Relaxin Alone and in Conjunction with Interferon-γ Decreases Collagen Synthesis by Cultured Human Scleroderma Fibroblasts

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Fibroblasts derived from the involved skin of scleroderma patients frequently display a phenotype of supernormal collagen expression when cultured. Fibroblasts displaying this phenotype derived from seven patients were treated with relaxin (1–100 ng/ml) and interferon-γ (1-100 U/ml), individually and in combination, to assess the relative abilities of these cytokines to down-modulate collagen synthesis and secretion. Scleroderma fibroblasts displayed varying sensitivities to both relaxin and interferon-γ. Relaxin (100 ng/ml) decreased expression of collagen by six of seven lines tested from 8 to 59% compared to untreated cultures. Interferon-γ (100 U/ml) depressed collagen secretion by all seven lines in a range from 7 to 89%. When relaxin and interferon-γ were used in combination, relaxin augmented IFN-γ-induced decreases in collagen secretion in four of seven lines. In three of these lines, the use of relaxin in conjunction with suboptimal doses of interferon-γ resulted in decreases equivalent to or greater than that seen with a tenfold higher concentration of interferon-γ. This study demonstrates the ability of relaxin to directly alter the excessive collagen-producing phenotype of scleroderma fibroblasts. In addition, in some cases, combining relaxin and interferon-γ resulted in a cooperative effect in decreasing collagen expression by scleroderma cells in vitro. J Invest Dermatol 99:337–342, 1992

Connective tissue turnover is normally an orderly process in which the breakdown of extracellular matrix molecules is offset by the synthesis of new elements. This balance, which is critical to normal growth and development, is temporarily altered in response to traumatic or pathologic tissue injury to effect tissue repair. However, in certain pathologies, such as scleroderma (progressive systemic sclerosis), this balance is chronically skewed in the direction of matrix over-production, resulting in excessive fibrosis of the skin, gastrointestinal tract, and other organ systems [1]. Fibroblasts cultured from scleroderma lesions synthesize increased amounts of collagen [2,3], glycosaminoglycans [4], and fibronectin [4,5]. This unregulated expression of extracellular matrix molecules is thought to be responsible for much of the tissue and organ damage manifest in this disease.

We have previously shown that synthetic human relaxin can down-regulate the collagen secreting phenotype of normal human dermal fibroblasts, especially when the cells are stimulated to overproduce collagen, such as by treatment with the cytokines transforming growth factor-β (TGF-β) or interleukin-1β (IL-1β) [6]. This occurs in conjunction with a dose-dependent increase in the secretion of the metalloproteinase procollagenase and a small decrease in tissue inhibitor of metalloproteinases, suggesting that relaxin may be able to modulate connective tissue turnover in favor of net collagen breakdown in situations of collagen overexpression. Previously published work has shown that interferon-γ (IFN-γ) is a potent down-regulator of collagen expression in scleroderma [7], normal [8–10], and rheumatoid synovial [10] fibroblasts. These findings were the bases of the current work, in which scleroderma fibroblasts were treated with relaxin alone and in conjunction with IFN-γ to see: i) whether relaxin could down-regulate collagen secretion in these fibroblasts and ii) whether the combination of relaxin and IFN-γ could be as or more effective than either agent individually in effecting a significant decrease in collagen secretion.

METHODS

Cell Culture Scleroderma fibroblast cell lines were derived from full-thickness skin biopsy specimens from patients who fulfilled the American Rheumatism Association criteria for the diagnosis of scleroderma [11]. Paired skin biopsies from the uninvolved skin of patients were also available in some cases. Explant cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine until a confluent monolayer of fibroblasts had been established. Cultures were then expanded using a 1:3 split in DMEM-FBS until an adequate number of cells was obtained for experiments. Normal dermal fibroblasts obtained from explant culture of normal human skin obtained from rhytidectomy or from the American Type Culture Collection (ATCC CRL1471) were used between passages 1 and 7. Cells were counted at each passage using a hemocytometer to assess saturation densities of the different lines.
The presence of bacterial collagenase and IFN-γ, which are dependent on normal cell killing, cultures were subjected to infection with purified bacterial collagenase at 0.1 U/ml. Linearized, adherent cultures were digested with 7% trypsin and 4% EDTA. Supernatants were collected after 30 min. Parallel controls were set up in parallel cultures. Cells were collected by scraping and centrifuged at 1,200 X g for 10 min.

