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Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour–stroma interaction

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Background: Interleukin-6 (IL-6) has an important role in cancer progression, and high levels of plasma IL-6 are correlated with a poor prognosis in a variety of cancers. It has also been reported that tumour stromal fibroblasts are necessary for steps in cancer progression, such as angiogenesis. There have been few reports of a correlation between fibroblast actions and IL-6 levels. In this study, we examined the correlation between cancer stromal fibroblasts and IL-6 and the utility of IL-6 as a therapeutic target in human colon cancer.

Methods: The expression levels of IL-6 and VEGF of fibroblasts and cancer cell lines were evaluated using real-time PCR and ELISA. The anti-angiogenic effect of inhibiting IL-6 signalling was measured in an angiogenesis model and animal experiment.

Results: We demonstrate that stromal fibroblasts isolated from colon cancer produced significant amounts of IL-6 and that colon cancer cells enhanced IL-6 production by stromal fibroblasts. Moreover, IL-6 enhanced VEGF production by fibroblasts, thereby inducing angiogenesis. *In vivo*, anti-IL6 receptor antibody targeting stromal tissue showed greater anti-tumour activity than did anti-IL6 receptor antibody targeting xenografted cancer cells.

Conclusion: Cancer stromal fibroblasts were an important source of IL-6 in colon cancer. IL-6 produced by activated fibroblasts induced tumour angiogenesis by stimulating adjacent stromal fibroblasts. The relationship between IL-6 and stromal fibroblasts offers new approaches to cancer therapy.

Interleukin-6 is a multifunctional cytokine that has a central role in the regulation of inflammatory and immune responses. IL-6 is produced by a variety of cells, primarily monocytes, macrophages and several tumour cells, and it binds to the IL-6 receptor. The IL-6 receptor has two forms: a membrane-bound form and a soluble form. As the expression of the membrane-bound IL-6 receptor is restricted to a few tissues such as the liver and some immune system cells, and the soluble form of the IL-6 receptor is present in human sera at high concentrations, it is generally believed that IL-6 trans-signalling is mainly dependent on the soluble form of the IL-6 receptor (Garbers *et al*, 2011; Rose-John, 2012). The IL-6/IL-6 receptor complex activates glycoprotein 130 that in turn upregulates Janus-activated kinase/signal transducers and activators of transcription (STAT; Kishimoto *et al*, 1995; Nilsson *et al*, 2005; Ara and Declerck, 2010).

Recently, it was reported that IL-6 has important roles in cancer progression, including proliferation, migration, and angiogenesis in several cancers, including colon cancer (Santer *et al*, 2010;

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Grivennikov *et al*, 2009; Liu *et al*, 2010), and it worsens cancer prognosis (Okugawa *et al*, 2010; Yeh *et al*, 2010). Among various phases of progressions, angiogenesis is one of the most important steps, as the aggressive growth of solid tumours is dependent on angiogenesis. Recently, IL-6 was characterised as an angiogenic cytokine (Nilsson *et al*, 2005). Several IL-6-related angiogenic pathways have been reported, including IL-6/HIF signalling (Anglesio *et al*, 2011) in ovarian cancer and Stat3/VEGF signalling in breast cancer (Niu *et al*, 2002). However, the mechanism by which IL-6 induces angiogenesis is not fully understood.

The concept of 'cancer-stroma interaction' (CSI) has been studied extensively (Fujita *et al*, 2009; Semba *et al*, 2009; Kitadai, 2010), and these studies have shown that stromal cells are indeed important for cancer progression. Tumours consist of cancer cells and stromal cells, and as much as 60–90% of the mass of colon cancers consist of stromal cells (Powell *et al*, 2005). In fact, the main cellular constituents of solid tumours are stromal fibroblasts (Worthley *et al*, 2010). Cancer stromal fibroblasts differ from normal fibroblast and have specific effects on tumour growth. Thus, they have been termed cancer-associated fibroblasts (CAFs) (Mueller *et al*, 2007; Paland *et al*, 2009; Fuyuhiro *et al*, 2011). CAFs are activated fibroblasts observed in cancer stroma. They have properties of myofibroblasts and are different from normal fibroblasts in the fact that they express α-SMA (Nakagawa *et al*, 2004). However, their origins are not understood. Some theories propose that they are cancer cells that have passed through the epithelial–mesenchymal transition (EMT) (Kalluri and Zeisberg, 2006). Others suggest that they are recruited from bone marrow (Mishra *et al*, 2008).

Several studies have shown that CAFs have a critical role in cancer progression. Some studies demonstrate that through their production of cytokines, growth factors and angiogenic factors, they lead to cancer cell proliferation, migration, angiogenesis and metastasis (Wu *et al*, 2011; Zhang *et al*, 2011).

Although both IL-6 and stromal fibroblasts have key roles in cancer progression, the relationships between IL-6 secretion and the activities of stromal fibroblasts (Hugo *et al*, 2012) have not been fully examined. In this study, we analysed the IL-6 responses of fibroblasts isolated from human colon cancer and a xenograft colon cancer cell line, with a focus on angiogenesis.

MATERIALS AND METHODS

Agents. Anti-IL-6 receptor antibody, MRA (tocilizumab: anti-human IL-6 receptor antibody) and MR 16-1 (anti-mouse IL-6 receptor antibody) were kindly provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Human recombinant IL-6 was purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Human recombinant soluble IL-6 receptor was purchased from Peprotech (Rocky Hill, NJ, USA).

IL-6 was supplemented with soluble IL-6 receptor (40 ng ml^{-1}). To certify the established cell lines as fibroblasts, anti-vimentin mouse monoclonal antibody (V9; Dako, Glostrup, Denmark), anti-cytokeratin mouse monoclonal antibody (AE1/AE3; Dako), anti-CD90 mouse monoclonal antibody (5E10; BD Biosciences Pharmingen, San Diego, CA, USA) and anti-α-SMA monoclonal antibody (1A4; Dako) were used for immunohistochemistry.

Cell lines. A human colon cancer cell line, HT-29, was purchased from the ATCC (Manassas, VA, USA). COLM-5 is a poorly differentiated human colon cancer cell line free of fibroblasts. It was kindly provided by H. Nakanishi (Aichi Cancer Center Research Institute, Aichi, Japan; Ito *et al*, 2010). Human dermal fibroblasts (DFs) and human umbilical vein endothelial cells (HUVECs) were purchased from KURABO (Osaka, Japan).

