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Interleukin-4 stimulates collagen gene expression in human fibroblast monolayer cultures

Potential role in fibrosis

P. Gillery[a], C. Fertin[a,b], J.F. Nicolas[c], F. Chastang[a], B. Kalis[b], J. Banchereau[d] and F.X. Maquart[a]

[a]*Laboratoire de Biochimie, CNRS URA 610 and* [b]*Laboratoire de Recherche Biomédicale en Dermatologie, Faculté de Médecine, 51 Rue Cognacq Jay, 51095 Reims, cedex, France,* [c]*INSERM U 346, Pavillon R, Hôpital Edouard Herriot, 69437 Lyon cedex 03, France* and [d]*Schering-Plough, Laboratoire de Recherches Immunologiques, BP 11, 69572 Dardilly, France*

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A role for the cytokines produced by tissue-infiltrated inflammatory cells (mainly T-lymphocytes and mast cells) in the pathophysiology of fibrosis has been suggested by several groups. Among the products of these cells, interleukin-4 (IL-4) might be one of the factors involved in the initiation of the fibrotic process. We studied the effects of recombinant human IL-4 on human fibroblast monolayer cultures. IL-4 (10 and 100 U/ml) induced a dose-dependent increase of collagen production. Non-collagen protein synthesis was not significantly altered. A concomitant increase of pro-α1(I) collagen mRNAs was observed, showing that IL-4 acts at a pre-translational level.

Interleukin-4; Fibroblast; Collagen synthesis; Collagen gene expression; Fibrosis

1. INTRODUCTION

Interleukin-4 (IL-4) is a cytokine, first described as growth factor for B-lymphocytes and now known to possess pleiotropic activities on a variety of cells (for a review see [1]). IL-4 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 by several groups [2–5]. In particular activated mast cells have been found to have increased in the affected skin of patients suffering from scleroderma (systemic sclerosis), especially in the cases of rapidly extensive fibrosis [6,7]. Several of the secretion products of mast cells have been reported to activate connective tissue cells. Heparin, for instance, was identified as a trigger for the secretion of endothelial cell-derived growth factor by endothelial cells [8]. Histamine was shown to stimulate proliferation and matrix synthesis in resting fibroblasts [9]. Mast cell tryptase was also reported as a mitogen for cultured fibroblasts [5].

In this paper we have investigated the effects of recombinant human IL-4 on human fibroblasts in monolayer cultures. We found that IL-4 was devoid of effects on fibroblast proliferation but stimulated collagen gene expression.

Correspondence address: P. Gillery, Laboratoire de Biochimie, CNRS URA 610, Faculté de Médecine, 51 Rue Cognacq-Jay, 51095 Reims cedex, France. Fax: (33) 26 05 87 40.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Purified recombinant human IL-4 derived from *E. coli* was obtained from Schering-Plough Research (Bloomfield, NJ), specific activity 10^7 U/mg. L-Ascorbic acid was obtained from Merck, β-aminopropionitrile from Sigma, L-glutamine and L-proline from Calbiochem. Clostridial collagenase (CLSPA grade) was bought from Worthington and purified in the laboratory [10]. Uniformly labelled L-[U-^{14}C]proline (specific activity 9.25 TBq·mmol^{-1}) was obtained from NEN. Other reagents were from Prolabo (analytical grade). All the culture media were from Gibco. Reagents for molecular biology were bought from Sigma, 5'-[α-^{32}P]deoxycytidine (110 TBq/mmol) from Amersham, and nick translation kit from Gibco-BRL. Sequence specific cDNA probes for hybridizations were used: pro α1(I) collagen cDNA, pH CAL 1U clone [11] was a generous gift from Prof. E. Vuorio (Turku, Finland) and 36B4 cDNA, corresponding to a ubiquitously expressed gene [12] was from Prof. P. Chambon (LGME U 184, Strasbourg, France).

2.2. *Cell cultures*

Fibroblasts were cultured from explants of normal skin obtained from reparative surgery and grown in Nunc tissue culture flasks using routine techniques [13]. The sterility of the cultures was regularly checked, especially concerning mycoplasmas. Cell counts were performed routinely with a Neubauer cell, and the Trypan blue test was used for checking viability.

