non-platinum antitumor agent and epidermal growth factor receptor tyrosine kinase inhibitors in 16 (43.2%), 7 (18.9%), 9 (24.3%) and 5 (13.5%) patients, respectively. Among these 37 patients, 35 were assessable for response. PR and SD were observed in 4 (11.4%) and 21 (60.0%) patients, respectively, yielding a disease control rate (PR+SD) of 71.4%. Although a much longer MST (17.5 months) was observed in the present study, the median PFS (4.3 months) was comparable to or tended to be shorter than that in previous studies (10,11), indicating that additional chemotherapy treatments following the failure of first-line combination treatment with S-1 and CDDP may have a favorable survival effect.

Accumulating evidence has shown that the histological types of NSCLC affect the clinical outcome of patients treated with anticancer drugs (16,17). A phase III study showed a significant survival difference in favor of CDDP/pemetrexed compared with CDDP/gemcitabine in patients with adenocarcinoma and large-cell carcinoma but not in those with squamous cell carcinoma (16). Consequently, pemetrexed is approved for use in combination with CDDP for first-line treatment in patients with advanced non-squamous NSCLC. Moreover, serious hemorrhagic events have been reported to occur more frequently among patients with squamous cell carcinoma treated by bevacizumab (17). Therefore, patients with advanced squamous cell carcinoma have been routinely excluded from bevacizumab treatment, while the addition of bevacizumab to carboplatin/paclitaxel in the treatment of patients with non-squamous NSCLC has a significant survival benefit. These findings indicate the disadvantages of NSCLC patients with squamous histology, since they cannot benefit from these drugs and have fewer treatment options than those with non-squamous histology. Notably, in the present study, a significantly higher RR was observed in patients with squamous cell carcinoma (55.5%) than in those with adenocarcinoma (9.1%) or other types of NSCLC (0.0%). Given the small sample size, these data should be interpreted with caution. It is noteworthy, however, that combination chemotherapy with S-1 plus CDDP may confer treatment benefit on lung cancer patients with squamous cell histology. One potential explanation for this outcome may relate to thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) expression levels in NSCLC histological types. The ratio of TP to DPD is considered to be a useful predictor of the efficacy of chemotherapy with 5-FU, since TP converts 5'-deoxy-5-fluorouridine to 5-FU and DPD inactivates 5-FU in tumor tissue (18). Moreover, the ratio of TP to DPD in NSCLC tissue was reported to be higher in squamous cell carcinoma than in adenocarcinoma (19). These findings indicate that chemotherapy with 5-FU is potentially more effective in lung cancer patients with squamous cell carcinoma than in those with adenocarcinoma. To the best of our knowledge, this is the first study to show the significant treatment benefit of this combination in lung cancer patients with squamous cell histology as compared to non-squamous NSCLC. Further prospective randomized controlled clinical trials are required to confirm these results.

In platinum-doublet chemotherapies, which are the standard first-line regimen for advanced NSCLC, grade 3/4 neutropenia and gastrointestinal toxicity have been reported in 57-76 and 4-35% of patients, respectively (12,13). By contrast, combination chemotherapy with S-1 plus CDDP is reportedly less toxic than standard platinum-doublet regimens. Findings of phase II studies of combination chemotherapy with S-1 and CDDP have shown grade 3/4 hematologic and non-hematologic toxicities in 22-35 and 4-13% of patients, respectively (8,9). In the present study, mild toxicity profiles were noted for combination chemotherapy with S-1 plus CDDP, consistent with previous reports (8,9). With regards to hematologic adverse events, grade 3/4 leukocytopenia, neutropenia, anemia and thrombocytopenia were observed in 3 (7.5%), 2 (5%), 6 (15%) and 1 (2.5%) patients, respectively. Only 1 patient (2.5%) developed febrile neutropenia. Regarding non-hematologic adverse events, grade 3 infection, anorexia, nausea/vomiting, general fatigue and gastric ulcer were observed in 2 (5%), 5 (12.5%), 3 (7.5%), 1 (2.5%) and 1 (2.5%) patients, respectively. No grade 4 level toxicity occurred, and no instance of irreversible toxicity or treatment-related death was noted. The results indicate the efficacy and safety of combination chemotherapy with S-1 and CDDP for advanced NSCLC.

In conclusion, we investigated the efficacy and safety of a combination regimen of S-1 with CDDP for the treatment of chemotherapy-naïve patients with advanced NSCLC. Results showed a potentially long MST with comparatively low toxicity, indicating that this regimen is a potentially useful alternative therapeutic strategy for patients with advanced NSCLC, particularly squamous cell carcinoma.

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Clinical Cancer Research

Human Cancer Biology

The EGFR Ligands Amphiregulin and Heparin-Binding EGF-like Growth Factor Promote Peritoneal Carcinomatosis in CXCR4-Expressing Gastric Cancer

Kazuo Yasumoto[1], Tadaaki Yamada[2], Atsuhiro Kawashima[3], Wei Wang[2], Qi Li[2], Ivan Shterev Donev[2], Shinji Tacheuchi[2], Hisatsugu Mouri[2], Kaname Yamashita[1], Koushiro Ohtsubo[2], and Seiji Yano[2]

Abstract

Purpose: Peritoneal carcinomatosis, often associated with malignant ascites, is the most frequent cause of death in patients with advanced gastric cancer. We previously showed that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis from gastric cancer. Here, we investigated whether epidermal growth factor receptor (EGFR) ligands are also involved in the development of peritoneal carcinomatosis from gastric cancer.

Experimental Design: The functional involvement of expression of the ErbB family of receptors and/or EGFR ligands was examined in CXCR4-expressing human gastric cancer cells and fibroblasts, clinical samples (primary tumors and ascites), and an animal model.

Results: High concentration of the EGFR ligands amphiregulin and heparin-binding EGF-like growth factor (HB-EGF), as well as of CXCL12, were present in malignant ascites. Human gastric cancer cell lines and primary gastric tumors, with high potential to generate peritoneal carcinomatosis, expressed high levels of EGFR and CXCR4 mRNA and protein. Both amphiregulin and HB-EGF enhanced the proliferation, migration, and functional CXCR4 expression in highly CXCR4-expressing gastric cancer NUGC4 cells. Amphiregulin strongly enhanced the proliferation of NUGC4 cells, whereas HB-EGF markedly induced the migration of fibroblasts. Moreover, HB-EGF and CXCL12 together enhanced TNFα-converting enzyme (TACE)-dependent amphiregulin shedding from NUGC4 cells. In an experimental peritoneal carcinomatosis model in mice, cetuximab effectively reduced tumor growth and ascites formation.

