Effects of Interleukin-4 on Extracellular Matrix Gene Expression in Normal Skin Fibroblasts Cultures

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Background **: Interleukin-4(IL-4) is a 20 kDa glycoprotein and is now known to possess fibrogenic activities.**

Objective **: The purpose of this study is to evaluate the effect of IL-4 on the extracellular matrix gene expression.**

Methods **: Quantitation of collagenous protein synthesis, Northern and dot-blot hybridization, transfection experiments and CAT assay in normal human skin fibroblasts were done.**

Results **: Maximal elevation of collagen synthesis was presented at the concentration of IL-4 being 5.0ng/ml. In Northern and dot-blot analysis, each level of type I collagens and fibronectin mRNA increased 3.0, and 2.8-fold, respectively in IL-4 treated fibroblasts. In CAT assay, the percentage of acetylation was 8.3% in the untreated control group and 23.1% in 5.0ng/ml of the IL-4 treated group in normal fibroblasts. The promoter activity was increased 2.8-fold in IL-4(5.0ng/ml) treated fibroblasts compared to the control groups.**

Conclusion **: IL-4 might be a fibrogenic cytokine that could be important in promoting the biogenesis of collagen proteins. This could be due to increased transcription.**

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Key Words : Interleukin-4, Extracellular matrix genes, Fibroblast

Interleukin-4(IL-4) is a 20 kDa glycoprotein, first described as a B-cell stimulating factor and now known to possess pleiotropic activities on a variety of the cells[1]. IL-4 is secreted by a restricted number of cells, such as, activated T-lymphocytes, mast cells and bone marrow stromal fibroblasts. It has been reported that these inflammatory cells infiltrate tissue at the early stages of fibrosis, and their possible role in the development of the fibrotic process has been suggested by several research groups[2-5]. Recently, it has been reported that IL-4 may be a fibrogenic cytokine. The effects of IL-4 on fibroblast functions may have important implications for a normal healing response to tissue injury as well as several human fibrosing diseases in which mast cells and T-lymphocytes play a central role[6-7]. IL-4 stimulates the synthesis of the extracellular matrix proteins, such as, type I, type III collagen and fibronectin, by human dermal fibroblasts in vitro, and it specifically increases the steady-state levels of types I, type III collagen and fibronectin mRNAs without any effect on cytoplasmic β-actin mRNA[8].

The interactions between IL-4 and the collagen gene promoter on normal skin fibroblasts have not been studied extensively yet. The purpose of this experiment is to evaluate the effect of IL-4 on the

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extracellular matrix gene expression including type I collagen and fibronectin in normal human skin fibroblasts. We checked the amount of protein and mRNA and compared the results with IL-4 and without IL-4 using the methods of quantitation of collagen protein synthesis, and Northern and dot blot hybridization. At the same time, we checked the CAT activity using the type I collagen promoter with and without IL-4.

MATERIALS AND METHODS

Fibroblast culture

Fibroblasts were obtained from skin biopsies of normal adult donors. Normal skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Co., Catalog No. 31600-034) with 10% fetal bovine serum, penicillin (100U/ml), streptomycin (100μg/ml), and amphotericine B(1μg/ml) at 37℃ in a humidified atmosphere of 5% CO_2. The trypan blue test was used to check cell viability.

Treatment of interleukin-4

Confluent fibroblasts in 100mm diameter petridishes were treated with six different dilutions (0.01, 0.1, 1.0, 2.5, 5.0, 10.0ng/ml) of IL-4 (Genzyme Co, Cambridge, MA, USA) for 48 hours in the absence of fetal bovine serum.

Assay of collagen production

Fibroblasts were plated at a density of 1×10^5 cell/well on a 24-well culture plate. Monolayer cells with around 95% of confluency were rinsed with Hank's balanced salt solution(HBSS) and incubated for the desired period in a labeling medium(1mM of DMEM with 50μg/ml of ascorbate and 2μCi of [^{3}H]proline, with or without IL-4). Collagen production was determined by measuring the incorporation of [^{3}H]proline into bacterial collagenase-sensitive protein synthesized by confluent fibroblast monolayer. The used method was a modification of Peterkofsky's and Diegelmann's method[9]. The relative rate of collagen production as a percentage of the total protein production was caculated using the following formula :

