Increased Production of a Fibroblast Growth Promoting Factor by Peripheral Lymphocytes from Patients with Active Connective Tissue Diseases

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Abstract: Human peripheral lymphocytes released a soluble factor with growth promoting activity on dermal fibroblasts, which seemed to be different from other growth factors previously reported. Supernatant from lymphocytes showed significantly higher growth promoting activity on fibroblasts than that from monocytes, and fractionation of lymphocytes revealed that the main cellular origin of this factor was non-T cells, especially CD24 positive cells. Supernatant from lymphocytes did not affect the proliferation of HeLa or KYM-1 cells. The fibroblast growth promoting activities significantly correlated with serum C-reactive protein levels or erythrocyte sedimentation rates in patients with connective tissue diseases. These data suggest that this factor may contribute to the pathogenesis of tissue fibrosis in inflammatory responses.

Keywords: lymphocyte, fibroblast, growth factor, non-T cell, fibrosis

Abbreviations: L-FGPF, lymphocyte-derived fibroblast growth promoting factor; IL-1, interleukin 1; PDGF, platelet-derived growth factor; TNF-α, tumor necrosis factor α, TGF-β, transforming growth factor β

INTRODUCTION

Fibroblasts, one of the most important elements in connective tissue, play a central role in the processes of wound healing and tissue remodeling.1,2) Specifically, the proliferative phase of fibroblasts is relevant to tissue repair under physiological conditions. On the other hand, under pathological conditions, the surplus fibroblast proliferative process can also cause tissue damage owing to tissue fibrosis and circulatory disturbance in organs such as the lungs and liver.3,4) Furthermore, fibroblasts may contribute to the pathogenesis of immunological or inflammatory diseases by means of cell-cell interactions and/or production of soluble mediators resulting in stimulation of the focal immune responses.5,6) An increasing amount of evidence suggests that the cell-cell interactions with immunocompetent cells or cytokines produced by these cells regulate the fibroblast functions,7,8) but regulatory mechanisms governing the fibroblast proliferation remain obscure.

In the present study, we investigated the effect of supernatants obtained from human peripheral blood cell cultures on fibroblast proliferation, and found that human lymphocytes release a soluble factor which predominantly stimulates the cell growth of human dermal fibroblasts. This factor seems to be different from other growth factors reported to have a proliferative effect on fibroblasts, such as interleukin 1 (IL-1), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β).

MATERIALS AND METHODS

Preparation of blood cell fractions
Mononuclear cell fractions (MNC) and polymorphonuclear leukocyte fractions (PMN) were separated from heparinized peripheral venous blood of 4 healthy controls and 13 patients with connective diseases including patients with rheumatoid arthritis (n=4), systemic sclerosis (n=4), polymyositis (n=1), systemic lupus erythematosus (n=2) and polymyalgia rheumatica (n=1) by Ficol-Hypaque density gradient centrifugation.

Lymphocyte fraction (Lym) was isolated by eliminating monocytes from MNC by the silica particle technique, as previously described.9)
Contamination of monocytes was always less than 1% as judged by esterase staining.

Lym was separated into subpopulations enriched with T lymphocytes and non-T lymphocytes by the rosetting and centrifugation technique and used as T lymphocyte fraction (T Lym) and non-T lymphocyte fraction (non-T Lym), respectively.

Monocyte fraction (Mono) was prepared from MNC by the adherent technique as previously described.9 The collected fraction consisted of over 96% esterase positive cells.

**Partial killing of lymphocyte subsets using monoclonal antibodies** To deplete the function of each subset of lymphocytes, lymphocyte fractions (7.5×10⁴/1.5 ml) from normal subjects were preincubated with 37.5 μl of anti-OKT4a, OKT8, OKNK or OKB2 antibodies (Ortho Diagnostic System Inc., Japan) for 30 min. 1.5 ml of rabbit complement (Low-tox-M Rabbit Complement; Cederlane Laboratories Ltd., Canada) was then added, and the incubation was continued for another 30 min. Cells, washed once with the culture medium (RPMI 1640 supplemented with 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B), were incubated for 12 h at a concentration of 2×10⁶/ml in the culture medium. The cells were washed again and used as Lym (CD4-), Lym (CD8-), Lym (CD16-) and Lym (CD24-), respectively.