2.3. *Cell proliferation*

Kinetic measurement of cell growth was performed in a series of 4 plastic Petri dishes of 3 cm diameter. Every dish was seeded on day 0 with 25×10^3 cells and incubated in DMEM supplemented with 5% fetal bovine serum (FBS). On day 1, IL-4 was added to 2 series while the other 2 received the same volume of Dulbecco's phosphate-buffered saline. Every following day, 2 dishes incubated with IL-4 and 2 controls were trypsinized and cells counted as described above.

2.4. *Protein synthesis*

The methods used to measure protein synthesis have been described in detail by Bellon et al. [14]. Cells were grown to confluence in a series of four 25 cm^2 flasks. The incubations lasted for 24 h at 37°C under an atmosphere of 95% air/5% CO_2, in DMEM supplemented with 0.2 mM β-aminopropionitrile, 0.28 mM ascorbic acid, 2 mM glutamine, 1% FBS, 7.4 kBq · ml^{-1} [^{14}C]proline diluted in 350 μM cold proline and a convenient concentration of IL-4. The culture medium and cell layer were then collected separately and DNA measured in the cell extract [15]. In both fractions, the incorporation of [^{14}C]proline into total proteins, collagen and non-collagen proteins was measured by the collagenase digestion technique of Peterkofsky and Diegelman [10]. Measurement of the amount, radioactivity and specific radioactivity of collagen hydroxyproline was also done after acid hydrolysis, derivatization of the amino acids with the fluorophore, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, and separation of the fluorescent derivatives by thin-layer chromatography. The percentage of hydroxylation of proline residues was measured in the secreted collagen using the following formula: % hydroxylation = (cpm hydroxyproline × 100)/(cpm proline + cpm hydroxyproline). The specific radioactivity of collagen was calculated by the ratio of cpm collagen hydroxyproline/nmol collagen hydroxyproline.

2.5. *Collagen mRNAs*

Total RNA was extracted from IL-4-treated monolayer cultures of fibroblasts according to Chomczynski and Sacchi [16]. Briefly, confluent monolayers were dissolved in a 4 M guanidinium isothiocyanate–2.5 M sodium citrate buffer, pH 7.0, containing 0.5% sarcosyl and 0.1 M β-mercaptoethanol. An equal volume of phenol and a 0.2 vol of chloroform/isoamylic alcohol (49/1) mixture were added in the presence of 0.3 M sodium acetate. After stirring, cooling on ice and centrifuging at 10,000 × g for 20 min, RNAs contained in the aqueous phase were precipitated by 2 vols. of ethanol. Their purity and integrity were checked by measurement of the $A_{260\ nm}/A_{280\ nm}$ ratio and agarose gel electrophoresis.

For dot-blot analysis, RNAs were dissolved in 15% formaldehyde and 10 × SSC (1 × SSC=0.15 M NaCl, 0.015 M sodium citrate) and denatured for 15 min at 65°C. Serial dilutions of RNA in 10 × SSC were spotted onto a nylon membrane (Biodyne transfer membrane, Pall Ultrafine Filtration Corp.) with a Bio-Rad Minifold device, and fixed by heating for 2 h at 80°C.

For Northern blot analysis, RNAs were separated by 1% agarose gel electrophoresis and transferred by capillarity on a nylon membrane treated as above. Standards of 18 S and 28 S ribosomal RNA were used during electrophoresis.

Membranes were pre-hybridized for 12 h at 42°C in a solution of 50% formamide, 5 × SSC, 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolinone, 0.02% Ficoll), 0.05% sodium dodecyl sulfate (SDS) and 100 μg · ml^{-1} sonicated salmon sperm DNA. Hybridization was carried out for 24 h at 42°C in the same medium, which in addition contained specific nick-translated cDNA probes (3 × 10^6 cpm · ml^{-1}). Membranes were then washed with SSC and exposed to audioradiography with Hyperfilm MP film (Amersham).

The intensity of hybridization was evaluated by densitometric scanning with a Desaga CD 60 densitometer (Heidelberg, Germany). Results of pro α1(I) collagen mRNAs were expressed by comparison with those obtained for 36B4 mRNA.

2.6. *Statistical analysis*

Experiments were done in quadruplicate. The results were calculated per 10 μg DNA and expressed as means ± 1 standard deviation. The Student's t-test and the non-parametric Mann and Withney U-test were used for checking statistical significance [17].

