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Scientific Research

Raw264.7 Cells Secrete Fibroblast Growth Stimulating Activity after Differentiation to Macrophages by Stimulation with Lipopolysaccharide

Jing-Yang Lai[1], Chung-Li Shu[1], Kazuhiro Morishita[2], Tomonaga Ichikawa[2], Yasuhisa Fukui[1*]

[1]Institute of Cellular and System Medicine, National Health Research Institute, Miaoli County, Taiwan
[2]Division of Tumor and Cellular Biochemistry, Department of Medical Sciences, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
Email: *990412@nhri.org.tw

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Abstract

Raw264.7 cells are monocytic cells that can differentiate to activated macrophages after lipopolysaccharide (LPS) stimulation. Here, we analyzed the factors secreted by Raw264.7 cells in response to LPS. The culture media of LPS-treated Raw264.7 cells was able to stimulate growth in MEF1F2 and NIH3T3 mouse fibroblast cell lines. We identified five secreted and LPS-induced chemokines, CCL2, CCL5, CCL12, CxCL2, and CxCL10, by microarray analysis and tested their stimulatory activity. We used commercially available bacterially expressed proteins, and found only CCL12, CxCL2 and CxCL10 stimulated growth in MEF1F2 and NIH3T3 cells. The saturation density of the cells was also increased. They were not able to stimulate growth in v-Src transformed MEF1F2 or SWAP-70 transformed NIH3T3 cells. We examined signaling pathways activated by these three factors. We found that ERK and p38 MAP kinase were activated and were required for the activity to stimulate the cell growth. Other pathways including phosophatidylinositol-3 kinase (PI3K), NFκB pathways were not activated. These results suggest that Raw264.7 cells secretes growth stimulation factors for fibroblasts when differentiated to macrophages implicating that fast growth of them is related to inflamation although the reason is still unclear.

Keywords

Raw264.7 Cells, Chemokine, Fibroblasts, Macrophage

*Corresponding author.

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1. Introduction

Inflammation in vascular tissues is part of a complex biological response to harmful stimuli, such as pathogens, damaged cells, or irritants [1]. An early step during inflammation is the activation of macrophages, which perform several key tasks during non-specific and specific defense pathways of vertebrates. They function to phagocytose, or engulf and then digest, cellular debris and pathogens. Macrophages also stimulate lymphocytes and other immune cells to respond to pathogens. However, they may also participate by secreting chemokines that affect the environment.

Chemokines are a family of small molecular weight cytokines, or signaling proteins secreted by cells [2] [3]. Chemokines are classified according to their structural characteristics such as their small molecular size and the presence of four conserved cysteine residues. Some chemokines are considered pro-inflammatory. They can be induced during an immune response and help to recruit immune cells to the site of infection. Other chemokines are considered homeostatic and are involved in controlling the migration of cells during tissue maintenance and development. CCL2 is a small molecular weight cytokine that belongs to the CC chemokine family. CCL2 recruits monocytes, memory T cells, and dendritic cells to sites of inflammation produced by either tissue injury or infection [4] [5]. CCL2 can bind to and activate either CCR2 or CCR5 receptor. CCL5 is an 8 kDa protein classified as either a cytokine or chemokine that is chemotactic for T cells, eosinophils, and basophils. CCL5 plays an active role in recruiting leukocytes to inflammatory sites [6]. CCL5 can bind to CCR1, CCR3 or CCR5 receptor. CCL12, is a small molecular weight cytokine belonging to the CC chemokine family, and its activity has been described in mice. CCL12 specifically recruits eosinophils, monocytes and lymphocytes. This chemokine can be greatly induced in macrophages and is predominately found in lymph nodes and thymus under a normal condition [7]. The corresponding receptor for CCL12 is CCR2. C-x-C motif chemokine CxCL2 is a small molecular weight cytokine belonging to the CxC chemokine family that is also called *macrophage inflammatory protein* 2-*alpha* (MIP2-alpha), *Growth-regulated protein beta* (Gro-beta) and *Gro oncogene*-2 (Gro-2). This chemokine is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells [8]-[10]. CxCL2 mobilizes cells by interacting with a cell surface chemokine receptor called CxCR2 [10].

