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HGF derived from Stromal Cells Enhances Angiogenesis in Human Colon Cancer Cell Lines

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ABSTRACT

Background: Previously we reported the critical role of Interleukin (IL)-1α in liver metastasis from colon cancer. However, its role in angiogenesis and metastasis, particularly as it relates to the interaction between tumor and stromal cells, was not clearly elucidated. Also, it has been suggested that HGF plays an important role as a paracrine factor in the invasion and metastasis in many cancers. The aim of this study was to investigate the cooperative role of HGF and IL-1α in metastatic processes promoted by interactions between colon cancer cells and stromal cells.

Materials and methods: Expression of IL-1α and HGF mRNA and protein was determined by RT-PCR and ELISA. The effect of HGF on metastatic potential of colon cancer cell lines was evaluated by proliferation, invasion, and angiogenesis assays using an *in vitro* system consisting of co-cultured tumor cells and stromal cells.

Results: IL-1α expression was closely correlated with metastatic potential, and cancer cell-derived IL-1α significantly promoted HGF expression by fibroblasts ($P<0.01$). HGF not only enhanced the invasiveness and proliferation of colon cancer cells, but also enhanced migration and proliferation of human umbilical vein endothelial cells (HUVECs). HGF signifi-

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cantly enhanced HUVEC tube formation ($P<0.01$). Furthermore, the high liver-metastatic colon cancer cell line (WiDr), which secretes IL-1α, significantly enhanced HUVEC tube formation compared to the low liver-metastatic cell line (Caco-2), which does not produce IL-1α ($P<0.01$).

Conclusions: Autocrine IL-1α and paracrine HGF co-enhance the metastatic potential of colon cancer cells via both IL-1α and HGF signaling pathways.

Key words: IL-1α, HGF, colon cancer, tumor-stromal interaction, angiogenesis

INTRODUCTION

Colon cancer is one of the most common cancers in the world with a high propensity to metastasize. The most common sites of metastasis from colon cancer are the regional lymph nodes, liver, lung, and peritoneum, in which liver being the most frequent site of metastases [1]. Because the majority of deaths with colon cancer are due to metastatic disease, inhibitions of growth and metastasis of colon cancer are expected to become effective treatment. Among them, anti-angiogenesis strategy has been growing and standard for treatment of patients with colon cancer, and the inhibition of tumor angiogenesis is likely to decrease frequency of metastasis [2].

Hepatocyte growth factor (HGF) is a mitogen for epithelial cells that regulates cell proliferation, migration, survival, tumor angiogenesis, and invasiveness [3]. It is known to be a pleiotropic cytokine that acts on epithelial cells in several organs [4]. Other studies have suggested that HGF plays an important role as a paracrine factor in the invasion and metastasis of oral squamous cell carcinoma, and that an elevated HGF serum level can be a predictive marker for metastasis in these patients [5]. HGF also has potent motogenic effects on various tumor cell types, and potently stimulates tumor invasion and metastasis [6-8]. Furthermore, HGF has been identified as a fibroblast-derived epithelial morphogen that induces branching tubular morphogenesis [9]. The c-Met/HGF receptor, which is a receptor tyrosine kinase, is expressed in a wide variety of tumor cells, including colon cancer cells [10]. Other investigators have suggested that in addition to autocrine HGF expression by the tumor cell itself, stromal (fibroblast) -derived HGF acting in a paracrine manner also plays an important role in tumor invasiveness and metastasis [11]. Moreover, HGF expression by fibroblasts has been shown to be regulated by malignant epithelial cells. For example, cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and basic fibroblast growth factor (bFGF) secreted by tumor cells may up-regulate HGF expression by fibroblasts, in turn leading to the acquisition of invasive growth potential by the tumor cells [12].

Interleukin-1 (IL-1α), an important pro-inflammatory cytokine, promotes inflammatory processes and modulates various immune, degradative, and growth-promoting pathways. This cytokine has been reported to be produced by cancer cell lines derived from carcinomas of the pancreas, lung, ovary, colon, and stomach [13-14]. Furthermore, we elucidated a role for IL-1α in pancreatic and colon cancer angiogenesis via interactions between tumors and their microenvironment [15-16].

In the present study, we developed a unique culture system to evaluate tumor-stromal cell interactions mediated by HGF. We first investigated whether human colon cancer cell-derived IL-1α promotes HGF secretion by stromal cell fibroblasts and, if so, whether and how HGF influences cell invasion, proliferation, and angiogenesis. Herein we report that human colon cancer cell-derived IL-1α enhances HGF levels secreted by fibroblasts to promote colon cancer invasion, proliferation, and angiogenesis by human umbilical vein endothelial cells (HUVECs) via the HGF/c-Met pathway.