Conclusions: Our results strongly suggest that the EGFR ligands amphiregulin and HB-EGF play an important role, interacting with the CXCL12/CXCR4 axis, in the development of peritoneal carcinomatosis from gastric cancer, indicating that these two axes may be potential therapeutic targets for peritoneal carcinomatosis of gastric carcinoma. *Clin Cancer Res; 17(11); 3619–30. ©2011 AACR.*

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide, with peritoneal carcinomatosis, often associated with malignant ascites, being the most frequent cause of death in patients with advanced gastric cancer (1–3). The 5-year survival rate of patients with peritoneal carcinomatosis, including those with intraperitoneal free cancer cells and without macroscopic peritoneal carcinomatosis, is only 2% (4). Peritoneal carcinomatosis in gastric cancer patients may be due to the direct dissemination of cancer cells into the peritoneal cavity. We have previously shown that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis with malignant ascites from gastric cancer (5). CXCL12 produced by the tumor environment, in particular by peritoneal mesothelial cells, may enhance the proliferation and/or survival of CXCR4-expressing gastric cancer cells in the peritoneal cavity, leading to the survival and peritoneal dissemination of gastric cancer cells. Other tumor-associated factors, in addition to CXCL12, may be necessary to promote the malignant potential and survival of cancer cells (6). Peritoneal fluid acts as a rich source of growth factor activity for cancer cells (7). Thus, activation of disseminated gastric cancer cells by cancer-activating factors, including CXCL12, may lead to the peritoneal dissemination of gastric cancers. The development of effective therapies targeting peritoneal carcinomatosis from gastric cancer requires further understanding of the processes and molecules leading to its initiation and progression.

Activation of the ErbB family of receptors has been associated with the progression of various tumor types.

Authors' Affiliations: Divisions of [1]Surgical Oncology and [2]Medical Oncology, Cancer Research Institute, Kanazawa University; and [3]Kanazawa Medical Center, Kanazawa, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Kazuo Yasumoto, Division of Surgical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan. Phone: 81-76-265-2794; Fax: 81-76-234-4524; E-mail: kyasu@staff.kanazawa-u.ac.jp

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Translational Relevance

Peritoneal carcinomatosis, often associated with malignant ascites, is the most frequent cause of death in patients with advanced gastric cancer. We have previously shown that the CXCR4/CXCL12 axis is involved in the development of peritoneal carcinomatosis form gastric cancer.

In this study, we showed that the EGFR ligands, amphiregulin and HB-EGF, are abundant in malignant ascites. Amphiregulin strongly enhanced the proliferation of CXCR4-expressing human gastric cancer NUGC4 cells, whereas HB-EGF markedly induced migration of fibroblasts. Moreover, HB-EGF and CXCL12 together enhanced TNFα-converting enzyme (TACE)-dependent amphiregulin shedding from functional CXCR4-expressing NUGC4 cells. Cetuximab, an anti-EGFR monoclonal antibody, effectively reduced tumor growth and ascites formation, and therefore dramatically prolonged survival in nude mice inoculated with NUGC4 cells. Our findings provide a novel insight into treatments that target tumor cells and their microenvironments during the development of peritoneal carcinomatosis from gastric cancer.

Epidermal growth factor receptor (EGFR) is a member of a family of closely related growth factor receptor tyrosine kinases, including EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Aberrant activation of EGFR signaling, such as that due to overexpression of EGFR or abnormal stimulation by autocrine growth factor loops, can contribute to constitutive EGFR signaling, resulting in the dysregulation of cell growth and ultimately leading to cancer (8). In particular, EGFR and ErbB2 have been recognized as targets in the treatment of various cancers (9). Although only about 20% of gastric cancers overexpress HER2, treatment with trastuzumab, a human monoclonal antibody specific for HER2, plus chemotherapy has shown survival benefits in patients with advanced, HER2-positive gastric cancer (10). To date, 7 ligands for EGFR have been identified: EGF, transforming growth factor α (TGFα); heparin-binding EGF-like growth factor (HB-EGF); amphiregulin; betacellulin; epiregulin; and epigen. Amphiregulin has been shown to play a crucial role in several types of tumors (11, 12), and HB-EGF has also been reported critical for tumor formation (13). However, the association between each EGFR ligand and tumor type, in particular the development of peritoneal carcinomatosis, is largely unknown. Moreover, there have been no comprehensive studies examining the role of EGFR ligands in gastric cancer progression driven by the tumor environment.

Here we report that the EGFR ligands amphiregulin and HB-EGF promote peritoneal carcinomatosis by stimulating the proliferation of tumor cells and their microenvironment and by enhancing functional CXCR4 expression in gastric cancers. Furthermore, these 2 EGFR ligands showed different biological activities in target cells. These findings indicate that EGFR and its ligands, especially amphiregulin and HB-EGF, are important in the development of peritoneal carcinomatosis in gastric cancer patients. Moreover, these findings provide a framework for future clinical trials of specific ErbB receptor inhibitors that target tumor cells and their environments.

Materials and Methods

Cell lines

The human gastric cancer cell lines, MKN28, MKN45, KKLS, NKPS (14), and NUGC4 (15), were purchased from the Health Science Research Resources Bank (Japan Health Science Research Resources Bank). All cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin G, and 100 units/mL streptomycin (Invitrogen Corp.). NKPS and NUGC4 are gastric cancer cell lines derived from malignant ascites or pleural effusions of patients with advanced gastric cancer and selectively express high levels of CXCR4. Following their inoculation into the abdominal cavity of nude mice, NUGC4 cells disseminate rapidly and form bloody ascitic fluid (16).

Tissue samples

Primary tumor tissue samples were obtained from the Division of Surgical Oncology, Cancer Research Institute, Kanazawa University, during gastrectomy of previously untreated gastric cancer patients. The histologic diagnosis of each patient was confirmed from the specimens. In addition, primary cultured fibroblasts were isolated from surgical specimens of human stomach and were identified by immunostaining with monoclonal antibodies against cytokeratin and vimentin. All patients provided written informed consent for molecular analysis of surgical samples.

Chemotaxis assay

Migration assays were carried out in 24-well Transwell plates (Costar Corp.) using inserts with 8 μm pore size membranes, as described (17). Cells were suspended in upper chambers at 1×10^6 cells/mL in 100 μL of RPMI 1640/0.1% bovine serum albumin. Where indicated, the cells were preincubated with 10 μg/mL monoclonal antibody against human CXCR4 (12G5, R&D) or EGFR (cetuximab, Merck Serono). The lower chambers contained 600 μL of RPMI 1640 supplemented with 100 ng/mL of CXCL12 (R&D Systems), 1 ng/mL of HB-EGF (R&D Systems), and 100 ng/mL of amphiregulin (R&D Systems). After incubation for 24 hours, the tumor cells remaining on the upper surface of the filters were removed by wiping with cotton swabs, and cells migrating to the lower surface of the membrane were stained with Giemsa solution (Wako) and at least 5 different fields were counted under a light microscope (original magnification, × 200). All assays were carried out in triplicate.

Proliferation assay

Gastric cancer cells (5×10^3) in RPMI 1640 supplemented with 10% charcoal treated FBS (Hyclone) were added to

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each well of a 96-well cell culture plate and cultured for 6 hours. Where indicated, the cells were preincubated with 10 μg/mL anti-EGFR monoclonal antibody (cetuximab, Merck Serono). The cells were stimulated with various concentrations of amphiregulin or HB-EGF and cultured for 72 hours. Two hours prior to the end of the assay, 10 μL of WST-8 (WST-8 Cell Counting Kit; Wako Pure Chemical) was added to each well, and the optical density at 450 nm was measured using an ELISA plate reader (BIO-RAD Laboratories, Inc.).

