Epidermal Growth Factor (EGF) Antagonizes Transforming Growth Factor (TGF)-β1-Induced Collagen Lattice Contraction by Human Skin Fibroblasts

Joon Seok Park,* Jeum Yong Kim, Jae Youl Cho,1 Jin Seok Kang,2 and Young Hyo Yu

Received June 26, 2000; accepted September 8, 2000

Wound contraction plays an important role in healing, but in extreme conditions, it may lead to excessive scar formation and pathological wound contraction. To date, the key regulator of excessive contraction is known to be transforming growth factor-beta (TGF-β1). In this study, we have evaluated epidermal growth factor (EGF) antagonism in fibroblast-populated collagen lattice (FPCL) gel contraction, which has been generally used as an in vitro model thought to mimic wound contraction in vivo. As expected, TGF-β1 treatment enhanced normal fibroblast-induced collagen gel contraction in a dose-dependent manner. In contrast, EGF did not affect normal gel formation, but significantly antagonized TGF-β1-induced gel formation (p<0.05 at 100 ng/ml), whereas the other growth factor, platelet-derived growth factor (PDGF), did not alter either normal or TGF-β1-induced gel contractions. Similarly, EGF treatment, but not PDGF, also significantly suppressed TGF-β1 release that was autologously elicited by TGF-β1 treatment (p<0.01 at 100 ng/ml). Therefore, the results suggest that EGF may negatively regulate the role of TGF-β1 through attenuating autologous release of TGF-β1.

Key words fibroblast-populated collagen lattice (FPCL); human skin fibroblast; TGF-β1; EGF; PDGF; ELISA

Wound contraction is one of the early events in the wound healing process in which the contraction of the fibroconnective tissue in the wound bed results in the reduction of wound volume. Although wound contraction is a normal event in wound healing, excessive contraction results in disfiguring scarring. The signals that trigger this contraction have not been fully elucidated; therefore, the fibroblast/collagen gel system has been employed by several investigators to analyze what the contraction signals are and how the contraction is regulated.

Growth factors such as transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are known to be essential throughout the wound healing process. However, their effects on the wound contraction process are not still clear. Even though TGF-β1 stimulates wound healing by increasing extracellular matrix synthesis, many reports that TGF-β1 induces scarring in animal and human are now acceptable. Thus, a therapeutic strategy to inhibit TGF-β1 may be an ideal target to prevent scarring.

We previously reported that EGF inhibited the TGF-β1-induced collagen gel contraction on rat skin fibroblast. In this study, to further study the antagonistic effect of EGF against TGF-β1 action in human fibroblast, a TGF-β1-induced collagen gel contraction populated with human skin fibroblasts was used and similar results are reported. In addition, to determine the inhibitory mechanism of EGF antagonism, a TGF-β1 immunoassay has been evaluated.

MATERIALS AND METHODS

Reagents and Culture Materials Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, an antibiotic-antimycotic (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B), and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY, U.S.A.). Recombinant human EGF (EGF) was obtained from Daewoong Pharm. Co. (Seoul, Korea). Daewoong EGF, expressed in E. coli with recombinant DNA techniques, showed almost similar characteristics to the rhEGF manufactured in E. coli by Boehringer Mannheim and Pierce. It was determined to have the same primary structure as natural hEGF by analysis of the amino acid sequencing, C-terminal amino acid residue, peptide mapping, and disulfide bond identification. It showed a half-maximal concentration (ED50, 0.7–1.0 ng/ml) for the stimulation of 3H-thymidine incorporation by NRK-49F fibroblasts. rhTGF-β1 (R&D systems, Minneapolis, MN, U.S.A.) has an ED50 of 0.02 to 0.06 ng/ml for the inhibition of IL-4-dependent 3H-thymidine incorporation in murine T-Helper cells (HT-2), rhPDGF B/B (Boehringer Mannheim, Mannheim, Germany) has an effective dose at 50% of 1 to 3 ng/ml for the stimulation of 3H-thymidine incorporation by NR-6T3 fibroblasts. An anti-TGF-β1 polyclonal antibody (R & D systems) was selected for its ability to neutralize the biological activity of TGF-β1. This antibody (0.005–0.01 μg/ml) will neutralize 50% of the bioactivity due to 0.25 ng/ml of rhTGF-β1.