CxCL10 is also a C-x-C motif chemokine with a molecular weight of 8.7 kDa in humans [11] [12]. CXCL10 is a ligand for the CxCR3 receptor, and is secreted by monocytes, endothelial cells and fibroblasts in response to IFN-γ [13]. CxCL10 activity is important in several processes including chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis [14] [15].

Raw264.7 monocytic cells differentiate to form activated macrophages, which are polarized toward that of the M2 macrophage phenotype, upon lipopolysaccharide (LPS) stimulation. Many studies have focused on the function of macrophages during inflammation, and PDGF, EGF, and some other factors have been shown to be secreted for growth of fibroblasts but there is still a room to be seen about other factors. Also during wound and healing, macrophages secrete growth factors [16] [17]. In this paper, we show that Raw264.7 cells secrete cytokines as they differentiate to activated macrophage and discuss the potential functions of these factors.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions

Raw264.7 and NIH3T3 cells were purchased from the Food Industry Research and Development Institute, Taiwan. NIH3T3/SWAP-70-590 cells were transformed with SWAP-70 as described before [18]. NIH3T3 related cells were cultured in high glucose DMEM supplemented with 200 mM glutamine and 10% calf serum. Raw264.7 cells were cultured in low glucose DMEM supplemented with 4 mM glutamine and 10% fetal bovine serum. MEF1F2 cells are normal mouse embryonic fibroblasts (MEF) lacking the SWAP70 gene [19]. MEF1F2 cells are cultured in normal low glucose medium and can maintain a normal phenotype for more than 7 years. In contrast, wild type MEFs usually transform after several months of culture. SWAP-70(-)/SWAP70/v-Src-1 cells are v-Src transformed MEF1F2 cells as described previously [19].

2.2. Preparation of Culture Media of Raw264.7 Cells

To obtain culture supernatants of Raw264.7 cells, they were stimulated with 100 ng/ml LPS and cultured for 24

hrs. The culture supernatant was collected. For the control culture supernatant, cells were cultured for 24 hrs and LPS was added just after harvesting the culture supernatant.

To test activated Raw264.7 cells continuously secrete the growth stimulating factors, confluent Raw264.7 cells were incubated with or without 100 ng/ml LPS and after 24 hours the culture supernatant was harvested. Then, new medium without LPS was added to the culture, incubated for 24 hours, medium was changed again, incubated for another 24 hrs, and the culture medium was harvested.

2.3. Bacterially Expressed Chemokines, Antibodies, and Reagents Used in This Study

Bacterially expressed chemokines, CCL2, CCL5, CCL12, CxCL2, and CxCL10 were purchased from R & D system (Minneapolis, MN, USA). Anti-ERK, anti-phospho ERK, p38 MAP kinase, anti-phospho p38 MAP kinase, Akt, anti-Phospho Akt, anti-IκB, and anti-phosphoIκB antibodies were purchased from Cell Signaling Co Ltd (Danvers, MA, USA). An ERK inhibitor, PD59089 and a p38 MAP kinase inhibitor, SB203580, were purchased from WAKO Co. Ltd (Tokyo, Japan).

2.4. PCR Reaction and Western Blotting

RNA extraction was performed using RNA extraction kit (Zymo Research, Irvine, CA, U.S.A.). Reverse transcriptase reaction was performed using the PrimeScript (Takara, Tokyo, Japan). PCR was done using ExTaq polymerase (Takara). Presence of chemokine receptors was analyzed by PCR using the following primers: ATGGAGATTTCAGATTTCACAGAAG and TCAGAAGCCAGCAGAGAGCTCATGTTC (CCR1); ATG GAAGACAATAATATGTTACC and TCACTTACTTTACAACCCAACCG (CCR2); GGCATTCAACACAG ATGA AATCAAG and CTAAAACACCACAGAGATTTCTTGC (CCR3); ATGGATTTTCAAGGGTCAGT TCCG and TCATGTTCTCCTGTGGATCGGGTATAG (CCR5); CAAAGATGGGAGAATTCAAGGTGG and CTTTAGAGGGTAGTAGAGGTGT (CxCR2); and ATGTACCTTGAGGTTAGTGAACG and TTACAAGC CCAGGTAGGAGGCC (CxCR3).

