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Original Article

Effects of Th2 cytokines and eosinophils on stem cell factor production by lung fibroblasts

Yuji Miyakuni, Shigeru Takafuji and Takemasa Nakagawa

Department of Internal Medicine, St Marianna University School of Medicine, Kanagawa, Japan

Abstract

Background: Although eosinophils and fibroblasts are believed to play important roles in the pathogenesis of chronic allergic inflammation, the interaction between eosinophils and fibroblasts has not been thoroughly elucidated. Stem cell factor (SCF) is one of the important cytokines produced by fibroblasts. In the present study, we examined the effects of some cytokines and eosinophils on SCF production by human lung fibroblasts.

Methods: Fibroblasts were cultured with or without interleukin (IL)-4 for up to 48 h. In some experiments, eosinophils were added to the wells after incubation with or without IL-5 for 3 h and cells were cultured for up to 48 h. At the end of the culture period, SCF in the supernatants was measured by ELISA. In addition, the expression of SCF mRNA was examined using reverse transcription–polymerase chain reaction analysis.

Results: Interleukin-4 significantly enhanced SCF production, whereas tumor necrosis factor-α, interferon-γ and IL-1α showed suppressive effects on SCF production. When fibroblasts were cultured with IL-4 plus IL-5-activated eosinophils, SCF production was significantly enhanced in comparison with IL-4 alone. Experiments using polymerase chain reaction amplification revealed that IL-5-activated eosinophils enhanced SCF production by fibroblasts through transcriptional gene activation.

Conclusions: These results suggest that some factor from activated eosinophils may interact in a synergistic fashion with IL-4 to further augment SCF production by fibroblasts and that eosinophils may play an important role in the activation of fibroblasts in chronic allergic inflammation.

Key words: eosinophil, fibroblast, interleukin-4, interleukin-5, stem cell factor.

Correspondence: Dr Shigeru Takafuji, Department of Internal Medicine, Toyoko Hospital, St Marianna University School of Medicine, 3-435 Kosugi, Nakahara-Ku, Kawasaki City, Kanagawa 211-0063, Japan.
Email: takafuji@marianna-u.ac.jp

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Introduction

Stem cell factor (SCF) is a stromal cytokine and is produced by fibroblasts, myofibroblasts, endothelial cells and epithelial cells in peripheral tissues.[1–5] It has been reported that interleukin (IL)-6 or tumor necrosis factor (TNF)-α induce SCF production in human primary foreskin fibroblasts or synovial fibroblasts in patients with rheumatoid arthritis, respectively.[6,7] In addition, transforming growth factor (TGF)-β or basic fibroblast growth factor enhanced SCF production in murine cell lines and leukemia inhibitory factor or IL-4 increased SCF mRNA in these cell lines.[8] Stem cell factor exists in two different forms, soluble and membrane bound, and is the ligand for the c-kit receptor that is found on primitive hematopoietic cells, mature tissue mast cells and eosinophils.[9–11] Interaction of the c-kit receptor with SCF stimulates the growth and early differentiation of hematopoietic cells[9] and sustains mast cell growth and differentiation in cultures of mouse bone marrow and human cord blood.[12,13] Furthermore, SCF primes mature dispersed human lung mast cells for both augmented exocytosis of secretory granules and cytokine production[14,15] and activates directly mouse bone marrow-derived mast cells to stimulate both exocytosis and eicosanoid generation.[16] Furthermore, SCF augments eosinophil adhesion to the very late antigen (VLA)-4 ligands fibronectin and vascular cell adhesion molecule

(VCAM)-1.[11] Therefore, it is suggested that SCF affects not only mast cells, but also eosinophils in allergic inflammation.

Eosinophils are believed to contribute to allergic diseases by releasing cytotoxic cationic eosinophil granule proteins or by generating lipid mediators and cytokines.[17–19] In recent years, it has been reported that eosinophils have some effects on fibroblast function. Eosinophils are capable of both adhering to and releasing mitogens for fibroblasts *in vitro*.[20] Eosinophil major basic protein (MBP) interacts in a synergistic fashion with IL-1α or TGF-β to further augment fibroblast IL-6-type cytokine production.[21] It has been reported that human eosinophil sonicate or supernatants of cultured eosinophils enhance lung fibroblast proliferation and this activity is partially inhibited by anti-TGF-β antibody.[22] However, the interaction between eosinophils and fibroblasts has not been thoroughly elucidated.