3. RESULTS

Kinetics measurement of cell growth (Fig. 1) showed no significant difference between control cells and cells incubated with 100 U of IL-4 per ml. The Trypan blue test showed no alteration of cell viability (data not shown).

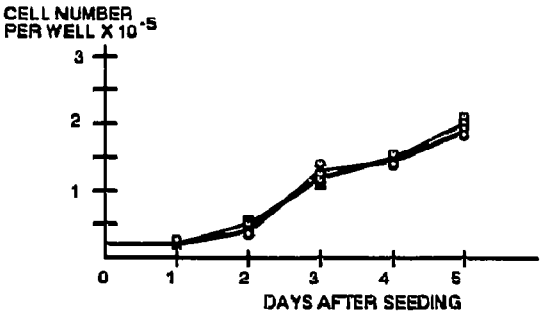

Fig. 1. Kinetics of human skin fibroblast proliferation in the absence (□) or presence (○) of IL-4, 100 U/ml. 25 × 10^3 cells were seeded in every dish on day 0.

IL-4, 10 and 100 U/ml, significantly stimulated collagen synthesis (Fig. 2), as indicated by [^{14}C]proline incorporation into collagenase-digestible proteins (Fig. 2B) and by fluorometric measurement of collagen hydroxyproline (Fig. 2C). The increase was about +60% at 10 U/ml and +100% at 100 U/ml. [^{14}C]Proline incorporation into non-collagen proteins was not significantly altered (Fig. 2A). The percentage of hydroxylation of proline residues was 37% in control cultures. It was not altered by IL-4 (Table I). No significant alteration of the specific radioactivity of collagen was observed in cells incubated with IL-4 (Table I).

Northern blot (Fig. 3A) and dot blot (Fig. 3B) analysis of the collagen pro α1(I) mRNAs showed a dose-dependent increase in cells incubated with 10 and 100 U/ml. This increase was quantified by comparison with the expression of control 36B4 mRNA (Fig. 3C). It was +50% at 10 U/ml and +120% at 100 U/ml.

Table I

Measurement of the percentage of hydroxylation of proline (pro) residues and of the specific radioactivity of hydroxyproline (hyp) in the secreted collagen

	Controls	IL-4	
		10 U/ml	100 U/ml
$\frac{\text{cpm pro} \times 100}{\text{cpm pro + cpm hyp}}$	36.8 ± 9.4	37.2 ± 6.6	39.5 ± 13.8
$\frac{\text{cpm hyp}}{\text{nmol hyp}}$	1,545 ± 680	1,362 ± 379	1,643 ± 723

Every data is the mean of 4 determinations ± 1 standard deviation. No significant difference was found.

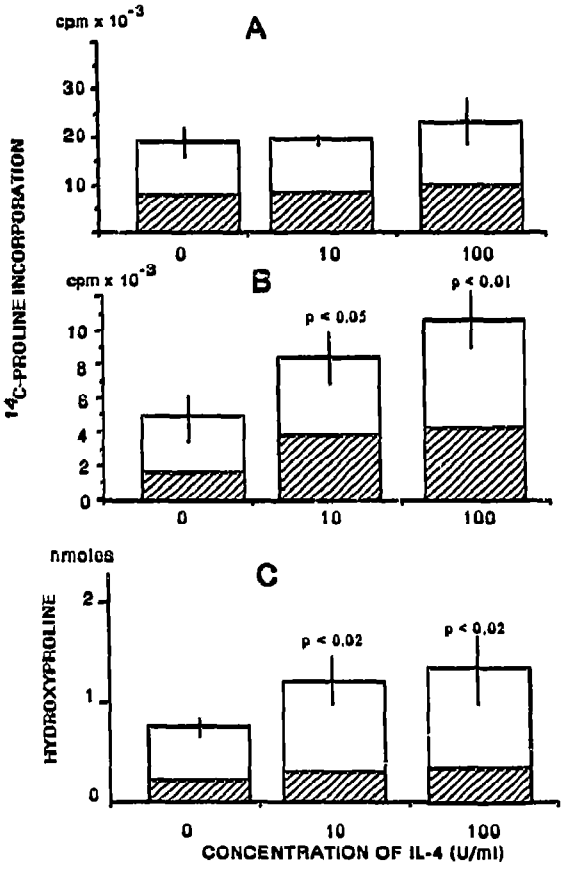

Fig. 2. Effects of IL-4 on (A) [^{14}C]proline incorporation into total proteins, (B) collagenase-digestible proteins and (C) on the amount of collagen hydroxyproline. Each bar represents the mean of 4 determinations ± 1 standard deviation. Open bars, culture medium; hatched bars, cell layer.