Procollagen expression by normal cultures of dermal fibroblasts was significantly greater than normal type III collagen isotypes. A densitometrically measured average of the cultures. Seven lines secreted 1.5-4.3 times more procollagen than normal cells on a per cell basis.

Production of procollagen bands by sensitivity to bacterial collagenase and IFN-γ quantification of procollagen expressed by normal and scleroderma cultures. (A) Collagenase- and IFN-γ-labeled proteins secreted by normal and scleroderma cells were scanned densitometrically. Densities were plotted such that the average amount of procollagen expressed by normal cells was equiva-

Procollagens. Procollagens were precipitated from both normal and scleroderma cultures. were scanned densitometrically. Densitometric values were normalized for differences in saturation density of the cultures. Seven lines secreted 1.5-4.3 times more procollagen than normal cells on a per cell basis.

Quantitation of procollagens expressed by normal and scleroderma lines. Cultures treated with relaxin and IFN-γ were precipitated and electrophoresed on a 7% polyacrylamide gel using a 3% stacking gel. Autoradiograms, which included procollagen, were hybridized with high molecular weight probes for types I and III procollagen and exposed to X-ray film (Kodak). The autoradiograms were scanned using the National Institutes of Health Image 1.23 h program on a Macintosh II computer (Apple Computers). The areas under the bands were measured, and the areas under the total bands were normalized for differences in lane density and quantified as the relative effects of relaxin and IFN-γ on the secretion of procollagens.

To assess the relative effects of relaxin and IFN-γ on the secretion of procollagens, cells were incubated with relaxin and IFN-γ. Collagen bands were detected on autoradiograms. The amount of protein secreted was calculated by integrating the areas under the bands. A densitometric scan of the autoradiograms was obtained using the National Institutes of Health Image 1.23 h program on a Macintosh II computer (Apple Computers). The areas under the bands were measured, and the areas under the total bands were normalized for differences in lane density and quantified as the relative effects of relaxin and IFN-γ on the secretion of procollagens.

For experiments, all cells were seeded at 6.25 X 10^4 cells/cm² in DMEM + FBS in 24-or 48-well plates. After 24 h, the cells were washed and the medium replaced with DMEM supplemented with 0.2% lactalbumin hydrolysate and 2 mM glutamine. Cells were treated with cytokines in this serum-free medium for 48 h. Radiolabeled proteins were tested for biosynthetic labeling and SDS-PAGE. For analysis of procollagens, cells were labeled with 3H-proline as described in the Materials and Methods section. Labeled procollagens were also labeled with [13C]proline as described in the Materials and Methods section. Labeled procollagens were also characterized according to migration on a 5% polyacrylamide gel following limited pepsin digestion and delayed electrophoresis on 7% polyacrylamide gel using 8.3% stacking gel. The gel was dried and exposed to X-ray film (Kodak). The autoradiograms were scanned using the National Institutes of Health Image 1.23 h program on a Macintosh II computer (Apple Computers). The areas under the bands were measured, and the areas under the total bands were normalized for differences in lane density and quantified as the relative effects of relaxin and IFN-γ on the secretion of procollagens.

Figure 1. (A) Identification of procollagen bands by sensitivity to bacterial collagenase and (B) quantification of procollagen expressed by normal and scleroderma cultures. (A) Collagenase- and IFN-γ-labeled proteins secreted by normal and scleroderma cultures were subjected to infection with purified bacterial collagenase at 0.1 U/ml. Linearized, adherent cultures were digested with 7% trypsin and 4% EDTA. Supernatants were collected after 30 min. Parallel controls were set up in parallel cultures. Cells were collected by scraping and centrifuged at 1,200 X g for 10 min. (B) Collagen bands were detected on autoradiograms. The amount of protein secreted was calculated by integrating the areas under the bands. A densitometric scan of the autoradiograms was obtained using the National Institutes of Health Image 1.23 h program on a Macintosh II computer (Apple Computers). The areas under the bands were measured, and the areas under the total bands were normalized for differences in lane density and quantified as the relative effects of relaxin and IFN-γ on the secretion of procollagens.