In the present study, we examined the effects of several cytokines and eosinophils on SCF production by human lung fibroblasts. As a result, we have found that IL-4 enhances SCF production by fibroblasts and that IL-5-activated eosinophils further augment SCF production stimulated by IL-4.

METHODS

Purification of human eosinophils

Eosinophils were prepared from the venous blood of informed healthy volunteers with eosinophil percentages ranging from 2 to 6%. Eosinophil preparations were purified by two-step density centrifugation, as described previously.[23] Erythrocytes in the preparation were lysed by hypotonic water lysis. Eosinophils were further purified by removal of CD16-positive cells (neutrophils) using immunomagnetic selection by a magnetic-activated cell sorter system (Miltenyi Biotec, Bergisch-Gladbach, Germany).[24] The purity of eosinophils was $>95\%$, as determined by microscopy of cytocentrifuge preparations stained with May–Giemsa. Purified eosinophils were suspended in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin, 0.1% human serum albumin (Miles, Kankakee, IL, USA).

Reagents

Recombinant human (rh) IL-4, rhTNF-α, rhIL-6, rhIL-1α, rhIL-5 and rhIL-13 were obtained from Genzyme (Cambridge, MA, USA) and IFN-γ was from Sigma Chemical (St Louis, MO, USA). The rhTGF-β was obtained from Life Technologies. Anti-MBP monoclonal antibody (mAb) was the kind gift of Dr Gerald J Gleich (Mayo Clinic, Rochester, MN, USA). Anti-TGF-β mAb and anti-IL-6 mAb were from Genzyme. These mAb were selected because of their ability to neutralize the activities of MBP, TGF-β or IL-6. These compounds were stored in small aliquots at –70°C and were thawed just before use.

Culture of HFL-1

Human lung fibroblasts (HFL-1; American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 1% penicillin/streptomycin. Cells in their 15th to 20th passages, which have the same characteristics as other soft tissue fibroblasts (i.e. production of collagens type I and type III),[25] were plated on 24-well tissue culture plates at $1.5–2 \times 10^5$ per well for SCF assays and cultured in a humidified atmosphere at 37°C with 5% CO_2. The monolayer achieved confluency at days 2–3.

Stem cell factor production

Once confluent, the fibroblasts were cultured with or without recombinant human cytokines for up to 48 h. In some experiments, 500 μL eosinophils (5×10^4) was added to the wells after incubation with or without IL-5 for 3 h and washing with medium. Then, the fibroblasts were cultured for up to 48 h. In neutralization experiments, mAbs (10 μg/mL) were added to the wells at the start of the culture. The concentration of mAbs was decided according to our previous report.[23] Namely, this concentration was enough to block mediator release from cells. At the end of the culture period, supernatants from the wells were collected and stored at –70°C until SCF measurements were performed. The SCF was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

Polymerase chain reaction of SCF mRNA

After the culture period, total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan). The SCF and β-actin mRNA of each sample were first reverse transcribed (RT) into complementary DNA using dithiothreitol (DTT), dNTP, diethylpyrocabonate-treated (DEPC)-dH_2O and oligo dT-primer, which was then subjected to

conventional polymerase chain reaction (PCR) amplification with the specific primer designed by SIGMA Genosys Japan kk (Hokkaido, Japan). Nucleotide sequences for primers were as follows: SCF, 5′-CCC AGG CTC TTT ACT CCT GAA-3′ and 5′-CTG CCC TTG TAA GAC TTG GCT G-3′; β-actin, 5′-AGT CCG CCT AGA AGC A-3′ and 5′-AGC CAT GTA CGT TGC TA-3′. The PCR products were then resolved by Agarose gel electrophoresis, stained with ethidium bromide, visualized and photographed under ultraviolet light and finally analyzed by the National Institutes of Health image analyzer. The PCR product size for SCF was 348 bp.

