Leukemia inhibitory factor enhances mast cell growth in a mast cell/fibroblast co-culture system through Stat3 signaling pathway of fibroblasts

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Abstract Leukemia inhibitory factor (LIF) enhanced mast cell growth in a mast cell/3T3 fibroblast co-culture system, however the precise mechanisms have not been defined. Western blot analysis showed that bone marrow-derived mast cells failed to express both LIF receptor (LIFR) and gp130, whereas 3T3 fibroblasts expressed both LIFR and gp130. This result indicates that the activity of LIF for mast cell growth is mediated by 3T3 fibroblasts. Signal transducer and activator of transcription (Stat) 3-transfected 3T3 fibroblasts enhanced mast cell growth. In addition, dominant-negative Stat3-transfected fibroblasts blocked LIF-mediated mast cell growth in the co-culture system. In conclusion, LIF-induced mast cell growth in the co-culture system is mediated by an indirect pathway via 3T3 fibroblasts through activating Stat3 signaling pathway in 3T3 fibroblasts.

Key words: Bone marrow-derived mast cell; Co-culture; Leukemia inhibitory factor; Signal transducer and activator of transcription 3; 3T3 fibroblast

1. Introduction

Mast cells are tissue-residing cells which play a key role in some host-defense mechanisms as well as in IgE-mediated immediate-type hypersensitivity reaction. Mast cells originate from hematopoietic stem cells in the bone marrow [1], however, their development is characteristic when compared with other hematopoietic lineages, such as basophils and eosinophils. Mast cell precursors differentiate from the hematopoietic stem cells under strong influence of a stromal cell-derived growth factor, stem cell factor (SCF) [2]. The precursor is released from bone marrow, and further differentiates into mature mast cells after reaching tissues by tissue-oriented mechanisms: in rodents, the precursor differentiates into mucosal mast cell by T cell-derived cytokine, interleukin (IL)-3, IL-4, IL-9 and IL-10 or into connective tissue-type mast cell by stromal cell-mediated cytokine, SCF.

In skin, mast cells reside in the dermal layer closely associated with dermal fibroblasts, and regulate microvascular circulation through release of a series of mediators and cytokines. Although SCF expressed on dermal fibroblasts plays a key role in the development of mast cells, the mechanisms that regulate mast cell proliferation or differentiation in pathological conditions of the skin are not fully understood. As an in vitro model of skin mast cells, the mast cell/fibroblast co-culture system has been used to investigate the mechanisms of mast cell differentiation [3]. Recent studies have revealed that fibroblasts have an essential role for mast cell differentiation in the mast cell/fibroblast co-culture system [4,5]. On the other hand, our previous study also defined that a potent fibroblast growth factor, platelet-derived growth factor, enhances mast cell proliferation in the co-culture system not directly but via fibroblasts, indicating that activation of fibroblasts is important for mast cell growth [6]. In addition, fibroblast-activating cytokines, IL-1α and tumor necrosis factor-α, also have mast cell growth-enhancing activity in the co-culture system through induction of prostaglandins [7]. Furthermore, by analyzing the conditioned medium of squamous cell carcinoma cell line (KCMH-1) cells for mast cell growth-enhancing activity, we identified leukemia inhibitory factor (LIF) as a potent mast cell growth-enhancing factor [8]. The mode of action of LIF for mast cell growth-enhancement might also be indirect and mediated by fibroblasts. In the present study, we investigated the role of LIF receptor (LIFR) and its signaling pathway in fibroblasts to understand the mechanisms of LIF-mediated mast cell growth-enhancement.

2. Materials and methods

2.1. Cells

Bone marrow-derived mast cells (BMMC) were prepared from 6-week-old male mice (WBB6F1-+/+) according to the method described previously [9]. Briefly, bone marrow cells were cultured in α-MEM (Gibco BRL, Rockville, MD, USA) containing 10% fetal calf serum (FCS), 100 U/ml penicillin G and 100 μg/ml streptomycin (complete α-MEM) supplemented with 100 μM 2-mercaptoethanol, 5 ng/ml of recombinant mouse IL-3 (R&D Systems, Minneapolis, MN USA) and 2 ng/ml of recombinant mouse IL-4 (R&D), and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were collected by centrifugation and resuspended

