Interleukin-10 Differently Regulates Type I Collagen and Stromelysin-1 Promoter Activities in Dermal Fibroblast Cultures

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***Background*: Interleukin-10(IL-10) was initially discovered and isolated on the basis of its ability to suppress cytokine by Th1 helper T cell. Recently, the effect of IL-10 has been reported in cultured connective tissue cells.**

***Objective*: To further investigate the mechanisms of IL-10 on extracellular matrix(ECM) homeostasis, we evaluated the expression of type I collagen and stromelysin-1 at the transcriptional level and also observed their promoters activities.**

***Methods*: We examined that effect of the recombinant human IL-10 on the expression of genes involved in extracelluar matrix(ECM) synthesis and remodelimg in human dermal fibroblast cultures. Quantification of collagen synthesis, Northern blot analysis, transient transfection and a chloramphenicol acetyl transferase(CAT) assay were performed.**

***Results*: In studying the dose and time response effect of IL-10 on collagen synthesis, maximal reduction was seen at 1.0 ng/ml concentration and 24 hours incubation. The synthesis of type I collagen mRNA was downregulated, while stromelysin-1 gene expression was enhanced by IL-10. In the transient transfection and CAT assay, the type I collagen promoter/CAT reporter gene construct showed downregulation by IL-10, while the stromelysin-1 gene promoter activities were upregulated.**

***Conclusion*: It is suggested that IL-10 differently regulates the transcriptional levels of type I collagen and stromelysin-1 gene and denoting IL-10 seems to take part in the homeostasis of ECM at pretranscriptional level. (Ann Dermatol 10:(1) 6~12, 1998).**

Key Words : IL-10, Type I collagen, Stromelysin-1, CAT assay

An enormous amount of information on interleukin-10(IL-10) has been gathered since its original description as a cytokine synthesis inhibition factor(CSIF) several years ago. These earlier studies on IL-10 have mainly concentrated on its effects on the various immunocompetent cells. IL-10 is mainly downregulatory towards T lymphocytes and monocytes[1]. For example, IL-10 is a powerful suppressor of the synthesis of IL-1 , IL-1 , IL-6, IL-8, TNF- , and granuloctye colony stimulating factor produced by activated monocytes[2,3].

The epidermis is a rich source of proinflammatory cytokines and growth factors, including IL-10. These factors may act a crucial role in maintenance of equilibrium between deposition and degradation of the extracellular matrix(ECM), which is essential to normal tissue development, repair of wounds and inflammatory responses in the skin[4].

Recently there have been some reports about the effects of IL-10 on the ECM gene expression[5]. To understand how ECM gene transcription is selectively controlled under physiological conditions, a number of studies have recently focused on the identification and characterization of cis-acting DNA regulatory elements in mammalian collagen

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genes[6]. In broad terms, these investigations have released 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. We are greatly interested in how IL-10 will affect the promoter activity and whether IL-10 will modulate the cis-acting DNA sequences controlling transcription of the type I collagen and stromely-sin-1 genes or not. As a first step towards this goal, a transfection experiment and CAT assay were performed to determine whether IL-10 might affect any cis-acting DNA regulatory element of promotor genes which regulate DNA sequences encoding type I collagen and stromelysin-1.

In this study, we noticed some different mechanisms which IL-10 modulates including in vitro expression of the collagen and stromelysin-1 genes. These are involved in ECM synthesis and breakdown in human dermal fibroblasts.

MATERIAL AND METHODS

Cell culture

Adult human skin fibroblast cultures were established from tissue specimens obtained during a dermatological surgery. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, penicillin(100 U/ml), streptomycin (100μg/ml), and amphotericine B(1μg/ml) at 37℃ in a humidified atmosphere of 5% CO_2.

Treatment of IL-10

Recombinant human IL-10(molecular mass 18.5kD) obtained from Genzyme Co (U.S.A.) in lyophilized form was dissolved in water and further dissolved in DMEM at varying concentrations, ranging from 1 to 20ng/ml, for 24 hours in the absence of fetal bovine serum.