Fibroblast Culture Five different human newborn foreskins were washed three times with Hank’s balanced salt solution containing an antibiotic-antimycotic, and cut into approximately 1×1×1 mm pieces. Subsequently, these pieces were put into 90 mm plastic dishes (Nunc, Naperville, IL, U.S.A.) with DMEM containing 10% FBS for primary culture. The pieces were cultured at 37℃ with 5% CO2 in a humidified incubator. Subculturing was carried out according to the ordinary method until enough fibroblasts had proliferated. The second through eighth cell passages were used for the experiments. Cells were released from the culture with trypsin-EDTA and used for the fibroblast-populated collagen lattice (FPCL) assay, cell proliferation and TGF-β1 immunoassay.

FPCL Assay Type I collagen (0.6 ml, 3 mg/ml, Boehringer Mannheim) was reconstituted with 0.15 ml DMEM concen-
treated four times, then mixed with 1.0×10^4 cells suspended in 0.75 ml DMEM with 10% FBS. The mixture was dispensed into a well of a 12-well plate. After polymerization at 37°C for 90 min, the gels were gently detached from the walls. Next, 50 μl media, which contain each growth factor, were added. Then 125 μl FBS and 325 μl serum free DMEM were added for a total volume of 2 ml in each well. The plates containing collagen gels were placed in an incubator at 37°C. The surface areas were calculated by using an image analyzer system (Advanced American Biotechnology, Fullerton, CA, U.S.A.). The measurements were read every 24 h for 3 d.

**Measurement of Cell Proliferation** The proliferation of cells was measured using a tetrazolium-based colorimetric assay (MTT assay). Briefly, samples of 5×10^3 cells were seeded into 96-well plates in DMEM with 10% FBS. Confirming 80—100% confluence after 24 h, they were incubated for an additional 24 h in DMEM with 0.5% FBS. The media were then changed with DMEM only, DMEM with 10 ng/ml TGF-β1, 100 ng/ml EGF, and 100 ng/ml PDGF, and incubated for 3 d. On the third day of incubation, 20 μl of 5 mg/ml MTT solution was added into each well and incubated for 4 h. Supernatants were removed by suction and 200 μl of dimethylsulfoxide solution was added. After shaking for 10 min, the absorbance of 560 nm was measured.

**ELISA of TGF-β1** The levels of TGF-β1 produced by fibroblasts and the effect of exogenous TGF-β1 and EGF on these levels were measured with a human TGF-β1 immunoassay (Biotrak, Amersham) with a sensitivity of 15.6 pg/ml. After 3 d of FPCL culture, supernatants were collected, and triplicate samples and activated and non-activated forms (total) were assayed. Activation was performed by adding 0.2 ml of 1 M HCl to 1 ml of cell supernatants for 10 min, followed by neutralization with 0.2 ml of 1.2 M NaOH/0.5 M HEPES. To determine the amount of adherence of exogenous TGF-β1 to collagen, 10 ng/ml TGF-β1 was incubated in collagen gel without cells, and the medium was assayed after 3 d. The samples containing exogenous TGF-β1 were diluted 1:5 to adjust the concentration of TGF-β1 to the linear range of the kit. The optical density of wells was determined with a microtiter plate reader set to 450 nm absorbance. The readings at 570 nm were subtracted from readings at 450 nm to correct for nonspecific absorbance.

**Statistical Analysis** All experiments were performed in triplicate and results expressed as mean±standard error. The significance of differences was calculated by the Duncan multiple range test (PHARM/PCS, Ver. 4.1).

**RESULTS**

**Effect of TGF-β1 and EGF on Human Skin FPCL Contraction** To gain insight into the optimal concentration of the growth factors used in this assay, we tested the effect of TGF-β1, EGF and PDGF on a collagen contraction populated with fibroblast in a dose- and time-dependent manner. The collagen gel contraction was measured as the reduction in gel surface area and expressed as a percentage of gel contraction. A full range of concentration of growth factors, i.e. TGF-β1 (0.1 to 10 ng/ml), EGF (1 to 100 ng/ml), and PDGF (1 to 100 ng/ml), were first tested in order to secure a complete picture of the responsiveness of the fibroblast. The representative kinetics of human skin fibroblast contraction in FPCL have been presented, respectively, in Fig. 1. TGF-β1 stimulated the FPCL contraction in a dose-dependent manner over the concentration range of 1—10 ng/ml (p<0.05), as shown in a previous report,^8^ whereas EGF showed a bell-shaped dose-responsive effect but was not significant at all doses. PDGF used as a negative control showed a similar profile with TGF-β1 and significantly stimulated contractions (p<0.05 at 100 ng/ml). From these results, we found that the effective concentration of growth factor in FPCL was approximately 100 times higher than their biologically effective concentrations, consistent with other results.⁹¹⁰ Thus, the defined concentration, i.e., TGF-β1 (10 ng/ml), EGF (100 ng/ml) and PDGF (100 ng/ml), was used as the standardized condition for the sequential studies.