Cell proliferation was also measured using the MTT dye reduction method (18). Cells at 80% confluence were harvested, seeded at 2×10^3 per well in 96-well plates, and incubated in RPMI 1640 with 10% FBS for 24 hours. Various concentrations of amphiregulin and HB-EGF were added to each well, and incubation was continued for an additional 72 hours. A 50 μL aliquot of MTT solution (2 mg/mL; Sigma) was added to each well followed by Incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL dimethyl sulfoxide. Absorbance was measured using an ELISA plate reader (BIO-RAD). Percent growth was measured relative to untreated controls. Each condition was assessed in triplicate wells, and each experiment was carried out at least 3 times.

Antibodies and Western blot analysis

Cells were incubated in 10 mL RPMI 1640 supplemented with 10% FBS in the presence or absence of HB-EGF or amphiregulin. The cells were washed twice with PBS, harvested in cell lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 μg/mL, leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride], and flash-frozen on dry ice. After allowing the cells to thaw, the lysates were collected with a rubber scraper, sonicated, and centrifuged at 14,000 × g (4°C for 20 minutes). Total protein concentrations were measured using a Pierce BCA Protein Assay kit (Pierce). For Western blotting analysis, 40 mg of total protein were resolved by SDS-polyacrylamide gel (Bio-Rad) electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad). After washing, the membranes were incubated for 1 hour at room temperature with Blocking One (Nacalai Tesque, Inc.) and then overnight at 4°C with the following primary antibodies: anti–phospho EGFR (Y1068), anti-ErbB2, anti-ErbB4, anti-Akt, phospho-Akt (Ser473), and anti-TACE (each 1:1,000 dilution, Cell Signaling Technology). The membranes were also incubated with anti-human EGFR (1 mg/mL), anti-CXCR4 12G5 (10 μg/mL), anti-human/mouse/rat extracellular signal-regulated kinase 1/2 (ERK1/2; 0.2 μg/mL), and anti–phospho-ERK1/2 (T202/ Y204; 0.1 μg/mL; R&D Systems); with anti-amphiregulin (1 μg/ml; Abcam), and with polyclonal anti–glyceraldehyde 3 phosphate dehydrogenase (anti-GAPDH; 1:1,000 dilution; Trevigen). After 3 washes, the membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with Super Signal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each assay was carried out independently in triplicate.

RNA interference

Duplexed Stealth RNA Interference (Invitrogen) against *EGFR* (5′-UUUAAAUUCACCAAUACCUAUUCCG-3′ and 5′-CGGAAUAGGUAUUGGUGAAUUUAAA-3′), *ErbB4* (5′-UAUAGAUGUUUCCUGCGCUGAUUUC-3′ and 5′-GAAAUCAGCGCAGGAAACAUCUAUA-3′), and TNFα-converting enzyme (*TACE*) (5′-AUGAGUUGUAACCAGGUACGCUUCC-3′, and 5′-GGAAGCUGACCUGGUUACAACUCAU-3′) and Stealth RNA Interference Negative Control Low GC Duplex 3 (Invitrogen) were used for RNA interference assay. Briefly, aliquots of 1×10^5 NUGC4 cells or human normal fibroblasts in 2 mL antibiotic-free medium were plated on 6-well plates and incubated at 37°C for 24 hours. The cells were transfected with small interfering RNA (siRNA; 250 pmol) or scramble RNA (siSCR) using Lipofectamine 2000 (5 μL) in accordance with the manufacturer's instructions. After 24 hours, the cells were washed twice with PBS and incubated with or without recombinant human amphiregulin (100 ng/mL) and/or HB-EGF (1 ng/mL) for an additional 72 hours in antibiotic-containing medium. These cells were then used for the proliferation (MTT) assay as described above. *EGFR*, *ErbB4*, and *TACE* knockdown were confirmed by Western blotting analysis. Each experiment was carried out at least 3 times using triplicate samples.

Animal studies

Pathogen-free, 6-week-old, female BALB/c (*nu/nu*) mice were obtained from Japan SLC (Hamamatsu) and quarantined for 1 week under pathogen-free conditions at the Advanced Science Research Center of Kanazawa University to confirm the absence of diseases. All animal experiments complied with the Guidelines for the Care and Use of Laboratory Animals in Kanazawa University. Mice weighting 18 to 22 g were injected intraperitoneally with 1×10^6 NUGC4 cells in 200 μL of PBS. NUGC4 cells can rapidly disseminate in the abdominal cavities of nude mice on i.p. inoculation and form bloody ascitic fluid (16). Beginning 3 days after NUGC4 cell injection, the mice were injected i.p. with 2 mg/kg cetuximab (19), a specific monoclonal anti-EGFR neutralizing antibody, or normal IgG (R&D Systems) twice weekly for 2 weeks. No *in vivo* toxicity of cetuximab was detected in these mice. To monitor the extent of development of peritoneal carcinomatosis, body weights were routinely measured. Mice were sacrificed 40 days after NUGC4 cell injection. Ascitic fluid was collected from each mouse, and its volume was recorded. Macroscopic peritoneal tumor dissemination and the size and number of tumors in the abdomen were also determined.

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ELISA

The concentrations of CXCL12, EGF, TGFα, HB-EGF, and amphiregulin in ascitic fluid were measured using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol. Optical density at 450 nm was measured using an ELISA plate reader (BIO-RAD).

Immunohistochemistry

Surgical specimens of primary gastric tumors were routinely fixed in formalin and embedded in paraffin. Tissue sections (3 μm) were deparaffinized in xylene, rehydrated in an ethanol series, and treated for 30 minutes with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The sections were subsequently washed with PBS, and sections being tested for EGFR expression were unmasked in citrate antigen unmasking solution (Target Retrieval Solution High pH; Dakocytomation) in an autoclave for 20 minutes at 120°C. Sections being tested for CXCR4 were not unmasked. The samples sections were immunohistochemically stained for EGFR with the EGFR pharmDx kit (Dakocytomation) or for CXCR4 with anti-human CXCR4 monoclonal antibody (44716; R&D Systems) using a standard indirect avidin-biotin HRP method. Sections were regarded as positively stained when the intensity of staining was 10% or more.

Statistical analysis

All parameters are reported as mean ± SD, with between-group differences compared using Student's t test. $P < 0.05$ was considered statistically significant.