MATERIALS AND METHODS

Cell lines and culture conditions

WiDr, HT-29, Caco-2 and COLO 320 cells were obtained from the American Type Culture Collection (Rockville, MD). WiDr and Caco-2 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). COLO 320 cells were maintained in RPMI-1640 supplemented with 10% FCS. HT-29 cells were maintained in McCoy's 5A supplemented with 10% FCS. HUVECs were obtained from Kurabo Co. (Osaka, Japan). HUVECs were cultured in HuMedia-EB2 medium supplemented with 2% FBS, 5 ng/mL bFGF, 10 μg/mL heparin, 10ng/mL epidermal growth factor, and 1 μg/mL hydrocortisone according to the supplier's instructions (Kurabo Co.). Fibroblasts were obtained from Lonza Walkersville Inc. (Walkersville, MD) and maintained in FBM-2 medium supplemented with 2% FBS, 1 ng/mL bFGF, and 1 μg/mL insulin according to the supplier's instructions. All cell lines were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air.

Reagents and antibodies

Recombinant human HGF and anti-HGF antibodies were purchased from R&D Systems (Minneapolis, MN), recombinant human IL-1α was provided by Diaclone (Beasancon, France), and recombinant human IL-1 receptor antagonist (IL-1ra) was provided by Pepro Tech EC Ltd (London, UK).

RT-PCR analysis

Total RNA was extracted from the four colon cancer cell lines using the Isogen Kit (Nip-

pon Gene, Tokyo, Japan), and quantities were determined spectrophotometrically. Total RNA aliquots (5 μg) were pretreated with Random Hexamers and dNTP Mix and were incubated at 65℃ for 5 minutes, chilled on ice, and then reverse-transcribed into cDNA using the SuperScript Ⅲ RT System (Invitrogen, San Diego, CA). One-μL cDNA aliquots were used as PCR templates. Forward and reverse primer pairs were designed using Primer 3 software. Primer sequences and PCR conditions are described previously [17]. Amplification reactions were performed using a DNA Thermal Cycler (model TP300; Takara PCR Thermal Cycler MP, Takara Bio Inc., Shiga, Japan). Amplified DNA fragments were observed by electrophoresis in 1.5% agarose gels containing ethidium bromide.

Enzyme-linked immunosorbent assay

All cells lines were 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 hours. The medium was collected and microcentrifuged at 1,500 rpm for 5 min to remove particles, and the supernatants were frozen at −80℃ until performance of enzyme-linked immunosorbent assay (ELISA). Concentrations of IL-1α and HGF were measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions. We also evaluated the influence of IL-1α on fibroblast HGF production. Fibroblast cultures were stimulated by 10 ng/mL of IL-1α and were incubated for an additional 48 hours; HGF concentration was then measured by ELISA. To further investigate the synergistic effect of the tumor-stromal interaction, we examined the effect of colon cancer cell-derived IL-1α on fibroblast HGF 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 IL-1α or IL-1ra, and then co-cultured with 5×10^4 WiDr or Caco-2 cells into inserts with 0.45-μm pores (Kurabo Co.). Co-culture systems were incubated for an additional 48 hours, and HGF concentrations were subsequently measured as described above.

In vitro proliferation of colon cancer cells stimulated with HGF

Each colon cancer cell line was seeded at a density of 2×10^3 cells/100 μL in 96-well plates and allowed to adhere overnight, and the medium was then exchanged with medium alone (control) or media containing different concentrations of HGF. After 72-hour incubation, colon cancer cell proliferation was determined using the WST-1 Cell Proliferation Assay System (Takara Bio Inc.). Absorbance was determined using a microplate reader (Molecular Devices, Sunnyvale, CA) at a test wavelength of 450 nm and reference wavelength of 690 nm.

Proliferation of HUVECs pretreated with HGF or conditioned media

To make conditioned media, 1×10^5 fibroblasts were plated into 24-well plates with FBM-2 medium with 2% FBS, while 5×10^4 WiDr cells were seeded into trans-well chambers containing polycarbonate membranes with 0. 45-μm pores with 2% FBS in RPMI or DMEM medium, and the trans-well chambers were then plated into 24-well plates. The co-culture system was incubated for 48 hours. Culture medium was collected and microcentrifuged at 1, 500 rpm for 5 minutes to remove particles. Supernatants were frozen at −80℃ until use in proliferation assays. HUVECs were seeded at a density of 2×10^3 cells/100 μL into 96-well plates with HUVEC basal medium and allowed to adhere overnight. Cells were then cultured with HUVEC basal medium only (control), 10 ng/mL of HGF, or conditioned media (supernatants from fibroblasts or from the WiDr/fibroblast co-culture system mixed with HUVEC basal medium [1: 1]) under the condition of with or without (w/o) 10μ/ml of HGF Ab. Culture media was exchanged every 24 hours, and after 72 hours of incubation, HUVEC proliferation was measured by WST-1 assay.