Cancer cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1% antibiotic-antimycotic solution (Sigma). DFs were cultivated in DMEM containing 5% FBS and 1% antibiotic-antimycotic solution. HUVECs were cultivated in HuMedia-EB2 (KURABO, Osaka, Japan) containing 2% FBS and 0.1% gentamicin and amphotericin B. All cell lines were incubated at 37 °C in air containing 5% CO_2.

Isolation and culture of human colon fibroblasts. We established fibroblast cell lines from a specimen resected from a 64-year-old Japanese male patient with well-differentiated colon cancer. The technical procedure was similar to a previous report (Mueller *et al*, 2007). After receiving written informed consent, tissues were retrieved from two separate regions: colon carcinoma tissue and non-malignant colon tissue. To avoid contamination, these tissues were approached from the serosal side, taking special care not to pierce the mucosa. After fragmenting with scissors, the tissue was incubated in DMEM containing 1000 U ml^{-1} Dispase (Godo Shusei, Tokyo, Japan) for 2 h. The fragments were cultivated in DMEM containing 5% FBS and 1% antibiotic-antimycotic solution and incubated at 37 °C in air containing 5% CO_2. To confirm that the cultivated cells were fibroblasts, the cells were immunostained for vimentin, CD90 and cytokeratin. Isolated cells were fixed with 10% formalin for 10 min, then blocked with 3% bovine serum albumin (BSA)/phosphate-buffered saline (PBS). Then, the primary antibodies (vimentin 1 : 80, CD90 1 : 100, cytokeratin 1 : 80) were applied for 60 min at 37 °C. HRP-conjugated secondary antibody (Dako Envision + System; Dako) was applied for 60 min at 37 °C. The cells were stained with a DAB substrate and counterstained with hematoxylin. We confirmed that all the cells were positive for vimentin and CD90 expression and negative for cytokeratin expression. We termed fibroblasts isolated from malignant regions or benign regions as CAFs or normal fibroblasts (NFs). These fibroblasts were used in their third through sixth passage in culture.

Dual immunofluorescence staining. Fresh frozen tissues embedded in OCT compound (Sakura Finetek, Tokyo, Japan) were cut by Cryostat (Leica, Germany) with 4-μm thickness and were air dried and fixed in 10% formalin PBS for 10 min. These sections were blocked with 1% BSA/PBS and first reacted with human a-SMA mouse monoclonal antibody (Nichirei, Tokyo, Japan) for 60 min at room temperature and then Alexa Fluor 568 conjugated goat anti-mouse IgG for 30 min as secondary antibody. Next, sections were reacted with Zenon Alexa fluor 488 (Invitrogen, Eugene, OR, USA) conjugated human IL-6 mouse monoclonal antibody and counterstained with DAPI (1 : 2000) after washing with PBS.

For dual immunofluorescence staining of cultured CAF and NF cells grown in chamber slides, cells were fixed with 10% formalin for 10 min, followed by 0.2%Tritonx-100 for 10 min and blocked with 1% BSA/PBS, and slides were reacted by human α-SMA mouse monoclonal antibody and Alexa fluor 488-conjugated human IL-6 mouse monoclonal antibody sequentially as described above. After washing with PBS, DAPI was applied for 10 min at room temperature. These slides were mounted with ProLong Gold Antifade Reagent (Invitrogen) and observed under a TS100 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a digital camera (Ds-Ri1). Images were captured on a Nikon Digital sight (Nikon DS-L3).

Quantitative real-time RT–PCR. To compare the expression level of each gene, we performed RT–PCR. Cells (1×10^5) were seeded into individual wells of six-well plates. Following overnight incubation, these cells were exposed to each of the conditions described in the following section for 24 h with 5% FBS in DMEM.

Then, total RNA from each sample was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA was reverse-transcribed using Super Script III (Invitrogen Carlsbad, CA, USA). Real-time RT–PCR was performed with the 7900HT Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using a Taqman assay for *IL6* (Hs99999032_m1) and *VEGFA* (Hs009000055_m1; detects human and mouse gene expression) (Invitrogen). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905, Invitrogen) was used as an internal standard to normalise mRNA levels.

ELISA. The expression of IL-6 and VEGF by each cell line was evaluated with ELISA. Fibroblasts and cancer cell lines were seeded into six-well plates at a density of $1 \times 10^5\,ml^{-1}$. Following overnight incubation, these cell lines were exposed to each of the conditions described in the following section, and incubated with 5% FBS in DMEM. After 48 h, culture supernatants were collected, centrifuged to pellet any detached cells, and measured using a VEGF ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA) or an IL-6 ELISA Kit (R&D). The ELISAs were performed according to the manufacturer's instructions. Culture supernatants were diluted twice (VEGFA) or 20 × (IL-6).

Expression of IL-6. IL-6 expression and secretion from fibroblasts and cancer cell lines were evaluated using RT–PCR and ELISA. NFs, CAFs, HT29 and COLM5 were incubated in 1 ml 5% FBS/DMEM. IL-6 expression of each cell type was measured using RT–PCR after 24 h and using ELISA after 48 h in each condition.

Cell-mediated stimulation of IL-6 expression by fibroblasts and cancer cell lines. IL-6 expression by fibroblasts or cancer cell lines was analysed under various conditions. Lipopolysaccharide (LPS, from *Escherichia coli*: $100\,ng\,ml^{-1}$, O-111, Wako Pure Chemical, Osaka, Japan) was added to 5% FBS/DMEM as a phlogogenic agent. Recombinant human IL-1β (one $ng\,ml^{-1}$, Peprotech) and tumour necrosis factor α (TNF-α; $1\,ng\,ml^{-1}$, Wako Pure chemical) were added to 5% FBS/DMEM as inflammatory cytokine. Fibroblasts and cancer cell lines were separately co-cultured with cancer cell lines in the upper chamber (BD cell culture insert; BD Biosciences Pharmingen) (10^4/insert) in 5% FBS/DMEM. IL-6 expression of each cell type was measured using RT–PCR and ELISA as described above.

Effect of IL-6 on VEGF expression. Cells were treated with IL-6 ($1\,ng\,ml^{-1}$)/sIL-6 receptor ($40\,ng\,ml^{-1}$) and MRA ($10\,\mu g\,ml^{-1}$) for 24 h (RT–PCR) or 48 h (ELISA). Expression of *VEGFA* mRNA was measured using RT–PCR as described above. VEGFA in culture supernatants was measured using ELISA as described above.

