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ORIGINAL ARTICLE

Basic Study

Fibroblast-derived CXCL12/SDF-1α promotes CXCL6 secretion and co-operatively enhances metastatic potential through the PI3K/Akt/mTOR pathway in colon cancer

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Abstract

AIM

To investigate the underlying mechanism by which CXCL12 and CXCL6 influences the metastatic potential of colon cancer and internal relation of colon cancer and stromal cells.

METHODS

Western blotting was used to detect the expression of CXCL12 and CXCL6 in colon cancer cells and stromal cells. The co-operative effects of CXCL12 and CXCL6 on proliferation and invasion of colon cancer cells and human umbilical vein endothelial cells (HUVECs) were determined by enzyme-linked immunosorbent assay, and proliferation and invasion assays. The angiogenesis of HUVECs through interaction with cancer cells and stromal cells was examined by angiogenesis assay. We eventually investigated activation of PI3K/Akt/mTOR signaling by CXCL12 involved in the metastatic process of colon cancer.

RESULTS

CXCL12 was expressed in DLD-1 cancer cells and fibroblasts. The secretion level of CXCL6 by colon cancer cells and HUVECs were significantly promoted by fibroblasts derived from CXCL12. CXCL6 and CXCL2 could significantly enhance HUVEC proliferation and migration ($P < 0.01$). CXCL6 and CXCL2 enhanced angiogenesis by HUVECs when cultured with fibroblast cells and colon cancer cells ($P < 0.01$). CXCL12 also enhanced the invasion of colon cancer cells. Stromal cell-derived CXCL12 promoted the secretion level of CXCL6 and co-operatively promoted metastasis of colon carcinoma through activation of the PI3K/Akt/mTOR pathway.

CONCLUSION

Fibroblast-derived CXCL12 enhanced the CXCL6 secretion of colon cancer cells, and both CXCL12 and CXCL6 co-operatively regulated the metastasis *via* the PI3K/Akt/mTOR signaling pathway. Blocking this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer.

Key words: CXCL12/SDF-1α; CXCL6; Metastasis; PI3K/Akt/mTOR pathway; Colon cancer

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Core tip: This study has provided the first report of fibroblast-derived CXCL12 enhancement of CXCL6 secretion in colon cancer cells, and of both CXCL12 and CXCL6 co-operatively regulating metastasis through the PI3K/Akt/mTOR signaling pathway. Blockage of this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer. Our work might encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

Ma JC, Sun XW, Su H, Chen Q, Guo TK, Li Y, Chen XC, Guo J, Gong ZQ, Zhao XD, Qi JB. Fibroblast-derived CXCL12/SDF-1α promotes CXCL6 secretion and co-operatively enhances metastatic potential through the PI3K/Akt/mTOR pathway in colon cancer. *World J Gastroenterol* 2017; 23(28): 5167-5178 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5167.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5167

INTRODUCTION

Colon cancer is the fourth most frequently diagnosed cancer in the United States. In 2015, an estimated 93090 new cases of colon cancer occurred in the United States. During that same year, it was estimated that 49700 patients died from colon and rectal cancers[1]. The poor prognosis of colon cancer is attributable to its tendency of metastases. However, the precise mechanisms that determine the directional proliferation and invasion of cancer cells into specific organs remain to be established[2,3]. Therefore, exploring the fundamental mechanism of invasion, proliferation, metastasis and tumor biological behaviors at the level of cellular or molecular microenvironments is needed in clinical diagnosis and therapy.

Chemokines (chemotactic cytokines) form a complex family of small, secreted proteins that play an important role in innate and adaptive immunity, homeostatic processes, angiogenesis and tumorigenesis[4,5]. Based upon the position of conserved cysteine residues, chemokines are classified into four subfamilies (C, CC, CXC, CX3C)[6]. CXC chemokines have been proven to modulate tumor behaviors, especially in regulation of angiogenesis, activation of a tumor-specific immune response and stimulation of tumor cell proliferation in an autocrine or paracrine fashion[7]. However, updated research has shed new light on this subfamily of cytokines, indicating that its members have multifaceted roles in the microenvironment that consists of the tumor cells themselves and/or stromal cells, including infiltrating leukocytes, endothelial cells (ECs) and fibroblasts.

The functions of CXC chemokines in the tumor microenvironment depend considerably on the chemokine type and tumor and stromal cells' characteristics. In addition, there are cases in which chemokines have been implicated as having tumor-inhibiting gene activities, and there are many more examples of CXC chemokines with tumor-promoting roles[8-11]. Two of the most famous members are the stromal cell-derived factor-1 (SDF-1/CXCL12/IL12) and chemokine ligand 6 (CXCL6). Numerous studies have shown that their activities would increase the establishment of tumorigenesis, invasion, proliferation and metastases.

Recent analysis has shown that CXCL12 supports the survival or growth of a variety of normal or malignant cell types, including hematopoietic progenitors, germ cells, leukemia B cells and breast carcinoma cells[12-15]. Other studies have shown that the CXCL12/CXCR4 and related axis are involved in tumor metastasis to sites which are characterized by high production of CXCL12, such as liver, lung and bone marrow[16,17]. Activation of the CXCL12/CXCR4 signaling axis leads to chemotaxis, cell survival, and/or proliferation; however, the downstream signaling cascades are tissue-specific and not well characterized in EC[18].

CXCL6, a small cytokine belonging to the CXC chemokine family, is also known as granulocyte chemotactic protein 2. As its former name suggests, CXCL6 is a chemoattractant for neutrophilic granulocytes[13-14]. It elicits its chemotactic effects by interacting with the chemokine receptors CXCR1 and CXCR2. This tumor progression may occur as a function of the regulation of angiogenesis, cell motility, immune cell infiltration, cell growth and survival in the microenvironment, and modulation of local anti-

tumor immune responses[19]. As evidenced by various experiments, CXCL6 is over expressed in colorectal, breast, lung and thyroid cancers. Actions of tumor cells in the microenvironment were also regulated by complicated molecular mechanisms[20-23].

Different chemokines played their specific roles. Both the angiogenesis-promoting effect of CXCL6 and chemotactic effect of CXCL12 play important roles in tumorigenesis and metastasis[24,25]. However, the molecular mechanisms of the active signaling pathway by which CXCL12 and CXCL6 co-operatively regulate metastasis of colon cancer remain to be clarified.

The purpose of this study was to investigate the co-operative promotion of metastatic potential and the underlying mechanism of CXCL12 and CXCL6 in order to better understand the interaction between colon cancer cells and stromal cells. Furthermore, our study provided data to demonstrate that phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway plays an important role in CXCL12 simulation and that this process is involved in the development and metastasis of colon cancer. Understanding the biologic mechanisms responsible for regulation of chemokines may enable better molecular targeted therapies to treat patients with metastatic colon cancer.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human CXCL6 and CXCL12 were purchased from R&D Systems (Minneapolis, MN, United States). Neutralizing monoclonal anti-human CXCL12 (anti-CXCL12 Ab), anti-human CXCL6 (anti-CXCL6 Ab), anti-human CXCR4 (anti-CXCR4 Ab) were obtained from Carbiochem (San Diego, CA, United States).