In vitro invasiveness of colon cancer cells following pretreatment with HGF or co-culture with fibroblasts

The *in vitro* invasion assay was performed using BioCoat Matrigel Invasion Chambers (Becton Dickinson [BD], Bedford, MA) according to the manufacturer's instruction. Each colon cancer cell line (WiDr or Caco-2) was seeded at a density of 1×10^5 cells into Matrigel pre-coated trans-wells containing polycarbonate membranes with 8-μm pores. Trans-well chambers were then plated into 24-well plates with basic medium alone (control), or medium pretreated with 10 ng/mL HGF, with/without 10 μg/mL anti-HGF antibody. After a 24-hour incubation, the upper surfaces of the trans-wells were wiped with a cotton swab and invasive cells were fixed and stained using the Diff-Quik kit. The invasive cells were counted in five microscope fields (×100). We also investigated whether fibroblast-derived HGF increased the invasive potential of colon cancer cells. Colon cancer cell invasion assays were performed using a double-chamber method. Fibroblasts were seeded at a density of 1×10^5 cells into 24-well plates with FGM-2 medium; at the same time, trans-well chambers (containing 1×10^5 colon cancer cells/chamber) were plated into 24-well plates and allowed to incubate for 24 hours. Colon cancer cell invasion was then determined as described above.

Migration of HUVECs pretreated with HGF or co-cultured with fibroblasts under treatment with/without anti-HGF antibody

The *in vitro* HUVEC migration assay was performed using BioCoat Matrigel Invasion Chambers as previously described [18]. First, HUVECs were seeded at a density of 1.0×10^5

cells into Matrigel pre-coated trans-wells containing polycarbonate membranes with 8-μm pores, and then trans-wells chambers were placed in 24-well plates with basic medium alone (control), or medium pretreated with 10, or 100 ng/mL HGF with or without 10 μg/mL anti-HGF antibody. After 24-hour incubation, the upper surfaces of the trans-wells were wiped with a cotton swab, and invasive cells were fixed and stained using a Diff-Quik kit. Invasive HUVECs were counted in five microscope fields (×100). To further investigate whether fibroblast-derived HGF increased HUVEC migration capability, a HUVEC migration assay was performed using a double-chamber method. Fibroblasts were seeded at a density of 1×10^5 cells/well into 24-well plates with FGM-2 medium with or without 10 μg/mL anti-HGF antibody and/or 10ng/ml IL-1α, at the same time, trans-well chambers (containing 1.0×10^5 HUVECs/chamber) were plated into 24-well plates and allowed to incubate for 24 hours. The numbers of invasive HUVECs were determined as described above.

In vitro angiogenic activity of HUVECs during co-culture with colon cancer cells

We previously reported that HGF enhances HUVEC tube formation under the co-culture system of HUVEC and fibroblast [17]. Also, we previously investigated the influence of colon cancer cell lines with different metastatic potential on HUVEC tube formation using double-chamber cell culturing methodology, and revealed that WiDr with high liver metastatic potential enhanced HUVEC tube formation higher compared with Caco-2 with low liver metastatic potential. So we next examined whether HGF plays an important role in enhanced angiogenesis by colon cancer [16]. Colon cancer cells (WiDr or Caco-2), HUVECs, and fibroblasts were co-cultured using a double-chamber method in24-well plates. WiDr or Caco-2 cells (1×10^4 cells) were seeded into trans-well chambers consisting of polycarbonate membranes with 0.45-μm pores, and allowed to adhere overnight. Trans-well chambers were then placed in the HUVEC/fibroblast co-culture system with or without 10ng/mL IL-1 ra, or 10 μg/mL anti-HGF antibody, and exchanged on the sixth day. All cells were cultured for a total of 11 days. HUVEC tube formation was measured as described above. This assay allowed us to quantitatively evaluate angiogenesis and to examine tumor-stromal interactions.

Statistical analysis

Data are presented as means ± standard deviations (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 to be statistically significant. Mean values and SDs were calculated for experiments performed at least three times.

RESULTS

Expression of HGF and IL-1α in colon cancer cell lines and fibroblasts

We previously classified colon cancer cell lines into two groups using an animal metastasis assay: high liver-metastatic cell lines (WiDr and HT-29) and low liver-metastatic cell lines (Caco-2 and Colo 320). In the present study, RT-PCR experiments revealed that IL-1α mRNA was expressed in the high liver-metastatic colon cancer cell lines WiDr and HT-29, but was not detected in the low liver-metastatic cell lines Caco-2 and Colo 320. HGF mRNA was only expressed by fibroblast (Figure 1A). Similarly, ELISA experiments revealed that IL-1α protein secretion was detected only in high liver metastatic colon cancer cell lines (WiDr and HT-29) (Figure 1B). Also, HGF secretion was only detected in Fibroblast (Figure 1C).

Effect of recombinant human IL-1α and colon cancer cell-derived IL-1α on fibroblast HGF secretion levels

Consistent with the RT-PCR results, secreted IL-1α protein was detected in WiDr, and Caco-2 culture supernatants, while secreted HGF protein was only present in fibroblast supernatant (Figure 1B, 1C). IL-1α appeared to increase fibroblast production of HGF. Likewise, co-culture with WiDr cells significantly enhanced fibroblast HGF secretion ($^*P<0.01$) (Figure 2A), while co-culture with Caco-2 cells did not have a significant effect. However, IL-1α treatment significantly increased HGF secretion levels in this co-culture system (Figure 2 B). Furthermore, the enhanced HGF production elicited by co-culturing with WiDr cells was significantly inhibited in the presence of IL-1ra ($^*P<0.01$).