Results

Presence of amphiregulin and HB-EGF in malignant ascitic fluid

Peritoneal carcinomatosis is often associated with malignant ascites, a rich source of growth factor activity for various cancer cells (7). To determine whether EGFR ligands are present in malignant ascites from patients with gastric cancer, we assayed the concentrations of the EGFR ligands EGF, TGFα, HB-EGF, and amphiregulin in these samples and in nonmalignant peritoneal exudates (Table 1). As we previously reported (5), the mean concentration of CXCL12 protein was markedly higher in ascitic fluid from gastric cancer patients (4,667 pg/mL) than in nonmalignant peritoneal exudates (<2,000 pg/mL). Although EGF and TGFα were barely detectable in any of these samples, the mean concentrations of amphiregulin (3,055 pg/mL vs. 784.2 pg/mL) and HB-EGF (346.5 pg/mL, vs. 69.7 pg/mL) were markedly higher in malignant ascites than in nonmalignant peritoneal exudates, suggesting that amphiregulin and HB-EGF may promote tumor growth during peritoneal dissemination.

EGFR ligands, amphiregulin and HB-EGF, enhance the proliferation and migration of CXCR4-expressing human gastric cancer cells

We previously reported that the CXCR4/CXCL12 axis is involved in the chemotactic response, proliferation and survival of gastric cancer in the peritoneal cavity (5). Because NUGC4 cells exhibit the highest levels of CXCR4 expression and rapidly disseminate in the abdominal cavity of nude mice on i.p. inoculation (5), we used this cell line as our model of CXCR4-expressing gastric cancer. To determine whether the EGFR ligands amphiregulin and HB-EGF promote tumor growth during peritoneal carcinomatosis, we assessed the proliferation of NUGC4 cells in response to amphiregulin and HB-EGF. We found that both of these ligands significantly and dose dependently enhanced the proliferation of NUGC4 cells when the cells were maintained in suboptimal serum-free growth conditions for 72 hours (Fig. 1A and B). Proliferation in response to amphiregulin was much greater than proliferation in response to HB-EGF and/or CXCL12. Furthermore, the anti-EGFR neutralizing antibody cetuximab significantly blocked the proliferation of NUGC4 cells induced by amphiregulin and HB-EGF (Fig. 1C). When we tested the effects of amphiregulin and HB-EGF in another CXCR4-expressing gastric cancer cell line, NKPS (5), we observed similar results. Both amphiregulin and HB-EGF enhanced the proliferation of

Table 1. Presence of amphiregulin and HB-EGF in malignant ascitic fluids

	Malignant ascites		Nonmalignant ascites	
Growth factors	Mean	Range, pg/mL	Mean	Range, pg/mL
CXCL12	4,667	2,530–7,320	1,958	1,408–2,443
EGF	<4		<4	
TGFα	<4		<4	
HB-EGF	346	30–2,500	52	45–164
Amphiregulin	3,055	288–13,850	776	320–1,600

NOTE: Concentration of the EGFR ligands (EGF, TGFα, HB-EGF, and amphiregulin) and of CXCL12 in malignant ascitic fluids from patients with gastric cancer ($n = 20$) and in nonmalignant exudates ($n = 10$), as determined by ELISA.

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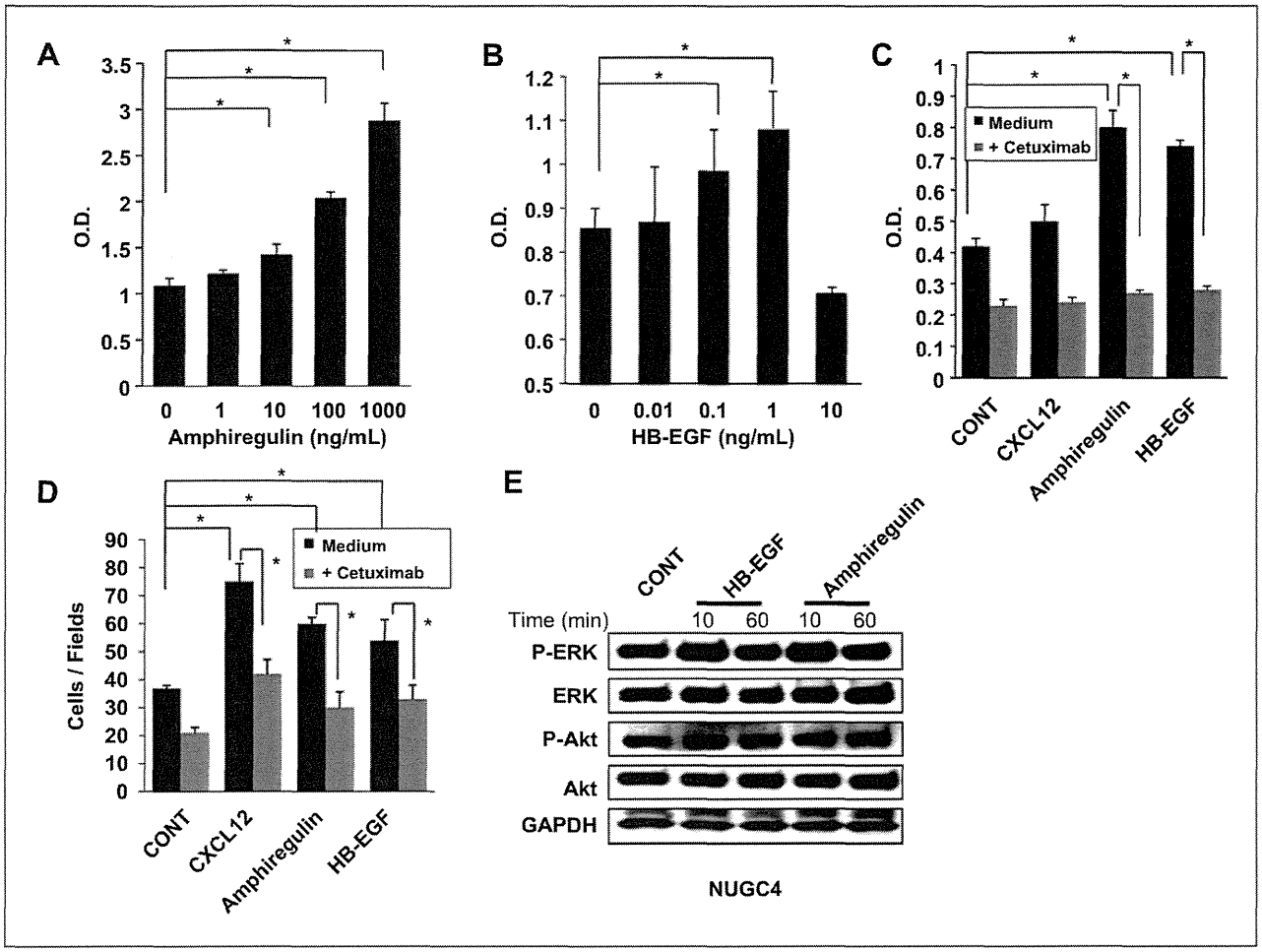

Figure 1. The EGFR ligands amphiregulin and HB-EGF enhance the proliferation, migration, and Akt and ERK phosphorylation of NUGC4 cells. NUGC4 cells were grown in medium containing 10% charcoal treated FBS, with or without amphiregulin (A) or HB-EGF (B). Cetuximab inhibits functional EGFR activities. NUGC4 cells were grown in serum-free medium with or without 10 μg/mL of the anti-EGFR antibody, cetuximab. Cetuximab significantly inhibited growth factor-induced cell proliferation (C). CXCL12, amphiregulin, and HB-EGF significantly enhance the migration of NUGC4 cells, a migration significantly inhibited by cetuximab (D). Immunoblotting analysis of Akt and ERK phosphorylation. NUGC4 cells were seeded in 10-cm dishes, and stimulated with HB-EGF (1 ng/mL) or amphiregulin (100 ng/mL) for 10 minutes and 1 hour. Whole cell lysates were electrophoresed, blotted onto filter membranes, and probed with primary antibodies against phospho-Akt, Akt, phospho-ERK, ERK, and GAPDH (E). Representative results from 3 separate experiments are shown. Bars, SD. *, $P < 0.05$.

