF-β1) in NFs and especially in KFs, and it may have application in decreasing excess scar formation.

Because of the many antagonisms of growth factor activities, it may be possible to correct a deficiency or overabundance with local application of another factor that modulates the wound cells' growth factor production profile. Once a modulator's (or combination thereof) autocrine growth factor stimulatory properties are known, it could be placed into a wound to achieve the desired healing response. Routine wound application of recombinant-produced or autologous-derived growth factors would be expensive. Using obtainable modulators such as tretinoin and copper tripeptide as cytokine stimulators would circumvent this problem.

In the larger scheme, using cytokine manipulations to vary the makeup of ECM components (such as collagen) might have a great impact in precisely controlling the wound-healing process. For example, if a person with diabetes has a nonhealing ulcer, the wound could be treated with a modulator that stimulates production of a fibrosis-producing growth factor. The appearance of the wound in this case is not as important as closure by scar tissue. Also, if an irradiated patient has an open wound because of poor tissue blood supply, the wound could be treated with a modulator that stimulates production of an angiogenic growth factor, which will cause local development of blood vessels. Finally, anyone undergoing surgery may benefit from wound treatment with a modulator causing production of a growth factor that causes an increase of collagen with tighter bundles, thus forming a smaller yet stronger scar.

In summary, the results of our study demonstrate that NFs treated with retinoic acid produce more bFGF than do controls, and this might partially explain the clinically observed tightening effects of tretinoin. Both NFs and KFs treated with copper tripeptide secreted less TGF-β1 than did controls, and this suggests possible clinical use for decreasing excessive scar and keloid formation.

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