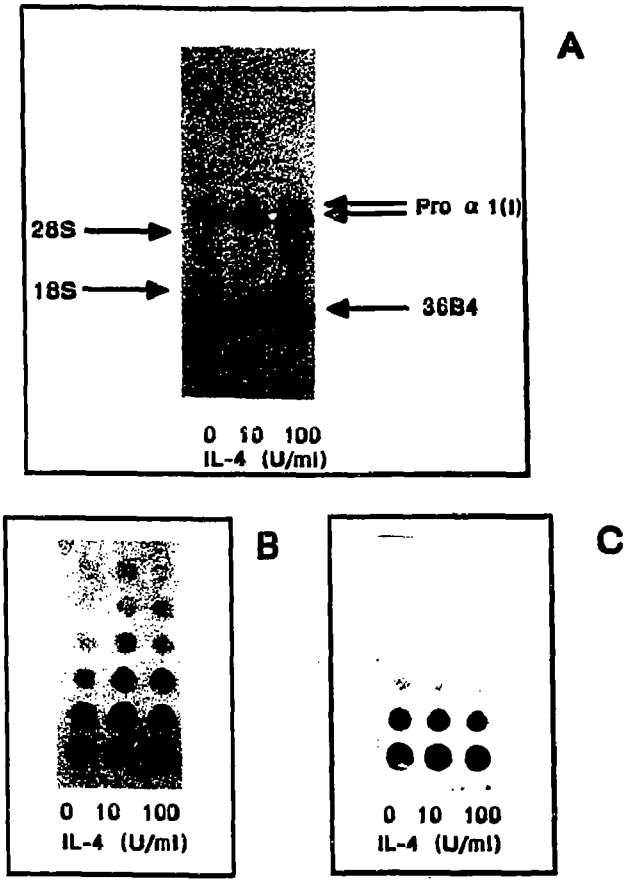

Fig. 3. Analysis of mRNAs extracted from fibroblast cultures incubated with IL-4, 0 (controls), 10 or 100 U/ml. (A) Northern blot analysis of pro α1(I) collagen and 36B4 mRNAs. 6 μg of total RNA were deposited in every lane, transferred and hybridized with specific cDNA probes. The position of pro α1(I) and 36B4 mRNAs, 18 S and 28 S ribosomal RNAs are indicated by arrows. (B,C) Dot-blot analysis. Serial dilutions of total RNA were deposited (10 to 0.3 μg) and hybridized with pro α1(I) cDNA probe (B) or control 36B4 cDNA probe (C).

4. DISCUSSION

The role of cytokines from tissue-infiltrated inflammatory cells in the pathophysiology of fibrosis has been suggested by several authors [2,9]. Among these cytokines IL-4 is a candidate for connective tissue cell activation. Up to now IL-4 was known mainly for its stimulating effects on the proliferation on human B-cells, T-lymphocytes, mast cells and haematopoietic progenitor cells [1]. A direct effect on extracellular matrix synthesis by fibroblasts has not yet been reported. Our results demonstrate that IL-4 is able to stimulate collagen synthesis by fibroblasts at physiological concentrations (100 U/ml=10 ng/ml). The combination of measurements of [^{14}C]proline incorporation into collagenase-digestible proteins and collagen hydroxyproline permitted the exclusion of any effect on the specific radioactivity of the secreted collagen or on its degree of hydroxylation. The intensity of the stimulation was of the same order of magnitude as that of transforming growth factor-β at the same concentration [18].