$$\frac{\text{dpm in collagen} \times 100}{(\text{dpm in non-collagenous protein}) \times 5.4 + \text{dpm in collagen}}$$

cDNA preparation

The following specific coding sequence(cDNAs) of human was used in this study: for type I collagen: a 1.8-kb proα1(I) cDNA ; for fibronectin: a 1.3-kb cDNA; for glyceraldehyde 3-phosphate dehydrogenase(GAPDH): a 1.2-kb cDNA. The cDNAs were labeled with ^{32}P-dCTP(NEG 036H, New England Nuclear, Boston, USA) by nick translation[10] to a specific activity of approximately 1×10^8cpm/μg.

RNA isolation

The total RNA was isolated from cultured normal skin fibroblasts using the methods of Chomczynski and Sacchi[11]. The total RNA was lysed directly by adding a Guanidium-thiocyanate buffer (4M Guanidium thiocyanate, 5mM Na-citrate, pH 7.0, 0.5% sarcosyl, 0.1M-2-mercaptoethanol, and 0.33% antifoam A emulsion), followed by phenol extraction and ethanol precipitation. A RNA pellet was suspended in diethylpyrocarbonate treated water and the concentration of RNA was determined by measuring the absorbance at 260nm and the purity of the nucleic acid preparation was assessed by the 260/280nm ratio.

Northern and dot-blot hybridization

The RNA was subjected to both Northern blot and dot-blot hybridization analyses. The total RNA was analysed by 1% agarose gel eletrophoresis(50 volt, 5 hours) after denaturing the samples with formaldehyde and formamide[12]. The obtained RNA transcripts were transferred to the nitrocellulose filter(Trans-Blot, BioRad, Ricmond, USA) in 20 X SSC overnight at 4℃[13]. The samples for the Dot-blot analyses were denatured with formaldehyde, and 4 different dilutions from 4.0, 2.0, 1.0, and 0.5 μg of total RNA and were dotted onto a nitrocellulose filter using a Dot-blot vacuum manifold(Milliblot, Millipore, Tokyo, Japan). Then each filter was prehybridized with a prehybridization mixture(50% formamide, 0.1% SDS, 3

X SSC, 1 X Denhart's solution, and 50μg/ml sonicated salmon sperm DNA) for 12-18 hours at 42 ℃ and hybridized with ^{32}P-labeled cDNA by ni-ck translation at 42 ℃ for 24 to 36 hours. Following hybridization, washing and autoradiography was performed according to conventional methods.

Transfection experiments

The construct containing the 3.5-kb COL1A2 promoter fused to the chloramphenicol acetyl transferase(CAT) gene(pMS-3.5/CAT) was derived from a 3.5-kb EcoRI/SphI genomic subclone that spans from position -3500 to +58 of the COL1A2 promotor[14]. Cultured normal human skin fibroblasts were transfected by the calcium-chloride/DNA co-precipitation method followed by a 1-min glycerol(15%) shock. Twenty-four hours before transfection, exponentially growing fibroblasts were harvested by trypsinization and replated at a density of 1-3 × 10^5 cells/60-mm dish. The cells were co-transfected with the construct and β-galactosidase vector(pUC18 vector) by the calcium chloride technique at the 70% of confluency. Calcium chloride precipitates were prepared according to Graham and Van der Eb[15] with plasmid DNA at a concentration of 5μg/ml. After twenty-two hours of transfection, the media were changed with 5ng/ml, 10ng/ml of IL-4 treated and non-treated testing media, and then fibroblasts were harvested after 48 hours. The cells were lysed by three cycles of 5 minutes' freeze-thawing in 100μl of 0.25M Tris-HCl, pH 7.8. The β-galactosidase activity was used for determinations of CAT activity using [^{14}C]chloramphenicol as the substrate[16]. The acetylated and non acetylated forms of radioactive chloramphenicol were separated by thin layer chromatography and visualized by autoradiography. The enzyme activity was quantified by cutting out pieces of thin layer chromatography plates containing different forms of [^{14}C]chloramphenicol converted to its acetylated forms. Each piece of data was corrected by β-galac tosidase activity.

