Endothelial cell growth factor and heparin regulate collagen gene expression in keloid fibroblasts

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Keloids are benign cutaneous tumours characterized by excess deposition of collagen, specifically type I collagen. We report here that collagen biosynthesis, as measured by hydroxyproline synthesis, was markedly inhibited by 65–80 % by the combination of endothelial cell growth factor (ECGF) supplement and heparin in keloid fibroblast cultures. Fibroblast cultures that were incubated with ECGF alone also demonstrated a measurable decrease of approx. 50 % in collagen synthesis compared with control cultures. The inhibition of collagen synthesis was related to the down-regulation of collagen gene expression. Quantitative measurements of mRNA–cDNA hybrids revealed that the gene expression of collagen type I was decreased by more than 80 % by heparin and ECGF. Markedly diminished levels of mRNA encoding collagen type I were also observed in cultures incubated with ECGF alone. The results show that ECGF and heparin elicit a negative regulatory effect on collagen production, and that this inhibition is due largely to the down-regulation of the pro- α 1(I) of type I collagen gene. Furthermore, ECGF has a potent suppressive effect, and heparin provides an additive effect to this inhibitory phenomenon.

INTRODUCTION

Keloids are cutaneous lesions composed of excess accumulation of extracellular matrix [1,2]. The predominant matrix component that is increased is collagen [3,4], specifically type I collagen [5,6], although a minor collagen, type VI, is elevated as well [7]. Keloid fibroblasts have been shown to be non-responsive to glucocorticosteroids [8], which are employed therapeutically to reduce the lesions. Indeed, cortisol is not effective in reducing collagen mRNA levels [8].

Heparin and endothelial cell growth factor (ECGF) supplement are capable of influencing various biological activities of many cell types [9–12]. Heparin, a highly negatively charged glycosaminoglycan, has been demonstrated to inhibit vascular smooth muscle cell proliferation [9,13–15]. ECGF, a potent mitogen, is expressed by various cell types [12]. Furthermore, ECGF has a strong binding affinity for heparin [12]. Heparin prolongs the half-life of the growth factor and potentiates its biological activities [16,17]. For instance, the proliferative effect of ECGF on vascular endothelial cells is potentiated by heparin [18].

In a previous study, we established that ECGF combined with heparin significantly decreases collagen production by human smooth muscle cells [19]. Furthermore, it was determined that ECGF and heparin exert a co-ordinate down-regulation of various matrix genes, including the collagen and fibronectin genes, in smooth muscle cells [20]. Our objective in the present study was to determine if a negative regulatory effect on gene expression could be elicited in keloid fibroblasts that produce excess levels of collagen. The results show that excess collagen production is inhibited by ECGF and heparin. The decrease at the protein level is explained largely by the dramatic inhibition of collagen gene expression.

MATERIALS AND METHODS

Cell culture

Human skin fibroblast cultures were established from excised keloid tissue from six patients, 20–30 years of age. These patients did not present with any known systemic conditions. Tissue samples were cut into small pieces and explanted in tissue culture flasks. Within 1–2 weeks, fibroblasts grew from the explants and were subcultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, U.S.A.) supplemented with 10 % fetal bovine serum, 200 units of penicillin/ml, and 200 μ g of streptomycin/ml (Gibco). Confluent cultures were trypsin-treated and subcultured. Cells in passages 6–10 were employed for the experiments described.

The cells were grown to early confluence before heparin and ECGF were added. Heparin was derived from pig intestinal mucosa (Grade I; Sigma Chemical Co., St. Louis, MO, U.S.A.). ECGF supplement was partially purified from bovine neural tissue and contains fibroblast growth factor activity (Sigma). Dose-dependence and time course studies were performed to determine the optimal concentration of ECGF and duration of incubation with ECGF.