Angiogenic effects of IL-6. We established an angiogenesis model using colon fibroblasts and HUVECs (Bishop *et al*, 1999). On day 0, 3×10^4 NFs were seeded in 24-well plates in 5% FBS/DMEM. After overnight incubation (day 1), 3×10^3 HUVECs were seeded as well. For each condition, two wells were used. On days 2, 5 and 8, cells were treated with IL-6 (one $ng\,ml^{-1}$) and/or MRA ($10\,\mu g\,ml^{-1}$) in a solution of 50% DMEM and 50% HUMEDIA-EB2. Then, the solution was supplemented with 5% FBS. IL-6 was supplemented with soluble IL-6 receptor ($40\,ng\,ml^{-1}$). On day 11, cells were fixed and immunostained with anti-CD31 antibody to measure the vascular area and to count the number of vascular joints as an indicator of vascular network formation. Cells were fixed in ice-cold 70% ethanol and blocked with 1% BSA/PBS. The primary anti-CD31 antibody (mouse anti-human CD31; KURABO) was applied at a dilution of 1 : 4000 for 60 min at 37 °C. Incubation with the second antibody (goat anti-mouse IgG alkaline phosphatase conjugate; KURABO) was performed for 60 min at 37 °C. They were visualised with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (Kurabo). The stained vascular area and the number of joints were measured as pixel density using Angiogenesis Image Analyzer (KURABO) according to the manufacturer's manual. The average vascular area and number of joints in eight fields (four regions per plate) at × 40 magnification were compared.

Animal experiments. To examine the effects of inhibiting IL-6 signalling, we performed an animal experiment using nude mice (Hara *et al*, 2008). COLM5 cells (5×10^5) were implanted subcutaneously (s.c.) into the inguinal region of mice. When tumours developed to approximately 5 mm in maximum diameter, treatment with intraperitoneal injection of MRA (anti-human IL-6R antibody; 100 μg per mouse, every 2 days) and/or MR16-1 (anti-mouse IL-6R antibody; 100 μg per mouse, every 2 days) was carried out for 5 weeks (five mice/group). MRA was used to inhibit IL-6 signalling in the human colon cancer xenograft. MR16-1 targeted IL-6 signalling in stromal fibroblasts. Each tumour's maximum diameter (L) and the maximal width at right angles to that axis (W) were measured once a week. Tumour volume was estimated by the following formula: $L \times W \times W \times 1/2$. Mice were killed after 5 weeks of treatment, according to the ethical guidelines of the UK Coordinating Committee on Cancer Research. Then, the tumours were removed and weighed.

Removed tumours were used for immunohistochemical observation and RNA extraction. Removed tumours were fixed in formalin-free zinc fixative (BD Biosciences Pharmingen, San Diego, CA, USA) for 24 h and embedded in paraffin and sectioned at a thickness of 4 μm. Immunohistochemical staining for blood vessels was carried out by the indirect immunoperoxidase method as described above using a rat monoclonal antibody to mouse CD31 (MEC 13.3; BD Biosciences Pharmingen) as the primary antibody. The micro-vessel density of each tumour was determined by light microscopy. Then, the number of structures positive for CD31 in the densest vascular field in each tumour was counted at a magnification of × 100 (Uzzan *et al*, 2004).

Statistical analysis. Data are presented as means and s.e.m. Differences between groups were compared by the Chi-square test and ANOVA. A P-value < 0.05 was considered significantly different.

RESULTS

IL-6 expression in colon cancer. Serial immunofluorescence staining of human colon cancer specimens showed that the IL-6-positive area was limited to the cancer stromal region (Figure 1A). Cytokine staining was not observed in the cytoplasm or the nucleus of the cancer cells. The IL-6- and α-SMA-positive regions were similarly positioned (Figure 1A and B). Merge images confirmed that in the tumour tissue both IL-6 and α-SMA were largely colocalised to the fibroblast-like cells in the stroma, and no significant expression by the tumour cells was observed (Figure 1C). On the other hand, regions of normal colon stroma were scarcely stained for IL-6 (Figure 1D). In benign colon samples, α-SMA was barely expressed except for the muscle layer and some cells, such as pericrypt myofibroblasts (Figure 1E). Furthermore, in the tumour tissue no significant CD45 expression was observed in the tumour stoma. (Figure 1G–I). These data indicated that fibroblasts in the tumour were different from those in the normal colon. Because α-SMA is an indicator of CAFs, these results showed that IL-6 was not provided by the cancer cells or by normal fibroblasts but by the cancer stromal fibroblasts activated by the cancer cells, that is, CAFs.

Isolation of fibroblasts from the human colon. We succeeded in culturing spindle cells from human colon specimens. All cultured cells were positive for vimentin and CD90 but negative for