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Abbreviations: BMMC, bone marrow-derived mast cells; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; IL-6, interleukin-6; JAK, Janus kinase; LIF, leukemia inhibitory factor; LIFR, LIF receptor; PBS, phosphate-buffered saline; SCF, stem cell factor; Stat3, signal transducer and activator of transcription 3

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in fresh medium every 7 days. After 4 weeks, more than 95% of the non-adherent cells were stained positively with alcian blue. NIH/3T3 fibroblasts (3T3 fibroblasts) were obtained from The American Type Culture Collection (CRL1658), and maintained in DMEM containing 10% calf serum, 100 U/ml penicillin G and 100 μg/ml streptomycin.

2.2. Mast cell/3T3 fibroblast co-culture

BMMC/3T3 fibroblast co-culture was performed as described previously [9]. Briefly, 2×10^4 3T3 fibroblasts suspended in 0.5 ml of complete α-MEM were seeded in each well of four-well multidishes (Nunc A/S, Roskilde, Denmark), and cultured until confluence. At reaching confluence, the medium was aspirated and 3×10^4 BMMC suspended with 500 μl of complete α-MEM containing various concentrations of recombinant mouse LIF (R&D) were added. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air and the culture medium was changed every 2 days.

2.3. Mast cell counting in co-culture

Adherent cells in the co-cultures were dispersed by incubation in trypsin-EDTA solution (Gibco BRL) for 10 min at 37°C, followed by addition of one tenth volume of FCS. The dispersed cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 1 ml of PBS containing 1% bovine serum albumin. Total number of cells in the suspension was determined using an improved Neubauer hemocytometer. Aliquots of cell suspensions were also spun in a cytocentrifuge (1500 rpm for 1 min) and resulting cell preparations on slide-glasses were stained with alcian blue. The alcian blue-positive cells (mast cells) in a culture dish were calculated using the following formula: number of mast cells/dish = $A\times C/B$, where A is total number of cells in a culture dish; B total number of cells in a cytocentrifuge specimen; and C number of alcian blue-positive cells in the same specimen.

2.4. Determination of histamine content in co-culture

Cultured cells in each well were denatured by adding 0.25 N $HClO_4$, and insoluble proteins were precipitated by centrifugation at $12\,000\times g$ at 4°C for 10 min. Histamine concentration was assayed with automated fluorometric high performance liquid chromatography system (Tosoh Corporation, Tokyo, Japan) as described by Tsuruta et al. [10].

2.5. Western blot analysis

Cells (3T3 fibroblasts and BMMC) were lysed in the sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) and electrophoresed by the method of Laemmli [11] with 5–20% polyacrylamide gel (Atto Corporation, Tokyo, Japan). The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). The membrane was incubated with rabbit anti-mouse LIFR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-mouse gp130 polyclonal antibody (Santa Cruz). After washed with TTBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody. The reacted antibodies were visualized by using ECL (Amersham Pharmacia Biotech, Buckinghamshire, UK) under a luminescent image analyzer (LAS-1000 plus, Fuji Photo Film Co., Ltd., Tokyo, Japan).

To detect LIF-induced tyrosine phosphorylation of LIFR and gp130, subconfluent cultures of 3T3 fibroblasts in 35 mm dishes were washed twice with PBS and stimulated with 100 ng/ml of LIF for indicated intervals. Immunoprecipitations were performed as previously described [12]. Briefly, cells were lysed in the lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 mM EDTA, 10 μg/ml leupeptin, 100 mM sodium fluoride, 2 mM sodium orthovanadate) at 4°C for 30 min. Cell lysates were clarified by centrifuging for 10 min at $10\,000\times g$ at 4°C and incubated with anti-mouse gp130 polyclonal antibody for 1 h at 4°C. Protein G-Sepharose 4FF (Amersham Pharmacia Biotech) was used to collect the antigen–antibody complexes. These immunoprecipitants were analyzed by immunoblotting using HRP-conjugated anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology, Lake Placid, NY, USA). For detection of tyrosine phosphorylation of the signal transducer and activator of transcription (Stat) 3 induced by LIF, cells were lysed and analyzed using PhosphoPlus® Stat3 (Tyr705) Antibody kit (New England BioLabs, Beverly, MA, USA) according to the manufacturer's instructions.