Effect of HGF on proliferation of colon cancer cell lines

A proliferation assay was performed to evaluate the effect of HGF on colon cancer cell proliferation. Colon cancer cell proliferation was enhanced by HGF in a concentration-dependent manner. HGF at 10 ng/mL significantly promoted the proliferation of colon cancer cells ($^*P<0.01$) (Figure 3A). Interestingly, the addition of anti-HGF antibody to the culture media significantly inhibited HGF-enhanced proliferation (data not shown).

Effect of HGF and conditioned media on vascular endothelial cell proliferation

We next investigated the effect of HGF on vascular endothelial cell proliferation. HUVEC proliferation was significantly enhanced by the addition of HGF ($^*P<0.01$ compared with control), and this enhancement was significantly blocked by anti-HGF antibody. Culture medium from fibroblast enhanced HUVEC proliferation, and the enhancement was signifi-

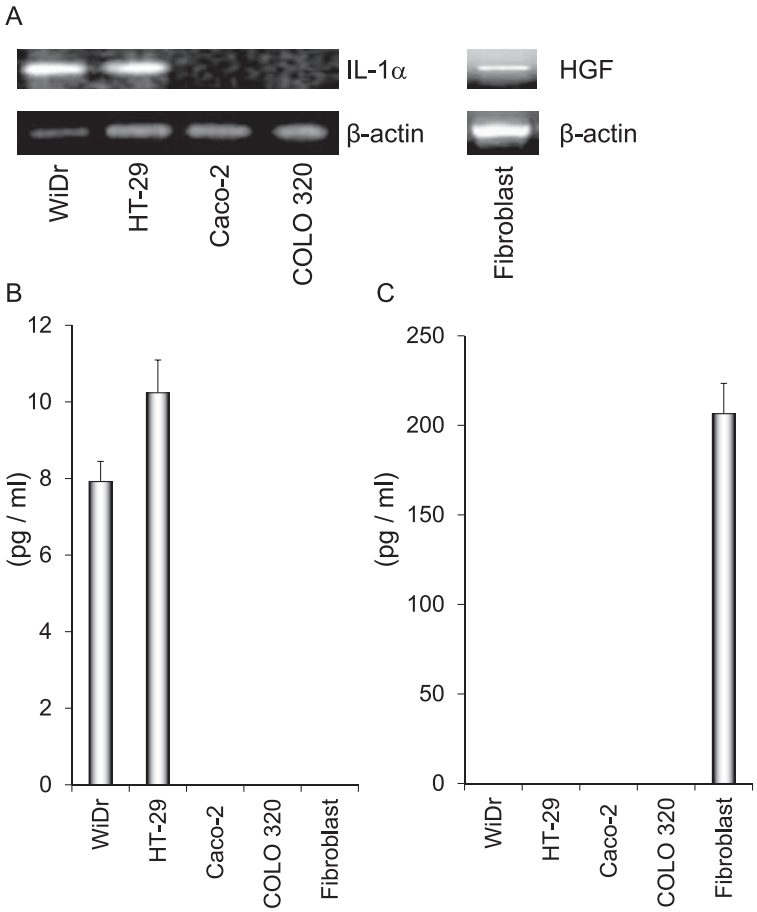

Figure 1 Expression of IL-1α and HGF in colon cancer cell lines and stromal cells. (A) Detection of IL-1α and HGF in colon cancer cells and stromal cells. PCR products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. (B) Secreted IL-1α proteins were detected in supernatants from colon cancer cells and stromal cells. All cells were cultured for 48 hours, and concentrations of both cytokines were measured by ELISA. (C) Secreted HGF proteins were detected in supernatants from colon cancer cells and stromal cells. All cells were cultured for 48 hours, and concentrations of both cytokines were measured by ELISA.

cantly inhibited by the addition of the anti-HGF antibody. Moreover, the conditioned medium from the WiDr and fibroblast co-culture system significantly increased HUVEC proliferation compared with the media from fibroblast alone ($^{*}P<0.01$) (Figure 3B).

Effect of HGF or fibroblast co-culture on colon cancer cell invasiveness

To confirm the interaction between colon cancer cells and stromal cell-derived HGF in