Cell lines and culture condition

The human colon cancer cell lines HT29, WiDr, CaCo-2, DLD-1 and Colo320 were obtained from the American Type Culture Collection (Rockville, MD, United States). DLD-1, WiDr and CaCo-2 were maintained in minimum essential medium (Eagle's; (Sigma Chemical Co., St. Louis, MO, United States) with high glucose and 10% fetal bovine serum (FBS). HT-29 and Colo320 were maintained in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% FBS. HUVECs were obtained from Kurubo Co. (Osaka, Japan) and maintained in HuMedia-EG2 medium supplemented with 2% FBS, 5 ng/mL basic fibroblast growth factor, 10 μg/mL heparin, 10 ng/mL epidermal growth factor, and 1 μg/mL of hydrocortisone, according to the supplier's instruction (Kurubo Co.). All cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 in air.

Western blot analysis

Cells were cultured in the media supplemented with 1% FBS for 1 d. After the indicated treatments, the cells were lysed in lysis buffer [25 mmol/L Tris (pH 7.8) with H_3PO, 2 mmol/L CDTA, 10 mmol/L DTT, 10% glycerol, 1% Triton® X-100, 2 mmol/L PMSF, 1 mmol/L sodium orthovanadate, and 10 μmol/L leupeptin]. The protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, IL, United States). The amounts of samples were 30 μg per lane. The lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membrane (Immobilo PVDF; Nihon Millipore Ltd, Tokyo, Japan). The membrane was incubated in the blocking buffer for 60 min at room temperature. The blocking buffer consisted of 5% nonfat dry milk dissolved into Tris-buffered saline containing 0.1% Tween 20 (TBS-T). After washing the membrane with TBS-T, the membrane was immunoblotted with each primary antibody (Ab) diluted into 1:1000-2000 overnight at 4 ℃. Afterward, membranes were washed with TBS-T three times, and subjected to HRP-conjugated secondary Ab for 60 min at room temperature. Protein Ab complexes were visualized with an ECL western blotting detection and analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom). β-actin western blots served as controls.

Enzyme-linked immunosorbent assay

All cancer cell lines and fibroblasts were separately seeded at a density of 3×10^5 cells/mL into 12-well plates containing medium with 10% FBS and allowed to adhere overnight. The medium was exchanged, and cells were cultured for an additional 48 h. The medium was collected and microcentrifuged at 1500 rpm for 5 min to remove particles, and the supernatants were frozen at -80 ℃ until performance of enzyme-linked immunosorbent assay (ELISA). Concentration of CXCL6 was measured by ELISA kit (R&D Systems) according to the manufacturer's instructions.

In order to further investigate the synergistic effect of the tumor-stromal interaction, we tested the effect of fibroblast-derived CXCL12 on cancer cell CXCL6 production using a double-chamber method in 24-well plates. Fibroblasts were seeded at a density of 1×10^5 cells/well into 24-well plates, and allowed to adhere overnight. The medium was exchanged with or without CXCL12 Ab, and then co-cultured with 5×10^4 HT-29, WiDr, CaCo-2, DLD-1 and fibroblasts cells that had been placed into inserts with 0.45-mm^2 pores (Kurabo Co.). The co-culture systems were incubated for an additional 48 h, and CXCL6 concentration was subsequently measured as described above. Each condition was assessed using 5 independent samples.

Proliferation assay

To confirm the effect of chemokines on HUVEC proliferation, we performed the proliferation assay according to the manufacturer's instructions. HUVECs were seeded at a density of 5×10^3 cells/100 μL in

96-well plates and allowed to adhere overnight. Then, cultures were re-fed with fresh media containing various concentrations of CXCL6 or CXCL12. After 72 h incubation, 10 μL WST-1 reagent was added to each well and cells were incubated for another 4 h at 37 ℃, then the cell proliferation was measured by the WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan). The absorbance was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, United States) at a test wavelength of 450 nm and reference wavelength of 690 nm.

Invasion assay

The effects of CXCL12, CXCL6 and co-cultures with fibroblasts or colon cancer cells (DLD-1) on invasive capability of HUVECs were determined by Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA, United States) according to the manufacturer's instructions. This system is separated by a PET membrane coated with Matrigel Matrix such that only invasive cells can migrate through the membrane to the reverse side. HUVECs (5×10^4 cells/mL) were suspended in medium containing 2% FBS and seeded into the Matrigel pre-coated transwell chambers consisting of polycarbonate membranes with 8-μm pores, and fibroblasts or DLD-1 cells were seeded at a density of 2×10^5 cells/well into the inner chambers in 24-well plates, then the transwell chambers were then placed into 24-well plates, into which we added basal medium only or basal medium containing gradient concentrations of CXCL6 (0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 10 ng/mL + 10 μg/mL CXCL6 Ab) or CXCL12 (0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 10 ng/mL + 10 μg/mL CXCL12 Ab). After incubating for 24 h and HUVECs for 16 h, the upper surface of the transwell chambers was wiped with a cotton swab and the invading cells were fixed and stained with Diff-Quick stain. The number of invading cells was counted in five random microscopic fields of the low filter surface under a microscope at 200 × magnification. Each condition was assessed in triplicate.

Angiogenesis assay

To investigate the influence of CXCL6 on tubule formation by HUVECs, HUVECs and fibroblasts were co-culture in the basal medium using an angiogenesis kit (Kurabo Co.) according to the manufacturer's protocol. First, HUVECs and fibroblasts were co-cultured in 24-well plates with basal medium. The media were exchanged every 2 d, with co-incubation continuing for a total of 11 d. The co-culturing system was stained with anti-CD31 Ab. The areas of angiogenesis were measured quantitatively over ten different microscopic fields for each well using an image analyzer (Kurabo Co.).

Angiogenic activity during co-culture with colon cancer cells in vitro

To further investigate the influence of colon cancer cell-derived CXCL12 on tubular formation by HUVECs, the colon cancer cells (DLD-1 secreting CXCL12 or CaCo-2 and HT-29 not secreting CXCL12), HUVECs, and fibroblasts were co-cultured using a double-chamber method in 24-well plates. DLD-1, CaCo-2 or HT-29 cells (5×10^4 cells) were seeded into transwell chambers, consisting of polycarbonate membrane with 0.45-m pores and allowed to adhere overnight. Transwell chambers were then placed in the HUVECs/fibroblasts co-culture system with or without 10 ng/mL of CXCL12 or CXCL12 Ab and exchanged on the sixth day. All cells were cultured for a total of 11 d. HUVEC tubular formation was described as above. This assay allowed us to evaluate angiogenesis quantitatively and examine tumor-stromal interactions through soluble cytokines.