2.6. Establishment of dominant-negative Stat3-stable transformants

The expression vector encoding hemagglutinin peptide (HA)-tagged Stat3 (pEF-Bos-HA-Stat3) was a gift from Dr. Nakajima (Department of Immunology, Osaka City University Graduate School of Medicine, Osaka, Japan). The mutageneses for dominant-negative Stat3, Glu434/435 to Ala (Stat3D) and Tyr705 to Phe (Stat3F) [13], were performed by QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, using the mutagenic primers as follows: Stat3D: sense primer 5′-GCC TCC TTG ATC GTG ACT GCG GCG CTG CAC CTG ATC ACC TTC G-3′, antisense primer 5′-CGA AGG TGA TCA GGT GCA GCG CCG CAG TCA CGA TCA AGG AGG C-3′; Stat3F: sense primer 5′-CCA GGT AGT GCT GCC CCG TTC CTG AAG ACC AAG TTC ATC TG-3′, antisense primer 5′-CAG ATG AAC TTG GTC TTC AGG AAC GGG GCA GCA CTA CCT GG-3′. 3T3 fibroblasts (1×10^6) were transfected with 19.5 μg of pEF-Bos (a gift from Dr. Nagata, Department of Genetics, Osaka University Medical School, Osaka, Japan), pEF-Bos-HA-Stat3, pEF-Bos-HA-Stat3D or pEF-Bos-HA-Stat3F together with 0.5 μg of pSV2*neo* (Clontech Laboratories, Palo Alto, CA, USA) by electroporation, and the transfected cells were selected with 1 mg/ml G418. The exogenous expression of HA-tagged proteins of each stable transformant was tested by Western blot analysis using anti-HA-tag polyclonal antibody (MBL, Nagoya, Japan).

3. Results

3.1. LIF indirectly enhanced mast cell growth in the mast cell/3T3 fibroblast co-culture system

When BMMC were cultured in the co-culture system in the presence of a series of concentrations of LIF, LIF increased both mast cell number and total histamine content in a dose dependent manner (Fig. 1). The increase of total histamine contents in the wells was parallel to the increase of mast cell number, thus total histamine contents were utilized for mast cell proliferation assay in subsequent experiments. In contrast, LIF had no effect on mast cell growth when BMMC were cultured in the absence of fibroblasts (data not shown).

Since the receptor for LIF is composed of a LIF-specific receptor subunit (LIFR) and a signal transduction molecule, gp130 [14,15], expression of these molecules was examined in BMMC and 3T3 fibroblasts in order to investigate the mech-

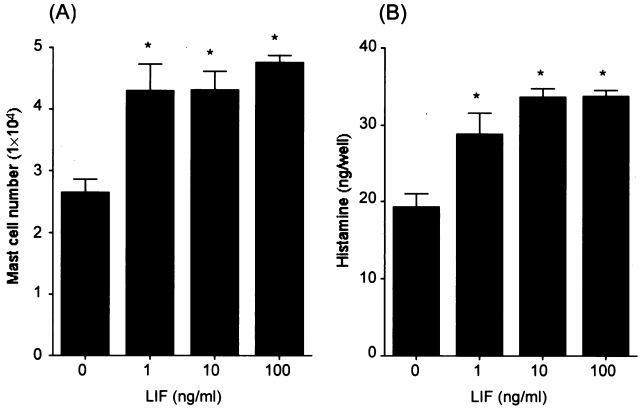

Fig. 1. LIF enhanced mast cell growth in the mast cell/fibroblast co-culture system. 3×10^4 BMMC were seeded and co-cultured with 3T3 fibroblasts in the presence of a series of concentrations of LIF. Mast cell numbers (A) and histamine contents (B) were determined on day 6. Data are mean ± S.E.M. of three independent experiments done in duplicate ($*P<0.05$).