RESULTS

The effect of IL-4 on collagen synthesis

The time response effect of IL-4 on collagen

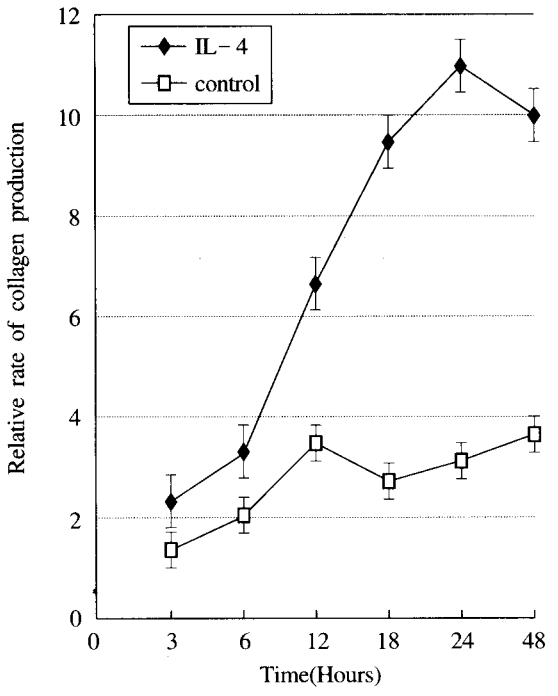

Fig. 1. Time response effect of IL-4 on collagen synthesis by culture of normal skin fibroblasts.
The maximum effect was observed at 24 hours after IL-4 treatment.

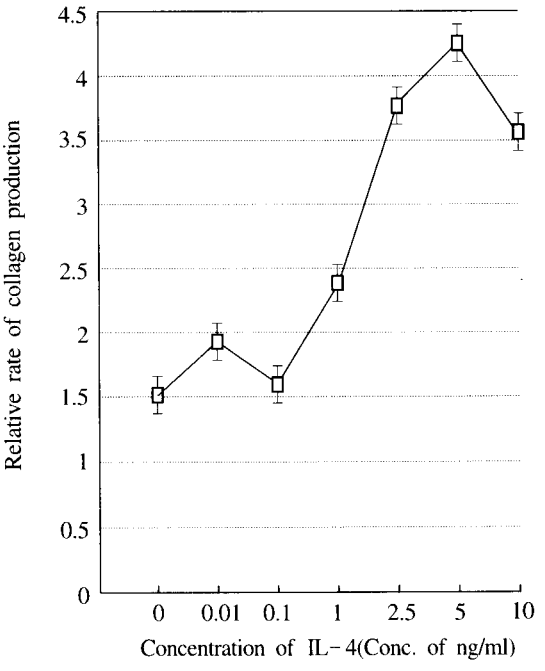

Fig. 2. Dose response effect of IL-4 on collagen synthesis by culture of normal skin fibroblasts.
It shows dose-dependent increase of collagen productions.

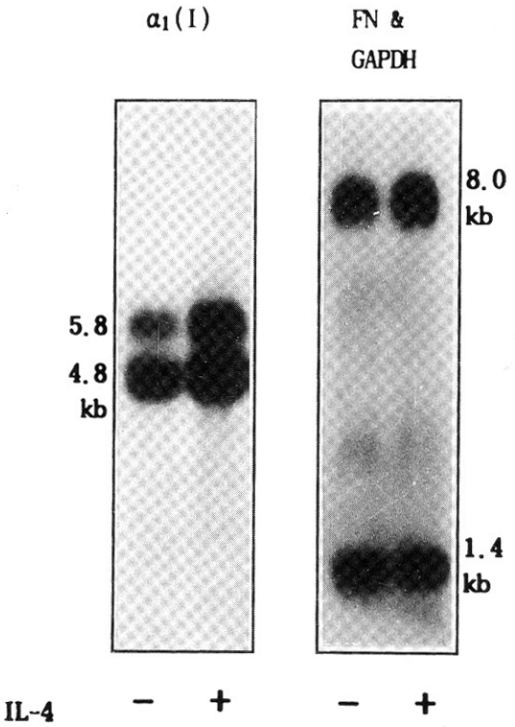

Fig. 3. Northern transfer analysis of proα1(I) collagen, fibronectin(FN) and GAPDH mRNA in IL-4 treated(5ng/ml) and non-treated groups in skin fibroblasts cultures.