Statistical analysis

Data are presented as mean ± SD. Differences in the mean of two groups were analyzed by an unpaired *t*-test. Multiple group comparisons were performed by one-way ANOVA with a post hoc test for subsequent individual group comparisons. $P < 0.05$ was considered statistically significant. Mean values and SD were calculated for experiments performed in triplicate (or more).

RESULTS

Expression of CXCL12, CXCL6 and CXCR4 proteins in colon cancer cell lines and stromal cells

Western blotting results revealed that CXCL12 protein was only expressed in fibroblasts and DLD-1, but not in HT29, WiDr, CaCo-2, Colo320 and HUVECs. CXCR4 and CXCL6 were expressed in all colon cancer cell lines, fibroblasts and HUVECs (Figure 1).

Effect of CXCL12 on secreted level of CXCL6 from colon cancer cell lines and HUCVECs

The secreted CXCL6 level was measured by ELISA assay in colon cancer cell lines and stromal cells. On the basis of this assay, secretion of CXCL6 was higher in DLD-1 and HT-29 cell supernatants than in supernatants from CaCo-2, WiDr and HUVECs. The addition of recombinant CXCL12 significantly enhanced CXCL6 production in CaCo-2 (2.54-fold *vs* control, $P < 0.01$; Figure 2A), WiDr (2.07-fold *vs* control, $P < 0.01$; Figure 2B), HT-29 (1.87-fold *vs* control, $P < 0.01$; Figure 2C) and HUVEC (2.79-fold *vs* control, $P < 0.01$; Figure 2E). Likewise, co-culture with fibroblast cells also significantly enhanced CaCo-2 (1.89-fold *vs* control, $P < 0.01$), WiDr (1.67-fold *vs* control, $P < 0.01$), HT-29 (1.62-fold *vs* control, $P < 0.01$) and HUVEC (2.15-fold, *vs* control, $P < 0.01$) cells' secretion of CXCL6. On the other hand, recombinant CXCL12 and co-culture with fibroblasts did not promote the CXCL6 secretion in DLD-1 culture supernatants (Figure 2D). Co-culture with DLD-1 cells significant

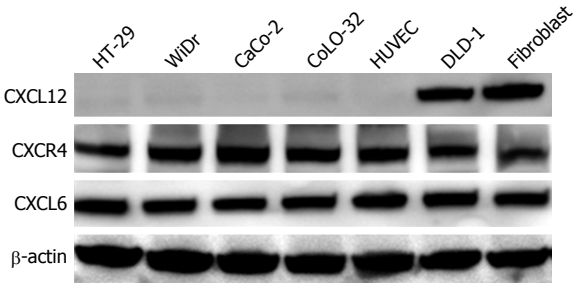


Figure 1 Expression levels of stromal cell-derived factor-1, CXC chemokine receptor 4 and granulocyte chemotactic protein-2 in colon cancer cell lines and stromal cells. The protein expression levels of CXCL2, CXCR4 and CXCL6 in colon cancer cell lines and stromal cells were determined in whole-cell lysates by western blotting analysis. Thirty micrograms of total cell lysate were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with antibodies to CXCL12, CXCR4 and CXCL6. β-actin was used as a loading control. CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; CXCR4: CXC chemokine receptor 4.

enhanced CXCL6 secretion level in the HUVEC culture supernatants as well ($P < 0.01$), because fibroblasts could secrete CXCL12 protein. Furthermore, the enhanced CXCL6 production elicited by co-culturing with fibroblast cells and recombinant CXCL12 were significantly inhibited in the presence of CXCL12 Ab ($P < 0.01$).

HUVEC proliferation following treatment with CXCL6, CXCL12 and fibroblast cell-cultured supernatants

To create stromal cell supernatants, fibroblast cells were seeded to a final number of 5×10^6 cells/5 mL into 100-mm dishes containing medium with 10% FBS, and were cultured overnight. Cells were then cultured in medium containing 2% FBS for 48 h. The culture media were collected and microfuged at 1500 rpm for 5 min to remove any particles, and the supernatants were used in proliferation assays. Recombinant CXCL6 elicited enhanced proliferation of HUVECs in a dose-dependent manner, and co-culture with fibroblasts caused significantly enhanced HUVEC proliferation ($P < 0.05$, $P < 0.01$; Figure 3A). Recombinant CXCL6 also promoted the proliferation of HUVECs in a concentration-dependent manner ($P < 0.05$, $P < 0.01$; Figure 3B).

CXCL6 and CXCL6 promotion of colon cancer cell and HUVEC invasiveness

The invasion assay was used to investigate whether CXCL12 and CXCL6 influence invasiveness of colon cancer cell lines. The invasive capacity of HT-29 cells was promoted by stimulation using recombinant CXCL6 (Figure 4A) and CXCL12 (Figure 4B) in a concentration-dependent manner ($P < 0.05$, $P < 0.01$), and 10 ng/mL of CXCL6 and CXCL12 significantly promoted cancer cell invasion ($P < 0.01$). Interestingly, CXCL6 (Figure 4C) and CXCL12 (Figure 4D) also significantly enhanced the invasion of HUVECS in a dose-dependent manner ($P < 0.05$, $P < 0.01$). However, the invasive behavior of HUVECs upon CXCL6 stimulation was more significant than upon CXCL12 stimulation. Enhancement of invasive ability of HUVECs by CXCL6 and CXCL12 stimulation were blocked by pre-incubating HUVECs with neutralizing anti-CXCL6 and anti-CXCL12 Ab, respectively ($P < 0.05$, $P < 0.01$; Figure 4C).

Effect of co-culturing with fibroblasts and DLD-1 cells on HUVEC invasiveness

To investigate the interaction between colon cancer and stromal cell-derived CXCL12 in the tumor microenvironment, we next examined the role of cell-derived CXCL12 on HUVECs' invasiveness using the Matrigel double culturing chamber invasion assay. The invasive capability of HUVECs were enhanced by co-cultivation with fibroblasts ($P < 0.01$; Figure 4E) and DLD-1 ($P < 0.01$; Figure 4F); meanwhile, the enhancement of HUVEC invasive behavior was inhibited by neutralizing anti-CXCL12 Ab ($P < 0.01$), and the addition of recombinant CXCL6 significantly enhanced HUVECs invasiveness in co-cultivation with fibroblasts system as well ($P < 0.01$; Figure 4E). At the same time, co-cultivation with CaCo-2 cells did not significantly increase the invasion of HUVECs.