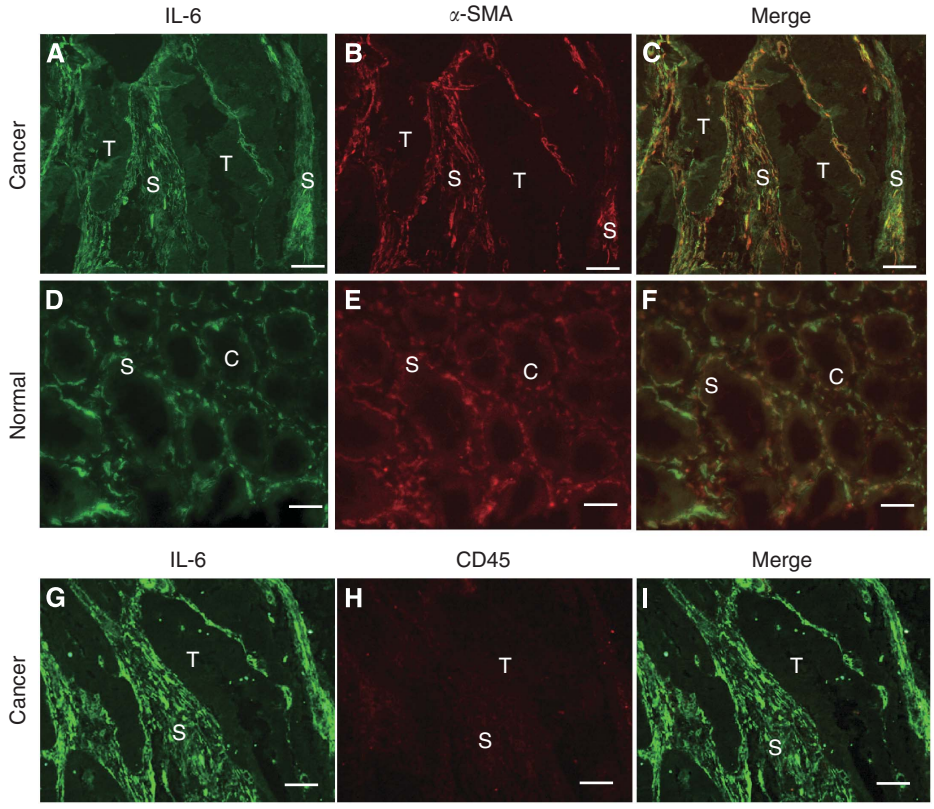

Figure 1. Localisation of IL-6 and α-SMA in the stroma of colon cancer tissues and normal colonic mucosa as revealed by double immunofluorescent staining of fresh frozen tissues. (**A** and **B**) In the tumour tissue, both IL-6 and α-SMA were largely colocalised in the cancer stromal compartment, and no significant expression by the tumour cells was observed. (**C**) Merged image demonstrates coexpression of both proteins mainly in the stromal fibroblast-like cells. (**D–F**) In normal colonic mucosa, IL-6 and α-SMA expression was weaker than tumour tissue, but the still weakly observed expression colocalised in the pericryptal and stromal fibroblast-like cells. (**G–I**) In the tumour tissue, no significant CD45 expression was observed in the tumour stoma. Bars = 100 μm. S = stroma, C = crypt, T = tumour tissue.

cytokeratin expression (Figure 2A). Fibroblasts isolated from malignant regions or benign regions were CAFs or NFs, respectively. Immunofluorescent staining revealed that almost all CAFs were positive for both IL-6 and α-SMA (Figure 2B), whereas NFs were weakly stained for α-SMA and barely positive for IL-6. Thus, α-SMA-positive CAFs expressed high levels of IL-6, but these cells were not seen in normal colon stroma (Figure 2B).

IL-6 was released mainly from fibroblasts and not from colon cancer cell lines *in vitro*. To investigate the origin of IL-6, gene expression and secretion of IL-6 protein were evaluated with RT–PCR and ELISA studies of fibroblasts and cancer cell lines (Figure 2C). RT–PCR showed that *IL6* expression levels in CAFs were about 12 times higher than in NFs. However, cancer cell lines HT29 and COLM5 expressed minimal to undetectable levels of *IL6*.

Consistent with the RT–PCR analysis, ELISA showed that whereas secretion of IL-6 from fibroblasts occurred at high levels those from cancer cell lines were minimal. These results revealed that more IL-6 was produced from fibroblasts than from colon cancer cells. Among these fibroblasts, CAFs expressed higher level of IL-6 than did benign NFs.

Cancer cells or inflammation promote IL-6 expression by stromal fibroblasts. Figure 2 showed that IL-6 secretion from DFs or NFs was significantly less than that from CAFs. This indicated that some conditions might stimulate fibroblast production of IL-6. To clarify whether the expression of IL-6 could be upregulated to the levels seen in CAFs, we compared the expression levels of IL-6 in DFs, NFs and cancer cell lines under several conditions (Figure 3).

RT–PCR showed that the expression of *IL6* mRNA in colon stromal fibroblasts was enhanced by both the presence of either LPS or cancer cell lines. Co-culture of stromal fibroblast with cancer cell lines (in separate chambers) strongly promoted the fibroblasts expression of *IL6* (Figure 3A and B).

The ELISA assessment of IL-6 secretion showed similar results. Co-culture of colon stromal fibroblasts and cancer cell lines increased production of IL-6 protein compared with the control (Figure 3D and E). On the other hand, IL-6 production by cancer cell lines was minimal even in the presence of LPS (Figure 3C and F). IL-1β and TNF-α significantly increased both mRNA expression and IL-6 production from stromal fibroblasts.

IL-6 promoted VEGF expression by fibroblasts but not by cancer cell lines. To clarify whether IL-6 promoted angiogenesis, we evaluated the expression of VEGFA from fibroblasts as a function of IL-6 stimulation. RT–PCR and ELISA showed that IL-6 upregulated colon fibroblasts' expression of *VEGFA* mRNA and VEGFA protein. Specifically, IL-6 significantly increased the expression of *VEGFA* mRNA in both CAFs and NFs, and *VEGF* expression was suppressed by anti-IL-6 receptor antibody to the same level as the control group (Figure 4A and B). In ELISA analyses, IL-6 induced fibroblasts to produce VEGFA. Moreover, MRA suppressed it to a level similar to that observed with RT–PCR (Figure 4D and E). Cancer cell lines produced VEGFA without stimulation, and IL-6 barely enhanced the expression when assessed by ELISA and RT–PCR (Figure 4C and F). Similar results were observed in an angiogenesis model *in vitro* (Figure 5). Both vascular area and vascular network formation were significantly increased by 1 ng ml^{-1} IL-6 and were decreased significantly by