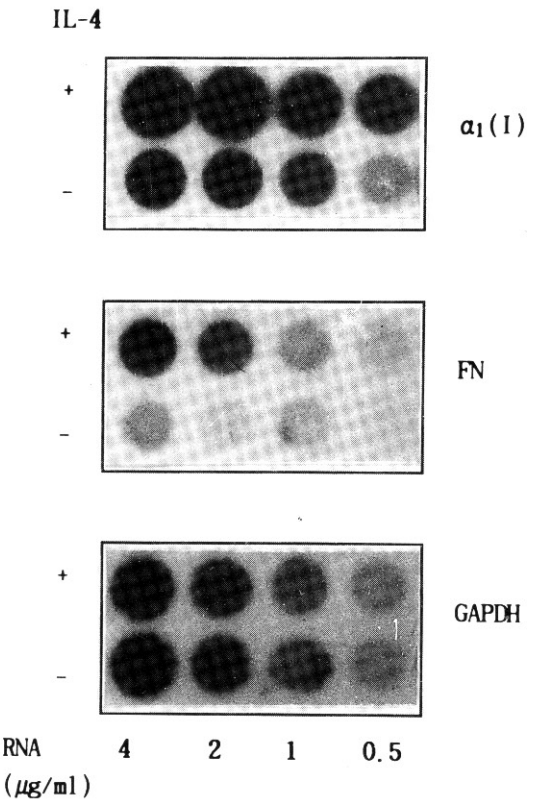

Fig. 4. Dot-blot analysis of proα1(I), fibronectin(FN) and GAPDH mRNA levels in IL-4 treated (5ng/ml) and non-treated groups in skin fibroblasts cultures.

synthesis by cultured normal skin fibroblasts was maximized after an incubation period of 24 hours (Fig. 1). After incubation for 24 hours, purified IL-4 stimulated production of collagen by dermal fibroblasts in a dose-dependent fashion with maximal stimulation occurring at 5.0ng/ml from normal fibroblasts. Compared to IL-4 non-treated fibroblasts, collagen production was increased 2.7 fold(Fig. 2).

Northern blot analysis of proα1(I) collagen and fibronectin mRNA

In the Northern blot analysis of cultured normal skin fibroblasts with or without IL-4 treatment, ^{32}P labeled Proα1(I) collagen, fibronectin, and GAPDH cDNA probes hybridized with each mRNAs

Table 1. Relative steady-state levels of mRNA in normal skin fibroblast cultures incubated with (5 ng/ml) and without IL-4

mRNAs	Nc	Nt	Fold Stimulation
proα1(I)/GAPDH	0.43 ± 0.03	1.29 ± 0.13*	3.00
fibronectin/GAPDH	0.9 ± 0.05	2.55 ± 0.17*	2.80

The values are expressed as densitometric absorbance unit.
Nc : normal fibroblast incubated without IL-4
Nt : normal fibroblast incubated with IL-4
* : $p<0.05$, compared with Nc

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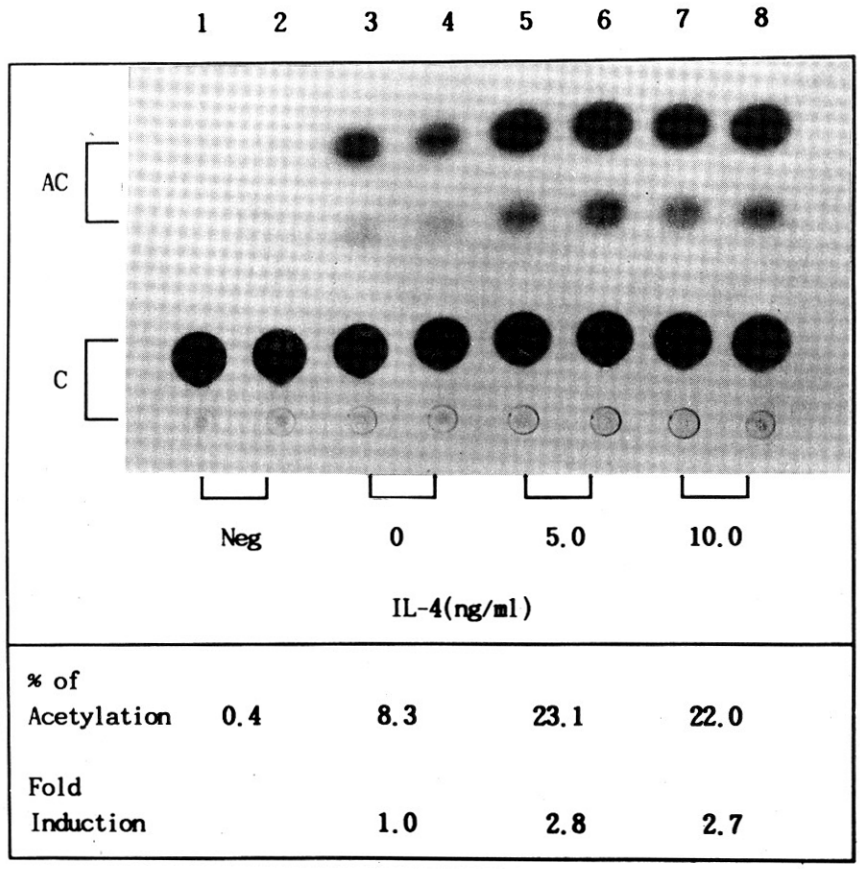