**Effect of EGF on FPCL Contractions Induced by TGF-β1** To test the antagonistic effect of EGF in TGF-β1 action, such as scar formation, TGF-β1-induced collagen gel contractions in the presence of EGF were examined. The result was also compared with those of PDGF and anti-TGF-β1.
Figure 2 shows that EGF significantly inhibited TGF-β1-induced contractions for all experiment periods \( p < 0.05 \), and this contractile degree was similar to that of the control \( p > 0.05 \). The inhibitory potency of EGF was similar to previous reports that tranlast and interferon-α, which have been developed as anti-scarring agents, significantly attenuated collagen gel contractions (up to 10%).\(^{14-16}\) In contrast, PDGF did not affect the TGF-β1-induced contraction \( p > 0.05 \), which rather significantly contracted vs. the control \( p < 0.05 \). From this result, it is suggested that EGF may attenuate the biological activity of TGF-β1 in the case of a collagen gel contraction. According to the previous report that both the initial action and autologous production of TGF-β1 were completely blocked by a neutralizing antibody to TGF-β1,\(^{15}\) we next tested an anti-TGF-β1 antibody to confirm that the potentiated contraction was induced by TGF-β1 action. As expected, the anti-TGF-β1 antibody (10 μg/ml) significantly suppressed TGF-β1-induced contractions \( p < 0.05 \), as shown in EGF, suggesting that the excessive contraction is due to TGF-β1 action and its proper concentration (Fig. 2).

### Effect of Growth Factors on Cell Proliferation

The number of skin fibroblasts initially plated was 5.0×10\(^3\) cells/well. After a 3-day culture period, the cell number in the control increased an average of 125%. Since some variation in absorbance in the MTT assay was observed, all values were normalized to the absorbance levels of their own respective control cells not exposed to growth factors. For all growth factors, no significant differences in cell number or viability between the control and treated cells were observed at the end of experiment (Table 1). Thus, the effect on the FPCL contraction caused by these growth factors cannot be due to cytotoxicity or the inhibition of proliferation.

### Effect of EGF on TGF-β1 Production

Since our results suggest that exogenous TGF-β1 at a proper concentration plays an important role in contractions, we hypothesized that the attenuating effect of EGF on the collagen gel contraction was related to TGF-β1. To examine this hypothesis from a molecular aspect, the effect of EGF on TGF-β1 release was assessed under collagen gel culture conditions by an enzyme-linked immunosorbent assay (ELISA). Previous studies have shown that TGF-β1 is present in the extracellular matrix, both in active and latent forms, but only the active form is able to bind to the TGF-β receptor.\(^{16}\) As the latent form can be activated in vitro by acidification,\(^{17}\) we quantified both active and latent forms of TGF-β1 in culture medium. Table 2 shows that the addition of TGF-β1 to fibroblast-populated collagen gel autologously induced 2.5 times the TGF-β1 production (3448 ± 184 pg/ml), compared with the TGF-β1 level in a medium control group (only DMEM without fibroblasts, 1424 ± 69 pg/ml) to which the same amount of TGF-β1 was added. Furthermore, a large proportion (85%) of TGF-β1 released was in an active form (2896 ± 140 pg/ml), as shown in the literature reported previously (Table 2).\(^{18}\) On the other hand, the active form was not determined from the culture supernatant of fibroblasts in the absence of TGF-β1, although a small amount of TGF-β1 was detected, suggesting not only that normal gel contraction was not due to active TGF-β1 action, but that the TGF-β1-induced potentiation of a gel contraction was mediated by exogenous TGF-β1. However, interestingly, EGF or anti-TGF-β1 antibody treatment significantly suppressed TGF-β1 production \( p < 0.01 \) vs. TGF-β1 treatment, suggesting that EGF may affect TGF-β1 production, as shown in the anti-TGF-β1 antibody treatment.

### DISCUSSION

EGF is well known to accelerate epithelialization in animal models,\(^{19}\) as well as in human partial-thickness skin wounds\(^{20}\) and chronic wounds.\(^{21}\) Since Kang et al. (1996) reported that EGF inhibited scar formation in a guinea pig wound model,\(^{22}\) the growth factor has been considered to be a possible candidate for use in scar treatment, although the mechanism in the wound contraction process was not clear. Our data also seem to support the possibility that EGF may be clinically useful for preventing scarring caused by severely contracting wounds, since the use of an anti-TGF-β1 antibody is regarded as a therapeutic strategy to prevent scar formation. Namely, the main finding of this study is that EGF inhibits TGF-β1-induced contractions of human skin fibroblast populated collagen lattices through blocking TGF-β1 production. Our data did not seem to be due to a non-specific effect of EGF, because PDGF did not affect gel formation in the presence or absence of TGF-β1 under the same conditions. In addition, the suppressive effect of EGF against TGF-β1-induced gel formation appears to be selective to TGF-β1 action, since EGF itself did not affect normal colla-