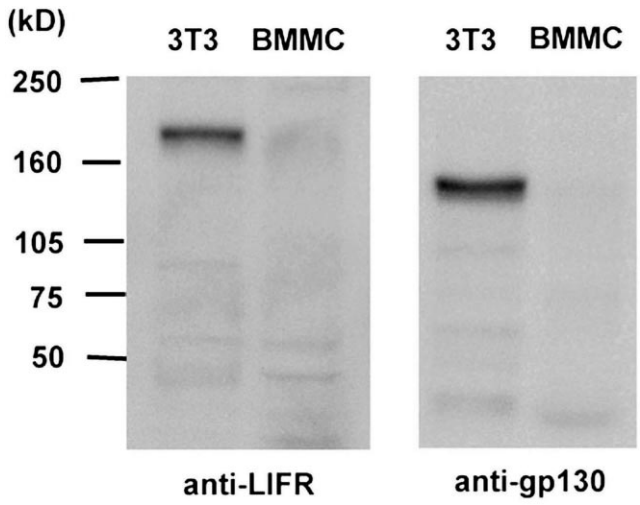

Fig. 2. Detection of LIFR and gp130 in BMMC and 3T3 fibroblasts. Cell lysates were prepared from 3T3 fibroblasts and BMMC, and 10 μg of these samples were analyzed by immunoblotting using anti-mouse LIFR and gp130 polyclonal antibody.

anisms of LIF-induced BMMC proliferation. Western blot analysis revealed that 3T3 fibroblasts express both LIFR and gp130, whereas mast cells express neither LIFR nor gp130 (Fig. 2). These results indicate that LIF indirectly enhances mast cell growth via 3T3 fibroblasts.

3.2. *Tyrosine phosphorylation of gp130 and Stat3 in the LIF-stimulated 3T3 fibroblasts*

Binding of LIF and LIFR induces heterodimerization of LIFR and gp130 [16], followed by activation of a Janus kinase (JAK)–Stat pathway [17]. In LIFR and gp130-mediated signal transduction, Stat3 is known to play a central role [17], activation of gp130 and Stat3 was determined in LIF-stimulated 3T3 fibroblasts by using immunoprecipitation and Western blot. LIF induced tyrosine phosphorylation and heterodimerization of both LIFR and gp130 5 min after stimulation (Fig. 3). Tyrosine phosphorylation of Stat3 was also detected in LIF-stimulated 3T3 fibroblasts 5 min after stimulation (Fig. 4). On the other hand, LIF failed to phosphorylate gp130 and Stat3 in BMMC (data not shown).

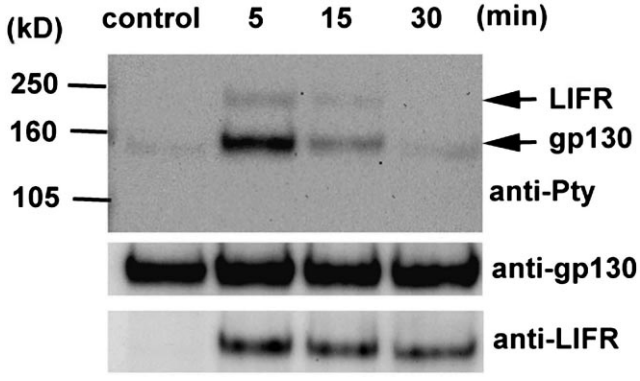

Fig. 3. LIF-induced tyrosine phosphorylation and heterodimerization of LIFR and gp130. 3T3 fibroblasts were stimulated with 100 ng/ml of LIF for indicated intervals, then lysed and immunoprecipitated by using anti-gp130 polyclonal antibody and Protein G-Sepharose. These antigen–antibody complexes were collected and analyzed by immunoblotting. Upper panel: immunoblotting with HRP-conjugated anti-phosphotyrosine antibody (4G10), middle panel: immunoblotting with anti-gp130 antibody, and lower panel: immunoblotting with anti-LIFR antibody.

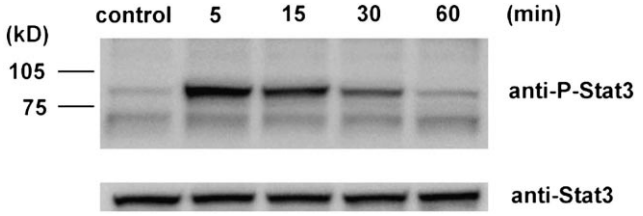

Fig. 4. LIF-induced tyrosine phosphorylation of Stat3 in 3T3 fibroblasts. 3T3 fibroblasts were stimulated with 100 ng/ml of LIF, then lysed and analyzed using PhosphoPlus® Stat3 (Tyr705) Antibody kit according to the manufacturer's instructions.