CXCL6 and CXCL12 enhancement of tube formation

To further determine the role of CXCL12 and CXCL6 in the living cell microenvironment, we focused on the interaction between tumor cells and stromal cells by characterizing angiogenic activity in co-cultured fibroblasts and vascular ECs, and the effect of CXCL6 and CXC12 in this system. Initially, we measured the influence of CXCL6 and CXCL12 on tube formation by HUVECs. HUVEC tube formation was significantly enhanced in a dose-dependent manner following treatment CXCL6 ($P < 0.01$; Figure 5A) and CXCl12 ($P < 0.01$; Figure 5B). The enhanced angiogenesis of HUVECs was inhibited by the addition of neutralizing anti-CXCL6 and anti-CXCL12 Ab ($P < 0.01$).

Effect of colon cancer cells with or without CXCL12 on tube formation by HUVECs

In order to explore the different secreted CXCL2 of colon cancer cells influence on tube formation by HUVECs, we cultured three cell lines using double chamber methods to determine the interaction among them. The tubular formation was significantly enhanced by co-culture with DLD-1 cells compared with control (HUVECs and fibroblasts only) or co-culture with HT-29 and CaCo-2 cells, respectively ($P < 0.01$; Figure 5C). Moreover, the CXCL12 and CXCL6 could significantly promote the tubular formation in co-culture with HT-29 and CaCo-2 cells system ($P < 0.01$). In contrast, the enhanced tubular formation by HUVECs was significantly inhibited by addition of anti

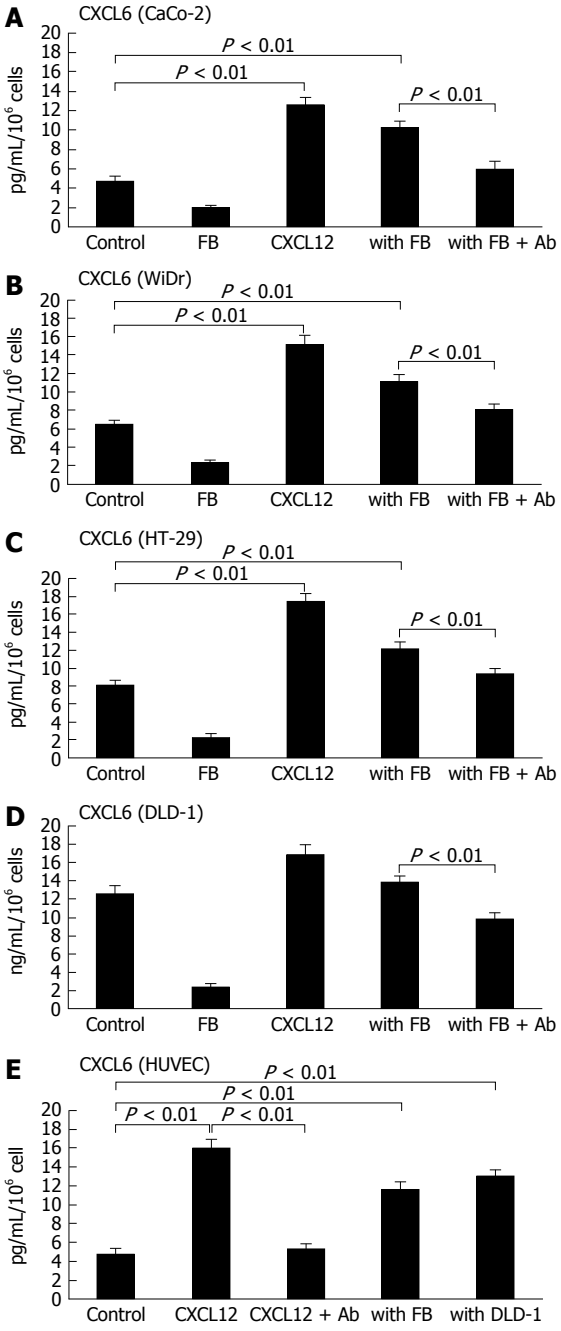


Figure 2 Enhancement of secreted granulocyte chemotactic protein-2 levels in colon cancer cell lines and stromal cells by recombinant stromal cell-derived factor-1 and co-culture with fibroblasts. The alteration of CXCL6 secretion from colon cancer cell lines [CaCo-2 (A), WiDr (B), HT-29 (C) and DLD-1 (D)] by recombinant CXCL12 stimulation or co-culture with fibroblasts (FB) were determined by enzyme-linked immunosorbent assay in cell culture medium. Meanwhile, colon cancer cells were treated with anti-CXCL12 antibody (Ab) for 2 h, and the concentration of CXCL6 was measured by ELISA in supernatants from colon cancer cells. Effect on secretion of CXCL6 from HUVECs stimulated by recombinant CXCL12 in co-culture system with fibroblasts and the colon cancer cells DLD-1 are shown (E). The experimental detail is described in the "Materials and Methods" section. Control: colon cancer cells only; FB: fibroblasts only; CXCL12: treated with recombinant CXCL12; with FB: colon cancer cells co-cultured with fibroblasts; with FB + Ab: colon cancer cells co-cultured with fibroblasts and pre-treated with anti-CXCL12 Ab. The values are expressed as mean ± SD. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