Figure 2. Relaxin dose-dependent decreases in procollagen expression by conditioned media. Collagenases were purified from the conditioned media of cells treated with relaxin and IFN-γ. Collagen bands were detected on autoradiograms. The amount of protein secreted was calculated by integrating the areas under the bands. A densitometric scan of the autoradiograms was obtained using the National Institutes of Health Image 1.23 h program on a Macintosh II computer (Apple Computers). The areas under the bands were measured, and the areas under the total bands were normalized for differences in lane density and quantified as the relative effects of relaxin and IFN-γ on the secretion of procollagens.
Table I. Responsiveness of Scleroderma Lines to Maximum Individual Doses of Relaxin and IFN-γ

<table>
<thead>
<tr>
<th>Scleroderma Line</th>
<th>Effect on Collagen Synthesis*</th>
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<tbody>
<tr>
<td></td>
<td>Relaxin† (%Δ)</td>
</tr>
<tr>
<td>SD5</td>
<td>−14%</td>
</tr>
<tr>
<td>SD10</td>
<td>−10%</td>
</tr>
<tr>
<td>SD11</td>
<td>−31%</td>
</tr>
<tr>
<td>SD14</td>
<td>−8%</td>
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<tr>
<td>SD16</td>
<td>−59%</td>
</tr>
<tr>
<td>SD17</td>
<td>−76%</td>
</tr>
<tr>
<td>SD18</td>
<td>0%</td>
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* Collagen bands on autoradiograms were scanned densitometrically to quantitate effects of relaxin and IFN-γ on collagen protein expression by seven scleroderma (SD) lines. All lines were subjected to nine treatment regimens in duplicate in at least two experiments each. Each experiment was run in duplicate to assess the consistency between experiments for each line. %Δ was calculated by obtaining the average change in collagen expression by cultures treated in duplicate with that of the untreated control culture in one representative experiment. Standard error was less than 10% in each case.

† Relaxin effect was assessed at 100 ng/ml.
‡ IFN-γ effect was observed at 100 U/ml.

RESULTS

All scleroderma lines included in this study demonstrated enhanced collagen expression when compared to normal dermal fibroblasts or fibroblasts from the uninvolved skin of the same patient, when available, as ascertained by densitometric analysis of labeled collagen bands on autoradiograms, as described below. Normal and scleroderma fibroblasts were labeled in DMEM in the absence of serum for 18 h with [3H]-proline in the presence of ascorbate, and BAPN, an agent that inhibits collagen cross-linking, as described in the Materials and Methods section. Biosynthetically labeled secreted proteins from normal and scleroderma cultures were precipitated from equal volumes of conditioned media and electrophoresed on 7% polyacrylamide gels. Procollagen was identified as the two most prominent proline-incorporating bands on the radiogram by sensitivity to digestion by purified bacterial collagenase (Fig 1A). To provide a normal reference for relative quantity of collagen synthesized by scleroderma fibroblast cultures, procollagen precipitated from a normal fibroblast culture was electrophoresed alongside those from scleroderma cultures. Procollagen bands were scanned densitometrically, as described in the Materials and Methods section; these areas were plotted as relative numbers such that the amount of collagen secreted by a normal dermal fibroblast culture was equal to 1.0. Differences in the saturation densities of the cell lines at confluence were noted (data not shown); therefore, values for relative amount of collagen synthesized were normalized for cell number per culture dish. Scleroderma lines were compared with normal cultures over two-four experiments and lines whose comparative collagen expression values were higher than normal by at least 50% were selected for this study. Seven scleroderma lines selected 1.5–4.3 times more collagen than normal fibroblasts (Fig 1B) and were analyzed for responsiveness to cytokine treatment.