When the 3T3 fibroblast clone that stably expressed exogenous HA-Stat3 was established by the electroporation method, and BMMC were co-cultured with the stable Stat3 transfectant, the Stat3-transfected fibroblasts significantly enhanced mast cell growth compared with mock-transfected fibroblasts (Fig. 5).

3.3. *Dominant-negative Stat3 blocked LIF-induced mast cell growth*

To confirm the role of Stat3 in the LIF-induced mast cell growth, we established two 3T3 fibroblast clones that express exogenous dominant-negative Stat3 (HA-Stat3D and HA-Stat3F) and examined the effect of these mutants Stat3 on LIF-induced mast cell growth. When the HA-Stat3D- and HA-Stat3F-transfected fibroblasts were used for the feeder layer in the BMMC/3T3 fibroblast co-culture system, both HA-Stat3D- and HA-Stat3F-transfected fibroblasts failed to mediate LIF-induced mast cell growth compared with mock-transfected fibroblasts (Fig. 6). Tyrosine phosphorylation of Stat3 was undetectable in HA-Stat3F-transfected fibroblasts even when stimulated by LIF (Fig. 7).

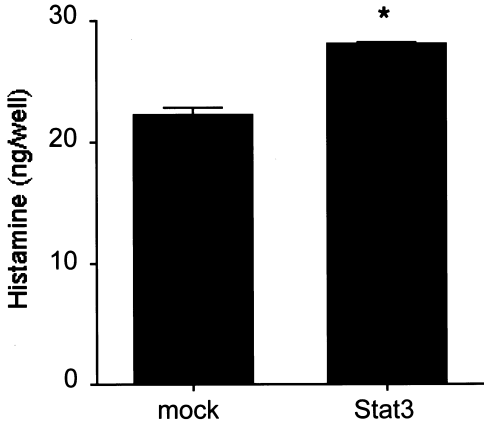

Fig. 5. Stat3-transfected fibroblasts enhanced mast cell growth in the co-culture. BMMC were co-cultured with mock-transfected fibroblasts and Stat3-transfected fibroblasts, and histamine contents were determined on day 6. Data are mean ± S.E.M. of three independent experiments done in duplicate ($*P < 0.05$).

4. Discussion

Through the characterization of mast cell growth-enhancing activity found in KCMH-1 cell conditioned medium, we have previously identified LIF as a mast cell growth-enhancing factor. Studies on the action of LIF suggested that LIF-induced mast cell proliferation in the co-culture was not by direct action on mast cells, but was mediated by the 3T3 fibroblasts in the BMMC/3T3 fibroblast co-culture system. To address the molecular mechanisms for LIF-induced mast cell growth-enhancement in 3T3 fibroblasts, we investigated the expression of LIFR and gp130 in 3T3 fibroblasts and BMMC by Western blotting. BMMC express neither LIFR nor gp130, whereas 3T3 fibroblasts expressed both LIFR and gp130, clearly indicating the indirect action of LIF for mast cell growth-enhancement.

Gp130 is a common signal transduction molecule shared by the receptors for IL-6, IL-11, oncostatin M, ciliary neurotrophic factor, and cardiotrophin 1. The signal from gp130 is transduced by two different pathways; one is mediated by phosphorylation of JAK followed by the activation of Stat3, and the other mediated with mitogen-activated protein kinase, extracellular signal-regulated kinase (ERK). To assess the mediation of Stat3 in LIF-induced mast cell proliferation, we made two forms of Stat3 mutant that block the activation of Stat3 in a dominant-negative manner. Mast cell proliferation-signal induced by LIF was mediated through the Stat3 molecule, since both mutants of Stat3, Stat3D and Stat3F completely and specifically blocked LIF-induced mast cell growth-enhancement (Fig. 6). In addition, an increased expression of Stat3 in the 3T3 fibroblasts enhanced mast cell growth in the co-culture system (Fig. 5), although transient expression of ERK in the 3T3 fibroblasts failed to enhance mast cell growth (data not shown), suggesting that activation of the Stat3 pathway is enough to induce mast cell proliferation.