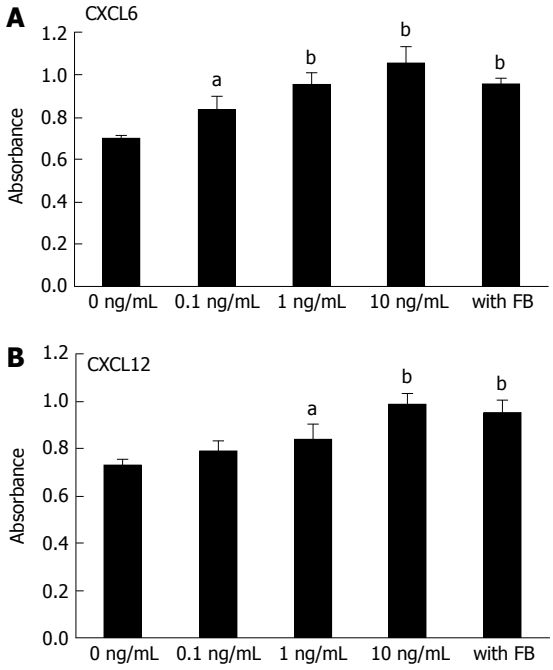
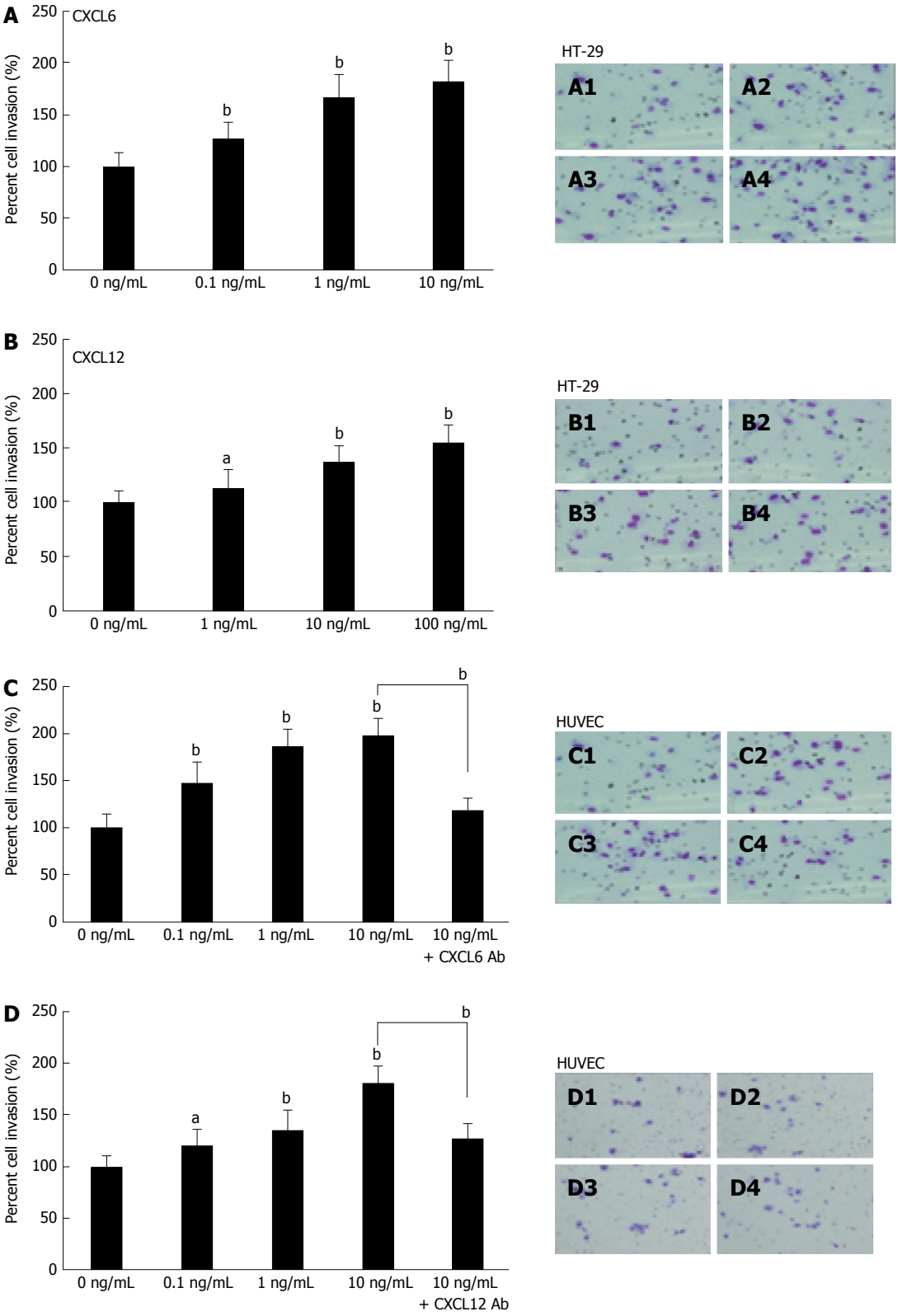


Figure 3 Effect of stromal cell-derived factor-1, granulocyte chemotactic protein-2 and conditioned medium from fibroblasts on human umbilical vein endothelial cell proliferation. HUVECs were cultured in medium containing different concentrations of CXCL6 (A), CXCL12 (B) and conditioned medium from fibroblasts. After 72 h of incubation, HUVEC proliferation was assessed using premixed WST-1 cell proliferation assay (column mean absorbance reading; Bars = SD). Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{a}P < 0.05$, $^{b}P < 0.01$. CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

CXCL12 Ab in co-culture with DLD-1 cells ($P < 0.01$).

Activation of the PI3K/Akt/mTOR signaling pathway after CXCL12 stimulation in colon cancer and stromal cells

We used the colon cancer cell HT-29 and stromal cell HUVECs to examine activation of the PI3K/Akt/mTOR signaling pathway, a downstream target of CXCL12. The stimulation by 10 ng/mL of CXCL12 could increase Akt (Figure 6A), PI3K (Figure 6B) and mTOR (Figure 6C) phosphorylation in a time-dependent manner in HT-29 cells and HUVECs. To determine the role of mTOR, we investigated the effect of CXL12 and PI3K/Akt inhibitor on the activation of mTOR in colon cancer cells and HUVECs; we looked at the effects of IGF-1 and/or PI3/Akt kinase inhibitors on the activation of mTOR in these cells. HT-29 and HUVECs were pre-treated for 60 min with PI3K/Akt inhibitors and then stimulated overnight with CXCL12 (100 ng/mL). The extracted proteins were separated by SDS-PAGE, transferred to membranes, and the membranes probed with Ab directed against phospho-mTOR and total mTOR. We found that by CXCL12-mediated increase phospho-mTOR was inhibited by 50 μmol/L PI3K inhibitor (LY294002) and 50 μmol/L Akt kinase inhibitor. These data indicate that CXCC12 regulates the PI3K/Akt/mTOR signaling pathway activity and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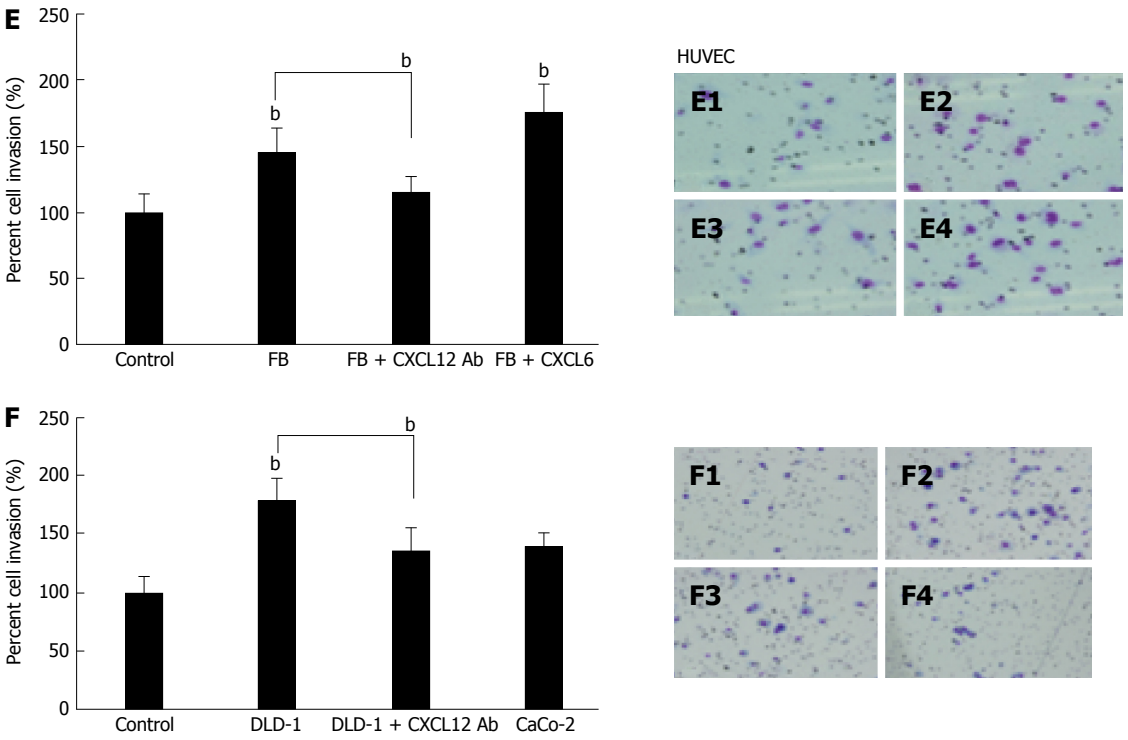

