Interferon-γ Inhibits Collagen Synthesis by Human Tenon's Capsule Fibroblasts In Vitro

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Collagen deposition is largely responsible for scar formation, an undesirable outcome of glaucoma filtering surgery. Because interferon-γ (IFN-γ) has been reported to inhibit collagen synthesis in a variety of cells, the authors examined its effect on collagen synthesis by Tenon's capsule fibroblasts cultured from three patients. In addition, the authors studied the effects of IFN-γ on fibroblast proliferation. IFN-γ inhibited collagen synthesis at dosages of 1–10,000 U/ml. The average median effective dose (ED<sub>50</sub>) was 6.60 U/ml. In contrast, IFN-γ had no significant effect on cell proliferation in dosages of 0.001–10,000 U/ml. These results suggest that IFN-γ may be a useful agent for modifying the wound healing response, particularly after glaucoma filtering surgery. Invest Ophthalmol Vis Sci 32:2964–2969, 1991

Excessive wound healing commonly causes failure of glaucoma filtering surgery. Histopathological studies of eyes after failed glaucoma filtering procedures show that the proliferation of fibroblasts and concomitant deposition of collagen eventually results in wound contraction and scar formation. Because the process of wound healing is composed of a complex cascade of interrelated events, it can be modulated by pharmacological intervention at various stages. A myriad of drugs have been demonstrated to inhibit important events in wound healing. For example, low doses of colchicine were found to inhibit fibroblast migration and proliferation in vitro. Both dexamethasone and triamcinolone acetate at low concentrations stimulate fibroblast proliferation; at high concentrations these agents inhibit fibroblast proliferation. In addition, the anticoagulant heparin, when administered with cortisone, inhibits angiogenesis. The antineoplastic agent 5-fluorouracil helps to improve the success of glaucoma filtering surgery in monkeys when injected subconjunctivally, and in rabbits when administered via bioerodible polymers. Currently, it is the only drug commonly used in patients at high risk for failure after glaucoma filtering surgery. Unfortunately, its use subconjunctivally frequently is associated with toxic side effects, such as corneal epithelial defects and conjunctival wound and needle track leaks.

In our search for a less-toxic alternative, we considered interferon-γ (IFN-γ), which has been shown to inhibit collagen synthesis in vitro in a variety of cell types. As a potent lymphokine, IFN-γ may be able to inhibit collagen synthesis by ocular fibroblasts in vitro at relatively low doses, thus minimizing adverse effects. To test this hypothesis, we investigated the effect of various doses of IFN-γ on collagen synthesis relative to total protein synthesis in Tenon's capsule fibroblasts from three different human cell lines.

Materials and Methods

Cell Culture

Tenon's capsule specimens were obtained from three ocular surgery cases with the approval of the UCLA Human Subject Protection Committee. Patient ages were 36, 61, and 82 yr. The tissues were processed within 12 hr after surgery by rinsing thoroughly in phosphate buffered saline (PBS) (Dulbecco's without magnesium and calcium, Flow Laboratories, McLean, VA), mincing into 1-mm cubes, and plating in 35-mm diameter tissue culture dishes (Falcon tissue culture, Becton Dickinson, Lincoln Park, NJ). A few drops of Minimal Essential Media (MEM) (Flow Laboratories) supplemented with 15% fetal bovine serum (FBS) (Dulbecco's without magnesium and calcium, Flow Laboratories, McLean, VA), mincing into 1-mm cubes, and plating in 35-mm diameter tissue culture dishes (Falcon tissue culture, Becton Dickinson, Lincoln Park, NJ). A few drops of Minimal Essential Media (MEM) (Flow Laboratories) supplemented with 15% fetal bovine serum (FBS), 100 IU/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B were placed over the tissue, which was then covered with a coverslip and an additional 2 ml of antibiotic and serum-containing MEM. This antibiotic combination has been shown to have no net effect on cell
growth in tissue culture. The dishes were then placed in a humidified 5% CO₂ incubator at 37°C. Fibroblasts usually appeared within 3–7 days and reached confluence within 2–4 weeks. The confluent cultures were passaged by washing with PBS, detaching with 0.05% trypsin (Sigma Chemical Co., St. Louis, MO) at 37°C for 10 min, adding MEM containing 10% FBS to stop trypsinization, centrifuging the cell suspension at 1,000 rpm, and transferring the pellet resuspended in 10% FBS-supplemented MEM into three 75-cm² flasks. The cell lines were passaged after becoming confluent and maintained in 150-cm² flasks with MEM containing 10% FBS.

**Gamma-Interferon Treatment**

Freshly trypsinized fibroblasts at the seventh or eighth passage were plated in 16-mm well diameter 24-well plates (Costar, Cambridge, MA) at a density of 30,000 cells per well. Viable cell number was quantified by staining with trypan blue and counting with a hemocytometer. Cells were incubated with 1 ml of MEM supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere. Seven days after plating, cells were treated for the first 24 hr with 1 ml of fresh 10% FBS-supplemented MEM containing appropriate concentrations of recombinant human IFN-γ (Genzyme, Boston, MA). All treatments and controls were done in triplicate wells for each experiment. Three experiments were performed using three different cell lines.