Fig. 5. Activity of COL1A2 promotor(pMS 3.5/CAT) in cultured normal fibroblasts. The figure shows the CAT assay depiciting a separation of acetylated(AC) and unacetylated(C) forms of [^{14}C] chloramphenicol by thin layer chromatography.
Lane 1 & 2, pSVO/CAT(Negative control)
Lane 3 & 4, pMS 3.5/CAT
Lane 5 & 6, pMS 3.5/CAT with IL-4 5ng/ml
Lane 7 & 8, pMS 3.5/CAT with IL-4 10ng/ml

specifically. Proα1(I) collagen revealed two mRNA transcripts, whose sizes were 5.8-kb and 4.8-kb. Fibronectin and GAPDH revealed one transcript, 8.0-kb and 1.4-kb, respectively(Fig. 3). There have been no changes in the size of each transcript since the treatment of IL-4, which indicates no alteration of its quality.

The effect of IL-4 on the steady-state accumulation of type I collagen and fibronectin mRNA

Proα1(I) collagen and fibronectin mRNA levels were measured by quantitative dot-blot hybridization(Fig. 4). Steady-state levels of type I collagen and fibronectin mRNAs increased in both the IL-4 treated groups. In the cultured normal fibroblasts, there was a 3.0, and 2.8 fold increase, respectively, as compared to the IL-4 non-treated groups ($p<0.05$)(Table 1).

Evidence for transcriptional regulation of the type I collagen gene at the promoter level

Fibroblasts were transfected with the construct and IL-4 in a concentration of 5.0ng/ml and 10ng/ml was added to the culture media for 24 hours after glycerol shock. As the result of the CAT assay, the percentages of acetylation were

8.3% in the non-treated control, 22.0% in 10ng/ml of IL-4, and 23.1% in 5.0ng/ml of the IL-4 treated groups. The promoter activity was increased 2.8 fold in 5.0ng/ml of the IL-4 treated fibroblasts compared to the control groups. This indicates that IL-4 may be a powerful up-regulator of proα2(I) collagen promoter activity, suggesting transcriptional enhancement of gene expression (Fig. 5).

DISCUSSION

It has been suggested that the cytokines from tissue infiltrated inflammatory cells play a role in the pathogenesis of fibrosis. For example, the levels of the T-cell-derived T-cell growth factor, IL-2, are increased in the blood of patients with progressive systemic sclerosis. Monocytes obtained from patients with systemic sclerosis produce diminished amounts of IL-1[17]. In addition, transforming growth factor-β involves extracellular matrix gene expression in the fibrotic process[18]. Scleroderma sera have been more frequently measured quantities of IL-4, IL-6, and IL-2. IL-4 causes adult dermal fibroblasts to proliferate and to make IL-6. IL-6 has been shown to stimulate fibroblast synthesis of collagen and glycosaminoglycans[19]. Among the cytokines, IL-4 is a candidate for connective tissue cell activation. Recently, it has been reported in the studies of human fibroblasts that IL-4 has fibrogenic activity in response to IL-4[6]. It is secreted by a restricted number of cells, mainly T-lymphocytes and mast cells. Tissue infiltration by these inflammatory cells has been reported at the early stages of fibrosis, and their possible involvement in the development of the fibrotic process has been suggested[2-5]. IL-4 stimulates the synthesis of the extracellular matrix proteins, e.g. type I and III collagen and fibronectin, by human dermal fibroblasts in human fibroblast monolayer cultures without change in collagenase synthesis[20]. The stimulation of collagen production by human recombinant IL-4 was also demonstrated in several fibroblastic synovial cell lines obtained from patients with rheumatoid arthritis and osteoarthritis.