LIF has a diversity of functions to various types of cells. LIF was first identified as a factor causing differentiation of myeloid leukemic M1 cells into macrophages in vitro and in vivo [18]. LIF also induces differentiation of megakaryocytes in vivo [19], production of acute phase proteins by hepatocytes [20], cholinergic neuronal differentiation [21], proinflammatory cytokine expression in a broad spectrum of cell types [22], and ear swelling and leukocyte infiltration in the mouse ear in vivo [23]. On the other hand, LIF suppresses spontaneous differentiation of murine embryonic stem cells in vitro [24]. Recently, Shih et al. have revealed that LIF induces hematopoietic stem cell expansion in a stromal-based culture system, and this action of LIF is indirect and mediated by induction of growth factors for hematopoietic stem cells in stromal cells [25]. Furthermore, by analysis of the supernatant of LIF-treated stromal cells, they have ruled out the contributions by six known cytokines, IL-3, IL-6, granulocyte macrophage-colony stimulating factor, SCF, flt-3 ligand and thrombopoietin, suggesting involvement of a novel growth factor. Our finding resembles to their facts in that LIF induces bone marrow-derived cell proliferation in a stromal-based culture system. In this study, a molecule causing mast cell growth which is induced on 3T3 fibroblasts has not yet been defined. One possible cause for the mast cell growth is an enhanced expression of SCF in 3T3 fibroblasts; another possibility is the expression of an unknown mast cell growth factor on the surface of 3T3 fibroblasts. This remains to be determined.

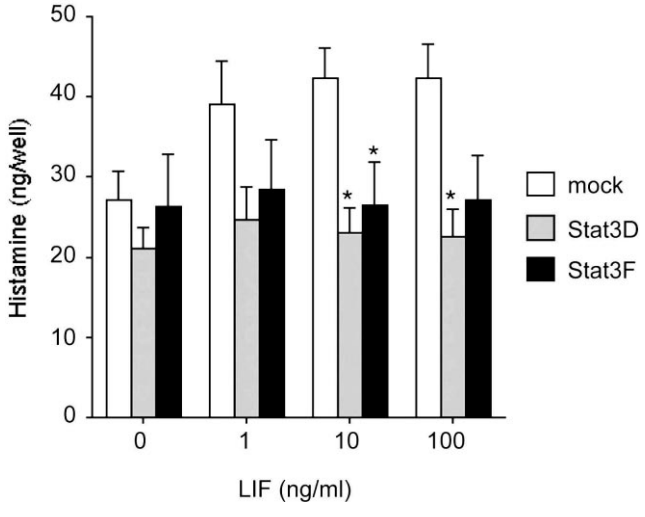

Fig. 6. Specific inhibition of LIF-induced mast cell growth by dominant-negative Stat3 (Stat3D/F). BMMC were co-cultured with mock-, Stat3D- and Stat3F-transfected fibroblasts in the presence of a series of concentrations of LIF, and histamine contents were determined on day 6. Data are mean ± S.E.M. of three independent experiments done in duplicate (*$P < 0.05$).

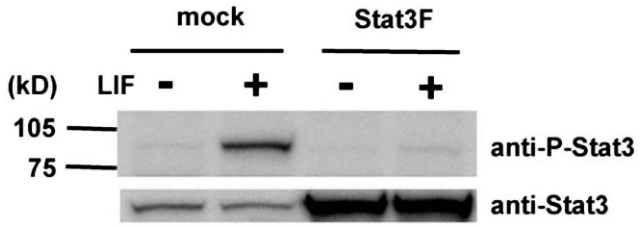

Fig. 7. Dominant-negative Stat3F inhibited LIF-induced tyrosine phosphorylation of endogenous Stat3. Mock- and Stat3F-transfected fibroblasts were stimulated with 100 ng/ml of LIF for 5 min, then cells were lysed and analyzed using PhosphoPlus® Stat3 (Tyr705) Antibody kit according to the manufacturer's instructions.

Although we have identified LIF as a keratinocyte-derived mast cell growth-enhancing factor [8], LIF is also produced from activated mast cells [26]. Mast cells, thus, may regulate their proliferation by themselves through the secretion of LIF.

In conclusion, we demonstrated that LIF-induced mast cell growth in the mast cell/fibroblast co-culture is mediated not by direct action of LIF to mast cells but by the indirect pathway via 3T3 fibroblasts through activating Stat3 signaling pathway in 3T3 fibroblasts.

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