Cells were harvested after stimulation of the chemokines. Cells were lysed with a buffer containing 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM sodium vanadate, and 1 mM PMSF. Aliquots of the samples were loaded on the SDS-PAGE and Western blotting was done as described before [20].

2.5. Examination of Cell Growth

Cells were plated at a density of 3×10^4 cells per dish in 3.5 cm dishes. To maintain growth factor activity, the media were changed every day. Number of cells in each dish were counted and expressed in graphs. To test effect of concentration of chemokines 1×10^5 cells were seeded and cultured for two days in the presence of various concentrations of chemokines.

2.6. Microarray

RNA was prepared from confluent cultures of MEF1F2, MEF1F2-SWAP-1, and MEF1F2-SWAB-7 cells. For microarray analysis, oligoarray type array (Agilent SurePrint G3 Mouse GE 8 x 60K) was used. Total RNA (0.2 mg) was labeled with Cy3-CTP using Agilent low input quick-Amp labeling kit. Hybridization was done by Agilent one-color microarray-based gene expression analysis low input quick-amp labeling kit v6.5 using 0.6 µg of cRNA. Genes whose expression levels were increased two fold compared with control cells were selected with p-value cut off of 0.05.

3. Results and Discussion

3.1. Activated Raw264.7 Cells Secrete Factors That Stimulate Growth in Normal Fibroblasts

Raw264.7 cells were stimulated with 100 ng/ml LPS and their culture medium was harvested after 24 h. Subsequently, SWAP-70(-) and NIH3T3 cells were incubated overnight with the harvested media. We found that this treatment led to growth stimulation in fibroblasts, but the effects were not sustained for longer than 24 h. We speculated that this was due to the potential instability of growth factors in the harvested medium. Indeed, daily replenishment of harvested media led to sustained growth stimulation of fibroblasts (**Figure 1(a)**).

3.2. The Secreted Growth-Stimulating Factors Are Chemokines

Secreted factors from LPS-treated Raw264.7 cells maintained their growth stimulatory activity for several days (**Figure 1(b)**). These results suggested that the factors were continuously secreted and therefore, transcriptionally upregulated. Accordingly, we performed microarray analysis of LPS-stimulated Raw264.7 cells. We found that many genes were over-expressed after LPS treatment. The number of the genes expressed two folds or more of the control cells was 254 out of 28,853 genes. Among these, we selected secretable proteins containing signal peptides for secretion. They were chemokines, CCL2, CCL5, CCL12, CxCL2 and CxCL10. They increased 8.29, 22.5, 3.45, 11.07, 2.61 fold each compared with control cells. We suspected that these proteins stimulated the cell growth of the fibroblasts and tested the activities of commercially available proteins. Only CCL12, CxCL2 and CxCL10 exhibited the activities and CCL2 and CCL5 did not (**Figure 2**).

3.3. Growth of Transformed Cells Is Not Affected by Secreted Chemokines

We tested the effect of CCL12, CxCL2 and CxCL10 on transformed MEF1F2 or NIH3T3 cells. The cells grown in the presence of these chemokines showed fast growth compared to the control cells, suggesting that they stimulate cell growth. However, they exhibited contact inhibition at the higher cell densities, suggesting that the cells are not transformed **(Figure 3(a)** and **Figure 3(b))**. This higher saturation density was outstanding. Unlike normal cells, growth was not stimulated by these chemokines in transformed cells (**Figure 4(a)** and **Figure 4(b)**).

3.4. Expression of Chemokines Receptors in MEFs and NIH3T3 Cells

To confirm that MEF1F2 and NIH3T3 cells expressed the receptors for these chemokines, we performed PCR. Bands were obtained using RNAs of MEF1F2 and NIH3T3 cells (**Figure 5**). These bands were cloned and sequenced. The results showed that these bands were indeed PCR products of CCR2, CCR3, CCR5, CxCR2, and CxCR3, which are the receptors for the secreted chemokines studied in this paper. Despite this, only a subset of chemokines was able to induce cell growth.