To assess the sensitivity of cultures of scleroderma cultures to relaxin, cells were cultured as described above and treated 72 h later in duplicate wells with relaxin (1, 10, and 100 ng/ml) for 48 h. At the end of this period, cells were labeled with [3H]-proline in the presence of cytokines, ascorbate, and BAPN for 18 h. All seven lines were tested in two separate experiments and response patterns were consistent between experiments for each line. Three lines, SD11, SD16, and SD17, were sensitive to relaxin treatment and manifested dose-dependent decreases in collagen expression, with maximum decreases of 29%, 59%, and 76%, respectively, at a relaxin dose of 100 ng/ml (Fig 2). Three other scleroderma lines demonstrated smaller decreases in collagen expression ranging from 8% to 14% in response to relaxin (100 ng/ml) (Table I). One line, SD18, showed no consistent response to relaxin. Total secreted protein, as assessed by [35S]methionine incorporation into quinine sulfate-SDS–precipitable material, increased 25% over control levels with the maximum dose of relaxin.

The seven scleroderma lines were tested individually for IFN-γ sensitivity (100 U/ml) and responded with decreases in collagen synthesis and secretion from ranging from 7 to 89% (Table I). These results are consistent with previously published reports on the effects of IFN-γ on collagen expression by scleroderma fibroblasts [9].

The ability of relaxin and IFN-γ to alter patterns of collagen expression when used in combination was then tested on the scleroderma fibroblasts. Scleroderma cultures were treated with relaxin and IFN-γ.
(1, 10, and 100 ng/ml) in combination with IFN-γ (1, 10, and 100 U/ml) such that all three relaxin doses were tested in conjunction with all three doses of IFN-γ, resulting in nine combination treatment groups. Four of seven lines tested demonstrated a cooperative effect of using both cytokines together in depressing collagen expression. Scleroderma line, SD5, which demonstrated responses of moderate magnitude to relaxin and IFN-γ individually (Table I), showed striking decreases in secreted collagen in the presence of both cytokines (Fig 3A,B, Table II). A suboptimal dose of IFN-γ (10 U/ml) in conjunction with low concentrations of relaxin (1 and 10 ng/ml) elicited decreases of 58% and 62% collagen depression. These decreases were not approached when maximum doses of either relaxin (100 ng/ml) or IFN-γ (100 U/ml) were used individually. The augmenting effect of relaxin on collagen depression occurred in a dose-dependent manner. Limited pepsin digestion and delayed reduction analysis of the collagen chains demonstrated roughly equivalent decreases in expression of the α1(I), α2(I), and α3(III) chains by treatment with the combination of cytokines (Fig 3C). The coordinate decreases in expression of all three chains that comprise types I and III procollagens were similar to changes induced by IFN-γ alone [10].

In the cases of SD11 and SD18, the addition of relaxin in conjunction with suboptimal concentrations of IFN-γ resulted in decreases in collagen secretion equivalent to that seen following treatment with a tenfold higher dose of IFN-γ (Fig 4). Treatment of SD11 fibroblasts with relaxin (100 ng/ml) or IFN-γ (10 U/ml) individually caused a 29% or 41% decrease in collagen expression, respectively (Fig 4A). The addition of the two agents together resulted in a 53% decrease, equivalent to that seen when IFN-γ was used at a tenfold higher concentration, 100 U/ml (Table II). SD18 showed no consistent response to treatment with relaxin alone but when relaxin was used in combination with IFN-γ, decreases in collagen expression exceeded that seen by using IFN-γ alone (Fig 4B). Another scleroderma line, SD14, which was relatively resistant to relaxin and IFN-γ when the cytokines were tested individually, demonstrated an additive response when they were used in combination. Whereas IFN-γ and relaxin individually effected 7% and 8% depressions in collagen expression, respectively, their combination resulted in a 17% decrease in collagen secreted (Table II). No additive or synergistic effects of the two cytokines were observed following treatment of scleroderma lines SD 10, 16, or 17. None of the combination treatments caused a change in total secreted protein (data not shown).