Indirect immunofluorescence

The fibroblasts were cultured on chamber slides (Lab Tek; Nunc Inc., Naperville, IL, U.S.A.) and grown to early confluency. The cells were incubated for 4 days (a) without ECGF or heparin (control), (b) with 75 μ g of ECGF/ml, (c) with 500 μ g of heparin/ml, or (d) with ECGF (75 μ g/ml) combined with heparin (500 μ g/ml). The media were changed on the third day; the cells were then fixed on the fourth day in cold (–20 °C) ethanol and pre-incubated for 15 min with Tris-buffered saline (TBS; 0.15 M-NaCl/0.05 M-Tris/HCl, pH 7.6) containing 1 % BSA to block

Abbreviations used: ECGF, endothelial cell growth factor; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

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non-specific antibody binding. The samples were exposed to rabbit antibody against human type I collagen (IgG; Pasteur Institute, Lyon, France) in appropriate dilutions in TBS/BSA overnight at 4 °C. The samples were washed in TBS for 1 h with five changes, and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Miles Laboratories, Inc., Elkhart, IN, U.S.A.). After a 1 h incubation at room temperature, the samples were washed with TBS for 1 h, rinsed with distilled water, mounted and examined with a fluorescence microscope (Nikon Optiphot) equipped with filters for detection of TRITC. In control reactions, the primary antibody was omitted or replaced with sera from non-immunized animals. Negligible background was observed in the control samples.

Assay for procollagen production

Human skin fibroblasts were grown to confluency in 25 cm² flasks. The cells were incubated (a) without heparin or ECGF (control), (b) with 75 µg of ECGF/ml, (c) with 100, 250 or 500 µg of heparin/ml, and (d) with combinations of 75 µg of ECGF/ml and 100, 250 or 500 µg of heparin/ml, in triplicate flasks for each of the culture conditions for a total of 4 days. The media were changed on the third day and replaced with one containing the identical respective supplements. Ascorbic acid (35 µg/ml) was added to each flask and preincubation was performed for 2 h prior to the addition of 20 µCi of L-[2,3,4,5-³H]proline (108.6 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.). The cells were incubated for an additional 18 h with the radioisotope at 37 °C. The incubation was terminated by removing the medium, cooling it at 4 °C and adding the following protease inhibitors to give final concentrations of 20 mM-Na₂EDTA, 10 mM-N-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride. The cell layer was rinsed with 2 × 0.5 ml of phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/4.3 mM-Na₂HPO₄/1.4 mM-KH₂PO₄, pH 7.4) and scraped into 2 ml of solution containing 0.4 M-NaCl, 50 mM-Tris/HCl (pH 7.4) and the same protease inhibitors. The cells were sonicated at 50 Hz for 30 s. To quantify the synthesis of [³H]hydroxyproline, an index of procollagen synthesis, aliquots of medium and cell fractions were dialysed against several changes of deionized water, hydrolysed in 6 M-HCl at 115 °C for 24 h and assayed for non-dialysable [³H]hydroxyproline, employing a specific radiochemical method [21]. The synthesis of [³H]hydroxyproline was normalized for cellular protein [22] and DNA [23] contents. Confluent triplicate flasks were analysed in parallel for each of the experimental conditions.

Quantification of mRNA levels by slot-blot analysis

Keloid fibroblasts were grown to confluency in 75 cm² flasks and were then incubated under the following conditions: (a) without heparin or ECGF (control), (b) with 75 µg of ECGF/ml, (c) with 100, 250 or 500 µg of heparin/ml, and (d) with a combination of 75 µg of ECGF/ml and 100, 250 or 500 µg of heparin/ml. Total RNA was isolated from confluent cell cultures by extracting the cells with 4 M-guanidinium isothiocyanate, pH 7.0, containing 5 mM-sodium citrate, 0.5 % (w/v) sarkosyl, 0.1 M-2-mercaptoethanol and 0.1 % Antifoam A emulsion. The samples were subjected to caesium chloride density-gradient ultracentrifugation [24] at 150 000 g for 18 h at 15 °C and the RNA was precipitated by ice-cold ethanol as described before [25]. The amount of RNA was quantified by spectrophotometric absorbance at 260/280 nm. Quantification of the mRNA transcripts was performed by slot-blot analysis. Serially diluted concentrations of total RNA from fibroblasts incubated in the various culture conditions were applied to nitrocellulose membranes, employing a vacuum manifold apparatus (Minifold

II; Schleicher and Schuell, Keene, NH, U.S.A.). The RNAs were immobilized by heat at 78 °C for 90 min under vacuum [25].