We demonstrated that IL-4 stimulates collagen gene expression at a pre-translational level. The increase of collagen mRNAs was of the same order of magnitude as that of the corresponding protein. It may correspond either to an increased gene transcription or to an increased stability of the mRNAs. The mechanism of signal transduction remains unclear. In human cells, no protein phosphorylation after binding of IL-4 was detected [19]. On the other hand, the rapid dephosphorylation of an 80 kDa phosphoprotein in cells treated with IL-4 was reported recently [20].

We found no significant effect of IL-4 on human fibroblast proliferation. A few years ago, Monroe et al. [21] reported that IL-4 stimulated [^{3}H]thymidine incorporation into murine fibroblasts. This discrepancy might be due to species specificity. Differences of methodology could also be implicated. Clement et al. [22] for instance, reported that [^{3}H]thymidine incorporation did not correlate with cell proliferation in cultured alveolar cells.

Our results support the involvement of T-cell or mast cell products on the accumulation of connective tissue proteins in fibrosis. The interactions of IL-4 with fibroblasts could, however, be modulated by other cytokines produced by inflammatory cells, for instance interferon-γ, which inhibits collagen synthesis [23] or interleukin-6 which was recently reported to stimulate collagen synthesis [24]. Further studies will be necessary to investigate these particular points.

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REFERENCES

[1] Banchereau, J. (1991) In: Hematopoietic Growth Factors in Clinical Applications (Mertelsmann, R. and Herrmann, F. eds) pp. 957–1019, Marcel Dekker, New York.
[2] Fleischmajer, R., Perlish, J.S. and Duncan, M. (1983) Arch. Dermatol. 119, 957–961.
[3] Seibold, J.R., Giorno, R.C. and Claman, H.N. (1990) Arthrit. Rheum. 33, 702–1709.
[4] Thompson, H.L., Burbelo, P.D., Gabriel, G., Yamada, Y. and Metcalf, D.D. (1991) J. Clin. Invest. 87, 619–623.
[5] Ruoss, S.J., Hartmann, T. and Caughey, G.H. (1991) J. Clin. Invest. 88, 493–499.
[6] Hawkins, R.A., Claman, H.N., Clark, R.A.F. and Steigerwald, J.C. (1985) Ann. Intern. Med. 102, 182–186.
[7] Claman, H.N. (1989) J. Invest. Dermatol. 92, 290–295.
[8] Gadjusek, C. (1984) J. Cell Physiol. 121, 13–21.
[9] Choi, K.L. and Claman, H.N. (1987) Immunol. Rev. 6, 145–152.
[10] Peterkofsky, B. and Diegelmann, F. (1971) Biochemistry 10, 988–994.
[11] Vuorio, T., Mäkelä, J.K., Kähäri, V.M. and Vuorio, E. (1987) Arch. Dermatol. Res. 279, 154–160.
[12] Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. and Chambon, P. (1982) Nucleic Acids Res. 10, 7895–7903.
[13] Maquart, F.X., Szymanowicz, A.G., Cam, Y., Randoux, A. and Borel, J.P. (1980) Biochimie 62, 93–97.
[14] Bellon, G., Randoux, A. and Borel, J.P. (1987) Collagen Rel. Res. 5, 423–425.
[15] Fiszer-Szafarz, B., Szafarz, D. and Guevara de Murillo, A. (1991) Anal. Biochem. 110, 165–170.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[17] Schwartz, D. (1983) Méthodes Statistiques à l'Usage des Médecins et des Biologistes, Flammarion Médecine-Sciences, Paris.
[18] Coustry, F., Gillery, P., Maquart, F.X. and Borel, J.P. (1990) FEBS Lett. 262, 339–341.
[19] McGarvie, G.M. and Cushley, W. (1989) Cell Signalling 1, 447–460.
[20] Mire-Sluis, A.R. and Thorpe, R. (1991) J. Biol. Chem. 266, 18113–18118.
[21] Monroe, J.G., Haldar, S., Prystowsky, M.B. and Lammie, P. (1988) Clin. Immunol. Immunopathol. 49, 292–298.
[22] Clement, A., Riedel, N. and Bordy, S. (1990) Am. J. Resp. Cell Mol. Biol. 3, 159–164.
[23] Jimenez, S.A., Freundlich, B. and Rosenbloom, J. (1984) J. Clin. Invest. 74, 1112–1116.
[24] Duncan, M.R. and Berman, B. (1991) J. Invest. Dermatol. 97, 686–692.