---

**Table 1. The Degree of Proliferation by TGF-β1 (10 ng/ml), EGF (100 ng/ml) and PDGF (100 ng/ml) Compared with the Control (%)**

<table>
<thead>
<tr>
<th>TGF-β1</th>
<th>EGF</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Proliferation</td>
<td>96.1±2.3</td>
<td>101.0±2.0</td>
</tr>
</tbody>
</table>

\( n=4 \)

**Table 2. TGF-β1 Content Affected by DMEM Only, TGF-β1, EGF + TGF-β1, and Anti-TGF-β1 Antibody (Ab) + TGF-β1**

<table>
<thead>
<tr>
<th>Control</th>
<th>TGF-β1 (T1)</th>
<th>T1 + EGF</th>
<th>T1 + anti-T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>765±52(^{a})</td>
<td>3448±184(^{a})</td>
<td>1721±92(^{a})</td>
</tr>
<tr>
<td>Active</td>
<td>&lt;15</td>
<td>2896±140</td>
<td>1326±68</td>
</tr>
</tbody>
</table>

\( a < 0.01 \) vs. control; \( b < 0.01 \) vs. TGF-β1; \( n=4 \).
gen gel contractions (Eq. 1), although several previous experiments using rabbit and ewe fibroblasts were different. However, the discrepancy may due to different species of fibroblasts, since Yang et al. (1997) showed that EGF has no inhibitory effect on collagen gel contraction by human fibroblasts.

TGF-β3 is known to be a TGF-β1 antagonist and potent anti-scarring agent. Particularly, the exogenous addition of TGF-β3 with TGF-β1 was reported to down-regulate TGF-β1 mRNA. The inhibition of TGF-β1 production was also shown in the treatment using EGF and anti-TGF-β1 antibody (Fig. 2). Thus, both EGF and anti-TGF-β1 antibody suppress the autologous production of TGF-β1 (Table 2), as assessed in a collagen gel culture condition by ELISA, to evaluate the active form from the fibroblast-populated collagen gel, even though an in vitro study to determine whether EGF affects TGF-β1 mRNA expression should be tested. The inhibitory effect of either growth factor on TGF-β1 release was correlated with the result of collagen gel contraction, although the exact mechanism of this result has not yet been elucidated. Thus, EGF almost completely blocked both total TGF-β1 production and the collagen gel contraction that is potentiated by TGF-β1 treatment. However, there are several possibilities regarding the mechanism of modulation of the TGF-β1 pathway by EGF: (1) EGF may inhibit the secretion and activation of TGF-β1 (2) EGF may change the expressions of a TGF-β1 receptor, and (3) the post-receptor signal transduction system may be modified by EGF. First of all, it may be considerable that the TGF-β1 receptor is a possible target of the EGF effect, because pretreatment of cells with 10 ng/ml EGF dose-dependently inhibited the receptor expression. It is likely that EGF may directly interrupt the mRNA expression level of TGF-β1. Indeed, several reports support such blocking of the gene expression level. For example, we and others have found that EGF significantly suppresses the alpha-SM-actin mRNA level from the scar-origin fibroblasts (data not shown), and alters the expression level of bax which is a member associated with apoptosis. To gain insight into the exact mechanism, however, these possibilities should be studied in a subsequent experiment, including direct action on the receptor expression or interference in signal transduction pathway, because the active form of TGF-β1 was detected in an exogenous TGF-β1 treatment group.

In conclusion, the present study shows that excessive collagen gel formation elicited by TGF-β1, which is one of the reasons for inducing scar formation, is antagonized by EGF, in part by decreasing TGF-β1 production, suggesting that EGF may be clinically useful in anti-scarring therapy, as shown in the anti-TGF-β1 antibody. Moreover, recent biotechnology allowed us to raise the production yield of recombinant human EGF, resulting in it being economically beneficial to develop to a medicine.

Acknowledgements We thank Dr. Seong Han Nam (Department of Plastic Reconstructive Surgery, Kyung Hee Pundang CHA Hospital, Sungnam, Korea) for kindly providing human skin.

REFERENCES

1) Present address: Department of Immunology, Windeyer Institute of Medical Sciences, University College London Medical School, 46 Cleveland Street, London W1P 6DB, U.K.
2) Present address: Korea Food and Drug Administration, Nokhun-dong 5, Eunpyung-gu, Seoul, S. Korea.