Assay of collagen production

An assay of collagen production was performed as described previously[7]. Collagen production was determined by measuring the incorporation of [^{3}H]proline into bacterial collagenase sensitive protein synthesized by confluent fibroblast monolayers. Then 250μl of 5× collagenase buffer containing 0.25 M Tris, 0.025 M $CaCl_2$ and 0.0125 M N-ethylmaleimide, pH 7.4 was added to the wells and the contents were sonicated for 25 to 30 seconds at 25% of maximum voltage with the microtip of the sonicator, keeping the sample placed on ice. The relative rate of collagen production as a percentage of total protein production was calculated by the following formula:

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

where disintegration per minute(dpm) in noncollagenous protein was determined by subtracting the dpm in collagen from the dpm in total protein[8]. Cellular DNA contents were measured by the method of Labarca and Paigen[9] and used as the base of protein synthesis.

cDNA preparation

The following human-sequence-specific cDNAs were utilized in this study: for α1(I) collagen; a 1.8kb α1(I) collagen cDNA[10] ; for stromelysin-1; a 1.0kb stromelysin-1 cDNA[11]; for GAPDH: a 1.2kb GAPDH cDNA[12]. The cDNAs were labelled with [^{32}P]-dCTP(NEG 036H, New England Nuclear, U.S.A.) by nick translation[13] to a specific activity of approximately 1×10^8cpm/μg.

Northern blot analysis

Total RNA was isolated by the methods of Chomzynski and Sacchi from cultured normal skin fibroblasts[14]. Total RNA was fractionated by 1% agarose gel electrophoresis(85 volt, 5 hours) after denaturating the samples with formaldehyde and formamide[15]. RNA transcripts obtained were transferred to the nitrocellulose filter(Trans-Blot, BioRad, Richmond, U.S.A.) in 20× SSC overnight at 4℃[16]. The filters were prehybridized for 12-18 hours at 42℃ with prehybridization mixture(50% formamide, 0.1% SDS, 3× SSC, 1× Denhart's solution, 50 g/ml ss-DNA) and hybridized with 32P-labeled cDNA by nick translation at 42℃ for 24 to 36 hours. Following hybridization, the filters were washed and autoradiography was performed.

Chimeric DNA constructs and expression vector

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 promoter[17]. The construct containing the 566-bp stromelysin-1 promoter gene(ST-560/CAT) was derived from a 560bp Xho/HindIII genomic subclone (kindly provided by Dr. Daisuke Sawamura) that spans from position -560 to +6 of the stromelysin-1 promotor[18].

Transfection and CAT assay

Cells were transfected with chimeric DNA constructs by the calcium phosphate technique followed by a 1-min glycerol(15%) shock[19]. To provide an internal control for DNA uptake, some experiments included co-transfection with the RSV-ß-galactosidase construct[20]. The cells were harvested after 24 hours of incubation and lysed by three cycles of freeze-thawing. An aliquot of the samples containing the same amount of beta-galactosidase activity was used with [^{14}C] chloramphenicol as a substrate, and the acetylated and nonacetylated forms of radioactive chloramphenicol were separated using thin layer chromatography[21]. One hundred micrograms of protein extract were used in each assay, as determined by Lowry et al[22].