Prehybridization and hybridization with the human cDNA probes described below were performed by labelling with [³²P]dCTP by nick translation [26] to a specific radioactivity of approx. 10⁸–10⁹ c.p.m./µg of DNA. The prehybridization and hybridization solutions contained 3 × SSC (1 × SSC = 0.15 M-NaCl/15 mM-sodium citrate), pH 6.8, 50 % formamide, 0.1 % SDS, 250 µg of denatured salmon sperm DNA/ml and 1 × Denhardt's solution (0.1 % polyvinylpyrrolidone, 0.1 % BSA and 0.1 % Ficoll). The hybridization was performed at 42 °C for 12–24 h [27]. The membranes were washed several times at 65 °C with a final stringency wash of 0.1 × SSC containing 0.1 % SDS.

Northern transfer analysis

To establish the specificity of the hybridizations with the human cDNA probes, Northern transfer analysis was conducted. Total RNA samples were electrophoresed in 1 % agarose gels under denaturing conditions and processed for Northern blotting as described previously [28]. The prehybridization and hybridization conditions were similar to those for slot-blot analysis.

The nitrocellulose filters from Northern transfer and slot-blot analysis were exposed to X-OMAT-AR films (Kodak, Rochester, NY, U.S.A.) between intensifying screens (Kodak). The autoradiograms were scanned with a He/Ne laser densitometer (LKB, Bromma, Sweden); the absorbance units were corrected for the specific activities of the probes and expressed as units/µg of RNA.

cDNA probes

The human cDNA probes employed in the slot-blot and Northern transfer analyses included: (i) a 1.5 kb pro-α1(I) (HF-677) cDNA corresponding to the C-terminal propeptide and the C-terminal portion of the triple helical region of the pro-α1(I) chain of human type I procollagen [29]; (ii) a 1.7 kb fibronectin (HF-771) cDNA corresponding to the C-terminal portion of human cellular fibronectin [30]; and (iii) a 1.7 kb β-chain (pHF A-1) cDNA [31].

RESULTS

ECGF was found to elicit maximal effects at 75 µg/ml. At this concentration, [³H]hydroxyproline synthesis was inhibited 52–57 % relative to control in keloid fibroblasts. Concentrations of less than 75 µg/ml (10 or 50 µg of ECGF/ml) resulted in inhibition of 37–43 % relative to control cultures (Table 1). The inhibition of collagen production appeared to be maximal after a 4 day incubation period, with fresh ECGF being replaced on the third day of the incubation period (Table 2).

Table 1. Dose-response study of the effects of ECGF after a 4 day incubation period

Values in parentheses are percentage inhibition relative to control.

Concentration of ECGF (µg/ml)	[³ H]Hydroxyproline synthesis	
	(10 ⁻⁶ × d.p.m./mg of DNA)	(10 ⁻⁵ × d.p.m./mg of cell protein)
0 (control)	6.5 ± 0.5 (0)	5.3 ± 0.5 (0)
10	4.1 ± 0.0 (37)	3.7 ± 0.8 (30)
50	4.1 ± 0.3 (37)	3.0 ± 0.3 (43)
75	3.1 ± 0.7 (52)	2.3 ± 0.2 (57)
100	3.2 ± 0.7 (51)	2.4 ± 0.6 (55)

Table 2. Time course study of the effects of ECGF

ECGF was added at a concentration of 75 µg/ml to early confluent cultures.

Incubation period (h)	10 ⁻⁷ × [³ H]Hydroxyproline synthesis (d.p.m./mg of DNA)		
	Control	ECGF	ECGF + heparin (100 µg/ml)
24	1.20 ± 0.28	1.21 ± 0.15	1.16 ± 0.05
48	0.64 ± 0.05	0.47 ± 0.09	0.26 ± 0.13
96	0.69 ± 0.02	0.37 ± 0.06	0.21 ± 0.05
144	0.44 ± 0.10	0.32 ± 0.02	0.27 ± 0.03

Effects of ECGF and heparin on [³H]hydroxyproline synthesis

Newly synthesized collagen synthesis, as measured by the amount of [³H]hydroxyproline synthesized and normalized by the amount of DNA or cellular protein content, was dramatically inhibited by ECGF and by the combination of ECGF and heparin (Table 3). ECGF caused a 51–59 % decrease in total [³H]hydroxyproline synthesis compared with control cultures ($P < 0.001$). The inhibitory effect of heparin (100, 250 and 500 µg/ml) was small; inhibition by 250 and 500 µg of heparin/ml was approx. 25–36 % relative to control cultures ($P < 0.05$). However, in cell cultures incubated with the combination of heparin and ECGF, the suppression of [³H]hydroxyproline