Presentation of data

All experiments were performed in duplicate and repeated at least three times with cell preparations from different donors. The effects of treatment examined within the same experiments were compared by Wilcoxon's signed-rank test for pairs.

Unless stated otherwise, data are presented as the mean ± SEM.

RESULTS

Effects of cytokines on SCF production by fibroblasts

Figure 1 shows the effects of various cytokines on SCF production by fibroblasts. Interleukin-4 (100 ng/mL) induced significant potentiation of SCF production by fibroblasts compared with control (400 ± 70 *vs* 283 ± 39 pg/mL, respectively; $n = 6$; $P < 0.01$), whereas IL-6 (10^3 U/mL) and TGF-β (10^{-9} mol/L) had no effect on SCF production. Tumor necrosis factor-α (10 ng/mL), IFN-γ (10^3 U/mL) and IL-1α (2.5 ng/mL) had suppressive effects on SCF production by fibroblasts compared with control (174 ± 19, 153 ± 17, 168 ± 13 and 283 ± 39 pg/mL, respectively; $n = 6$; $P < 0.05$). Interleukin-13 had the same effect as IL-4; that is, IL-13 (100 ng/mL) enhanced SCF production compared with control (308 ± 36 *vs* 220 ± 19 pg/mL, respectively; $n = 3$; $P < 0.05$). Interleukin-13 (100 ng/mL) plus IL-4 (100 ng/mL) had no further potentiating effect compared with either IL-13 alone or IL-4 alone (data not shown).

Dose–response to IL-4

Figure 2 shows the dose dependence of the effect of IL-4 on SCF production by fibroblasts. The enhancement of SCF production by IL-4 was concentration dependent, becoming maximal at 100 ng/mL IL-4.

Effects of IL-5-activated eosinophils on SCF production by fibroblasts

We examined the effects of unstimulated eosinophils or IL-5-stimulated eosinophils on SCF production by fibroblasts (Fig. 3). When fibroblasts were cultured with unstimulated eosinophils or IL-5-stimulated eosinophils, no effect on SCF production was observed. In addition,

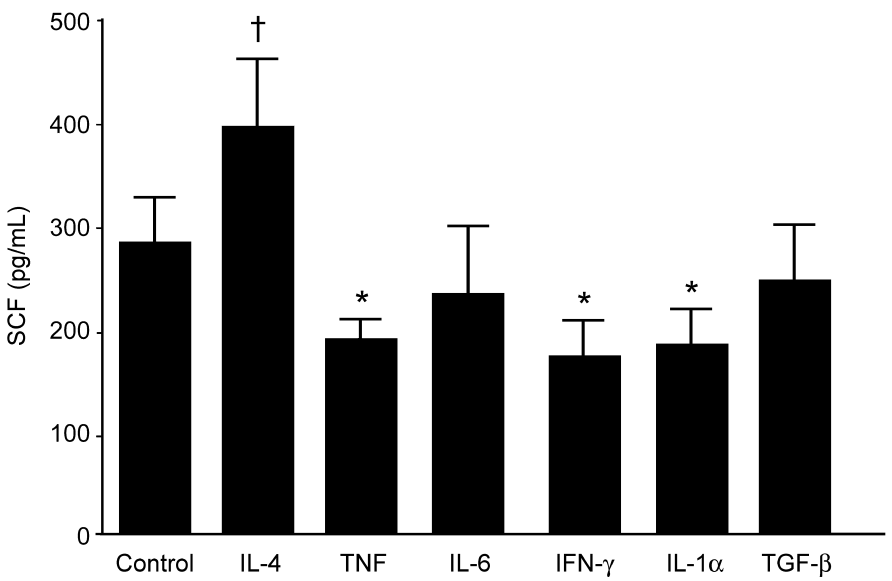

Fig. 1 Effects of cytokines on stem cell factor (SCF) production by fibroblasts. Confluent fibroblast monolayers were cultured with culture medium alone (control) or medium containing 100 ng/mL interleukin (IL)-4, 10 ng/mL tumor necrosis factor (TNF), 10^3 U/mL IL-6, 10^3 U/mL interferon (IFN)-γ, 2.5 ng/mL IL-1α or 10^{-9} mol/L transforming growth factor (TGF)-β for 48 h. The concentration of each cytokine was selected as the most effective concentration in preliminary experiments. Data are the mean ± SEM obtained from six experiments performed in duplicate. *$P < 0.05$, †$P < 0.01$ compared with control.