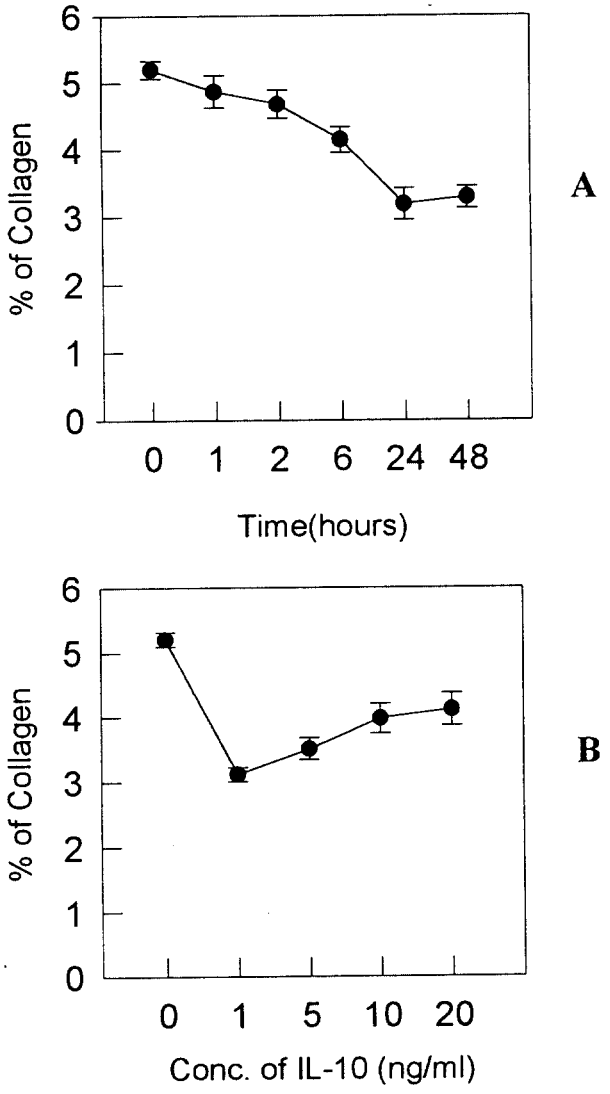

Fig. 1. Time(A), dose(B) response effects of IL-10 on collagen synthesis in cultured normal skin fibroblasts.

RESULTS

Effect of IL-10 on collagen synthesis

Purified IL-10 decreased production of collagen by dermal fibroblasts in a time dependent manner which was maximized after incubation of 24 hours. During the incubation time, collagen production decreased from 0.77 fold to 0.86 fold in the IL-10 treated cultured skin fibroblasts(Fig. 1A). The dose-response curve revealed that maximal stimulation occurred at 1ng/ml of IL-10. In comparison with non-IL-10 treated fibroblasts, collagen production decreased 0.93 to 0.63 fold(Fig. 1B).

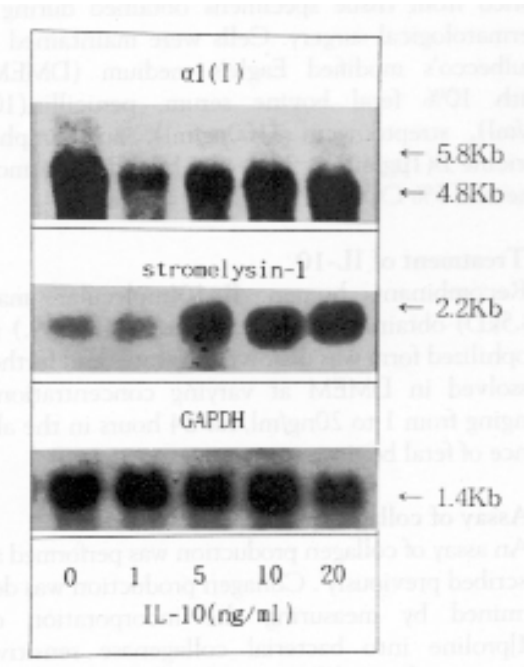

Fig. 2. Effects of different concentrations of IL-10 on type I collagen and stromelysin-1 mRNA levels in cultured normal skin fibroblasts.

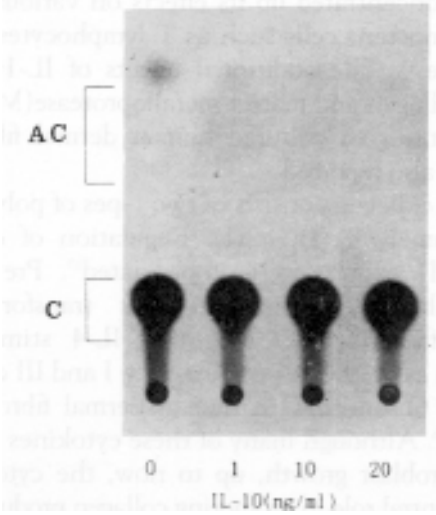

Fig. 3. Effects of IL-10 on type I collagen promoter activity in cultured fibroblasts. This figure shows a CAT assay depicting the separation of acetylated(AC) and unacetylated(C) forms of [^{14}C]chloramphenicol by thin layer chromatography.