synthesis was dramatic. Indeed, the combination of heparin (100, 250 or 500 µg/ml) and ECGF (75 µg/ml) caused a 65–77 % inhibition of collagen production relative to control cultures ($P < 0.001$). The inhibition by the combination of ECGF and heparin was more pronounced than that by ECGF alone, particularly at heparin concentrations of 250 µg/ml and 500 µg/ml ($P < 0.001$). At the highest concentrations of heparin and ECGF, collagen production was about 50 % that of cultures incubated with ECGF alone. At a lower concentration of 100 µg of heparin/ml plus ECGF, the inhibition of [³H]hydroxyproline synthesis relative to ECGF treatment alone was significant when the values were normalized to cell protein content ($P < 0.01$) (Table 3). Thus total [³H]hydroxyproline synthesis was inhibited significantly by ECGF, but was further decreased by the additional presence of heparin.

It was interesting to note that heparin caused increased accumulation of [³H]hydroxyproline in the cell layer relative to the control or ECGF-treated cells. The accumulation was dependent upon the concentration of heparin. ECGF alone did not promote accumulation in the cell layer. However, in cultures incubated with ECGF combined with increasing concentrations of 100, 250 and 500 µg of heparin/ml, a similar trend of enhanced accumulation of [³H]hydroxyproline, albeit much less than that observed with heparin alone, was found in the cell layer (Table 3).

In the medium compartment, ECGF decreased the amount of [³H]hydroxyproline synthesis by approx. 50 % or more compared with parallel control cultures (Table 3). Decreased quantities of

Table 3. Effects of ECGF and heparin on [³H]hydroxyproline synthesis by keloid fibroblasts

Confluent keloid fibroblasts were incubated in triplicate flasks for each of the sets of conditions for 4 days. Secretion (%) was calculated as: medium [³H]hydroxyproline/[³H]hydroxyproline of medium + cells. * Medium + cells values were analysed by two-way analysis of variance for comparison with control: ECGF, $P < 0.001$; heparin (250 µg/ml, 500 µg/ml), $P < 0.05$; ECGF + heparin (100 µg/ml, 250 µg/ml, 500 µg/ml), $P < 0.001$. † Medium + cells values were also analysed by two-way analysis of variance for comparison with ECGF: ECGF + heparin (100 µg/ml), $P < 0.01$; ECGF + heparin (250 µg/ml, 500 µg/ml), $P < 0.001$.

		ECGF	Heparin			ECGF (75 µg/ml) + heparin		
	Control	(75 µg/ml)	(100 µg/ml)	(250 µg/ml)	(500 µg/ml)	(100 µg/ml)	(250 µg/ml)	(500 µg/ml)
A. 10 ⁻⁵ × [³ H]Hydroxyproline (d.p.m./mg of DNA)								
Medium	72.7 ± 2.3	28.8 ± 3.9	59.1 ± 12.9	39.9 ± 6.0	33.7 ± 11.1	21.9 ± 6.2	14.4 ± 0.8	12.3 ± 0.7
Cells	3.6 ± 0.3	2.5 ± 0.2	14.7 ± 3.4	17.5 ± 1.5	18.6 ± 2.5	3.3 ± 0.7	4.3 ± 0.3	5.1 ± 0.3
Medium + cells	76.3 ± 2.6	31.3 ± 4.1*	73.8 ± 14.1	57.4 ± 6.8*	52.3 ± 13.2*	25.2 ± 5.5*	18.7 ± 1.0*†	17.4 ± 1.0*†
Secretion (%)	95.3	92.0	80.1	69.5	64.4	86.9	77.0	70.7
B. 10 ⁻⁴ × [³ H]Hydroxyproline (d.p.m./mg of cell protein)								
Medium	49.0 ± 5.2	23.2 ± 3.6	31.8 ± 7.9	22.6 ± 2.6	22.2 ± 6.7	15.5 ± 3.3	11.2 ± 0.9	9.0 ± 0.6
Cells	2.4 ± 0.3	2.0 ± 0.2	7.8 ± 1.3	10.0 ± 0.5	12.4 ± 0.5	2.4 ± 0.6	3.4 ± 0.1	3.7 ± 0.3
Medium + cells	51.4 ± 5.5	25.2 ± 3.8*	39.6 ± 9.2	32.6 ± 2.3*	34.6 ± 6.7*	17.9 ± 2.7*†	14.6 ± 1.0*†	12.7 ± 0.9*†
Secretion (%)	95.3	92.1	80.3	69.3	64.2	86.6	76.7	70.9

Table 4. DNA and cellular protein contents

Confluent keloid fibroblasts were incubated for 4 days in triplicate flasks under one of the growth conditions shown. The cell layer was extracted and analysed for DNA and protein contents as detailed in the Materials and methods section. Values represent the means ± S.D. of triplicate flasks, each of which was analysed in duplicate *Two-way analysis of variance for comparison with control; $P < 0.001$.