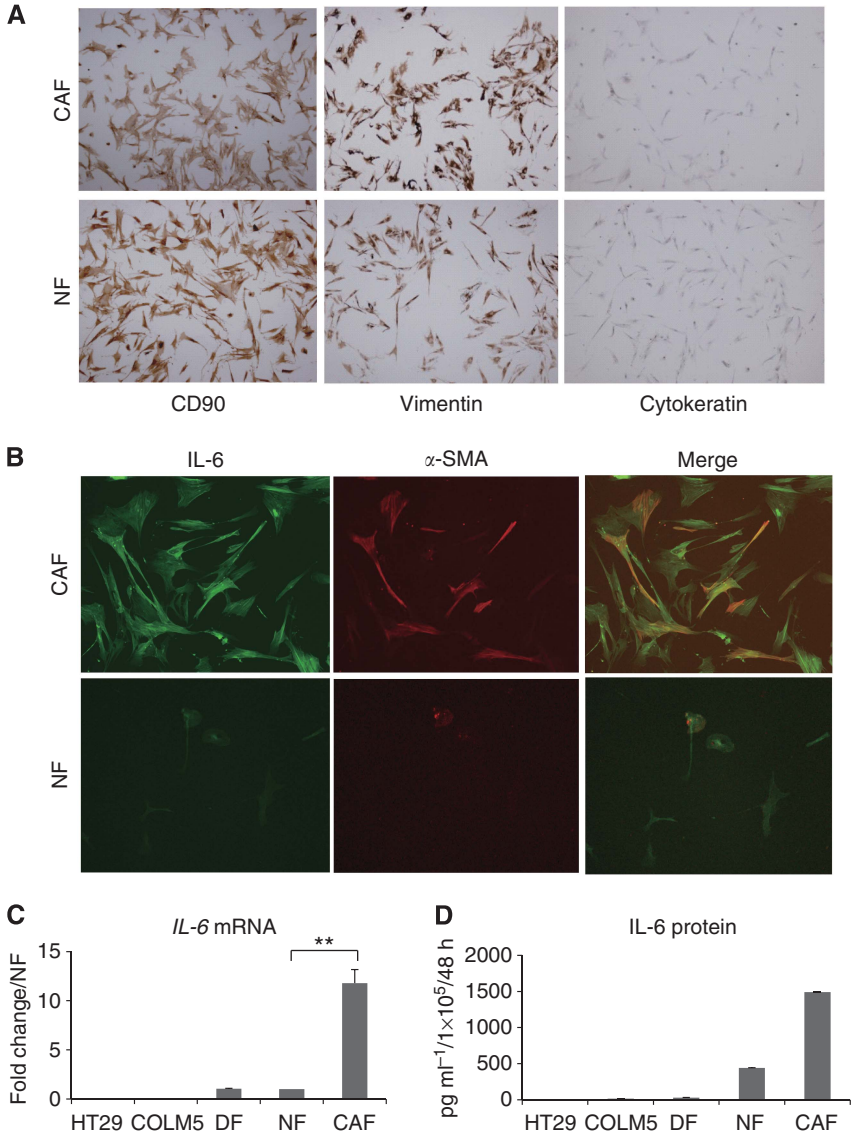

Figure 2. Isolated fibroblasts. (**A**) All cultivated cells were positive for expression of CD90 and vimentin, whereas all were negative for cytokeratin. IL-6 expression by cancer cells (HT29, COLM5), fibroblasts isolated from normal colon (NFs) and those from cancer (CAFs). (**B**) Immunofluorescent staining of IL-6 and α-SMA. Whereas CAFs stained for both IL-6 and α-SMA, NFs did not stain for either. (**C**) *IL6* mRNA levels measured with RT–PCR (relative to NFs as a control). *IL6* mRNA expression in CAFs was 11.8-fold higher relative to that in NFs. Compared with fibroblasts, *IL6* mRNA expression in cancer cells was $<1/100$ as great (COLM5: 0.01; HT29: not detected. $^{**}P<0.01$). (**D**) ELISA assay of IL-6 protein secretion of cancer cell lines (HT-29 and COLM5) and various fibroblasts. Whereas IL-6 secretion from cancer cell lines was barely detected, that from fibroblasts was significantly higher. Especially, IL-6 secretion from CAFs was 3.5-fold greater than NFs.

MRA. One ng ml^{-1} of IL-6 by itself did not directly support the proliferation of HUVECs (data not shown).

CAFs generally produced more VEGFA without IL-6 stimulation than did NFs. However, IL-6 stimulation had a greater effect on VEGFA production by NFs than by CAFs.

Inhibition of IL-6 signalling suppressed angiogenesis and tumour growth *in vivo*. The anti-cancer effect of inhibiting IL-6 signalling was examined in a s.c. tumour model in nude mice treated with anti-IL-6 receptor antibody. In this study, two kinds of anti-IL-6 receptor antibody were used: MRA, an anti-human IL-6 receptor antibody, was used to block the IL-6 signal in human cancer cell lines, and MR16-1, an anti-mouse IL-6 receptor antibody, targeted mouse stromal cells.

Xenografted tumour sizes and weights after 4 weeks of treatment were significantly smaller in groups treated with anti-mouse IL-6 receptor antibody (MR16-1 and the combination group) than those without the MR16-1 groups (MRA and the control group) ($P=0.03$). There is no significant difference between the MR16-1 group and the combination group. Although those in the MRA group were smaller than the control group, it was not statistically significant (Figure 6B). The data indicated that IL-6 signals modulating stromal cell responses were more important for tumour cell growth than those directly stimulating cancer cells. The expression level of *VEGFA* mRNA and micro-vessel density in the xenografted tumours showed similar results as did the analyses of tumour volume and weight (Figure 6C–E). Whereas those in the groups treated with MR16-1 produced significantly less *VEGF* mRNA and had lower micro-vessel densities, those treated with MRA were not significantly different from the control group. Thus, *VEGFA* expression was regulated by stromal production of IL-6. This indicated that blocking the IL-6 signal in stromal cells was more effective at inhibiting angiogenesis than blocking the signal in cancer cells.