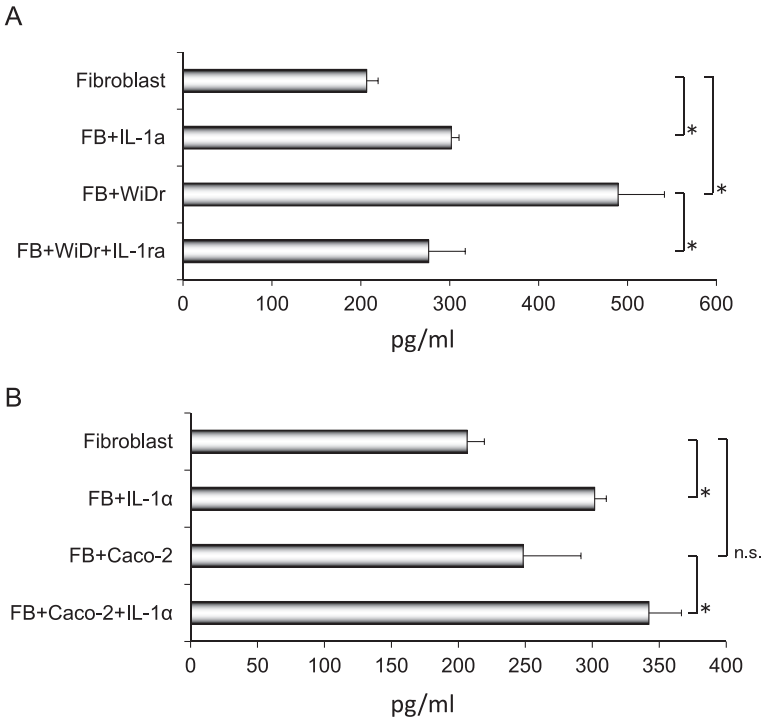

Figure 2　Effect of IL-1α or co-culture with colon cancer cells of different metastatic potential on fibroblast HGF secretion. Fibroblasts were stimulated by recombinant human IL-1α (10 ng/mL) or were co-cultured with colon cancer cells (WiDr [A], Caco-2[B]), and pretreated with IL-1α (10 ng/mL) or IL-1ra (10 μg/mL). Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Bars indicate SD, $^*P<0.01$. Values are expressed as mean ± SD.

the tumor microenvironment, we next examined the effect of HGF on colon cancer cell invasiveness using an invasion assay (Figure 4A; WiDr, Figure 4B; Caco-2). The invasive capability of colon cancer cells was enhanced by HGF, 10 ng/mL significantly promoted cancer cell invasiveness ($^*P<0.01$). Furthermore, co-cultivation with fibroblasts also significantly enhanced cancer cell invasiveness. The addition of anti-HGF antibody significantly reduced these enhancements by either recombinant and fibroblast (Figure 4).

Effect of HGF or fibroblast co-culture on HUVEC migration

The migrating ability of HUVECs was enhanced by HGF in a dose-dependent manner, and HGF 10 ng/mL significantly enhanced HUVEC invasiveness ($^*P<0.01$) (Figure 5A). Co-cultivation with fibroblasts significantly increased HUVEC migration in the fibroblast co-culture system. Moreover, IL-1α increased this enhanced HUVEC migration by fibroblast

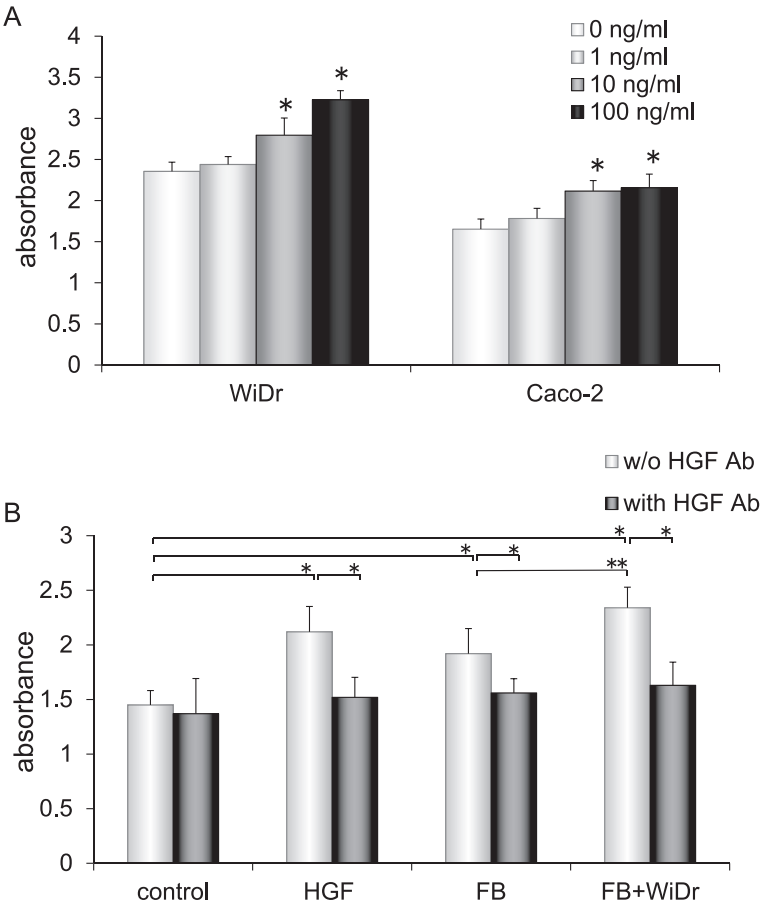

Figure 3 Effect of HGF pretreatment on proliferation of colon cancer cells (A) and HUVECs (B). (A) WiDr and Caco-2 cells were seeded at a density of 2×10^3 cells/100 (L in 96-well plates and allowed to adhere overnight. Media were then exchanged and cells were cultured in medium only (control) or in medium containing different concentrations of HGF. After 72 hours of incubation, cell proliferation was assessed using the premixed WST-1 cell proliferation assay. Absorbance was measured at 450 nm and 690 nm (column mean absorbance reading; bars, SD). Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $*P<0.01$. (B) Proliferation of HUVECs associated with HGF, fibroblast, and WiDr. HUVEC were treated with 10 ng/ml of HGF with or without 10 μg/mL anti-HGF antibody, HUVEC proliferation was also measured by WST-1 assay. Similarly, influence of different conditioned media on HUVEC proliferation was examined. Conditioned medium from the fibroblast co-cultured with or without WiDr significantly increased HUVEC proliferation and the enhancement was inhibited by the addition of 10 μg/mL anti-HGF antibody. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $*P<0.01$, $*P<0.05$.