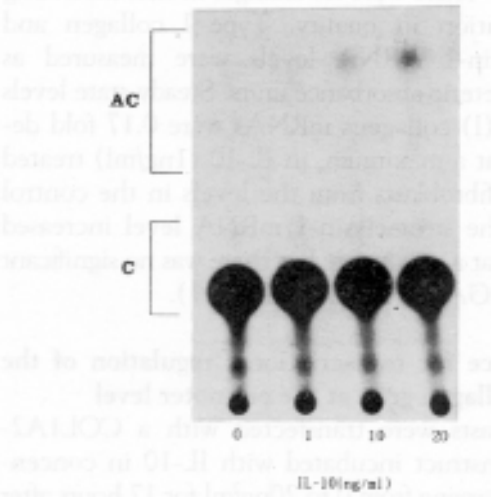

Fig. 4. Effect of IL-10 on stromelysin-1 promoter activity in cultured fibroblasts. This figure shows a CAT assay depicting the separation of acetylated(AC) and unacetylated(C) forms of [^{14}C]chloramphenicol by thin layer chromatography.

Table 1. Relative steady-state levels of type I collagen & stromelysin-1 mRNA in cultured normal skin fibroblasts incubated with different concentrations of IL-10

IL-10 (ng/ml)	Type I collagen/ GAPDH	Stromelysin-1/ GAPDH
0	283 ± 21.2	81 ± 7.1
1	50 ± 4.1*	102 ± 9.5
5	102 ± 18.1	123 ± 11.6
10	133 ± 15.9	298 ± 21.2
20	175 ± 15.2	321 ± 25.3*

The values are mean ±SD and expressed, as densitometric absor-bance units(* $p<0.05$).

Effects of IL-10 on the steady-state accumulation of type I collagen, and stromelysin-1 mRNA

In Northern blot analysis of cultured normal skin fibroblasts with or without IL-10 treatment, [^{32}P] labeled Pro 1(I) collagen, stromelysin-1 and GAPDH cDNA probes specifically hybridized with each mRNA. The incubation time response effect of IL-10 on the steady state levels of pro 1(I) collagen mRNA was maximized, as a 0.26 fold decrease, after 24 hours of incubation(data not shown). Pro- 1(I) collagen revealed two mRNA transcripts, 5.8-kb and 4.8-kb, in size which strolmelysin-1 and GAPDH revealed one tran-

Table 2. CAT assay of type I collagen and stromelysin-1 promoter activity in cultured normal skin fibroblasts incubated with different concentrations of IL-10

	Type I collagen		Stromelysin-1	
IL-10 (ng/ml)	CAT activity	fold difference	CAT activity	fold difference
0	10.2 ± 0.09	1.0	0.9 ± 0.01	1.0
1	1.8 ± 0.02*	0.17	1.7 ± 0.05	1.8
10	2.3 ± 0.03	0.23	10.2 ± 0.11	11.3
20	2.8 ± 0.01	0.27	20.4 ± 1.98*	22.6

The values of CAT activity are expressed as the percentage of acetylation(mean ± SD) of [^{14}C]chloramphenicol (* $p<0.05$).

script, with sizes of 2.2-kb and 1.4-kb respectively(Fig. 2). There were no changes in size, indicating no alteration in quality. Type I collagen and stromelysin-1 mRNA levels were measured as densitometeric absorbance units. Steady-state levels of pro- 1(I) collagens mRNAs were 0.17 fold decreased, at a maximum, in IL-10 (1ng/ml) treated cultured fibroblasts from the levels in the control group. The stromelysin-1 mRNA level increased 3.96 fold at a maximum, but there was no significant effect on GAPDH mRNA(Table 1).

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

Fibroblasts were transfected with a COL1A2-CAT construct incubated with IL-10 in concentrations varying from 0 to 20ng/ml for 17 hours after glycerol shock. The CAT assay, revealed that the percentages of acetylation were 10.2 ± 0.09% in untreated controls, and 1.8 °æ 0.02% in the IL-10 treated group. Thus the α2(I) collagen gene promotor activities were decreased 0.17 fold. This data suggests that IL-10 may be a stimulator of downregulation of the type I collagen gene promoter activity at the pretranscriptional level in the gene expression(Fig. 3, Table 2).

Evidence for transcriptional regulation of the stromelysin-1 gene at the promoter level

Fibroblasts were transfected with stromelysin-1-CAT construct and incubated with IL-10 in concentrations varying from 0.01 to 1 U/ml added to the culture media for 24 hours after glycerol shock. The CAT assay revealed that the percentages of acetylation were 0.9 °æ 0.01% in the untreated controls and 20.43 °æ 1.98% in IL-10 the treated group. Thus the stromelysin-1 gene promotor activities were increased 22.6 fold. This data suggests that IL-10 may be an upregulator of stromelysin-1 gene promoter activity and enhance pretranscriptional gene expression in human skin fibroblasts(Fig. 4, Table 2).