NKPS cells, though these effects were weaker than in NUGC4 cells (data not shown).

Assays of the chemotactic responses of NUGC4 cells to EGFR ligands showed that these cells were highly responsive to amphiregulin (100 ng/mL), HB-EGF (1 ng/mL), and CXCL12 (100 ng/mL), with the strongest responses to CXCL12. Furthermore, the neutralizing anti-EGFR antibody cetuximab significantly inhibited the chemotactic responses of NUGC4 cells to these EGFR ligands (Fig. 1D).

We also tested the effects of EGFR ligands on the phosphorylation of Akt and ERK, molecules involved in cell survival signaling (20). We found that both amphiregulin and HB-EGF rapidly and strongly enhanced the phosphorylation of Akt and ERK in NUGC4 cells (Fig. 1E), occurring as soon as 10 minutes after exposure.

HB-EGF markedly enhances migration and proliferation of human normal fibroblasts, accompanied by increased phosphorylation of ERK and Akt

Tumor-stroma interactions play an important role in tumor development and progression, and stromal fibroblasts are frequently associated with cancer progression. When we tested the effects of amphiregulin, HB-EGF, and CXCL12, on the migration and proliferation of primary cultured human normal fibroblasts, we found that HB-EGF, but not amphiregulin, strongly enhanced the proliferation of human fibroblasts, up to 1.5-fold, and that this proliferation was slightly inhibited by cetuximab (Fig. 2A). Interestingly, human fibroblasts showed marked chemotactic responses to HB-EGF, but not to CXCL12 or amphiregulin, with this chemotaxis significantly inhibited by

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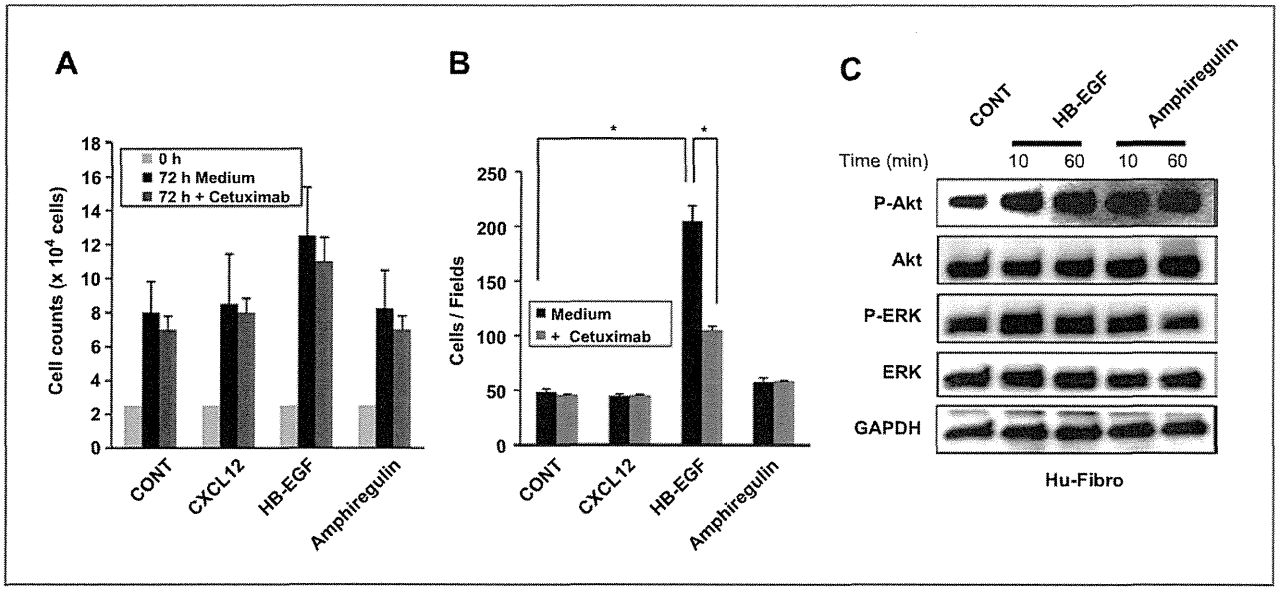

Figure 2. HB-EGF induces chemotactic response and cell proliferation, and rapid phosphorylation of Akt and ERK, in human normal fibroblasts. A, HB-EGF enhances proliferation of human normal fibroblasts. Normal human fibroblasts, seeded at 2.5×10^4 cells per well in 6-well plates, were stimulated with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 72 hours. HB-EGF stimulation resulted in an up to 1.5-fold increase in the number of human fibroblasts, compared with control or amphiregulin treated cells. Representative results from 1 of 3 independent experiments are shown. B, HB-EGF enhances migration of human normal fibroblasts. Induction of human fibroblast migration by CXCL12, HB-EGF, and amphiregulin. Neutralizing anti-EGFR antibody significantly blocked the enhancing effects of HB-EGF on migration of human normal fibroblasts. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$. C, HB-EGF enhances Akt and ERK phosphorylation of human normal fibroblasts. Immunoblotting analysis of the phosphorylation of Akt and ERK. Human normal fibroblasts were seeded in 10-cm dishes and stimulated with HB-EGF (1 ng/mL) or amphiregulin (100 ng/mL) for 10 minutes and 1 hour. Whole cell lysates were electrophoresed, blotted onto filter membranes, and probed with primary antibodies against phospho-Akt, Akt, phospho-ERK, ERK, and GAPDH. Representative results from 1 of 3 independent experiments are shown.

cetuximab (Fig. 2B). Moreover, stimulation of human fibroblasts with both HB-EGF and amphiregulin rapidly and strongly increased the phosphorylation of Akt and ERK (Fig. 2C).

Taken together, these findings showed that amphiregulin more effectively enhanced the proliferation of NUGC4 cells than HB-EGF, whereas HB-EGF more strongly enhanced human fibroblast migration than amphiregulin. These results strongly suggest that the EGFR ligands, amphiregulin and HB-EGF, both of which are abundant in malignant ascitic fluids, promote tumor progression driven by the tumor microenvironment during peritoneal dissemination.