**Culture of blood cell fractions** To study the effect of pretreatment with mitogens, Lym from normal subjects were stimulated with 5 μg/ml of concanavalin A (Con A), phytohemagglutinin (PHA) or pokeweed mitogen (PWM) in RPMI 1640 supplemented with 10% FCS for 90 min. Cells, washed three times with the RPMI 1640, were incubated in the serum-free RPMI 1640 for 4 days. To exclude growth factors derived from FCS, each blood cell fraction was incubated in serum-free RPMI 1640 supplemented with 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B for 4 days in all the experiments. All the supernatants from blood cell cultures were harvested after centrifugation.

**Determination of the cell proliferation activities** Primary culture of fibroblasts was prepared from forearm skin specimens from normal volunteers according to the methods previously reported.9 Fibroblasts of the 3rd to 6th subpassage were used in all the experiments. HeLa cells (human cervical carcinoma cell line) and KYM-1 cells (human undifferentiated rhabdomyosarcoma cell line) were obtained from American Type Culture Collection and Meneki Seibutsu Kenkyujo Co., respectively. Fibroblasts, HeLa cells and KYM-1 cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin G sodium, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B.

To count the number of cells, fibroblasts, HeLa cells and KYM-1 cells were harvested from stock cultures by tripisinization and preincubated for 2 days at a density of 2×10⁵/dish (gelatin coated 35 mm petri dishes; Falcon Plastics, Oxnard, CA). After removal of the conditioned media, cells, washed twice with serum-free RPMI 1640, were incubated continually for another 10 days in 1.5 ml of serum-free RPMI 1640 or undiluted supernatants from blood cell fractions. Final cell counts were done manually with a hemocytometer after tripisinization.

To study [³H]-thymidine incorporation, cells, seeded in multwell plates (S. B. Medical Co., Japan) at the density of 2×10⁴/well, were preincubated for 2 days. The cells, washed twice with serum-free RPMI 1640, were incubated in serum-free RPMI 1640 or undiluted supernatants from blood cell fractions for another 72 h before 2-h exposure to 1 μCi/ml [³H]-thymidine. On completion of incubation, the cells were dissolved in 0.1 N KOH and were neutralized with 0.1 N HCl. The radioactivity of the 10% trichloroacetic acid (TCA) precipitates of cells was counted in a liquid scintillation counter.

To exclude variation in experimental conditions, the activities of cell proliferation incubated in serum-free RPMI 1640 were used as the controls in all the experiments. The results for numbers of cells and [³H]-thymidine incorporation were given as percentages of data for the controls.

**Sephadex G 100 SF gel filtration** The supernatant from Lym was applied to a Sephadex G 100 SF (Pharmacia Fine Chemicals Inc., Sweden) column (2×30 cm) equilibrated with Ca and Mg free Dulbecco phosphate buffered saline with strict aseptic precautions. The supernatant (2.5 ml) was eluted from the column with RPMI 1640 at a flow rate of 0.5 ml/min and 2 ml fractions were assayed for the fibroblast prolifer-
Reversal phase high performance liquid chromatography (rpHPLC) analysis. Lym supernatants were partially purified by rpHPLC with a 20 × 250 mm Cosmosil 10C18-P column (Nacalai Tesque Co., Kyoto, Japan) eluted with a linear gradient at 0.1% trifluoroacetic acid/acetonitrile (100:0 to 0:100, v/v) at a flow rate of 13.0 ml/min. The elute was collected in 1 ml aliquots and each fraction was assayed for $[^3H]$-thymidine incorporation by fibroblasts.

Production of IL-1 from blood cell fractions

![Fig. 1](image1.png)

Fig. 1 Comparison of growth promoting activities on dermal fibroblasts in supernatants from lymphocyte culture (Lym), monocyte culture (Mo), T lymphocyte culture (T) and non-T lymphocyte culture (non-T). Vertical bars represent the mean ± SD.