**Collagen Synthesis**

Collagen synthesis was determined using a modified version of the method described by Diegelmann et al. After exposure to IFN-γ for 24 hr, the cells were pulsed with MEM containing 5% serum, 40 μCi/ml [³H]-proline (26 Ci/mmol, Amersham, Arlington Heights, IL), 50 μg/ml ascorbic acid, and various concentrations of IFN-γ. The cells were incubated for 30–34 hr in 5% CO₂ at 37°C. The pulse was terminated by heating the plate for 10 min at 90°C and sonicating for 30 sec per well at 20% power (Biosonik III, Rochester, NY). One-fifth of a milliliter of the cell homogenate was removed from each well, and 1 ml of a 1 mg/ml aqueous bovine serum albumin solution was mixed in by pipetting in and out to provide carrier protein. Total protein was precipitated with approximately 5% trichloroacetic acid (TCA) and washed six times by repeated centrifugation at 2,000 rpm (Beckman Model J-6B, Palo Alto, CA), aspiration of the supernatant, and precipitation with

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**Fig. 1.** Schematic of a modified version of the collagen synthesis microassay published by Diegelmann et al.²⁰
10% TCA in a 1-mM solution of cold L-proline (Sigma, St. Louis, MO).

Trichloroacetic acid was extracted by addition of cold ethanol:ethyl ether (3:1) after the last centrifugation, and the dried protein was incubated in 0.5 ml of an incubation buffer containing 60 μmol Hepes buffer (pH 7.2), 1.25 μmol N-ethylmaleimide, and 0.25 μmol CaCl₂ for 4 hr in a shaking water bath (80 cycles/min) at 37°C. Next, the protein suspensions were precipitated with 0.5 ml of a 5% TCA solution followed by 0.5 ml of a 10% TCA solution, and the supernatants were saved as the incubation blank. Total protein was again freed of TCA and dried. This was digested with 10 vial units of bacterial collagenase Form III (Advanced Biofractures, Lynbrook, NY) in 0.5 ml incubation buffer under the same conditions as described for the incubation blank.

Using this collagenase, an incubation time of 4 hr was demonstrated to be optimal for complete collagen digestion. Noncollagen protein (NCP) was precipitated in 5% and 10% TCA solutions, and the supernatants were saved as the collagenase-digestible protein (CDP). The pellets were dissolved with 0.5 ml of 0.2 N NaOH followed by 1 ml of 0.2 N HCl and saved as the NCP fraction. All manipulations after sonication were performed at 4°C. Aliquots of each incubation blank, collagen fraction, and noncollagen fraction were placed in miniature polyethylene scintillation vials (Packard, Downers Grove, IL) with Ready Protein scintillation cocktail (Beckman, Palo Alto, CA) in a 1:7 dilution, vortexed, and placed in total darkness overnight to minimize chemiluminescence. Radioactivity (disintegrations per minute) in each vial was counted for 1 min the next day using a Packard 1900 CA Tri-Carb liquid scintillation analyzer.

For each well, percent collagen synthesized relative to total protein was calculated with an equation similar to one used to assay chick embryo collagen synthesis.

\[
\frac{\text{net CDP total DPM}}{\text{net CDP total DPM} + (\text{NCP total DPM} \times 4.0)} \times 100
\]

The coefficient of 4.0 was derived from the known amino acid compositions of various human collagen and noncollagen proteins. In human collagen, proline and hydroxyproline constitute approximately 21.8% of the total amino acid residues, whereas noncollagen proteins contain an average of 5.5% proline and hydroxyproline. Therefore, human collagen contains approximately 4.0 times more proline and hydroxyproline than noncollagen protein.

Data points corresponding to any particular concentration of IFN-γ were plotted as percent adjusted control:

\[
\frac{X \% \text{ collagen synthesis} - \text{minimum } X \% \text{ collagen synthesis}}{\text{control } \% \text{ collagen synthesis} - \text{minimum } \% \text{ collagen synthesis}} \times 100
\]

Fibroblast Proliferation

The effect of a 3-day incubation with 0.001–10,000 U/ml of IFN-γ on Tenon’s capsule fibroblast proliferation was determined in a separate set of experiments. This was performed according to the method described by Givens et al using a different human cell line and a colorimetric assay that relates cell number to cytoplasmic hexosaminidase activity.

Statistics

Dose-response curves were compared by two-way analysis of variance (ANOVA), and the average median effective dose (ED₅₀), standard error (SE), and 95% confidence intervals were estimated by the interpolation method described by Finney. F ratios were calculated for two parameters: curve and dose. The accepted level of statistically significant difference between parameters was a corresponding P value of less than 0.05. The power of comparison was calculated for any corresponding P value of greater than 0.05.