3.5. Signaling of Cytokines That Stimulate Cell Growth of Fibroblasts

We tested whether several signaling pathways which are widely studied are activated upon stimulation by these cytokines. As shown in **Figure 6(a)**, ERK was clearly activated 5 to 10 min after stimulation. p38 MAP kinase

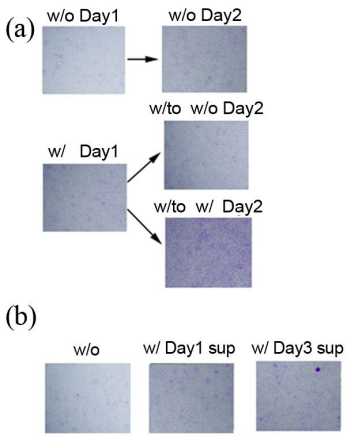

Figure 1. Daily replacement is required to maintain growth-stimulating activity of LPS-induced Raw264.7 culture media. (a) MEF1F2 cells were cultured with culture media of Raw264.7 cells. After cultivation with culture media of LPS-treated Raw264.7 cells for one day (w/Day1), the cell density of Raw264.7 cells was significantly higher than that of using LPS-untreated Raw264.7 cells (w/o Day1). Medium was replaced with culture media of LPS-treated Raw264.7 cells for 1 day and then replaced culture media from LPS-untreated (w/o Day2) or LPS-treated (w/Day2) culture media. Cells grew faster when cultured with media from LPS-treated Raw264.7 cells. (b) MEF1F2 was cultured with culture media of Raw264.7 cells stimulated with LPS (w/Day1 sup) or that obtained by further incubation without LPS stimulation for 72 hours (w/o Day3 sup). w/: culture supernatant of LPS-treated Raw264.7 cells; w/o: culture supernatant of LPS-untreated Raw264.7 cells.

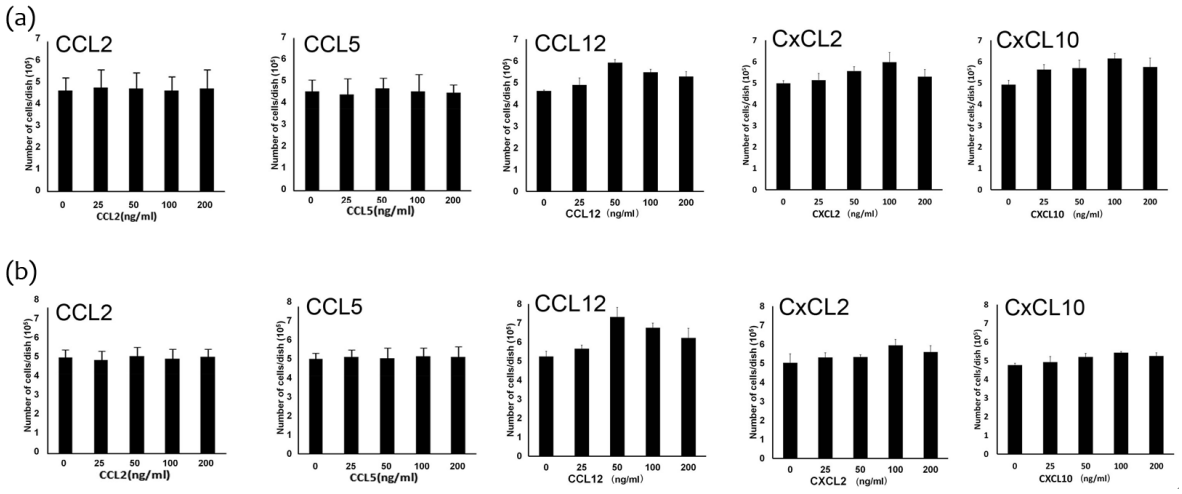

Figure 2. Concentration of chemokines required for fast cell growth. MEF1F2 (a) or NIT3T3 (b) cells were seeded at the density of 1×10^4 cells per 3.5 cm dishes. Various doses of CCL2, CCL5, CCL12, CxCL2, and CxCL10 were added to the cultures. The medium was changed to the ones containing the same concentration of chemokines on the following day. The numbers of the cells were counted 24 hrs after the medium change. The results are the averages of three independent experiments.