Expression of EGFR, ErbB4, and CXCR4 in human gastric cancer cell lines and primary gastric tumors with peritoneal carcinomatosis

Amphiregulin and HB-EGF show different biological activities in target cells (21), with amphiregulin mainly stimulating cell proliferation (22) and HB-EGF stimulating chemotaxis (23). Amphiregulin binds to ErbB1 (EGFR), whereas HB-EGF binds to both ErbB1 (EGFR) and ErbB4 (24). To determine the molecular basis underlying these functional differences, we assayed the expression of EGFR, ErbB4, and CXCR4 in human gastric cancer cell lines and primary tumors. Western blotting using antibodies specific to EGFR showed that 4 of 5 human gastric cancer cell lines (MKN28, MKN45, NKPS, and NUGC4) expressed high levels of EGFR protein, whereas the fifth (KKLS) expressed little EGFR protein (Fig. 3A). We also found that normal human fibroblasts, which are present in the tumor microenvironment of the stomach and peritoneal cavity, express high levels of EGFR protein (Fig. 3A). RT-PCR showed similar results for EGFR mRNA (data not shown). When we assayed ErbB4 expression in these cell lines and primary tumors (Fig. 3A), we found that NKPS cells expressed a low amount, whereas MKN28, MKN45, KKLS, and NUGC4 cells essentially expressed none. In contrast, normal human fibroblasts expressed high levels of ErbB4 protein (Fig. 3A). NUGC4 and NKPS cells, which are highly efficient in generating malignant ascites in nude mice on i.p. inoculation, selectively expressed CXCR4 mRNA and protein (5). In particular, NUGC4 cells, which express high amounts of CXCR4, as well as EGFR mRNA and protein, disseminate soon after inoculation into the abdominal cavity of nude mice and form massive amounts of bloody ascitic fluid (5). Immunohistochemical staining showed that, of 29 primary stage IV gastric carcinomas with peritoneal carcinomatosis, 23 (79%) and 22 (76%) were positive for EGFR and CXCR4, respectively (Fig. 3B), whereas none was positive for ErbB4 expression (Supplementary Fig. S1).

We also used specific siRNA for EGFR and ErbB4 to knock down the expression of these proteins (Fig. 3C) and examined the effects of the amphiregulin

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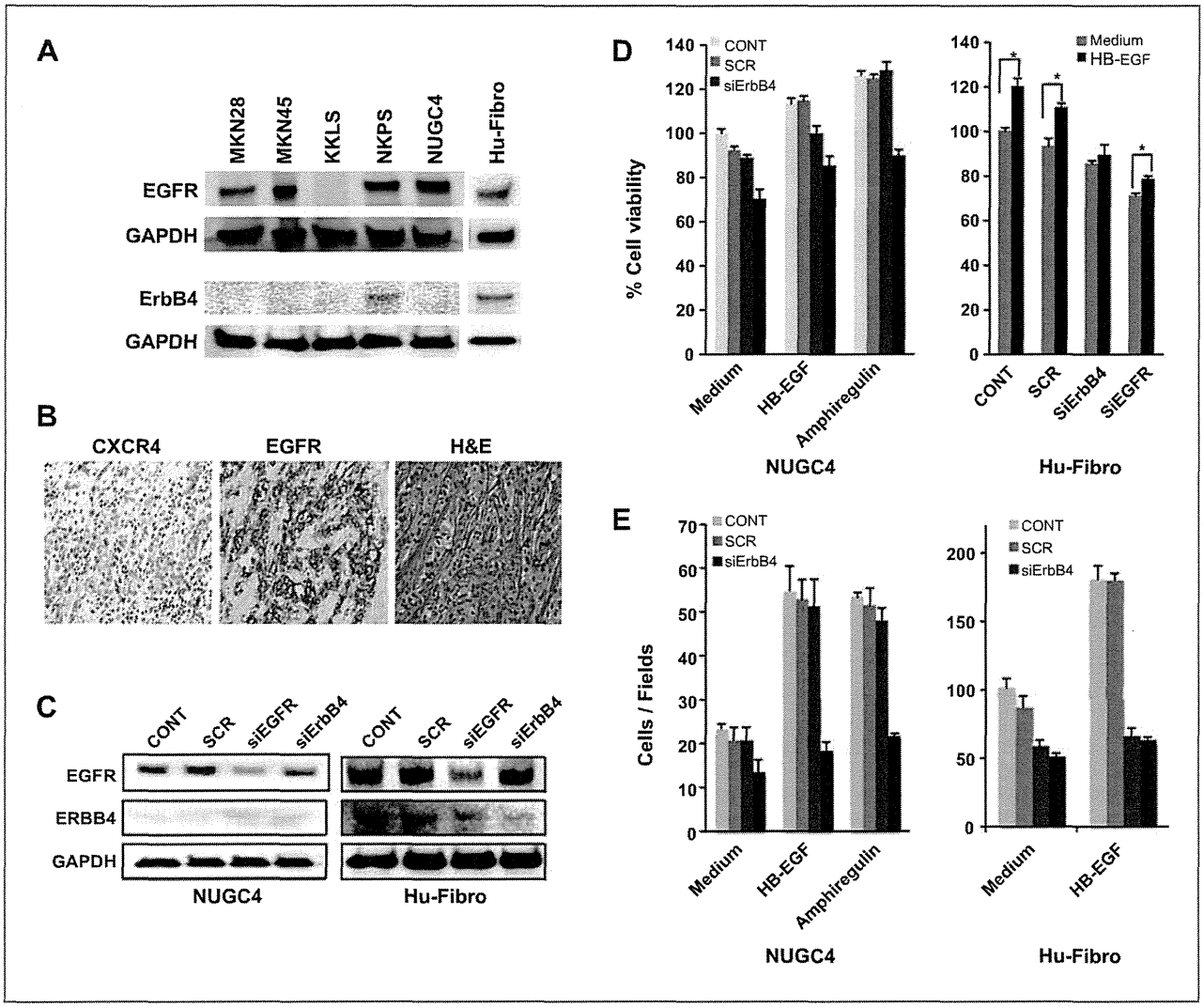

Figure 3. Expression of EGFR and ErbB4 in human gastric cancer cell lines and normal human fibroblasts. A, Western blotting analyses of EGFR and GAPDH expression in 5 human gastric cancer cell lines (MKN28, MKN45, KKLS, NKPS, and NUGC4) and normal human fibroblasts. The levels of EGFR protein in each sample were normalized relative to the levels of GAPDH protein. Representative results from 3 independent experiments are shown. B, immunohistochemical staining of CXCR4 and EGFR in human primary gastric tumors. Anti-CXCR4, anti-EGFR, and H&E staining of a representative CXCR4- and EGFR-positive gastric cancer. CXCR4 and EGFR were strongly localized in the membranes but weakly expressed in the cytoplasm of these cells. Original magnification, ×160. (C) Effects of EGFR and ErbB4 siRNA on NUGC4 cells and human normal fibroblasts. EGFR and ErbB4 knockdown was confirmed by Western blotting. Each experiment was done at least in triplicate and 3 times independently. D, knockdown of EGFR effectively reduced the amphiregulin-induced enhancement of NUGC4 cell proliferation, whereas knockdown of ErbB4 effectively reduced the HB-EGF enhancement of fibroblast proliferation. Cell proliferation was measured using the MTT dye reduction method. The percentage of growth is shown relative to untreated controls. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$. E, knockdown of EGFR effectively reduced the enhancing effects of amphiregulin and HB-EGF on NUGC4 cell migration, whereas knockdown of either EGFR or ErbB4 effectively reduced the enhancing effects of HB-EGF on fibroblast migration. Columns, mean number of migrated cells per field from a representative experiment in triplicate and repeated thrice; bars, SD. *, $P < 0.05$.