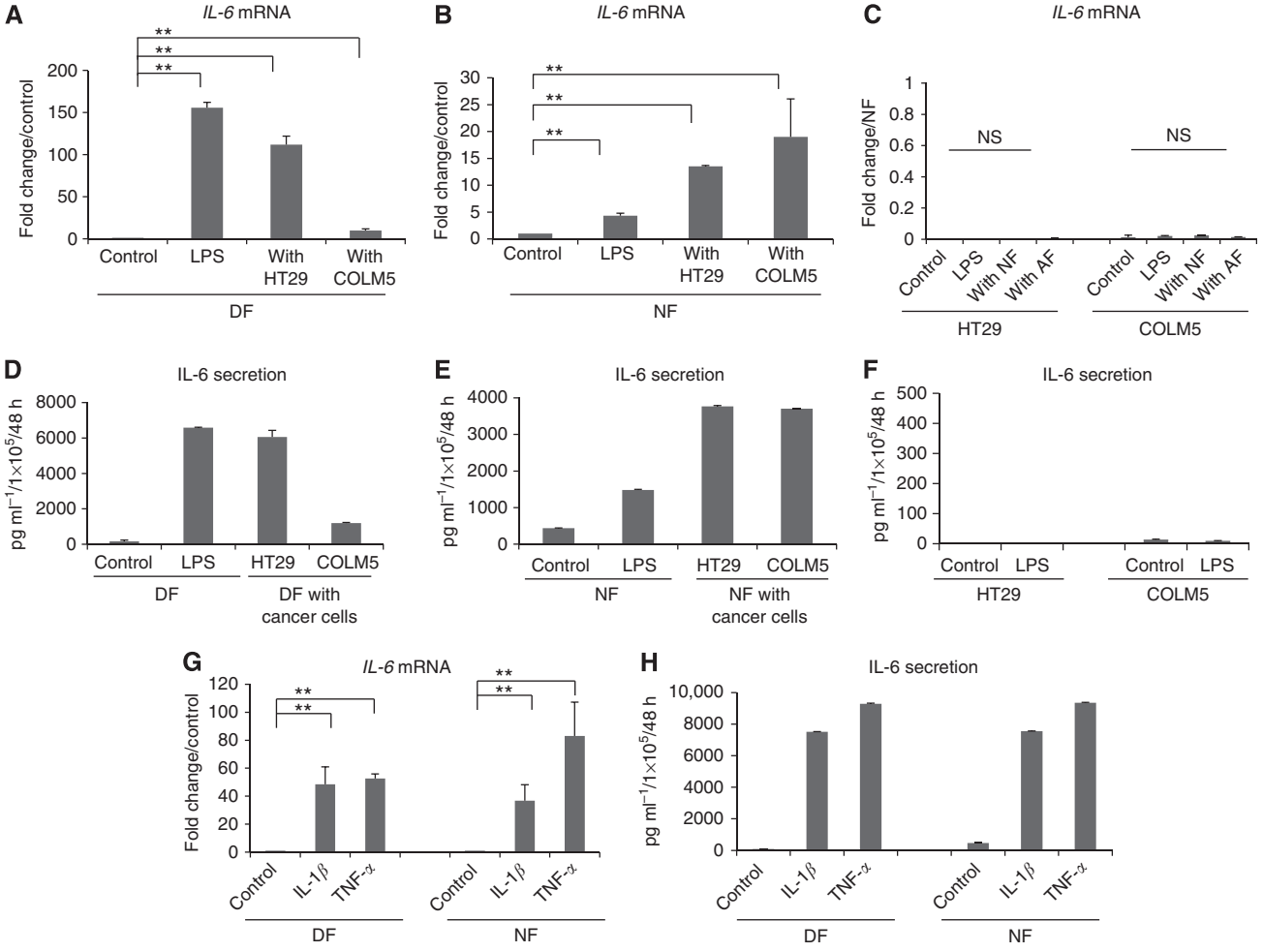

Figure 3. IL-6 secretion in response to various stimuli. *IL6* mRNA expression levels were calculated relative to fibroblasts without any stimunlation. (**A**–**C**) Co-culture of fibroblasts with cancer cells or stimulation by LPS increased the expression of *IL6* mRNA from fibroblasts significantly more than control. However, cancer cell lines were not induced by conditions that induced fibroblasts to express IL-6 (**$P<0.01$). (**D**–**F**) IL-6 protein levels in the supernatants measured with ELISA. As observed with mRNA, IL-6 protein levels secreted from fibroblasts cocultured with cancer cells were much higher than DFs or NFs alone. Cancer cell lines barely secreted IL-6 protein. (**G** and **H**) IL-1β and TNF-α significantly increased mRNA expression and IL-6 production of DFs and NF.

DISCUSSION

Fibroblasts produced IL-6 under inflammatory conditions and in the presence of cancer cells. Previous studies have shown that IL-6 has an important role in cancer progression. Some investigators have examined several kinds of cells such as T cells, myeloid cells and macrophages as producers of IL-6 (Grivennikov *et al*, 2009; Nilsson *et al*, 2005; Liu *et al*, 2011; Guo *et al*, 2012). For example, Nilsson *et al* (2005) reported that ovarian cancer cells produced IL-6. Furthermore, Liu *et al* (2011) reported that the co-culture of breast cancer cells and mesenchymal cells upregulated their IL-6 production. However, our results demonstrated that the secretion of IL-6 from cancer stromal fibroblasts was greater than that of cancer cells, both *in situ* and *in vitro*. In addition, we demonstrated that LPS or cancer cells induce normal colon fibroblasts to express elevated amounts of IL-6. This finding shows that CAFs as well as NFs produce significant amounts of IL-6 in the presence of cancer cells. It is believed that cancer cells cause fibroblasts to initiate an inflammatory response, promoting IL-6 expression. (Hugo et al 2012) In fact, colon cancer cell lines used in this study barely expressed IL-6 compared with normal fibroblasts. Furthermore, the amount of IL-6 was not increased by LPS, or other cells. Even though Nilsson *et al* (2005) reported that cancer cells produce IL-6 in cancer, our studies revealed significantly higher expression levels of IL-6 by fibroblasts, especially CAFs. These results show that most of the IL-6 produced in a colon tumour was generated by CAFs. As shown in Figures 2 and 3, cancer cell lines and LPS can upregulate IL-6 production by NFs to levels greater than by CAFs. Furthermore, IL-1β and TNF-α are representative inflammatory cytokines produced by cancer cells. As shown in Figures 3G and H, these cytokines increased IL-6 production of stromal fibroblasts. These results suggest that cytokines produced by cancer cells induced stromal fibroblasts to produce IL-6. To this point, only CAFs have attracted attention as central players in CSI. Our results suggest that cancer cell stimulation and/or inflammatory processes lead both CAF and normal stromal fibroblasts to have important roles in CSI, including IL-6 production or IL-6-mediated VEGFA production/angiogenesis. Until now, the origin of CAF has not been clear. Although bone marrow-derived mesenchymal cells and EMT are known to be associated with generation of CAF (Taketo, 2009; Kalluri and Zeisberg, 2006), our results strongly suggest that normal colon fibroblasts might become CAF under the stimulation of tumour cells.