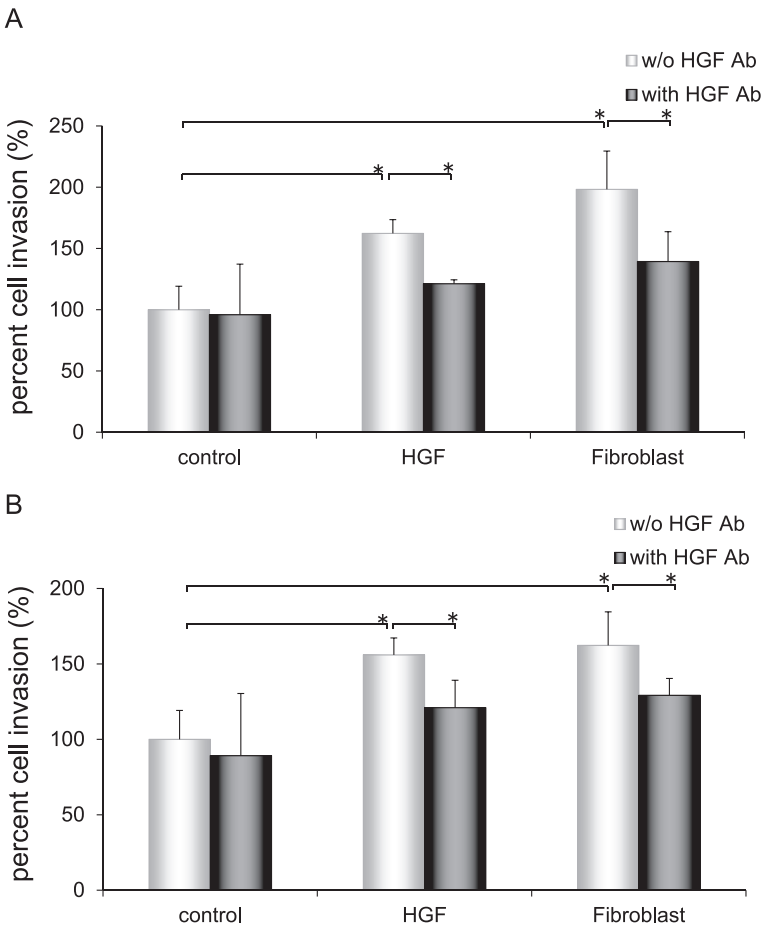

Figure 4　Effect of HGF or fibroblast co-culture on colon cancer cell invasiveness [WiDr (A) and Caco-2(B)]. The influence of 10 ng/ml of HGF or co-culture with fibroblast on colon cancer cell invasiveness was assessed using the BD Bio-Coat Matrigel invasion assay system (BD Biosciences) as described in the "Material and methods" section. Invading cells were fixed and stained with Diff-Quick stain. Invading cells were counted in five random microscopic fields (×200). Also, the effect of 10 μg/mL anti-HGF antibody on enhanced invasive ability of colon cancer cells was examined. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; *$P<0.01$.

much more. These enhanced migration ability were significantly inhibited by anti-HGF antibody (*$P<0.01$), (Figure 5B).