There are increased numbers of activated T-cells in early lesions of scleroderma, therefore T-cells produce greater amounts of TGF-β , IL-4, and lymphotoxin which stimulate fibroblast growth in scleroderma than in normal skin tissue. Therefore it is suspected that increased activated T-cells are responsible for the fibrosis process in fibrotic diseases. However, Fertin et al[7] reported that IL-4 was devoid of effects on fibroblast proliferation but that it stimulated collagen gene expression. Mast cells, which are a source of IL-4, are closely associated with inflammation and the fibrotic responses, especially scleroderma related syndromes such as toxic oil syndrome, eosinophilic fasciitis, chronic graft versus host disease, and bleomycin induced fibrosis[21]. In contrast, interferon-γ and tumor necrosis factor-α suppress collagen synthesis[22]. Even though many of these cytokines can affect fibroblast growth, TGF-β produced by T cells and platelets mainly plays a central role in increasing collagen production[18]. Because of the complex effects of cytokines on collagen synthesis, it will be an interesting experimental field to focus on the pathophysiological relationship between many cytokines, especially IL-4, and any of the other fibrotic diseases mentioned above. In this study, purified IL-4 stimulated the production of collagen by dermal fibroblasts in a dose-dependent fashion with maximal stimulation at 5.0ng/ml. Compared to IL-4 non-treated fibroblasts, collagen production was increased 2.7 fold in the IL-4 treated group of normal skin. Data from this study also showed that steady-state levels of type I collagen and fibronectin mRNAs were increased in IL-4 treated fibroblasts.

A deeper understanding of the fibrotic process may come when more is known about regulatory sequences in the 5' region of the collagen genes: what factors turn them on and off, how cytokines modulate their expression, and which sequences are important for interaction with cis- and trans-acting factors[23]. To understand how collagen transcription is selectively controlled in physiological conditions, a number of studies have recently focused on the identification and characterization of cis-acting DNA regulatory elements in mammalian collagen genes[24-25]. These investigations have revealed that collagen expression is regulated by unique arrangements of complex networks of interacting sequences which reside both downstream and upstream of the transcription initiation sites. It is of interest how IL-4 affects the promoter activity and whether IL-4 modulates the cis-acting DNA sequences controlling the tran-

scription of the type I collagen genes or not. As a first step toward understanding this, a transfection experiment and CAT assay were performed in this study on the basis that IL-4 might stimulate any cis-acting DNA regulatory elements of the promoter gene which regulates DNA sequences encoding type I collagen protein. The stimulatory effect of human recombinant IL-4 on fibroblast collagen synthesis was an increase in the steady-state levels of type I collagen mRNAs. Nuclear run-off transcriptional experiments indicated that IL-4 stimulated the rates of mRNA biogenesis. Based on these observations, we might conclude that IL-4 exerts its effect on collagen synthesis at the pretranslational level, and the transcriptional level, resulting in the synthesis and accumulation of these collagen in the tissue. We did not study the other cytokines, but based on our data, we presumed that IL-4 could have a stimulatory effect on collagen synthesis by itself or synergistically with other cytokines. The identification and characterization of cis-acting DNA regulatory elements has not been determined yet. An important question therefore, is how collagen transcription is controlled in the IL-4 stimulation and what the unique arrangements are in the complex networks of interacting sequences which reside both downstream and upstream of the transcription initiation sites.