when fibroblasts were cultured with IL-4 plus unstimulated eosinophils, no significant enhancement was shown. However, when fibroblasts were cultured with IL-4 plus IL-5-stimulated eosinophils, significant enhancement of SCF production by fibroblasts was observed compared with IL-4 alone (500 ± 107 *vs* 400 ± 70 pg/mL, respectively; $n = 6$; $P < 0.05$). Interleukin-5 alone had no effect on SCF production and IL-5 plus IL-4 had no further enhancing effect compared with IL-4 alone (data not shown). In order to examine what from IL-5-stimulated eosinophils affects SCF production by fibroblasts, we examined the effects of anti-MBP mAb, anti-TGF-β mAb or anti-IL-6 mAb by adding the mAb at the start of coculture of eosinophils and fibroblasts. These mAbs had no effect on SCF enhancement.

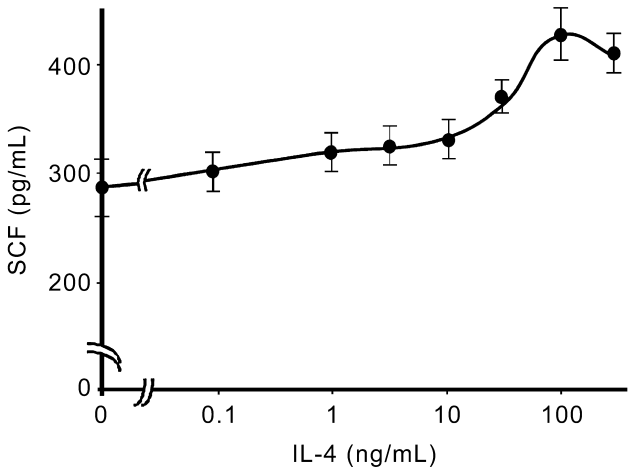

Fig. 2 Dose dependence of interleukin (IL)-4 on stem cell factor (SCF) production by fibroblasts. Fibroblasts were cultured with IL-4 at the concentrations indicated for 48 h. Data are the mean ± range of duplicate cultures in a representative of three separate experiments.

Kinetics of fibroblast response modulation induced by IL-4 and IL-5-activated eosinophils

Figure 4 shows the time-course of SCF production induced by medium alone, IL-4 or IL-4 plus IL-5-activated eosinophils. The enhancing effect of SCF production by IL-4 was observed after 12 h. The further enhancement of SCF production by IL-5-activated eosinophils in comparison with IL-4 alone became manifest after 24 h. In other experiments, the enhancing effect of IL-5-activated eosinophils plus IL-4 after 24 h was significantly greater than that of IL-4 alone (298 ± 42 *vs* 208 ± 31 pg/mL, respectively; $n = 6$; $P < 0.05$).

Expression of SCF mRNA

In order to confirm whether the increased production of SCF is accompanied by the transcription of the SCF gene, we examined the expression of SCF mRNA using