Cells	Control	ECGF (75 µg/ml)	Heparin			ECGF (75 µg/ml) + heparin		
			(100 µg/ml)	(250 µg/ml)	(500 µg/ml)	(100 µg/ml)	(250 µg/ml)	(500 µg/ml)
DNA (µg)	9.3 ± 0.7	14.7 ± 0.8*	8.6 ± 0.5	9.9 ± 0.2	9.9 ± 1.0	15.4 ± 1.9*	14.4 ± 0.7*	15.3 ± 0.5*
Protein (mg)	0.14 ± 0.01	0.18 ± 0.01*	0.16 ± 0.00	0.17 ± 0.01	0.15 ± 0.01	0.21 ± 0.01*	0.18 ± 0.01*	0.21 ± 0.01*

newly synthesized [^3H]collagen were also found in the media of cultures incubated with heparin alone and in combination with ECGF. The decrease in [^3H]procollagen in the medium was related to suppressed collagen synthesis as well as to decreased release of collagen into the medium by heparin (Table 3).

Other analyses

Cellular protein and DNA analyses (Table 4) revealed that ECGF in the absence and in the presence of heparin increased

DNA and cell protein contents significantly relative to control cells ($P < 0.001$). Heparin did not have such an effect.

Immunofluorescence microscopy of keloid skin fibroblasts incubated with ECGF and heparin

Previously we have noted that increased [^3H]hydroxyproline was associated with the cell layer in the presence of heparin. Immunofluorescence microscopy, using antibodies that recognized the type I collagen epitopes, revealed that there were

Fig. 1. Indirect immunofluorescence staining for type I collagen

Normal human skin (a–d) and keloid (e–h) fibroblasts were incubated for 4 days in DMEM and 10% fetal bovine serum under the following conditions: (a) and (e) control, no treatment, (b) and (f) 500 μg of heparin/ml, (c) and (g) 75 μg of ECGF/ml, (d) and (h) combination of 75 μg of ECGF/ml and 500 μg of heparin/ml. The cells were prepared for immunostaining with the antibody for type I collagen as described in the Materials and methods section. The exposure time was the same for all the frames and the prints were reproduced under identical conditions. Magnification $\times 112.5$.

major differences in the distribution of the type I collagen in the cell cultures maintained in ECGF and heparin as well as in those without the supplemental factors. Type I collagen was detectable in normal (Figs. 1*a*–1*d*) and keloid (Figs. 1*e*–1*h*) skin fibroblasts. In the cultures that were maintained in the presence of heparin, type I collagen was mostly intracellular (Figs. 1*b* and 1*f*). The immunofluorescence intensity was increased considerably in the heparin-treated cells (Figs. 1*b* and 1*f*) as compared with the ECGF-treated cultures (Figs. 1*c* and 1*g*) and those incubated with both heparin and ECGF (Figs. 1*d* and 1*h*). The cells that were incubated with 75 μg of ECGF/ml (Figs. 1*c* and 1*g*) demonstrated a lowered level of immunofluorescence, indicating a decreased amount of intracellular type I collagen. Interestingly, incubation with ECGF resulted in the deposition of the type I collagen into a fine extracellular network. In cultures that were incubated with both ECGF and heparin (Figs. 1*d* and 1*h*) there was intracellular accumulation of the collagen, but much less than that with heparin treatment alone (Figs. 1*b* and 1*f*); in

addition, a network of fibres was deposited extracellularly (Figs. 1*c*, 1*d*, 1*g* and 1*h*). Thus the type I collagen as seen in immunofluorescence microscopy in normal and keloid fibroblasts is distributed differently in response to ECGF and heparin.

