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Enhancement of Collagen Production in Skin Cells Via Cell Communication Signals

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Abstract

As people age, so does their skin, which becomes apparent as fine and deep wrinkles. Wrinkling is caused by the degradation and reduced synthesis of collagen. In order to overcome the effects of aging on appearance, people often turn to cosmetic intervention. One common treatment for wrinkling is to use collagen fillers to expand the skin. Collagen injection is, however, invasive and may result in pain, bruising, redness, allergic reactions, swelling, lumpiness, and asymmetry. A less intrusive method with fewer risks would be to stimulate collagen production in the patient's own skin cells. It's known that collagen production is stimulated through the activation of communication pathways initiated by transforming growth factor-beta (TGF-β) proteins. However, which other proteins may enhance collagen production is unclear. Here we test the hypothesis that collagen production will be enhanced by the activation of TGF-β signaling when combined with other signaling pathways. In mouse fibroblast cells we altered signaling pathways to test the activation and blockade of TGF-β signaling with and without the activation or blockade of an additional pathway, the p38 kinase cascade, and assessed the effects on collagen production. Our results indicate that TGF-β activation enhances collagen production compared to controls. This effect is reversed by the blockade of TGF-β signaling. We are now testing the effects of activation and blockade of the p38 kinase cascade independently and in conjunction with TGF-β activation. Our results will determine if these combined pathways can enhance collagen stimulation and perhaps provide an alternative to collagen fillers to combat wrinkling.

Introduction

As people age, so does their skin, which becomes apparent due to hallmark changes in appearance. When young, the skin is typically plump, smooth, and wrinkle-free; however, fine and deep wrinkles are present in aged skin. In order to overcome the effects of aging on appearance, people often turn to cosmetic intervention. The most common cosmetic interventions performed to combat wrinkling include the use of botulinum toxin and soft tissue fillers. Botulinum toxin, or Botox, is injected into the face to treat typically fine wrinkles through paralysis of small muscle groups of the face, which provides a youthful appearance (Helfrich et. al., 2008). Soft tissue fillers are typically used to treat deep wrinkling in the face. These fillers are injected to volumize and expand the skin, making wrinkle lines less visible. Both applications, however, come with certain risks. Botox may cause bruising, temporary eyelid and eyebrow drooping, and temporary facial asymmetry while injectable fillers may result in bruising, redness, allergic reactions, swelling, lumpiness, and asymmetry (Currie, 2007). The effects of Botox and fillers are typically temporary and will fade any time ranging from three months to about a year, thus requiring reinjections. Fillers and Botox are a costly, temporary solution that comes with a variety of risks. A less intrusive method to combat wrinkling with fewer risks would be to stimulate the cells in the region of wrinkling to produce more collagen intracellularly.

The human skin is made up of three layers: the epidermis, the dermis, and the subcutaneous layer. The epidermis is the top layer on the outside, the dermis is the middle layer, and the subcutaneous layer is the inside, third layer. Collagen, found in the dermal layer of the skin, is one of the main building blocks of the human skin, providing much of the skin's strength (Helfrich et. al., 2008). The youthful appearance of young skin is due to extracellular matrix proteins, including collagen. Increased breakdown and decreased synthesis of collagen is the cornerstone of aging, creating overall lower levels of collagen in the dermis.

There are ways to stimulate the production of collagen in the extracellular matrix through the use of cell signaling, a form of cell communication. Cell signaling is an intricate system of interactions and communications that provide the cell with information regarding its environment. The processing of this information dictates activities and actions within a cell. One mechanism to stimulate collagen production in the extracellular matrix involves the signal transduction pathway of a regulatory protein from the transforming growth factor-beta (TGF-β) superfamily. Specifically, the TGF-β1 isoform causes an increase in collagen formation in the extracellular matrix. TGF-β1 increases the levels of fibronectin and type I collagen expressed in order to control the composition and abundance of the extracellular matrix (Ignotz et. al., 1987). Similarly, it has been shown that TGF-β1 is a strong and rapid inducer of several genes coding for extracellular matrix components, such as type I collagen, by stimulating transcription of the human α2(I) collagen gene promoter (Inagaki et. al., 1994). TGF-β1 also has a strong influence on proliferation, differentiation, and expression in cells (Ignotz and Massagué, 1986). TGF-β1 not only increases collagen levels and collagen incorporation into the extracellular matrix, but also inhibits matrix degradation. TGF-β1 stimulates collagen production via a complex signal transduction pathway. However, it's likely that TGF-β1signaling does not act alone to invoke collagen production. It is hypothesized that collagen production will be enhanced by the activation of TGF-β signaling when combined with other signaling pathways.