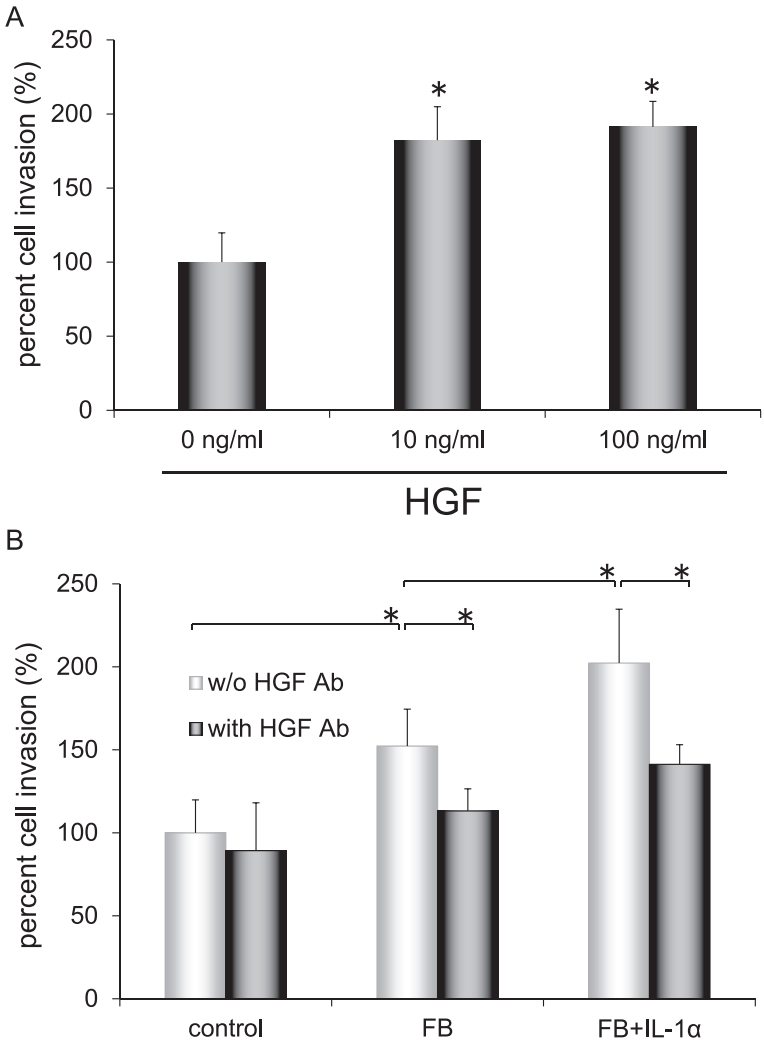

Figure 5 (A) Effect of HGF on HUVEC invasiveness. HUVECs were pre-treated with different concentrations of HGF and following a 24-hour incubation, the invading cells were fixed and stained with Diff-Quick stain. Invading cells were counted in five random microscopic fields (×200). Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Columns, relative invading cell number versus control (0 ng/mL). Bars indicate SD; $*P<0.01$, $**P<0.05$. (B) Effect of fibroblast co-culture on HUVEC invasiveness and role of IL-1α. To assess the influence of fibroblast-derived HGF on HUVEC migration, HUVECs were co-cultured with fibroblasts pretreated with or without 10 μg/mL anti-HGF antibody and/or 10 ng/ml IL-1α. After a 24-hour incubation, invading cells were fixed and stained with Diff-Quick stain. Cells were counted in five random microscopic fields (×200). Multiple comparisons were performed by one-way ANOVA followed by the SNK test. Bars indicate SD; $P<0.01$.

Effect of colon cancer cells with or without IL-1α or anti-HGF antibody on HUVEC tube formation

Tube formation was significantly enhanced by co-culture with WiDr cells compared to co-culture with Caco-2 cells ($*P<0.01$). Moreover, the enhanced tube formation of HUVECs was significantly inhibited by addition of IL-1ra in the WiDr cell co-culture system ($*P<0.01$), and was also inhibited by anti-HGF antibody in the WiDr and Caco-2 cell co-culture system ($*P<0.01$) (Figure 6).

DISCUSSION

Our this study clearly provides novel insight about cooperative interactions between colon cancer cells, endothelial cells, and fibroblasts with respect to the biological effects of cytokines. We have shown that colon cancer cell-derived IL-1α increases fibroblast-derived HGF secretion in a paracrine manner, and that enhanced HGF expression promotes cancer cell and HUVEC proliferation, invasion/migration, and tube formation.

In our previous studies, we classified colon cancer cell lines by metastatic potential into high liver-metastatic cell lines (WiDr and HT-29) and low liver-metastatic cell lines (Caco-2 and Colo 320). We then demonstrated that increased IL-1α expression is a feature of high liver-metastatic colon cancer cell lines, in contrast to low liver-metastatic colon cancer cell lines [16]. We next focused our attention on colon cancer angiogenesis and angiogenic factors. In the present study, we hypothesized that high liver-metastatic colon cancer cell-derived IL-1α might increase mature HGF production by fibroblasts in a co-culture system, thereby activating the HGF/c-Met pathway and enhancing colon cancer metastasis.

HGF, which was originally identified as a potent mitogen for hepatocytes, is a stromal cell-derived cytokine that induces a spectrum of biologic activity, including mitogenesis, motogenesis, morphogenesis, angiogenesis, and inhibition of apoptosis [18-21]. The multiple effects of HGF are mediated through binding to the cognate receptor, c-Met, a receptor tyrosine kinase that is generally known to be expressed on the surfaces of cells of epithelial origin. Various reports have implicated the HGF/c-Met system in cancer progression through tumor-stromal cell interactions [22]. As a structural component of tumor tissue, fibroblasts have been shown to be deeply involved in tumor proliferation and motogenic processes. Fibroblasts produce certain cytokines that influence neighboring cells, including malignant cells [23]. Herein, we investigated whether cancer cell-derived IL-1α influences fibroblast-derived HGF activity, thereby co-regulating the invasive potential of pancreatic tumors. Our initial experiments revealed that IL-1α is only expressed in high metastatic colon cancer cell lines (WiDr and HT-29), while HGF is only expressed in fibroblasts. One of the most salient observations of our study was that fibroblasts secrete higher levels of HGF than colon cancer cells.