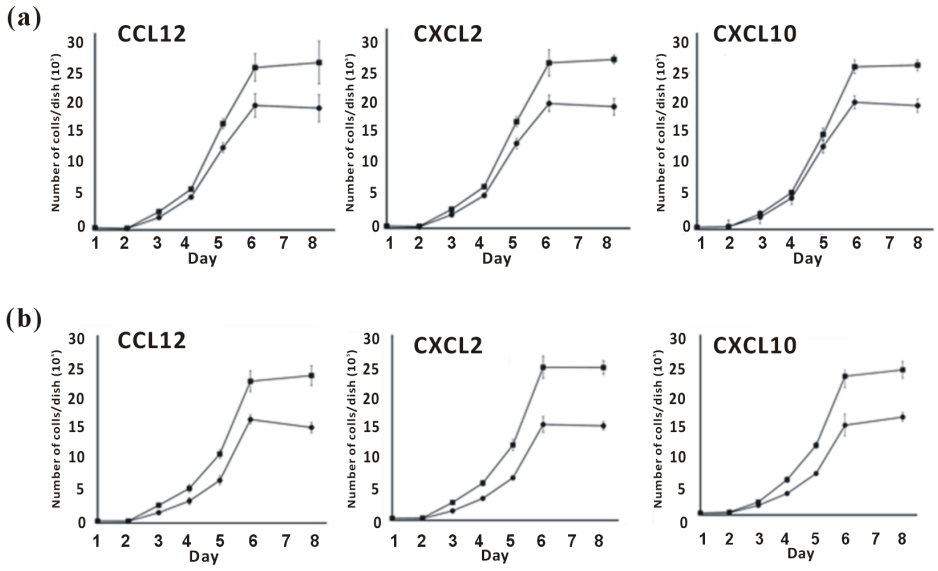

Figure 3. Growth curve of fibroblasts stimulated with culture media containing bacterially expressed chemokines. MEF1F2 (a) or NIH3T3 (b) cells were plated at the density of 3×10^4 cells/dish in 3.5 cm dishes. Commercially available chemokines (50 ng/ml for CCL12 and 100 ng/ml for CxCL2 and CxCL10) were added to the cells. The medium was changed every day. The number of cells in dishes were counted and plotted on the graph. Closed circles indicate controls, while closed squares highlight the growth curve of chemokine-treated cells. Error bars represent standard deviation. The results are the averages of three independent experiments.

was also activated. However, Akt and NFκB monitered by phosphorylation of IκB remained inactivated. We also monitored some other signaling molecules such as PKCα but none of them were activated (data not shown).

To see which pathway is related to fast growth of the cells, we used inhibitors for the signaling pathways and test growth stimulation activities of the cytokines. As shown in **Figure 6(b)**, PD59089, an inhibition of ERK, and SB203580, a p38 MAP kinase inhibitor, clearly inhibited growth stimulation, suggesting that these two pathways are necessary for stimulation of the cells to grow faster than the control cells. How these pathways