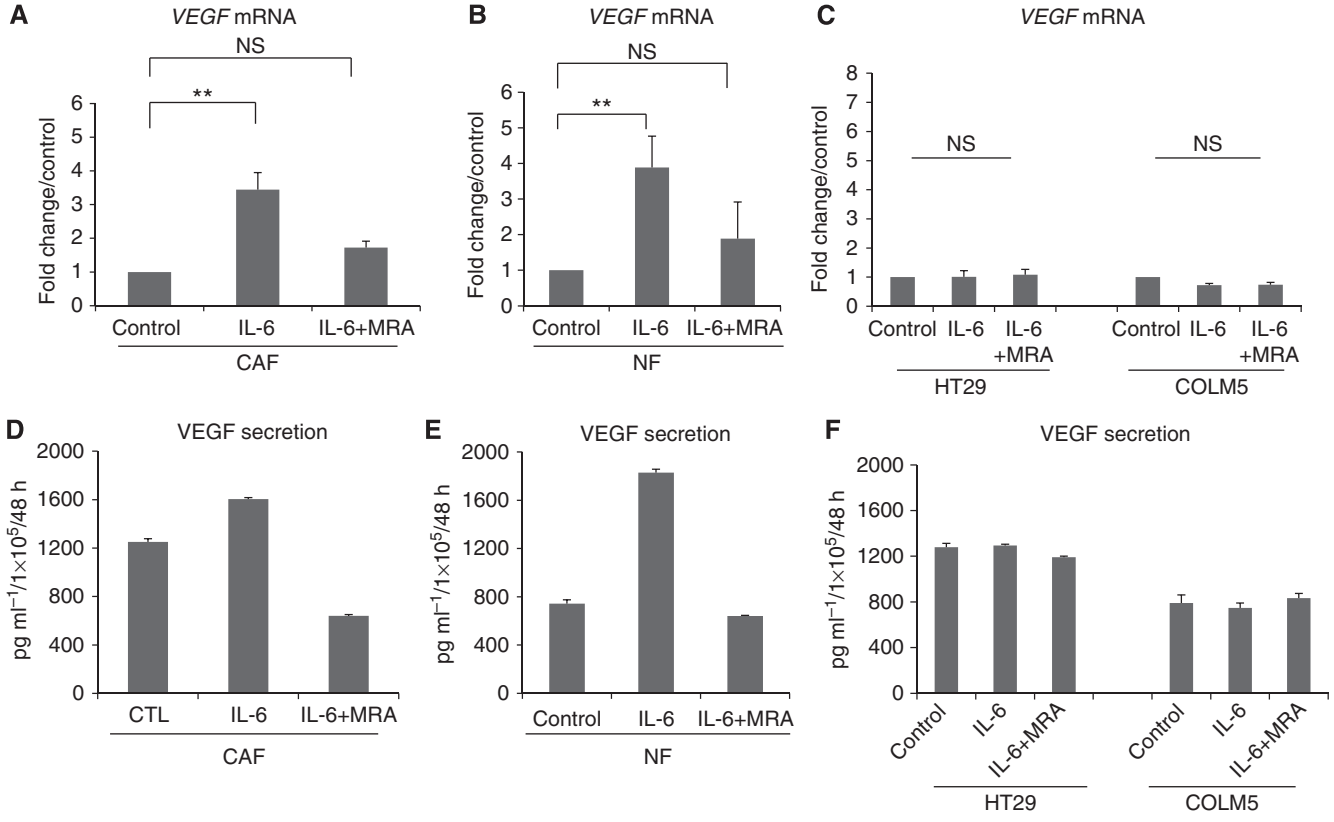

Figure 4. The expression and secretion of VEGF induced by IL-6. Expression levels of *VEGF* mRNA 24 h after treatment and VEGF protein levels 48 h after treatment with IL-6 and MRA were measured using RT–PCR (relative to control) and ELISA, respectively. *VEGF* mRNA levels in (**A**) CAFs and (**B**) NFs were increased by stimulation with IL-6 and decreased by treatment with MRA. IL-6 (1 ng ml^{-1}) induced *VEGF* mRNA expression in both CAFs and NFs. MRA inhibited IL-6-induced *VEGF* expression. (**D** and **E**) The increases in VEGF secretion mediated by IL-6 stimularion were more remarkable in NFs than CAFs. IL-6 induced VEGF secretion by both dermal and colon fibroblasts. MRA inhibited VEGF secretion of fibroblasts. (**C** and **F**) IL-6 scarcely induced colon cancer cell lines to express VEGF (**$P<0.01$).

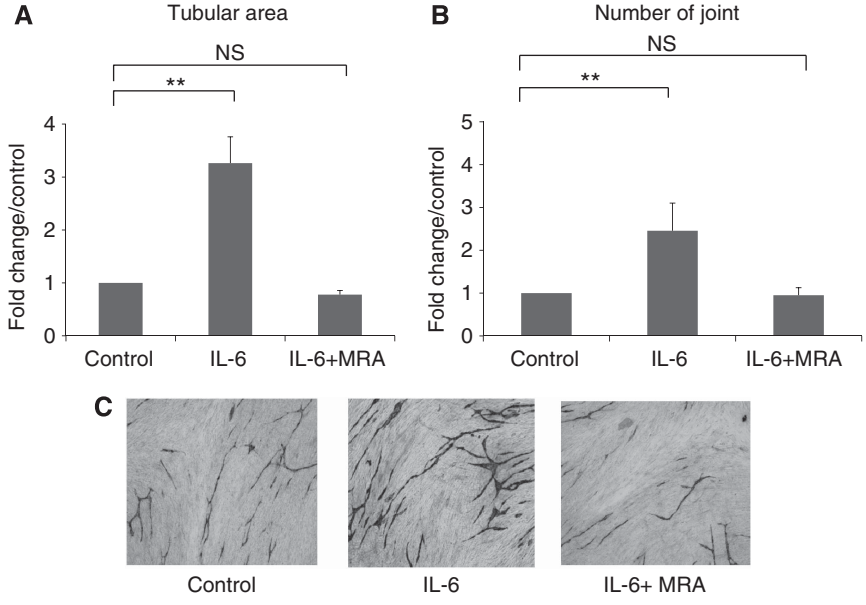

Figure 5. Angiogenic response to IL-6 stimulation. Stimulation of angiogenesis by IL-6 was estimated using an HUVEC plus colon fibroblast model (see Materials and Methods). Both (**A**) tubular area and (**B**) the number of joints were significantly increased in the presence of IL-6 ($P<0.05$). These increases were inhibited by the addition of anti-IL-6 receptor antibody, MRA, to the control level. The increases were not significantly diffferent between 1 and 20 ng ml^{-1} IL-6. (**C**) Representative images of immunohistochemistry 9 days after treatment with IL-6 and MRA.