DISCUSSION

IL-10(18.5-kD) which was selectively produced by Th2 clones affects the synthesis of IL-2 and interferon- by Th1 cells and macrophages. It was originally named cytokine synthesis inhibitory factor, as it was identified and its gene was cloned based on its cytokine synthesis inhibitory activities[23]. Most studies concentrated up its effects on various immunocompetent cells such as T lymphocytes and monocytes[24]. The additional effects of IL-10 on type I collagen and matrix metalloprotease(MMP) transcriptions in cultured human dermal fibroblasts was also reported[5].

Type I collagen consists of two types of polypeptides, namely α1(I), α2(I). Regulation of α1(I) and α2(I) expression is coordinated[25]. Previous studies have demonstrated that transforming growth factor-beta(TGF-ß) and IL-4 stimulate synthesis of the ECM proteins, type I and III collagen and fibronectin, in human dermal fibroblast cultures[26]. Although many of these cytokines stimulate fibroblast growth, up to now, the cytokine with a central role in promoting collagen production has been reported to be TGF-ß, which is produced by a variety of cells including T cells and platelets[27].

Matrix metalloproteases (MMPs) constitute a familly of structurally related proteolytic enzymes, but their specific activities are divergent[28]. For example, MMP-1(interstitial collagenase) is the major enzyme responsible for degradation of extracellular fibers comprised of collagen types Ⅰ, Ⅱ, or Ⅲ. MMP-3 (stromelysin-1) has a broad spectrum of proteolytic activities, including degradation of various gelatins, proteoglycan linked protein, fibronectin, and laminin, as well as native collagen types Ⅲ, Ⅳ, and Ⅸ[29]. Stromelysin-1 is also required for maximal activation of MMP-1 in physiological situations[29]. In the developing and adult organism, temporal and spatial expression of extracellular matrix genes is modulated by a variety of cytokines and hormones[30]. Likewise, transcription of collagen genes and MMP genes in tissue cultures can be greatly affected by the action of these substances and by chemical or viral transformation as well[31].

Cytokine mediated increases in collagen deposition and degradation in response to environmental stimuli are also the major histopathological feature of maintance of ECM[32]. Focusing on the pathophysiological relationship between many cytokines, especially IL-10, and the other tissue degrading conditions mentioned above will be an interesting experimental field. In the present study, the assay of collagen production showed that IL-10 decreased the collagen synthesis in normal skin fibroblasts cultures. The steady-state levels of pro

1(I) collagen mRNAs were decreased in IL-10 treated cultured fibroblasts, with maximal reduction at 1ng/ml concentration and 24 hours incubation, but there was no significant effect on GAPDH mRNA. These results are similar with previous reports by Reitamo et al[5].

Unlike type I collagen, the transcriptional levels of stromelysin-1 mRNA were increased by IL-10 in a dose dependent fashion. This data suggests that IL-10 may be a cytokine whose effects include both fibrosis suppression and fibrolysis activation that occur during embryonic development, uterine resorption, and wound healing as well as in diseases, such as rheumatoid arthritis and tumor invasion[33].

An understanding of the equilibrium of ECM may come when we know more about regulatory sequences in the 5' region of the pro α2 (I) collagen and stromelysin-1 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[34]. According to the CAT assay of promotor activity, the pro 2(I) collagen promotor activities were decreased 0.17 fold and the stromelysin-1 gene promotor activities were increased 22.6 fold. Thus IL-10 exerts its effect on collagen and stromelysin-1 synthesis at the pretranscriptional level. It has been reported that collagenase activated by IL-10, could be due to a post-transcriptional mechanism[5]. In this study, we found stromelysin-1 was activated by IL-10 at a pretranscriptional level and it may participate in the breakdown of ECM. Furthermore, it is possible to suggest that these results could have connections with the prior report that stromelysin is required for the maximal activation of collagenase[30].

However, we have not identified or charctacterized the cis-acting DNA regulatory elements as yet. There remain interesting and important questions of how collagen and stromelysin-1 transcription are controlled in IL-10 stimulation and what the unique arrangements of complex networks of interacting sequences are which reside both downstream and upstream of the transcription initiation sites. Further studies are needed to determine whether other factors are related to the IL-10 activity and the regulation of ECM.

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