**DISCUSSION**

The coordinated assembly and disassembly of collagen and other matrix elements allows for tissue growth and repair and provides developmental and differentiation cues that are critical to proper form and function of multicellular animals. When this precise regulation of connective tissue turnover is disrupted, severe problems in form and function do occur. Fibroblasts derived from the sclerotic skin of scleroderma patients can demonstrate markedly elevated levels of collagen synthesis and secretion as compared to normal dermal fibroblasts [2,3]. The overproduction of collagen and other extracellular matrix constituents [4,5] are thought to cause the progressive fibrosis that leads to tissue damage in this disease. Previously reported work has shown that IFN-γ can be effective in decreasing collagen synthesis in scleroderma fibroblasts [7]. These and subsequent studies that have shown that IFN-γ can decrease collagen accumulation in vivo [19–21] were the bases for the initiation of limited clinical trials to test the efficacy of IFN-γ in the treatment of scleroderma and keloids [22–24]. The hormone relaxin has been shown to be effective in decreasing collagen expression in normal dermal fibroblasts in vitro, particularly when significant collagen overexpression is induced by the cytokines TGF-β or IL-1β [6]. Recently, we have also shown that relaxin is capable of inhibiting fibrosis in two animal models [25]. In the present study, we have extended these findings by demonstrating that relaxin can down-regulate collagen expression in scleroderma fibroblasts and have also shown that the two collagen inhibitory cytokines, relaxin and IFN-γ, have greater potential utility when used in combination than when used individually.

Consistent with our previous findings that relaxin is a potent collagen down-regulatory cytokine under conditions when cells are overproducing collagen, relaxin can depress collagen expression by scleroderma fibroblasts that are stimulated to produce excess collagen. The basis for the differences in sensitivity of the scleroderma lines to relaxin treatment is unknown. We and others have observed a similar variability of sensitivity of lines to IFN-γ treatment. The differences may lie in receptor number or affinity, although differences in intracellular signaling pathways may also be relevant. Because the etiology and biochemical basis of the disease scleroderma are unknown, in what aspects these cells have deviated from normal are difficult to assess. The fact that the clinical designation of "sclero-
Table II. Summary of Relaxin and IFN-γ Cooperativity in Inhibiting Collagen Synthesis by Scleroderma Fibroblasts

<table>
<thead>
<tr>
<th>Scleroderma Line</th>
<th>Relaxin(^{\text{a}}) (%)Δ</th>
<th>IFN-γ(^{\text{a}}) (%)Δ</th>
<th>Combination(^{\text{a}}) (%)Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD5</td>
<td>-14%</td>
<td>-38%</td>
<td>-69%</td>
</tr>
<tr>
<td>SD11</td>
<td>-29%</td>
<td>-41%</td>
<td>-53%</td>
</tr>
<tr>
<td>SD14</td>
<td>-8%</td>
<td>-7%</td>
<td>-17%</td>
</tr>
<tr>
<td>SD18</td>
<td>0%</td>
<td>-37%</td>
<td>-88%</td>
</tr>
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\(^{\text{a}}\)Decreases in collagen expression were calculated as described in Table I.

The systemic administration of IFN-γ to humans at pharmacologic doses is accompanied by some adverse reactions, predominantly flu-like symptoms [23, 24, 27]. Consequently, it is desirable to keep the administered dose as low as possible while maintaining the desired effect. In the present study, we evaluated the effectiveness of the combination of IFN-γ and relaxin in decreasing collagen expression by scleroderma fibroblasts in vitro. We have found that in four of seven scleroderma lines, relaxin was able to augment the collagen-depressing effect of IFN-γ. In three of the lines, the addition of relaxin to cultures in combination with suboptimal concentrations of IFN-γ resulted in decreases in collagen expression equivalent to or better than that obtained by IFN-γ alone at a tenfold higher concentration. This demonstrates that using relaxin in conjunction with IFN-γ may allow for a reduction in the concentration of IFN-γ necessary to achieve a significant decrease in collagen expression. In another of the lines, which was relatively insensitive to either relaxin or IFN-γ when used individually, the combination of cytokines at the maximum in vitro doses effected an additive decrease in collagen secretion.

Because relaxin is a naturally occurring hormone of pregnancy [12], it is likely to be well-tolerated if given exogenously. In addition, studies published in the late 1950s and early 1960s reported some efficacy of partially purified proline relaxin in the treatment of scleroderma patients [28, 29], particularly with regard to increasing skin elasticity and healing of skin ulcers. However, the ability of relaxin to be evaluated further was limited by uncertainties concerning the source and purity of relaxin preparations [29]. The availability of highly purified, human recombinant relaxin will now allow a full evaluation of its potential role, alone and in combination with IFN-γ, as a modulator of excessive collagen synthesis in vitro and in vivo.

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REFERENCES