Figure 4 Effect of granulocyte chemotactic protein-2, stromal cell-derived factor-1 and co-culture with fibroblasts or DLD-1 on colon cancer cell and human umbilical vein endothelial cell invasiveness. The influence of different concentrations of CXCL6 (A), CXCL12 (B) or co-culture with fibroblasts on colon cancer cell invasiveness was measured by the BD Bio-Coat Matrigel invasion assay system (BD Biosciences). HT-29 (A and B) cells and HUVECs (C and D) were pre-treated with different concentrations of CXCL6 and CXCL12, and co-culture with fibroblasts (E) or DLD-1 (F), or pre-treated with or without anti CXCL6 or CXCL12 antibody, and following a 24-h incubation. The invading cells were fixed and stained with Diff-Quick stain. Invading cells were counted in five random microscopic fields (× 200). The experiment detail is described in the "Material and Methods" section. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{a}P < 0.05$, $^{b}P < 0.01$. A1: HT-29 cells only; A2: 0.1 ng/mL of CXCL6; A3: 1 ng/mL of CXCL6; A4: 10 ng/mL of CXCL6. B1: HT-29 cells only; B2: 0.1 ng/mL of CXCL12; B3: 1 ng/mL of CXCL12; B4: 10 ng/mL of CXCL12; C1: HUVECs only; C2: 1 ng/mL of CXCL6; C3: 10 ng/mL of CXCL6; C4: 10 ng/mL of CXCL6 treated with 10 μg/mL CXCL6 Ab. D1: HUVECs only; D2: 1 ng/mL of CXCL12; D3: 10 ng/mL of CXCL12; D4: 10 ng/mL of CXCL12 treated with 10 μg/mL CXCL12 Ab. E1: HUVECs only; E2: HUVECs co-culture with fibroblasts; E3: Co-culture with fibroblasts + 10 μg/mL CXCL12 Ab; E4: Co-culture with fibroblasts + 10 ng/mL CXCL6. F1: HUVECs only; F2: HUVECs co-culture with DLD-1 cells; F3: Co-culture with DLD-1 cells + 10 μg/mL CXCL12 Ab; F4: Co-culture with CaCo-2 cells. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

suggest that the PI3K/Akt/mTOR signaling pathway could participate in the regulation of metastatic behavior by colon cancer cells (Figure 6D).

DISCUSSION

Many tumors produce chemokines, which may explain the presence of the tumor-associated microenvironment. However, the role of these chemokines in tumor biology is still unclear. Chemokines form a complex family of small, secreted proteins that play important roles in innate and adaptive immunity, homeostatic processes, angiogenesis and tumorigenesis[4]. Recent exploration of the tumor microenvironment has become the crux of research aimed at explaining tumor behaviors, especially those involving metastasis of solid tumors as in colon, stomach, liver, lung and breast cancers.

The tumor microenvironment consists of tumor, stromal, immune and inflammatory cells, all of which produce cytokines, growth factors and adhesion molecules[26,27], and the abnormal expression of cytokines has been shown to have great effect on tumor behaviors, such as tumor progression and metastasis[28,29]. The CXC chemokine family of cytokines, which are founded in the microenvironment, represent a significant difference between tumors and normal tissues[30]. The tumor microenvironment contains secreted chemokines representing distinctive profiles, the components of each having specific target cells. The chemokine CXCL12, through its receptor CXCR4, positively regulates angiogenesis by promoting EC migration and tube formation. However, the relevant downstream signaling pathways in EC have not been defined.

Our previous studies elucidated that IL-1α is one of the most important inflammatory cytokines involved in the metastatic process of colon cancer. IL-1α contributed to the regulation of tumor growth, progression, and liver metastasis in primary gastric carcinoma and pancreatic cancer. Pancreatic cancer cell-derived IL-1α increases fibroblast-derived hepatocyte growth factor (HGF) secretion in a paracrine manner, and that enhanced HGF expression promotes invasion, proliferation and angiogenesis of cancer cells. In the living microenvironment of the tumor, the chemokines act as couriers or guides for promoting tumor development and the metastasis process[31-33].