Inhibition of collagen gene expression by ECGF and heparin

Slot-blot analysis using the human cDNA probe for pro- $\alpha 1(\text{I})$ of type I procollagen demonstrated a marked decrease in the mRNA level for type I procollagen in keloid fibroblast cultures incubated with 75 μg of ECGF/ml (Fig. 2). The mRNA level was decreased by 75 % by ECGF (Fig. 2*b*) compared with the control cultures (Fig. 2*a*). In cultures incubated with ECGF plus heparin (100 or 500 μg /ml), the expression of the collagen type I gene was inhibited by 80–90 % (Figs. 2*e* and 2*f* respectively). Heparin at a concentration of 100 μg /ml (Fig. 2*c*) caused a decrease of 37 % in the mRNA level as compared with the control cultures (Fig. 2*a*). However, with 500 μg of heparin/ml, there was no inhibition of expression of the mRNA encoding procollagen type I (Fig. 2*d*).

Thus, at the mRNA level, ECGF has a potent inhibitory effect on the expression of the pro- $\alpha 1(\text{I})$ of the type I procollagen gene. The negative regulatory effect on collagen gene expression is selective, since the mRNA level for β -actin was not altered by any of the treatment conditions (Table 5). In addition, the level of mRNA encoding fibronectin was not affected by heparin or ECGF or the combination thereof (Table 5).

Northern transfer analysis confirmed the specificity of the

Fig. 2. Inhibition of collagen gene expression by ECGF and heparin analysed by slot-blot analysis

Keloid fibroblasts cells were incubated at confluent growth in DMEM and 10 % fetal bovine serum under the following conditions: (a) without treatment (control), (b) 75 μg of ECGF/ml, (c) 100 μg of heparin/ml, (d) 500 μg of heparin/ml, (e) 75 μg of ECGF/ml combined with 100 μg of heparin/ml, and (f) 75 μg of ECGF/ml combined with 500 μg of heparin/ml for 4 days. Total RNA was isolated (see the Materials and methods section) and serially diluted concentrations of total RNA, beginning with 8, 4, 2 and 1 μg of RNA, were applied to nitrocellulose filters as illustrated. The samples were hybridized with a human cDNA probe for collagen pro- $\alpha 1(\text{I})$. Quantification of the slots was performed by laser densitometry and the data are expressed as absorbance units/ μg of RNA. The values represent means $\pm$ S.D.

Fig. 3. Effects of ECGF and heparin on keloid fibroblast expression of pro- $\alpha 1(\text{I})$ of type I procollagen mRNA analysed by Northern transfer analysis

Total RNA was extracted from confluent cultures of keloid fibroblasts incubated with the following: lane A, no treatment (control); B, 75 μg of ECGF/ml; C, 100 μg of heparin/ml; D, 250 μg of heparin/ml; E, 500 μg of heparin/ml; F, 75 μg of ECGF/ml combined with 100 μg of heparin/ml; G, 75 μg of ECGF/ml combined with 250 μg of heparin/ml; H, 75 μg of ECGF/ml combined with 500 μg of heparin/ml for 4 days. Total RNA (10 μg) was applied identically to each lane. Hybridization was performed with a human cDNA probe for pro- $\alpha 1(\text{I})$ collagen as detailed in the Materials and methods section.

Table 5. Fibronectin and β -actin mRNA steady-state levels in keloid fibroblasts incubated with ECGF and heparin for 4 days

mRNA level (absorbance units/ μg of RNA)	Control	ECGF		Heparin		ECGF (75 μg /ml) + heparin	
		ECGF (75 μg /ml)		(100 μg /ml)	(500 μg /ml)	(100 μg /ml)	(500 μg /ml)
Fibronectin	3.6 $\pm$ 0.6	3.2 $\pm$ 0.5		3.7 $\pm$ 0.4	3.4 $\pm$ 0.7	3.6 $\pm$ 1.2	3.6 $\pm$ 0.4
β -Actin	1.9 $\pm$ 0.4	2.1 $\pm$ 0.4		2.0 $\pm$ 0.3	1.8 $\pm$ 0.5	1.7 $\pm$ 0.3	1.8 $\pm$ 0.2