and HB-EGF on the proliferation and migration of NUGC4 cells and fibroblasts (Fig. 3D and E). In NUGC4 cells, knockdown of EGFR effectively reduced the enhancing effects of amphiregulin on proliferation compared with that of ErbB4. By sharp contrast, in fibroblasts, knockdown of ErbB4 effectively reduced the enhancing effects of HB-EGF on proliferation compared with that of EGFR (Fig. 3D). We also found that knockdown of EGFR, but not ErbB4, effectively reduced the enhancing effects of amphiregulin and HB-EGF on NUGC4 cell chemotaxis. Interestingly, in fibroblasts, knockdown of either EGFR or ErbB4 effectively reduced the chemotaxis enhancing effects of HB-EGF (Fig. 3E).

Collectively, these results indicate that, in NUGC4 cells, EGFR may be the predominant receptor mediating proliferation and migration induced by amphiregulin and HB-EGF. In fibroblasts, however, ErbB4 may be the predominant receptor mediating HB-EGF induced proliferation,

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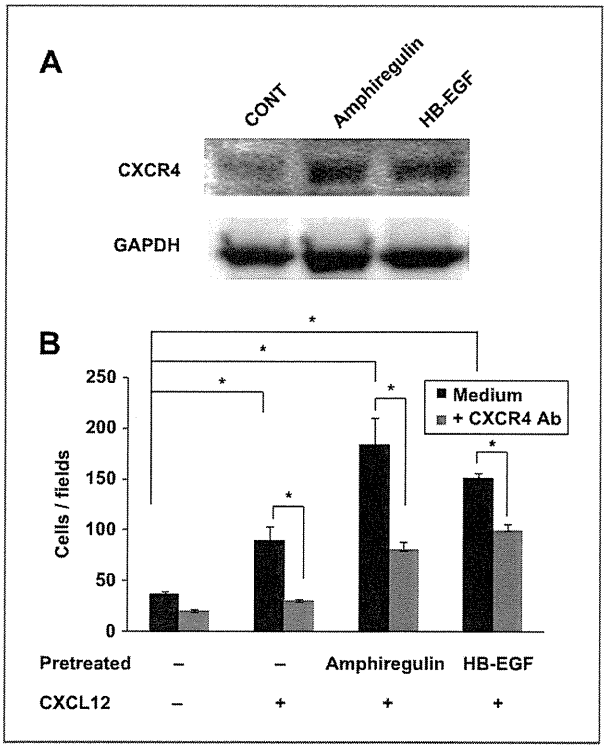

Figure 4. Amphiregulin and HB-EGF induce functional CXCR4 expression in NUGC4 cells. A, Western blot analysis of total proteins extracted from cells treated with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 1 hour and incubated with anti-CXCR4 antibody. B, pretreatment with amphiregulin (100 ng/mL) or HB-EGF (1 ng/mL) for 24 hours significantly enhanced the migration of CXCR4-expressing NUGC4 cells. Representative results from 1 of 3 independent experiments are shown. Bars, SD. *, $P < 0.05$.

whereas both ErbB4 and EGFR are important for HB-EGF induced migration.

Amphiregulin and HB-EGF markedly enhance functional CXCR4 expression in NUGC4 cells

Because CXCR4 expression by primary gastric cancers promotes the development of peritoneal carcinomatosis (5), we examined whether amphiregulin and HB-EGF modulate CXCR4 expression in NUGC4 cells. We found that both ligands strongly increased CXCR4 protein concentrations in NUGC4 cells (Fig. 4A). We also found that NUGC4 cells pretreated with both ligands for 24 hours showed a significant and much higher chemotactic response to CXCL12 than non-pretreated NUGC4 cells (Fig. 4B). Tumor environmental stimulation, such as hypoxia and CXCL12 (SDF-1α), a growth factor abundantly produced in the peritoneal cavity, have been found to induce CXCR4 protein expression in cancer cells (25–27). These findings suggest that amphiregulin and HB-EGF may upregulate functional CXCR4 expression as well as the proliferation and/or survival of CXCR4-expressing gastric cancer cells in the peritoneal cavity, leading to the survival and peritoneal dissemination of gastric cancers.

TACE (ADAM17) cleaves amphiregulin produced by CXCR4-expressing gastric cancer NUGC4 cells

Using enzyme-linked immunosorbent assays (ELISA), we found that amphiregulin was present in the conditioned media of CXCR4-expressing NUGC4 cells and human fibroblasts (Fig. 5A). In contrast, none of these CXCR4-expressing gastric cancer cell lines and fibroblasts produced EGF, TGFα, or HB-EGF (data not shown). Interestingly, HB-EGF enhanced amphiregulin secretion from NUGC4 cells, particularly in the presence of CXCL12, but not from human fibroblasts. We also confirmed that costimulation with HB-EGF and CXCL12 resulted in a marked increase in amphiregulin production by all CXCR4-expressing gastric cancer cell lines, including NUGC4 (data not shown). Because HB-EGF enhanced amphiregulin secretion from NUGC4 cells, we also determined whether HB-EGF acts by increasing amphiregulin shedding alone or on ErbB signaling in NUGC4 cells. We confirmed that neutralizing anti-amphiregulin antibody could not block the ability of HB-EGF to enhance the proliferation of NUGC4 cells (Supplementary Fig. S2).

TACE has been shown to be a key regulator of amphiregulin cleavage (28). Because TACE was expressed in NUGC4 cells (Fig. 5B), we assessed whether TACE is the key regulator of these endogenously produced growth factors. We therefore used siRNA to knock down the expression of TACE and measured growth factor secretion by transfected cells (Fig. 5B and C). We found that amphiregulin shedding by NUGC4 cells was proportional to their level of TACE expression. Furthermore, Western blotting showed that the expression of TACE was highly increased in NUGC4 cells stimulated with amphiregulin and HB-EGF, as well with CXCL12 (Fig. 5D), suggesting that TACE is the primary regulator of amphiregulin secretion in NUGC4 cells. Collectively, these findings suggest that the potent proliferative EGFR ligands, amphiregulin and HB-EGF, both of which are present in ascitic fluids, synergize with CXCL12 in promoting the dissemination of CXCR4-expressing gastric cancer cells in an autocrine and/or paracrine manner.