In order to test this hypothesis, I first needed to establish a model system to gauge collagen production. Here I describe an *in vitro* system where I established methods to activate TGF-β signaling and tested whether my system was adequate to enhance collagen production and hence achieve my experimental aims.

Materials and Methods

Cell Culture

The cell line NIH/3T3 (mouse fibroblast cells; ATCC) was used to test how i) the activation and ii) the blockade of TGF-β

signaling affect the production of collagen. NIH/3T3 cells were grown in Dulbeco's Modified Eagles Medium (DMEM) with 10% Fetal Calf Serum (FCS). Cells were incubated and split about every three days, when the cells were subconfluent. After splitting, the amount of FCS was decreased in a step-wise manner from 10% to 7.5%, 5%, and then 2.5% to decrease the effect of serum on our signaling results. For each experiment, cells were plated in a 24-well plate with 1 x 10^5 cells per well, totaling 500 µl of cells and media in each well.

TGF-β Signal Activation

A purified TGF-β protein (R&D Systems), hereafter termed TGF-β ligand, was used to activate TGF-β signaling. A TGF-β ligand titer was performed to determine the concentration range that would cause an effect on the cells, such as a change in cell morphology. One, 2.5 and 10 µg/mL of the TGF-β ligand were independently added to cells. As determined by the TGF-β titer, 10 µg/mL of the TGF-β ligand was shown to cause a consistent change in morphology and was added to NIH/3T3 cells for all subsequent experiments testing for collagen production (see below). For comparison and to establish a baseline, a control set of cells received no treatment.

The amount of collagen produced was assessed using Western Blots, a quantitative measure for protein. The amounts of collagen produced in the TGF-β ligand treatments were directly compared to the amount of collagen in the control cells –the baseline.

Western Blotting

Cells removed from the plate using trypsin, were transferred to Eppendorf tubes, and centrifuged at 1200 rpm for 8 minutes. Media was removed and cells were lysed in radioimmunoprecipitation (RIPA) buffer (0.05M Tris, 0.15M NaCl, 5mM EDTA, 1% NP40, 0.5% sodium-deoxycholate, 10% SDS; pH 7.4). Total protein concentrations were determined with a protein assay. Eight percent polyacrylamide gels were loaded with 20 µg of protein per sample and run at 50 volts for 15 minutes, 80 volts for 10 minutes, and 100 volts for 1 hour. Gels were transferred onto PVDF membranes using a semi-dry transfer solution (0.048M Tris, 0.039M glycine, 10% SDS, 20% methanol). The membrane was incubated overnight with Collagen I antibody (EMD Millipore; #AB765P) and β-actin antibody (1:1000) at 4°C. The antibodies were detected with species-specific horseradish peroxidase-labeled secondary antibody (0.5 µg/ml). ImmPACT VIP Substrate Kit (Vector Laboratories) was used to obtain a colorimetric reaction indicative of protein quantity. Bands of interest were normalized with β-actin protein as a loading control.

Band Quantification

Band quantification was performed using ImageJ software. The Western blot was scanned and converted into a jpeg. In ImageJ, a single region of interest was defined around the largest band with a frame. The frame was moved to each band and a measurement was recorded. A background measurement was taken with the frame. The pixel density for all data was inverted by subtracting the measurement from 255, the standard blank background reading. Net value was determined by subtracting the inverted background from the inverted band values. A ratio of net band value to net loading control value within each lane was calculated.

A brief outline of the experimental procedures is shown in Fig. 1.

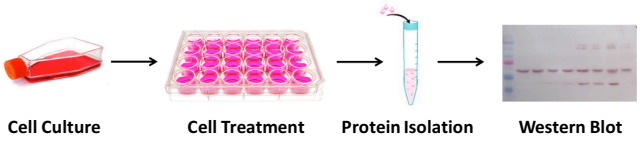

Figure 1. Methods for the growth, isolation, and quantification of collagen protein. See description in text.

Results

The main goal of my experiments were to determine if TGF-β ligand treatment singly or in combination with other signaling pathways, could increase the production of collagen from mouse fibroblast cells. First I tested if the TGF-β ligand was biologically active using NIH/3T3 fibroblast cells. This was done by assessing changes in cell morphology and cell number,

compared to untreated controls, and correlating these to the amount of collagen produced.