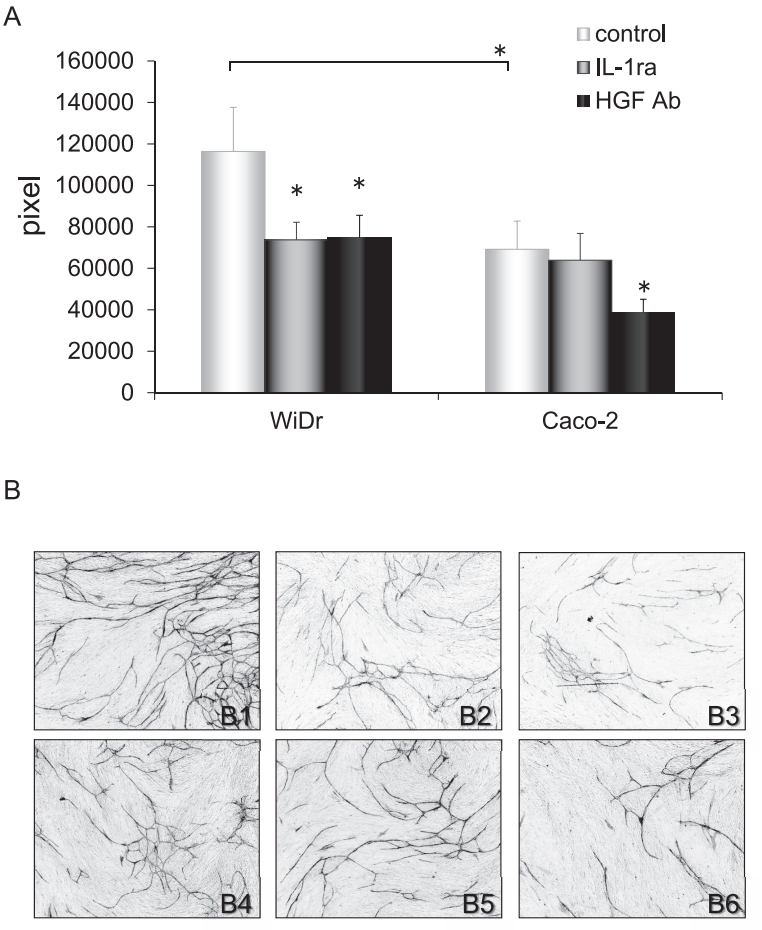

Figure 6 Effect of co-cultured colon cancer cells on angiogenesis and role of IL-1α and HGF. (A) Effect of colon cancer cells (WiDr or Caco-2) on HUVEC tube formation. Angiogenesis assay by cultivation of HUVECs/fibroblasts with WiDr or Caco-2 cells pretreated with or without (w/o) IL-1ra (10 ng/ml) or anti-HGF antibody (10 μg/mL) using the double-chamber method. HUVECs were stained with anti-CD31 antibody. Tube formation area was measured quantitatively using an image analyzer. (×40.) Multiple comparisons were performed by one-way ANOVA followed by the SNK test; *$P<0.01$. (B) Microscopic images of angiogenesis assay. These images are representative pictures from three independent experiments of HUVEC tube formation assay followed by staining with CD31 antibody. Magnification, × 40. B1, co-culture with WiDr; B2, co-culture with WiDr cells pretreated with 10 ng/mL IL-1ra; B3, co-culture with WiDr cells pretreated with 10 μg/mL anti-HGF antibody; B4, co-culture with Caco-2 cells; B5, co-culture with Caco-2 cells pretreated with 10 ng/mL IL-1ra; B6, co-culture with Caco-2 cells pretreated with 10 μg/mL anti-HGF antibody.

Interestingly, the levels of HGF secreted by fibroblasts were significantly enhanced by cancer cell-derived IL-1α in a co-culture system, suggesting that this highly expressed HGF binds to c-Met receptors on the surface of colon cancer cells, further enhancing their invasive capability. This suggests that a paracrine factor secreted by colon cancer cells increases HGF production to enhance the metastatic potential of cancer cells. Recently, the relationship between tumor invasion and stromal-derived HGF has received much attention. Most cancer cells express c-Met, and over-expression of c-Met is often observed in highly malignant cancer cells [24, 25]. HGF induces carcinoma cell invasion *in vitro* [12], and functional coupling between HGF and Met enhances invasion and metastasis in certain tumor cells [26]. Moreover, HGF may also be involved in neovascularization in tumor tissues [3, 5]. In our study, we observed a striking cooperative interaction between colon cancer cell-derived IL-1 α and fibroblast-derived HGF that promoted invasion, proliferation, and angiogenesis. These results indicate that the interaction between colon cancer cells and stromal cells may produce an important cytokine network to regulate these processes. So, as tumor cell-dependent angiogenesis was inhibited by IL-1ra, these data suggest that IL-1ra, alone or in combination with an anti-HGF antibody, may be of great clinical benefit for patients with various cancers that produce IL-1α.

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