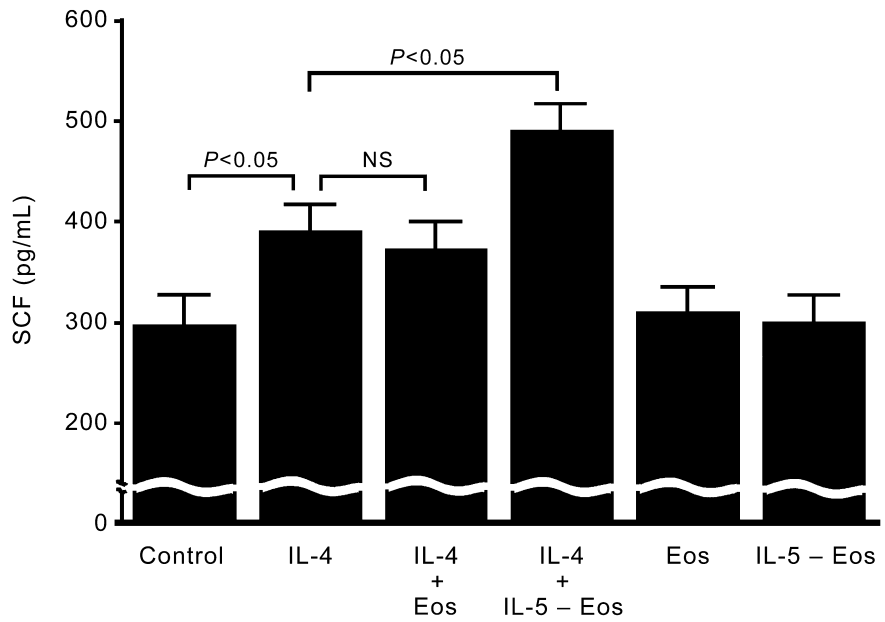

Fig. 3 Effects of eosinophils on stem cell factor (SCF) production by fibroblasts. Eosinophils (Eos) were incubated with or without 10 ng/mL interleukin (IL)-5 for 3 h. Then, fibroblasts were cultured with or without 100 ng/mL IL-4 in the absence or presence of unstimulated eosinophils or IL-5-stimulated eosinophils for 48 h. Data are the mean ± SEM obtained from six experiments performed in duplicate.

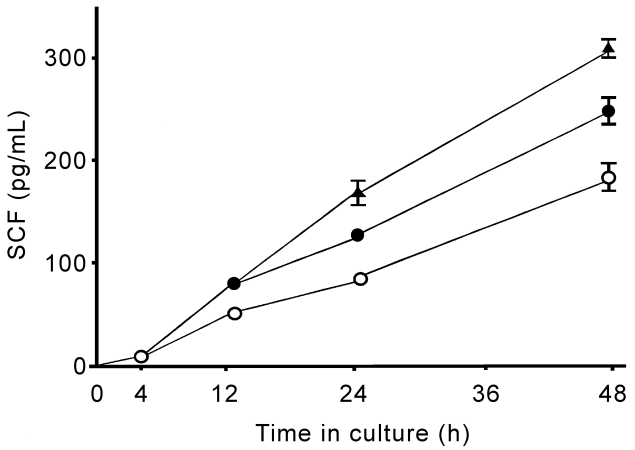

Fig. 4 Time-course of stem cell factor (SCF) production. Fibroblasts were cultured with interleukin (IL)-4 (100 ng/mL) in the absence (●) or presence (▲) of IL-5-treated (10 ng/mL for 3 h) eosinophils for the time indicated. As a control, fibroblasts were cultured without IL-4 in the absence of IL-5-treated eosinophils for various times (○). Data are the mean ± range of duplicate cultures in a representative of three separate experiments.