hybridization of mRNAs with the pro- $\alpha 1(I)$ collagen type I cDNA. The mRNA transcripts consisted of two bands of 5.8 and 4.8 kb, characteristic for pro- $\alpha 1(I)$ of type I collagen [29]. ECGF, at 75 $\mu\text{g/ml}$, caused a dramatic decrease in the mRNA level of pro- $\alpha 1(I)$ of type I collagen (Fig. 3, lane B) relative to the non-treated control samples (Fig. 3, lane A). Heparin at 100 $\mu\text{g/ml}$ (Fig. 3, lane C) suppressed collagen gene expression to a much lesser extent than did ECGF (Fig. 3, lane B). However, at 250 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ (Fig. 3, lanes D and E respectively), heparin had the opposite effect, inducing a greater abundance of mRNAs for type I collagen. This effect appears to be concentration-dependent. When ECGF at 75 $\mu\text{g/ml}$ was combined with 100, 250 or 500 μg of heparin/ml, the expression of the pro- $\alpha 1(I)$ of type I collagen gene was inhibited (Fig. 3, lanes F, G and H); the amount of the mRNAs was decreased to a level much less than that of ECGF alone (Fig. 3, lane B). These findings support the data from the slot-blot analysis. ECGF down-regulates the expression of the type I collagen gene and the effect is potentiated when ECGF is combined with heparin.

DISCUSSION

Overabundant production of collagen, specifically collagen type I, the major interstitial collagen of skin fibroblasts, is the hallmark of keloids [1,2,5,6]. Type I procollagen mRNA levels have been reported to be selectively increased in keloid fibroblast cultures [5] and keloid tissues [7].

In the present study, a consistent and dramatic inhibition of collagen synthesis was observed by biochemical and immunostaining methods in all strains of keloid fibroblasts that were incubated with ECGF alone and in combination with heparin. ECGF by itself was capable of decreasing collagen production substantially. Although ECGF is known to be mitogenic for various cell types [12] and was found to increase the DNA and cell protein of keloid fibroblasts, the growth factor was a potent inhibitor of collagen biosynthesis in keloid fibroblasts. The inhibitory effect is largely explained by the action of ECGF on the mRNA level. The gene expression of collagen type I was down-regulated by ECGF, whereas the β -actin and fibronectin genes were not regulated by the growth factor.

It is well known that heparin binds ECGF [32] and potentiates its effects, e.g. on the growth of human endothelial cells [18]. Several workers have shown that heparin prolongs the biological half-life of growth factors [17] by protecting them from proteolytic degradation [16], acid and heat inactivation [33]. In addition, heparin increases the affinity of ECGF for cell surface receptors by inducing conformational changes in the ECGF peptide [34]. In the present study heparin alone had a relatively small inhibitory effect on collagen synthesis. However, when heparin was combined with ECGF, collagen synthesis and expression of the type I collagen gene were decreased substantially, to a greater extent than was caused by ECGF alone, thereby suggesting that heparin potentiates the action of ECGF when it binds the growth factor.

Northern and slot-blot analyses showed that heparin at high concentrations, particularly at 500 $\mu\text{g/ml}$, increased the mRNA level for pro- $\alpha 1(I)$ of type I collagen. It would appear that the steady-state mRNA levels for type I collagen were not positively correlated with the total amount of collagen synthesis, strongly suggesting that heparin at various concentrations may have differential effects on the pre-translational, translational and post-translational activities.

The amount of newly synthesized [^3H]hydroxyproline was elevated in the cell layer of cultures incubated with heparin. Immunostaining with the antibody to type I collagen also

revealed the intracellular accumulation of collagen caused by heparin. Decreased amounts of hydroxyproline were released into the medium compartment, and the overall amount of newly synthesized collagen was lowered. The reason for the apparent decrease in the release of collagen into the medium is not known; it is possible that the intracellular collagen is abnormal or that the collagen binds to the internalized heparin and its fragments, and is not secreted at the normal rate. Previous studies have established that many types of collagen, including the interstitial collagen types I and III, are capable of binding to heparin [35,36]. It is clearly evident that the effects of heparin are complex and differential at the biochemical and molecular levels. Further studies are required to detail the mechanisms of action of heparin.

In summary, ECGF in the absence and presence of heparin potently inhibits collagen synthesis. The inhibition of collagen production by ECGF can be explained largely by a selective down-regulation of the pro- $\alpha 1(I)$ of type I collagen gene expression, resulting in diminished levels of the corresponding mRNA transcripts. The presence of heparin serves to potentiate the inhibitory effect of ECGF on the extracellular matrix macromolecules produced by keloid fibroblasts. This novel finding may, therefore, have therapeutic implications for decreasing excess collagen deposition in keloids.

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