Control NIH/3T3 fibroblast cells, lacking TGF-β ligand treatment (0 ng/ml), were flat and triangular in shape (Fig 2A). Cells treated with 1 ng/ml of the TGF-β ligand had a similar morphology to the control cells. The lowest concentration of TGF-β to provide a change in cell morphology was 2.5 ng/ml (Fig 2A). At 2.5 ng/ml of TGF-β, cells appeared more linear and elongated and were tightly packed together. A similar cell morphology was seen with 10 ng/mL of TGF-β, creating a meshwork pattern due to the numerous cells in the dish (Fig 2A).

To determine the effect of TGF-β on cell number, we counted the total number of cells within each treatment, from 0 to 10 ng/ml TGF-β. The average number of control cells (0 ng/ml TGF-β) was $9.8x10^4$ cells per dish (Fig 2B). The average number of cells treated with 1, 2.5, and 10 ng/mL of TGF-b was $2.5x10^5$, $3.2x10^5$ cells, and $5.0x10^5$ cells, respectively (Fig 3B). These data indicate that compared to the controls, as the concentration of TGF-β ligand increased, the average number of cells also increased approximately 5-fold, from 0 to 10 ng/ml TGF-β.

We wanted to determine if TGF-β treatments could enhance the production of collagen from NIH/3T3 fibroblasts. A Western Blot was performed to quantitate the amount of collagen produced by cells treated with the different concentrations of TGF-β ligand (Figure 2C). To determine the amount of collagen produced for each condition, a ratio of collagen protein compared to the β-actin loading control, as quantitated by band intensity, was calculated for each sample. Controls showed that the basal level of collagen isolated from the cells was 0; there were faint or no collagen bands detected (Fig 2C). The Western Blot shows that at the higher TGF-β ligand concentrations, 2.5 and 10 ng/ml, collagen bands of approximately 200 and 140 kDa were visible (Fig 2C). The ratio of collagen vs β-Actin, relative to controls, was 0.36 and 0.22 for 2.5 ng/ml treatments and 0.41 for the 10 ng/ml treatment (Fig 2C). These results indicate that higher concentrations of TGF-β ligand produce more collagen in NIH/3T3 cells.