![Fig. 2](image2.png)

Fig. 2 Effect of depletion of CD4, CD8, CD16 or CD24 positive cells with monoclonal antibodies and rabbit complement on the production of the fibroblast growth promoting activity by lymphocytes. Subpopulations of lymphocyte fraction were used as Lym (CD4-), Lym (CD8-), Lym (CD16-) and Lym (CD24-), respectively. Vertical bars represent the mean ± SD of the data for supernatants from lymphocyte cultures pretreated rabbit complement alone. *p < 0.05, Lym (CD24-) versus Lym (CD4-), Lym (CD8-) and Lym (CD16-).
Supernatants from blood cell cultures were stored at −70°C. IL-1-α and IL-1-β concentrations were determined by means of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka Pharmaceutical Co., Ltd., Japan). The detection limits of the IL-1-α and IL-1-β assays were 7.8 pg/ml and 15.6 pg/ml, respectively.

Statistical analysis Internal distribution data were analyzed by Student’s t-test. Correlation was measured by Pearson’s product moment correlation coefficient.

RESULTS

Effect of the supernatants from blood cell cultures on fibroblast proliferation in the case of normal subjects (n=4)
The number of fibroblasts incubated in the supernatant from MNC cultures increased to 262.2±37.5% of that of the controls incubated in serum-free RPMI 1640 (p<0.005), but no proliferative effect was observed in the supernatant from PMN cultures.

The supernatant from Lym cultures more strongly stimulated the fibroblast proliferation than that from Mono cultures (Fig. 1) (p<0.005). The supernatant from non-T Lym cultures remarkably stimulated the fibroblast growth activity in contrast to the supernatant from T Lym cultures (Fig. 1) (p<0.005).

Depletion of CD4, CD8 or CD16 positive lymphocytes did not affect the production of the fibroblast growth promoting activity by Lym. By contrast, the fibroblast proliferative effect of the supernatant from Lym was suppressed by depletion of CD24 positive Lym (Fig. 2) (p<0.05).

Effect of pretreatment of Lym cultures with mitogens (n=4)
The extent of [3H]-thymidine incorporation by fibroblasts in the supernatant from Lym cultures stimulated with Con A or PHA or PWM was measured by means of a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka Pharmaceutical Co., Ltd., Japan). The detection limits of the IL-1α and IL-1β assays were 7.8 pg/ml and 15.6 pg/ml, respectively. The data represent the mean±SD for analysis.

<table>
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<th>IL-1α (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
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<tr>
<td>Mono</td>
<td>69.5±17.3</td>
<td>77.5±10.8</td>
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<tr>
<td>Lym</td>
<td>&lt;7.8</td>
<td>&lt;15.6</td>
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Table 1. Comparison of concentrations of IL-1α and IL-1β in supernatants from monocyte culture (Mono) and lymphocyte culture (Lym). The detection limits of the IL-1α and IL-1β assays were 7.8 pg/ml and 15.6 pg/ml, respectively. The data represent the mean±SD for analysis.

Fig. 3 Comparison of number of cells and [3H]-thymidine incorporation in cultured dermal fibroblasts, HeLa cells and KYM-1 cells incubated in supernatant from lymphocyte culture. Vertical bars represent the mean±SD. *p<0.005 for number of cells, human skin fibroblasts versus both HeLa cells and KYM-1 cells. **p<0.01 for [3H]-thymidine incorporation, human skin fibroblasts versus both HeLa cells and KYM-1 cells.

Fig. 4 Elution profile of fibroblast growth promoting activity of supernatant from lymphocytes through Sephadex G100 SF column. Molecular weights of bovine serum albumin (BSA), chymotrypsinogen A, ribonuclease A and ACTH are 67,000 Da, 25,000 Da, 13,700 Da and 4,541 Da, respectively.
treatment was approximately equal to the results obtained with unstimulated Lym cultures.

**Effect of the supernatants from Lym on HeLa cells and KYM-1 cells (n=3)**

In contrast with fibroblasts, the supernatant from Lym did not affect the growth activities of HeLa cells or KYM-1 cells (Fig. 3).

**Concentrations of IL-1-α and IL-1-β in supernatant from blood cell fractions (n=4)**

The supernatant from Mono contained 69.5±17.3 pg/ml of IL-1-α and 77.5±10.8 pg/ml of IL-1-β, whereas concentrations of both in the supernatants from Lym were below the detection limits (Table 1).