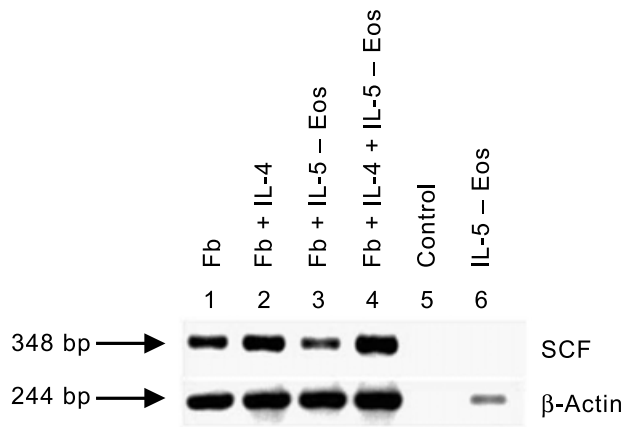

Fig. 5 Polymerase chain reaction (PCR) amplification of cDNA specific for stem cell factor (SCF). Fibroblasts (Fb) were stimulated with medium alone (lane 1), with interleukin (IL)-4 (lane 2), with IL-5-activated eosinophils (Eos; lane 3), or with IL-4 plus IL-5-activated eosinophils (lane 4) for 16 h. The cDNA was subjected to PCR amplification (35 cycles). The SCF-specific band was not observed in the case of medium alone (lane 5) and IL-5-activated eosinophils (lane 6). β-Actin gene expression was used as a control and results for SCF gene expression were analyzed as the ratio to β-actin gene expression.

PCR amplification. As shown in Fig. 5, SCF gene expression was intensified on IL-4 stimulation and this increased expression was further intensified by IL-5-activated eosinophils. (The ratio of SCF gene expression divided by β-actin gene expression was 0.66, 1.29, 0.64 and 1.79 for fibroblasts, fibroblasts + IL-4, fibroblasts + IL-5·eosinophils and fibroblasts + IL-4 + IL-5·eosinophils, respectively (a representative of five different experiments).)

Discussion

In the present study, we examined the effects of some cytokines and eosinophils on SCF production by human lung fibroblasts. Interleukin-4 significantly enhanced SCF production, whereas TNF-α, IFN-γ, and IL-1α showed suppressive effects on SCF production. When fibroblasts were cultured with IL-4 plus IL-5-activated eosinophils, significant enhancement of SCF production was observed in comparison with IL-4 alone. Therefore, it is suggested that some factor from activated eosinophils may interact in a synergistic fashion with IL-4 to further augment SCF production by fibroblasts.

In the present study, TNF-α, IFN-γ and IL-1α suppressed SCF production by human lung fibroblasts. The effect of TNF is different between the present study and a previous report in which TNF-α increased SCF production in synovial fibroblasts in patients with rheumatoid arthritis.[7] The reason for this difference is unclear. However, the difference may be due to that of fibroblasts used in the experiments and the conditions of the experiments.

It has been reported that fibroblasts express IL-4 receptors and that stimulation with IL-4 triggers upregulation of the expression of adhesion molecules and the production of IL-6 and monocyte chemoattractant 1 in the fibroblasts.[26] In addition, it has been shown that human mast cells stimulate fibroblast proliferation after cell–cell contact and that this stimulatory effect of mast cell coculture can be completely abrogated by preincubation of fibroblasts with anti-IL-4R mAb.[27] Thus, it is suggested that IL-4 from mast cells activates fibroblast function. In the present study, IL-4 activated fibroblasts to enhance the production of SCF, which primes mast cells for augmented cytokine production.[15]

It has been shown that IL-4Rα and IL-13Rα1 chains are present in three different fibroblast populations.[26] Interleukin-13 also upregulates the expression of adhesion molecules and cytokine production similarly

to IL-4.[26] In the present study, IL-13 had the same effect as IL-4, potentiating SCF production. It is possible that IL-13, as well as IL-4, may have an important effect on fibroblast function.

The present study has shown that IL-5-activated eosinophils further augment SCF production stimulated by IL-4. The enhancing effect of IL-5-activated eosinophils was also observed in the expression of SCF mRNA. It has been reported that eosinophil MBP interacts in a synergistic fashion with IL-Iα or TGF-β to further augment fibroblast IL-6-type cytokine production.[21] However, anti-MBP mAb had no effect on SCF enhancement in the present study. Recently, we have reported that cysteinyl leukotrienes (cysLTs) increase eotaxin production by fibroblasts in the presence of IL-4.[28] It is possible that cysLTs from eosinophils may have some effect on SCF production. Further investigation of the mechanism of SCF enhancement by IL-5-activated eosinophils is required.

In summary, the present study shows that IL-4 and IL-5-activated eosinophils increase SCF production by fibroblasts. It is possible that IL-4 and some factor from eosinophils stimulate fibroblasts to produce SCF, which activates mast cells, the important source of IL-4, and eosinophils,[11] leading to chronic allergic inflammation.

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