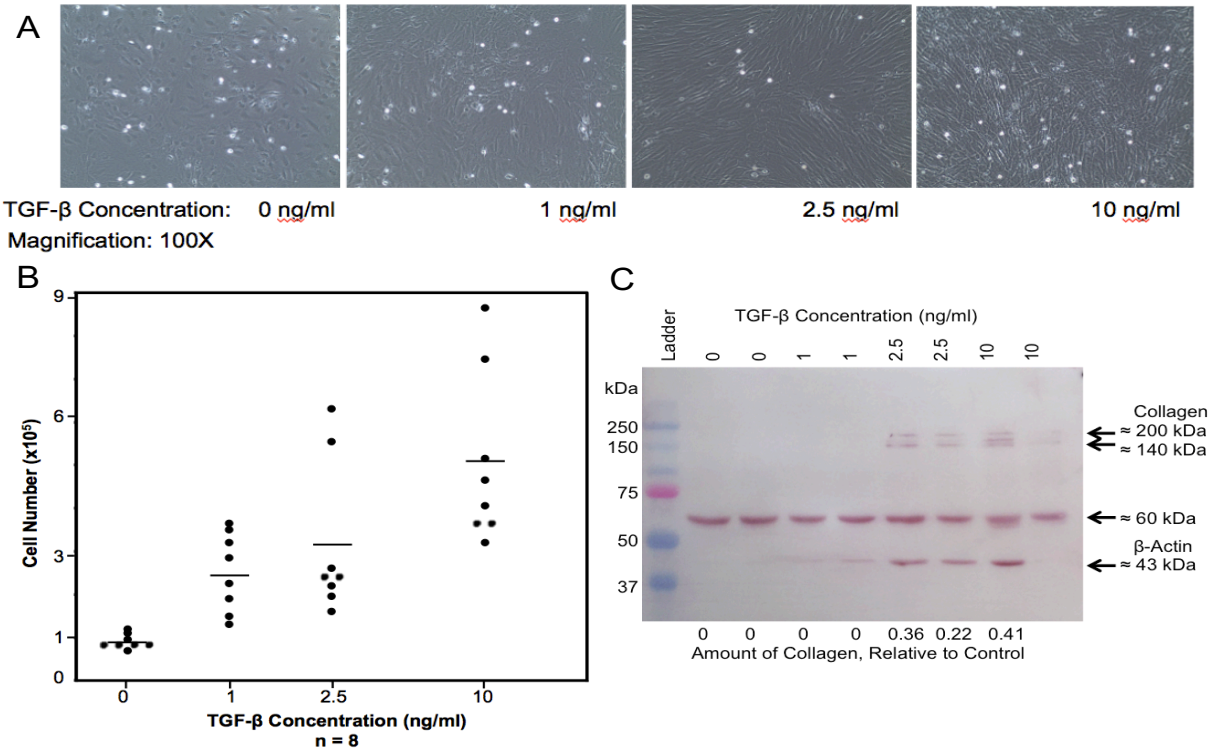

Figure 2. Morphology, cell number, and protein analysis of NIH/3T3 mouse fibroblast cells treated with varying concentrations of TGF-β ligand. (**A**) Cells were plated in a 24-well plate with $1x10^5$ cells per well. One, 2.5, and 10 ng/ml of TGF-β ligand were independently added to the cells and incubated for 3 days in DMEM with 2.5% FCS.

Control cells received no TGF-β treatment (0 ng/ml). Images of cells were taken at 100X magnification under a light microscope. (**B**) Total cell numbers for each treatment were quantitated with a hemocytometer. Every symbol represents the results from a single well. The horizontal lines indicate the average cell number for each treatment, from 8 independent experiments (n=8). (**C**) A Western blot was used to analyze proteins. Collagen protein represented by the double bands was detected at about 200 and 140 kDa. The β-Actin protein was used as a protein loading control for each sample. β-Actin protein was present at 43 kDa. A band around 60 kDa was apparent that was neither TGF-β nor β-Actin. This band is an unknown protein that was not quantitated during our analysis. Control and 1 ng/ml treatments yielded no detectable collagen. The relative amount of collagen vs β-Actin for each TGF-β treatment was 0.36 and 0.22 for 2.5 ng/ml and 0.41 for 10 ng/ml.

Discussion

In order to test the hypothesis that collagen production would be enhanced by the activation of TGF-β signaling when combined with other signaling pathways, I first needed to establish a model system to gauge collagen production. I established an *in vitro* cellular system where I activated TGF-β signaling and tested whether TGF-β activation was adequate to enhance collagen production.

I assessed changes in cell morphology and cell number to determine if my TGF-β ligand was biologically active. The change in morphology as well as the increase in average cell number as the concentration of TGF-β ligand increased showed that the protein was active and working appropriately (Fig 3). Ten ng/ml of TGF-β ligand had the greatest effect on the cells, as it yielded a larger average cell number and greater morphological change compare to 2.5 or 1 ng/ml treatments. It was determined that 10 ng/ml of TGF-β ligand would be used to stimulate TGF-β signaling for future experiments because it not only caused the greatest change, but was also a small enough concentration to allow for multiple replicates without being too expensive. I have now tested a TGF-β blocker, SIS3, for its biological activity and have determined the concentration required to alter cell morphology without causing a high level of cell death (data not shown). Perhaps NIH/3T3 cells require low levels of TGF-β signaling to avoid cell death.

With the *in vitro* system established, I tested if TGF-β signaling had an effect on collagen production. Western blots showed an increase in collagen production with increasing TGF-β ligand concentration. However, in some lanes detecting β-actin protein on the Western blot was difficult. This could be due to an incomplete transfer of proteins from the gel and onto the membrane prior to the Western blot. Probing the membrane for both collagen and actin at the same time could have caused this lack of β-actin detection as well. In future experiments, I will test different transfer methods and probe the protein separately to see if β-actin detection is more consistent.

With this system established for TGF-β signaling, I can test my hypothesis that collagen production will be enhanced by the combination of signaling pathways when combined with TGF-β, rather than with TGF-β signaling alone. Additional supporting experiments will test the effects of the activation and blockade of an additional pathway, the p38 kinase cascade, independently and in conjunction with TGF-β activation. At the end of my experiments, I will know which combinations of signals will best increase collagen production in NIH/3T3 cells. Future results will determine if combined pathways can enhance collagen stimulation and perhaps provide an alternative to collagen fillers to combat wrinkling in aging skin.

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