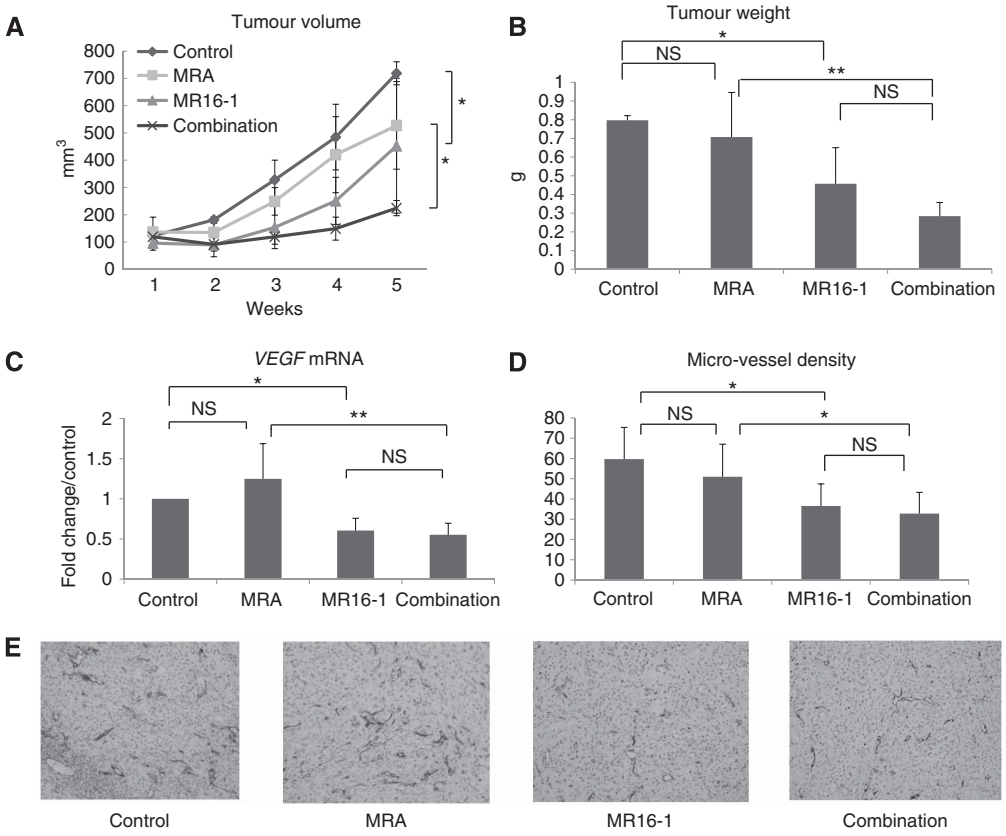

Figure 6. Anti-tumour effect of anti-IL-6 receptor antibody on xenografted colon cancer cell line. (**A**) Xenografted tumour volume was measured after various treatments (control, MRA: anti-human IL-6 receptor antibody, MR16-1: anti-mouse IL-6 receptor antibody, and combination). Harvested tumours after 5 weeks of treatment were examined. (**B**) Harvested tumours weights were significantly less in the groups with MR16-1 (MR16-1 and the combination group) ($P<0.05$). (**C**) *VEGF* mRNA expression and (**D** and **E**) micro-vessel density in harvested tumours were significantly less in the MR16-1 group and combination group. However, those in the MRA group were not significantly different from those of the control group (*$P<0.05$, **$P<0.05$).

IL-6 induced fibroblast expression of VEGFA, leading to angiogenesis. Progressive growth of malignant tumours is dependent on angiogenesis (Sakurai and Kudo, 2011). VEGFA, which is secreted by cancer cells, targets endothelial cells and is regarded as one of the most important angiogenic factors that directly mediate tumour angiogenesis.

This study demonstrated that IL-6 facilitated cancer angiogenesis via induction of fibroblasts' expression of angiogenesis-related proteins, including VEGFA. Although colon cancer cell lines used in this study produce some VEGFA, the increase in VEGFA expression by IL-6 stimulation was relatively small. On the other hand, VEGFA expression by fibroblasts was significantly increased by IL-6 stimulation. These results showed that stromal fibroblasts, not cancer cells, enhanced the expression of VEGFA. Mechanism of this angiogenesis is still unclear. However, Shao *et al* (2006) reported that Prostaglandin E2 promotes the secretion of VEGF from myofibroblasts. Taken together, these results suggest the possibility that IL-6 stimulates VEGF secretion by the mediation of PGE2 from myofibroblasts.

Nillson *et al* (2005) have reported that IL-6 signals stimulating cancer cells are important for tumour growth. As far as we know, there have been no previous reports that have shown the role of fibroblasts in mediating IL-6-induced tumour angiogenesis. Unlike previous reports, we used anti-human and anti-mouse IL-6 receptor antibodies to separate the blockade of IL-6 receptor signals in mouse stromal cells from human cancer cells xenografted into mouse. Our results showed that IL-6 modulation of stromal cells was more important for tumour growth and angiogenesis than was the direct effect of IL-6 on cancer cells.

Myofibroblasts expressing α-SMA (CAFs) reportedly mediate CSI, essential for tumour growth (Kalluri and Zeisberg, 2006). Our experimental results revealed the ability of normal fibroblasts to produce both IL-6 and VEGF. Therefore, in our IL-6/VEGF angiogenesis model not all fibroblasts are CAFs. From our results, the interaction between tumour cells and adjacent cells can be summarised as follows. Cancer cells or inflammation induces colon stromal fibroblasts to express IL-6. The secreted IL-6 induces surrounding fibroblasts to express VEGF in an autocrine or paracrine fashion. Secreted VEGF from fibroblasts subsequently induces angiogenesis. In this model, IL-6 has a primary role in cancer–stromal interactions.

Inhibition of IL-6 signalling between tumour cells and stroma is a potential novel therapeutic target. With regard to cancer progression, both IL-6 and fibroblasts are regarded as key players. In fact, several studies have reported that IL-6 and fibroblasts represent potential therapeutic targets in several cancers (Loeffler *et al*, 2006; Mueller *et al*, 2007; Coward *et al*, 2011; Hsu *et al*, 2011). However, few studies have examined the relationship between IL-6 and fibroblasts in colon cancer. For that reason, we analysed the interaction between colon tumour cells and stroma.

Conventional anti-cancer therapies directly target cancer cell proliferation. However, targeting cancer–stromal interaction is gaining attention (Shinagawa *et al*, 2013). Nevertheless, it is not clear which signals or factors should be targeted to inhibit angiogenesis. As described above, we demonstrated that cancer cells initiate inflammatory processes, including production of IL-6, which in turn induces angiogenesis. Anti-IL-6 receptor antibody

has been clinically used as a therapeutic agent for the treatment of rheumatoid arthritis (Suzuki *et al*, 2010; Hashizume and Mihara, 2011). Recently, its anti-inflammatory effect has attracted notice, and its potency as an anti-cancer agent has been investigated (Oguro *et al*, 2012). Here, we revealed that anti-IL-6 receptor antibody inhibited stromal IL-6 signalling and inhibited angiogenesis and tumour growth. Thus, anti-IL-6 receptor antibody could be a novel therapeutic agent capable of inhibiting interaction between colon tumours and adjacent stroma.

CONCLUSIONS

Inflammation and/or the presence of tumour cells upregulated the release of IL-6 by cancer stromal fibroblasts, thereby enhancing tumour angiogenesis. Anti-IL-6 receptor antibody inhibited tumour–stroma IL-6 signalling and suppressed tumour angiogenesis and growth by blocking cancer–stroma interaction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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