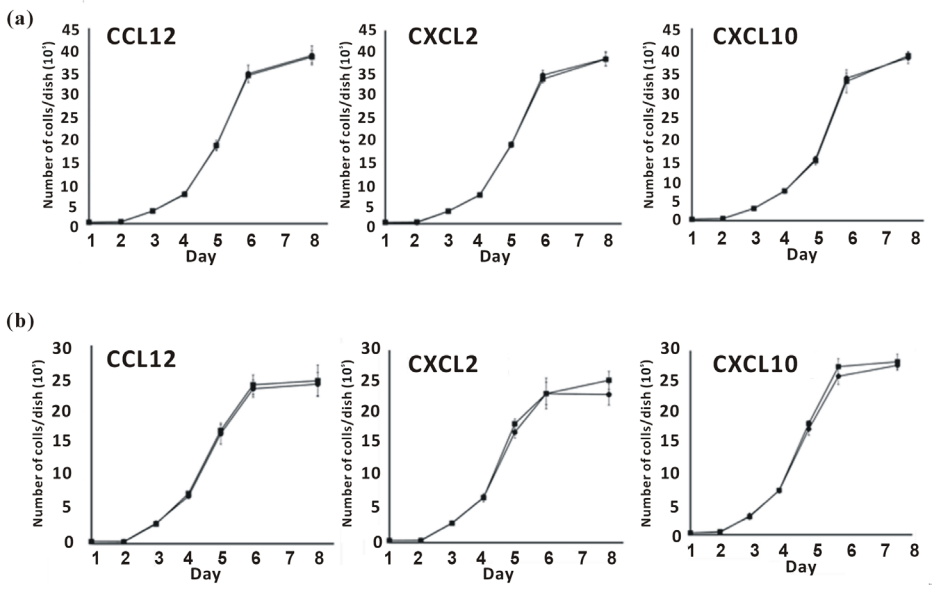

Figure 4. Growth of transformed fibroblasts is not stimulated by bacterially expressed chemokines. SWAP-70(-)/SWAP70/v-Src-1 (a) or NIH3T3/SWAP-70-590 (b) cells were plated at the density of 3×10^4 cells/dish in 3.5 cm dishes. Commercially available chemokines (50 ng/ml for CCL12 and 100 ng/ml for CxCL2 and CxCL10) were added to the cells. The medium was changed every day. The number of cells in dishes were counted and plotted on the graph. Closed circles indicate controls, while closed squares highlight the growth curve of chemokine-treated cells. Error bars represent standard deviation. The results are the averages of three independent experiments.

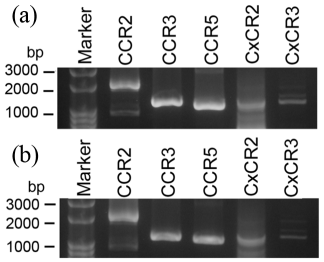

Figure 5. Expression of chemokine receptors. Expression of chemokine receptors in MEF1F2 (a) and NIH3T3 (b) cells was analyzed by PCR. The bands were cloned and sequenced to confirm the bands are really the signals of the chemokine receptors.

contribute to fast growth of the cells should be studied in the future.

Chemokines function as chemo-attractants during the process of inflammation. Moreover, they stimulate growth of some immune cells. However, their effects on the neighboring fibroblasts have not been fully appreciated. In this paper, we found that certain chemokines secreted from macrophages can stimulate growth of fibroblasts. The role of this activation is unclear, but it is possible that the growth of fibroblasts can support immune cell activity at the site of inflammation.

CCL12, CxCL2, and CxCL10 showed growth stimulation activity. To make sure that only CCL12, CxCL2, and CxCL10 have the activity, we cloned the genes for CCL2, CCL5, CCL12, CxCL2, and CxCL10 and expressed the proteins in 293T cells. Again CCL2 and CCL5 did not show the activity and other three showed significant activity, confirming the results of bacterially expressed protein.

The cells, which are stimulated with CCL12, CxCL2, and CxCL10 maintained contact inhibition activity, although the saturation cell number was bigger than that of the untreated cells. Therefore, the cells grow faster but they are not transformed. However it is possible that continuous activation of the cells might transform the cells. Further study is required to get the conclusion of this question.