**Gel filtration analysis (n=3)**

When the supernatant from Lym was applied to a Sephadex G 100 SF column, peak proliferation activity was eluted at a molecular weight of 15 kDa (Fig. 4).

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**Fig. 5** UV absorbance profile (A) and fibroblast proliferative activity of the lymphocyte-derived materials separated by HPLC. The UV absorbance profile is represented by a solid line and the acetonitrile gradient by a dotted line (A). Fractions were assayed in triplicate for [3H]-thymidine incorporation by fibroblasts. Vertical bars represent the mean±SD (B).
**DISCUSSION**

This study demonstrated that the supernatants from unstimulated human peripheral blood lymphocyte cultures strongly stimulated the growth activity of dermal fibroblasts. Lymphocyte cultures released a significantly larger amount of the growth promoting factor than monocyte cultures. Among subpopulations of lymphocytes, the main source of this factor seemed to be non-T lymphocytes, especially CD24 positive cells. To our knowledge, our study first demonstrated the existence of a growth factor synthesized predominantly by B cells rather than monocytes. Stimulation with mitogens did not affect the production of this lymphocyte-derived fibroblast growth-promoting factor (L-FGPF). The molecular weight of L-FGPF was considered to be approximately 15 kDa.

Previous studies revealed IL-1, PDGF, FGF, EGF, TNF-α and TGF-β as potent growth factors for fibroblasts which are derived from peripheral blood cells. IL-1 has a molecular weight of 17.5 kDa, which resembles that of L-FGPF, but, the major cellular sources of IL-1 are reported to be monocytes and macrophages, and such activity was unable to be detected in supernatants from unstimulated normal lymphocyte cultures in our studies. Our data clearly demonstrated the discrepancy between detected IL-1 levels and fibroblast growth promoting activities in supernatants from blood cell cultures, which clearly indicates that L-FGPF differs from IL-1.

L-FGPF seems to be different from PDGF (30 kDa dimer), FGF (18 kDa) and EGF (6 kDa) with respect to molecular weights and cellular sources. Unlike L-FGPF, PDGF, FGF and EGF also stimulate the proliferation of tumor cells including HeLa cells and KYM-1 cells as well as normal fibroblasts.
In contrast to L-FGPF, TNF-α (a biologically active trimer with 17 kDa subunits) causes cytoly &sis or cytostasis on tumor cell lines such as HeLa cells and KYM-1 cells.\(^{37-29}\)

In serum-free medium, TGF-β (25 kDa) by itself has been reported to have no mitogenic effect on fibroblasts.\(^{31,42}\)

These growth factors previously reported were therefore considered to be different from the L-FGPF reported in the present study.

L-FGPF, by itself and/or in co-operation with other cytokines, may contribute to host defense involving tissue remodeling by adaptive stimulation of fibroblast proliferation. By contrast, L-FGPF may possibly induce excessive activation of fibroblasts, which is associated with pathogenesis of diseases characterized by tissue fibrosis following inflammatory responses.

It was of particular interest to compare laboratory indices of inflammatory reaction such as CRP and ESR with L-FGPF in patients with connective tissue diseases. Indeed, as found in our study, a significant correlation was observed between L-FGPF activities and serum CRP concentrations or ESR. In vivo, circulating blood cells-fibroblast interactions are not mediated by the individual effects of a small number of soluble factors, but will result from interacting cytokines which provides stimulatory or inhibitory signals for fibroblast functions. Considering the existence of the complex networks of interacting cytokines, it is reasonable to assume that an increase in L-FGPF may be involved in the activated cytokine networks and cell-mediated effector mechanisms which are involved or regulate the amplitude and duration of inflammatory response. It is also possible that fibroblasts not only receive instructions from inflammatory cells, but in turn, feed back to regulate inflammatory cell functions.

The findings of the present studies emphasize the need to purify L-FGPF and identify its specific inhibitors. Purification of this factor may provide a novel therapeutic agent to repair tissue more rapidly and efficiently than was previously possible. In addition, such inhibitors have the potential to serve as useful tools for the treatment of tissue fibrosis including that in skin and lungs such as glucocorticoid therapies cannot regulate.

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