REFERENCES

1. Banchereau J: Human interleukin-4 and its receptor. In Mertelsmann R, Herrman F(eds): Hematopoietic growth factors in clinical applications. Marcel Dek ker Inc, New York, 1991, pp957-1019.
2. Fleischmajer R, Perlish JS, Duncan M: Scleroderma. A model of fibrosis. Arch Dermatol 119:957-961, 1983.
3. Seibold JR, Giorno RC, Claman HN: Dermal mast cell degranulation in systemic sclerosis. Arthrit Rheum 33:1702-1709, 1990.
4. Thompson HI, Burbelo PD, Gabriel G, et al: Murine mast cells synthesize basement membrane components: A potential role in early fibrosis. J Clin Invest 87:619-623, 1991.
5. Ruoss SJ, Hartmann T, Caughey GH: Mast cell tryptase is a mitogen for cultured fibroblasts. J Clin Invest 88:493-499, 1991.
6. Gillery P, Fertin C, Nicholas JF, et al: Interleukin-4 stimulates collagen gene expression in human fibroblast monolayer cultures: Potential role in fibrosis. FEBS Lett 302:231-234, 1992.
7. Fertin C, Nicholas JF, Gillery P, et al: Interleukin-4 stimulates collagen synthesis by normal and scleroderma fibroblasts in dermal equivalents. Cell Mol Biol 37:823-829, 1991.
8. Postlethwaite AE, Holness MA, Katai H, et al: Human fibroblast synthesize elevated levels of extracellular matrix protein in response to interleukin-4. J Clin Invest 90:1470-1485, 1992.
9. Peterkofsky B, Diegelmann R: Use of mixture of proteinasefree collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10:988-994, 1971.
10. Rigby PWJ, Dieckmann M, Rhodes L, et al: Labeling deoxyribonucleic acid to high specific in vitro by nick-translation with DNA polymerase. J Mol Biol 113:237-251, 1977.
11. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidilm, thiocyanate-phenolchloroform extraction. Anal Biochem 162:156-159, 1987.
12. Whal GM, Stern M, Starck GR: Efficient transfer of large DNA fragments from agarose gel to diabenzylloxymethyl paper and rapid hybridization by using dextran sulfate. Proc Natl Sci USA 76:3683-3687, 19 79.
13. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Sci USA 77:5201-5205, 1980.
14. Dickson LA, DiLiberto M, Weil D, et al: Analysis of the promoter region and N-propeptide domain of the human pro α 2(I) collagen gene. Nucleic Acids Res 12:6117-6125, 1985.
15. Graham FL, Vander Eb AJ: A new technique for the assay of infectivity of adenovirus 5 DNA. Virology 57:456-467, 1973.
16. Gorman CM, Moffat LF, Howard BH: Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044-1054, 1982.
17. Whicher JT, Gilbert AM, Westacott C, et al: Defective production of leukocytic endogenous mediator(interleukin-1) by peripheral blood leukocytes of patients with systemic sclerosis, SLE, rheuamtoid arthritis, and connective tissue disease. Clin Exp Immunol 65:80-89, 1986.
18. Roberts AB, Sporn MB, Assoian RK, et al: Transforming Growth factor type beta: Rapid induction

of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83:4167-4171, 1986.

19. Smith EA: Connective tissue metabolism including cytokines in scleroderma. Curr Opin Rheumatol 4:869-877, 1993.
20. Uitto J, Bauer EA, Eisen AZ: Scleroderma: Increased biosynthesis of triple helical type I and type III procollagen with unaltered expression of collagenase by skin fibroblast in culture. J Clin Invest 64:921-930, 19 79.
21. Kahari VM, Chen YQ, Su MW, et al: Tumor necrosis factor-α and interferon-γ suppress the activation of human type I collagen gene expression by transforming growth factor-β 1. J Clin Ivest 86:1489-1495, 1990.
22. Claman HN: Mast cells and fibrosis. Rheum Dis Clin North Am 16:141-151, 1990.
23. Vuorio T: Activation of type I collagen genes in cultured scleroderma fibroblasts. J Cell Biochem 28:105 -113, 1985.
24. Schmidt A, Rossi P, Crombrugghe B: Transcriptional control of the mouse 2(I) collagen gene: functional deletion analysis of the promotor and evidence for cell specific expression. Mol Cell Biol 6:347-354, 1986.
25. Boast S, Su MW, Ramirez F, et al: Functional analysis of cisacting DNA sequences controlling transcription of the human type I collagen genes. J Biol Chem 265:13351-13356, 1990.