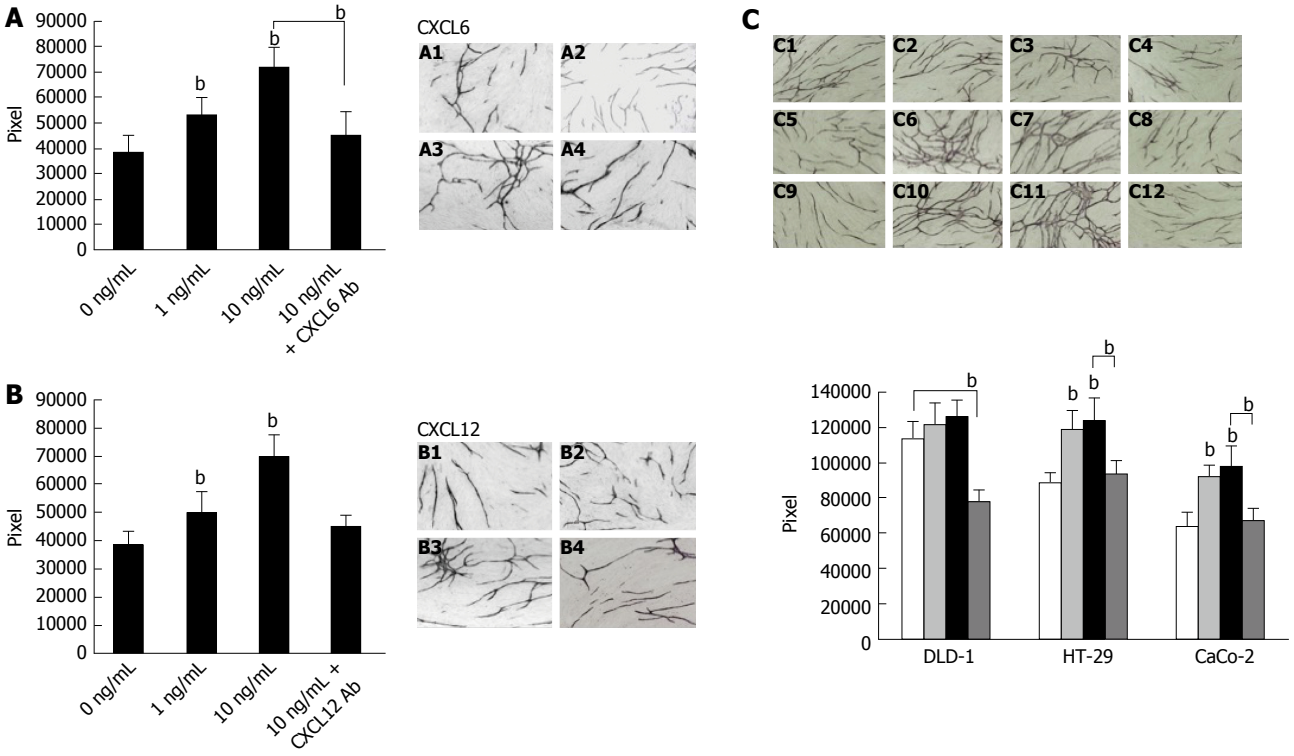

Figure 5 Effect of granulocyte chemotactic protein-2, stromal cell-derived factor-1 and co-culture with colon cancer cells on angiogenesis. The treatment of CXCL6 (A) and CXCL12 (B) influence HUVEC tube formation. After incubation of the HUVEC/fibroblast co-culture system in the presence or absence of CXCL6 or anti-CXCL12 Ab, then co-culture for 7 d, the HUVEC/fibroblast co-culture system was stained with anti-CD31 antibody. Tube formation area was measured quantitatively using an image analyzer. A1: Control; A2: 1 ng/mL CXCL6; A3: 10 ng/mL CXCL6; A4: 10 ng/mL CXCL6 + 10 μg/mL of CXCL6 Ab. B1: Control; B2: 1 ng/mL CXCL12; B3: 10 ng/mL CXCL12; B4: 10 ng/mL CXCL12 + 10 μg/mL of CXCL12 Ab. Effect of colon cancer cells (DLD-1, HT-29 or CaCo-2) on HUVEC tube formation is shown (C). Angiogenesis assay by HUVEC/fibroblast co-culture with DLD-1, HT-29 or CaCo-2 cells was conducted using the double-chamber method. Detection of tube formation by HUVECs was described in the "Material and Methods" section. C1: Co-culture with DLD-1; C2: Co-culture with DLD-1 + 10 ng/mL of CXCL6; C3: Co-culture with DLD-1 + 10 ng/mL of CXCL12; C4: Co-culture with DLD-1 + 10 μg/mL of CXCL12 Ab; C5: Co-culture with HT-29 cells; C6: Co-culture with HT-29 cells pre-treated with 10 ng/mL CXCL6; C7: Co-culture with HT-29 cells pre-treated with 10 ng/mL CXCL12; C8: Co-culture with HT-29 + 10 μg/mL of CXCL12 Ab; C9: Co-culture with CaCo-2 cells; C10: Co-culture with CaCo-2 cells pre-treated with 10 ng/mL CXCL6; C11: Co-culture with CaCo-2 cells pre-treated with 10 ng/mL CXCL12; C12: Co-culture with CaCo-2 cells pre-treated with 10 μg/mL anti-CXCL12 antibody. Columns, mean pixels of HUVEC tube formation area; Bars = SD. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{b}P < 0.01$ *vs* control. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

As a structural component of tumor tissue, fibroblasts have been shown to be deeply involved in tumor proliferation and the mitogenic processes. Fibroblasts produce certain cytokines that influence neighboring cells, including malignant cells[4]. The precise role of chemokines in neovascularization during inflammation or tumor growth is not yet fully understood. We investigated here whether cancer cell stromal cell-derived CXCL12 influences colon cancer CXCL6 secretion, thereby co-regulating the metastatic potential of colon cancer. Our results revealed that CXCL12 was expressed in DLD-1 and fibroblasts, while CXCL6 and CXCR4 were expressed in all cell lines.

The most salient observations of our study were that the secreted CXCL6 levels by colon cancer cells and HUVECs were significantly promoted by cancer cell (DLD-1)- and stromal cell (fibroblast)-derived CXCL12 in the co-culturing system, and that the enhanced CXCL6 production could be significantly inhibited by CXCL12 Ab. Similar results were reported for other effects through the up-regulation of MMP-9, providing a possible mechanism mediating the effect of CXCL6 on metastasis[34]. In our study, CXCL6 and CXCL12 not only co-operatively enhanced proliferation and invasion of HUVECs, but also promoted the invasion of colon cancer cells. Similarly, CXCL6 has been reported to be up-regulated in colon cancer, and plays key roles in the induction and maintenance of gut inflammation, enhancing the development and growth of colitis-associated colorectal cancer[35].

To further investigate the inaction between CXC chemokines and cancer cell living microenvironment, we focused on the interaction between tumor cells and stromal cells by characterizing angiogenic activity in co-cultured fibroblasts and vascular ECs, and the effect of CXCL6 and CXC12 in this system. HUVEC tube formation was significantly enhanced by CXCL6. We aimed to explore the influence of different secreted CXCL2 from colon cancer cells on tube formation by HUVECs. The tubular formation was significantly enhanced by co-culture with DLD-1 cells, as compared with colon cancer cells, and this is related to the produced CXCL12. In contrast, the enhanced tubular formation by HUVECs was significantly inhibited by