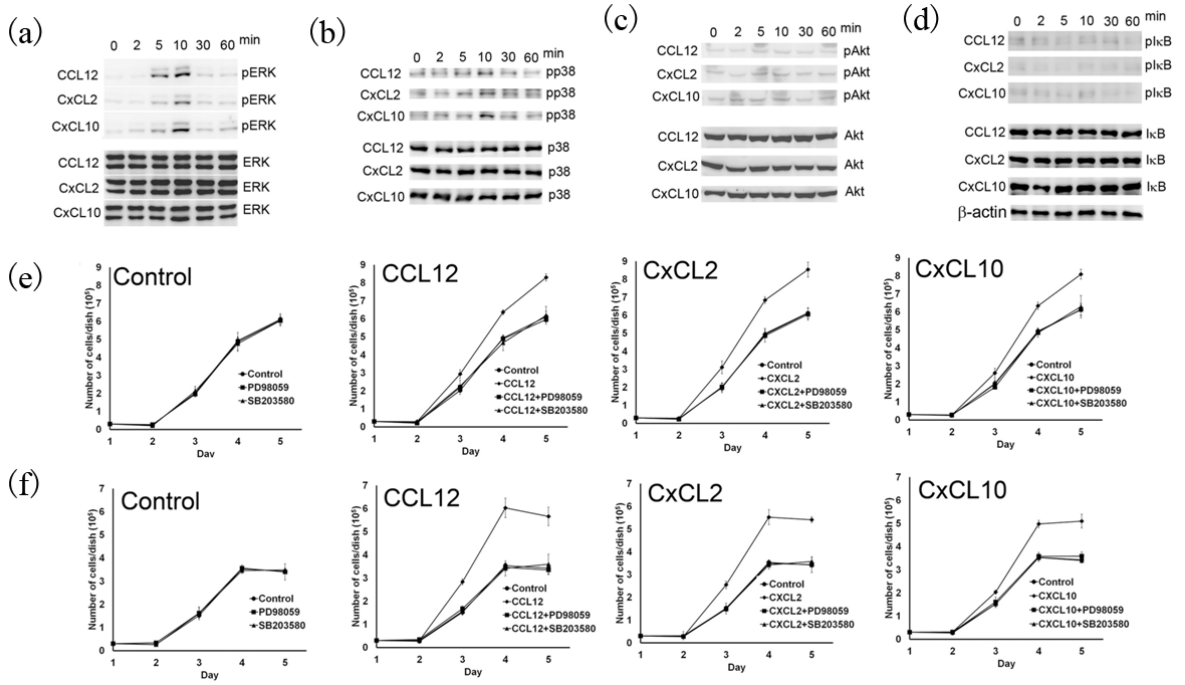

Figure 6. Signaling of chemokine stimulation and requirement of the signal for fast growth of the cells. MEF1F2 cells were stimulated with the chemokines shown in the figure (50 ng/ml for CCL12 and 100 ng/ml for CxCL2 and CxCL10). Cells were harvested at the time indicated in the figure. Activation of ERK (a), p38 MAP kinase (b), Akt (c), and NF*κ*B (d) were monitored. (e) MEF1F2 cells were plated at the density of 3×10^4 cells/dish in 3.5 cm dishes. Commercially available chemokines (50 ng/ml for CCL12 and 100 ng/ml for CxCL2 and CxCL10) were added. Also, an ERK inhibitor PD98059 (10 μM) or a p38 MAP kinase inhibitor, SB203580 (10 μM), was added in some dishes. The medium was changed every day. The number of cells in dishes were counted and plotted on the graph. (f) The same experiment as (e) was done using NIH 3T3 cells. The symbols are shown in the figures. The results are the averages of three independent experiments.

The interplay between chemokines and their corresponding receptors is complex. We found that CCR2, CCR3, CCR5, CxCR2, and CxCR3 are expressed in MEFs and NIH3T3 cells, however the complex interplay of signaling networks downstream of these receptors have complicated the functional analysis of chemokines. We found that the MAP kinase and p38 MAP kinase pathways were important for the cells to grow fast, however Akt and NF*κ*B were not. CCL12, CxCL2, and CxCL10 take different receptors however the signaling was very similar, suggesting that these receptors play a similar role for signal transduction. Here, we found that CCL2 and CCL12 elicit distinct biological responses, although they bind to the same receptor. Why CCL2 does not show growth stimulation activity is a mystery. It is possible that CCL12 reacts with unknown receptor which is required for cells growth or CCL2 reacts with another unknown receptor which inhibits stimulation of cell growth. For instance, activation of CCR5 might inhibit fast growth of the cells.

In this study we found that chemokines, CCL12, CxCL2, and CxCL10 can stimulate growth of fibroblasts. However, they have to be added every day maybe because of their instability. Therefore, Cell growth of normal fibroblasts will be stimulated only when chemokines are supplied by macrophages *in vivo*. For the fast growth of the cells, activation of the ERK and 38 MAP kinase pathways was required. However, cell growth of transformed cells was stimulated, and probably the ERK and the 38 MAP kinase pathways are already activated. Stimulation of growth of fibroblasts may be limited to very specific condition, which we do not know yet. It is interesting that there are appropriate concentrations of chemokines to stimulate the cell growth. This result may implicate that there is a fine regulation of growth of fibroblasts by macrophage. This may be an important finding to consider what is happening *in vivo*. Further study is required to understand the meaning of the observation we made in this paper.

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