Results

Interferon-γ caused collagen synthesis to decrease in a dose-response manner. At the maximal tested doses, collagen synthesis inhibition ranged from 40% to 54% relative to controls. The dose-response curves are shown in Figure 2, and the corresponding ED₅₀s and 95% confidence intervals are summarized in Table 1.

Dose-dependent inhibition of collagen synthesis in all three experiments was confirmed by dose-parameter F ratios corresponding to P values of less than 0.05. However, experiments 2 and 3 (donor ages 36 and 61 yr) differed significantly from experiment 1 (donor age 82 yr) when curve parameters were compared. Experiments 2 and 3 did not differ significantly from each other in this parameter, and their power of comparison was 0.212. The average ED₅₀ for the three experiments was 6.60 U/ml, with an overall 95% confidence interval between 1 and 53 U/ml.

Interferon-γ had no significant effect on fibroblast proliferation at any of the concentrations of IFN-γ tested (Fig. 3), and no toxicity was apparent microscopically.
Effect of gamma-interferon on collagen synthesis

![Graph showing the inhibitory effect of IFN-γ on collagen synthesis by three cell lines. Average SES = 10.6, 13.3, and 5.74 for experiments 1, 2, and 3, respectively.]

Discussion

Extracellular matrix collagens produced by fibroblasts are largely responsible for scar formation, an undesirable outcome of glaucoma filtration surgery. Using a recently developed microassay, we investigated the effect of a range of IFN-γ doses on collagen synthesis by Tenon’s capsule fibroblasts in culture. The advantages of using this procedure over other published protocols are many. Because all manipulations were performed in the original well, this microassay measured total collagen synthesis encompassing intracellular and both extracellular matrix and nonmatrix collagen, and eliminated experimental variability associated with transferring materials into different containers. In addition, it involved a 10-min heating step at 90°C, which terminated radiotracer uptake, destroyed proteolytic activity, and facilitated cell membrane disruption. In general, the protocol was well suited for our objectives. We modified the published procedure for our cell lines according to trial and error and communication with the authors. In our system, we found the presence of serum and a pulsing time beyond 5 hr necessary for adequate incorporation of [3H]-proline into cell proteins.

Human IFN-γ is a 40–50 kD glycoprotein primarily produced by mitogen- or antigen-stimulated T lymphocytes. It has antiviral and antitumor activity and is a potent immunomodulator. In concentrations as low as 1 U/ml, IFN-γ has been demonstrated to cause physiological effects, such as the induction of Class II surface antigen expression.

In agreement with others who studied the effect of IFN-γ on collagen synthesis by various cell types, we found an inhibitory effect at concentrations greater than 1 U/ml. By using fibroblasts from three patients, we also found that dose-dependent collagen synthesis inhibition by IFN-γ could be demonstrated in different patient cell lines. At the time of writing this manuscript, this is the first report of IFN-γ’s inhibitory effect on collagen synthesis by any type of ocular fibroblast. Analyses of variance reveal that curves of experiments 2 and 3 are not statistically different, although their power of comparison is relatively low. The curve of experiment 1 is significantly different from the curves of the other two experiments, reflecting a lesser sensitivity to IFN-γ’s inhibitory effect on collagen synthesis. However, small sample size (n = 3) precludes a meaningful analysis of variable cell line sensitivities to IFN-γ.

Table 1. Median effective doses of gamma-interferon on independent cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Donor age (yrs)</th>
<th>Surgical case</th>
<th>ED50 (U/ml)</th>
<th>(95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>Glaucoma</td>
<td>46.5</td>
<td>(41.4–52.3)</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>Glaucoma</td>
<td>3.01</td>
<td>(2.71–3.34)</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>Strabismus</td>
<td>2.05</td>
<td>(1.18–3.58)</td>
</tr>
</tbody>
</table>
In a variety of clinical studies, IFN-γ is used to treat patients with cancer. Commonly reported side effects after subcutaneous or intravenous administration include fever, chills, fatigue, and headache. Mild side effects are thought to be attributable to poor absorption of IFN-γ from tissue sites. Because a regional decrease in collagen deposition is desired after glaucoma filtration surgery, low-dose local IFN-γ delivered to the eye may avoid causing adverse systemic effects and inhibit postoperative scarring.

The ability to inhibit collagen synthesis by Tenon's capsule fibroblasts without affecting fibroblast proliferation implies potential clinical usefulness for IFN-γ as an adjunct to glaucoma filtration surgery. Being a physiological substance, IFN-γ may be a suitable alternative to 5-FU by inhibiting wound healing more specifically. In the eye, it may elicit its biological activities locally and efficaciously. If adverse side effects are minimal, IFN-γ may be a useful adjunct to glaucoma filtering surgeries with poor surgical prognoses, as well as the treatment of proliferative vitreoretinopathy and other ocular scarring disorders.

Key words: collagen synthesis, fibroblasts, interferon-γ

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References