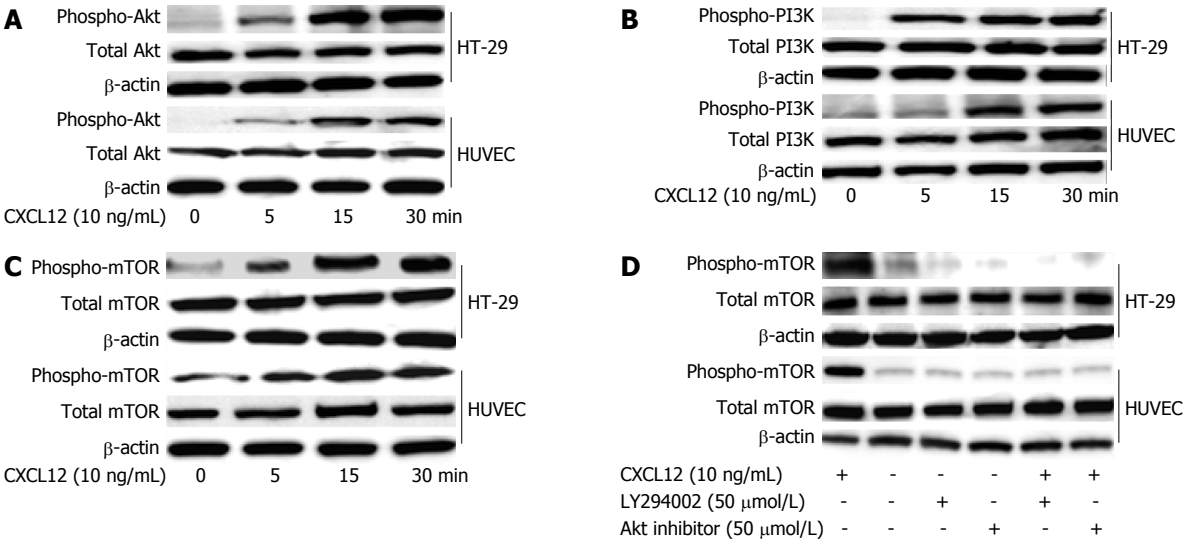

Figure 6 Stromal cell-derived factor-1-induced phosphorylation of PI3K/Akt/mTOR signaling in colon cancer cell lines and stromal cells. HT-29 cells and HUVECs were treated with 10 ng/mL of CXCL12 cultured for 5, 10 and 30 min. The cells were collected and lysed by lysis buffer. Aliquots of 30 μg of lysed protein were subjected to immunoblotting with a phospho-Akt (A), phospho-PI3K (B) and phosphor-mTOR (C) Abs. Detection of total Akt, PI3K or mTOR levels aided in loading control. HT-29 cells or HUVECs, after being pre-treated with 50 μmol/L Akt inhibitor and 50 μmol/L LY294002 for 1 h, were incubated with 10 ng/mL CXCL12 for 1 h. Results of immunoblotting using the mTOR Ab is shown (D). Detection of total mTOR levels served as loading control. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

addition of anti-CXCL12 Ab in co-culture with DLD-1 cells (Figure 5). CXCL12 should be the initial factor secreted by fibroblasts, and the target colon cancer cells enhanced the secretion of CXCL6 after CXCL12 combined with its receptor CXCR4. The proliferation and invasion of colon cancer cells and HUVECs have been activated and enhanced after a series of complicated biochemical reactions.

Breakthroughs of insights into the tumor microenvironment have made great contributions towards clinical treatment. All kinds of anti-carcinoma chemotherapeutics have been based upon this mechanism, and there is no exception among the newly targeted cancer therapies or the gene therapies; proof of effects on critical pathways in proliferation or differentiation are sought. Chemokines are chemo-attractant cytokines that regulate genetic activity of leukocytes and other cell types, including tumor and stromal cells[36]. It is already known that mTOR is an atypical intracellular serine/threonine protein kinase regulated by PI3K. The activated mTOR pathway has been identified in several human malignancies, thus being an attractive target for anticancer therapy[37].

Our results showed that CXCL12-enhanced secretion level of CXCL6 co-regulation of invasion, proliferation and angiogenesis were dependent on PI3K-Akt-mTOR signaling activation. However, both of these factors up-regulation of PI3K-Akt-mTOR survival signaling were decreased by selective inhibitors of PI3K and Akt. All these results suggest that both the CXCL12 factor and the enhancement of CXCL6 expression serve to co-operatively promote metastatic potential in colon cancer cells. CXCL12-induced activation of this signaling pathway could be inhibited by PI3K/Akt inhibitor, consistent with the inhibition of metastatic capabilities of colon cancer cells. This cascade may be a key pathway for colon cancer cells to metastasize.

Crosstalk between CXCR4, CXCL12 and PI3K/mTOR has been previously described in peritoneal disseminated gastric cancer and pancreatic cancer. The solid tumors indicate an interconnection between CXCL12 and mTOR signaling. Interfering with mTOR signaling has abolished chemotaxis towards CXCL12[38]. The mTOR will enhance cell growth and proliferation *via* promotion of the ribosome S6 protein kinase (p70S6K) and inhibition of the eIF4E-binding proteins (4E-BP1), and can even enhance the secretion of vascular endothelial growth factor and angiogenesis by promoting expression of the transcription factor hypoxia-inducible factor 1 and its downstream target genes. Under a series of exterior and interior factors, cancer cell proliferation and invasion can be induced and cell apoptosis can be avoided by initiating the PI3K/Akt/mTOR pathway[39].

In conclusion, this is the first report on the concomitant involvement of CXC12 and CXCL6 both transducing the mTOR pathway, affecting progression and spreading of human colon cancer cells, ultimately suggesting that targeting of CXCR4 and mTOR may improve therapeutic efficacy and prevent mTOR-targeting agents' resistance. Our work should encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

COMMENTS

Background

Colon cancer is the fourth most frequently diagnosed cancer worldwide. The poor prognosis of colon cancer is attributable to its tendency of metastases.

However, the precise mechanisms of metastasis are still unknown. The target of this study was to investigate the underlying mechanism by which CXCL12 and CXCL6 influences the metastatic potential of colon cancer and the internal relation of colon cancer and stromal cell, as well as to investigate the interaction with CXCL12/CXCL6/PI3K/Akt/mTOR signaling in the metastatic process.

Research frontiers

The functions of CXC chemokines in the tumor microenvironment depend considerably on the chemokine type and tumor and stromal cell characteristics. Both the angiogenesis-promoting effect of CXCL6 and chemotactic effect of CXCL12 play important roles in tumorigenesis and metastasis. However, the molecular mechanisms of the activity signaling pathway by which CXCL12 and CXCL6 co-operatively regulate metastasis of colon cancer remain to be clarified.

Innovations and breakthroughs

This research provides the first demonstrations of fibroblast-derived CXCL12 enhancing CXCL6 secretion of colon cancer cells. CXCL6 and CXCL12 not only co-operative enhanced proliferation and invasion of HUVECs, but also promoted the invasion of colon cancer cells *via* the PI3K/Akt/mTOR signaling pathway. Blocking this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer. This work might encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

Applications

The concomitant involvement of CXC12 and CXCL6 transduces the mTOR pathway, affecting progression and spread of human colon cancer cells. The authors suggest that targeting CXCR4 and mTOR may improve therapeutic efficacy and prevent mTOR- targeting agents' resistance. The authors' work should encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

Terminology

The CXC chemokine family of cytokines, found in the microenvironment, represent the significantly distinctive profiles of tumors and normal tissues. The tumor microenvironment contains secreted chemokines representing distinctive profiles, the components of each having specific target cells. The chemokine CXCL12, through its receptor CXCR4, positively regulates angiogenesis by promoting endothelial cell (EC) migration and tube formation.

Peer-review

The results of this study for the relationship between CXCL6 and CXCL12 in colorectal cancer and ECs seem to be of interest to many readers, and the experiment is well planned. But, before publication, several issues have to be considered.

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