Cetuximab, an EGFR monoclonal antibody, inhibits ascites formation by NUGC4 cells in nude mice

On i.p. inoculation into nude mice, CXCR4-expressing NUGC4 cells efficiently produce peritoneal carcinomatosis (5). We utilized this model to test whether EGFR blockade by cetuximab (19) affects the development of experimental peritoneal carcinomatosis in mice. Forty days after tumor inoculation, mice administered cetuximab showed consistently smaller disseminated tumors in the greater omentum and mesenterium than did mice administered control IgG (Fig. 6A). Furthermore, treatment with cetuximab significantly decreased the mean volume of ascitic fluid (0.24 ± 0.54 mL vs. 5.48 ± 1.86 mL, $P < 0.01$; Fig. 6B), resulting in significantly longer survival, than control IgG-treated mice (Fig. 6C).

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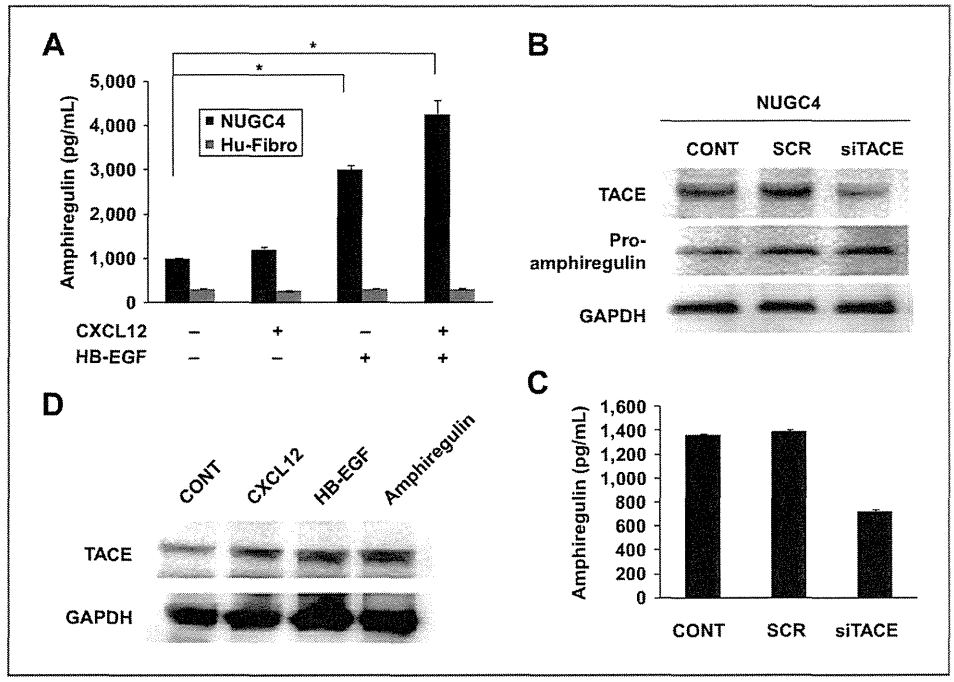

Figure 5. Presence of an autocrine amphiregulin loop in CXCR4-expressing NUGC4 cells and fibroblasts. A, synergistic production of amphiregulin by HB-EGF and CXCL12 proteins in NUGC4 cells, but not in fibroblasts. Bars, SD. *, $P < 0.05$. B and C, ELISA analysis of amphiregulin shedding in NUGC4 cells transfected with TACE siRNAs. Ligand shedding was proportional to the level of TACE expression. D, Western blot analysis showing strongly increased TACE protein levels in NUGC4 cells stimulated with amphiregulin (100 ng/mL), HB-EGF (1 ng/mL), and CXCL12 (100 ng/mL) for 1 hour. Representative results from 1 of 3 independent experiments are shown.

Discussion

We have shown here that the EGFR ligands amphiregulin and HB-EGF play important roles in the development of peritoneal carcinomatosis in patients with gastric cancer (Supplementary Fig. S3). In addition to CXCL12, amphiregulin and HB-EGF are abundantly present in malignant ascites from gastric cancer patients, whereas EGF and TGFα are not. Amphiregulin, HB-EGF, and CXCL12 stimulate CXCR4-expressing gastric cancer cells to migrate into the peritoneal cavity, as well as their proliferation. HB-EGF and CXCL12 cooperate and stimulate TACE expression, thereby upregulating amphiregulin secretion from gastric cancer cells. Secreted amphiregulin further stimulates the migration, proliferation, and amphiregulin production of gastric cancer cells in an autocrine and/or paracrine manner.

Gastric cancer and ovarian cancer are 2 major causes of malignant ascites (5, 29). In ovarian cancer, only HB-EGF is present at higher concentrations in malignant than in nonmalignant ascites (29), whereas, in gastric cancer, both HB-EGF and amphiregulin are present at higher concentrations in malignant than in nonmalignant ascites. Interestingly, though HB-EGF concentrations were about 10-fold higher in malignant ascites from ovarian than from gastric cancer patients, amphiregulin concentrations were 10-fold higher in malignant ascites from gastric than from ovarian cancer patients (3,055 pg/mL vs. 200 pg/mL; ref. 29). Moreover, amphiregulin enhanced the proliferation and/or survival signals (phosphorylated ERK and Akt) of CXCR4-expressing NUGC4 cells more strongly than did HB-EGF. These findings suggest that, though both amphiregulin and HB-EGF are involved, amphiregulin may be the predominant EGFR ligand in the development of malignant ascites in gastric cancer. Although HB-EGF did not enhance amphiregulin-stimulated proliferation or migration (data not shown) *in vitro*, it enhanced amphiregulin secretion from NUGC4 cells, particularly in the presence of CXCL12, suggesting that HB-EGF may cooperate with amphiregulin and facilitate malignant ascites formation *in vivo*.

Recent studies have revealed the process underlying the production of EGFR ligands. Amphiregulin and HB-EGF are synthesized as type I transmembrane protein precursors and expressed on the cell surface as pro-amphiregulin and pro–HB-EGF, respectively (30, 31). Shedding of pro-amphiregulin and pro–HB-EGF results in the plasma-membrane-anchored remnant carboxyl-terminal fragments amphiregulin-CTF and HB-EGF-CTF, as well as soluble EGFR ligands (32, 33). Interestingly, cleavage of membrane-anchored amphiregulin and HB-EGF induces translocation of their CTFs from the plasma membrane to the nucleus and regulates the cell cycle and/or global transcription (34, 35).

Amphiregulin is reported crucial in several types of tumors, including breast cancer, colon cancer, and hepatocellular carcinoma (11, 12). Although the expression of amphiregulin mRNA and protein has been assessed in gastric cancers (36), its role in gastric cancer pathogenesis, especially in the development of peritoneal carcinomatosis, remains poorly understood. We found that amphiregulin was present in malignant ascites from gastric cancer patients and that it could stimulate the proliferation and migration of gastric cancer cells. Amphiregulin is synthesized as a transmembrane precursor and is proteolytically processed to its mature secreted form (32) by TACE, also called disintegrin and metalloprotease-17

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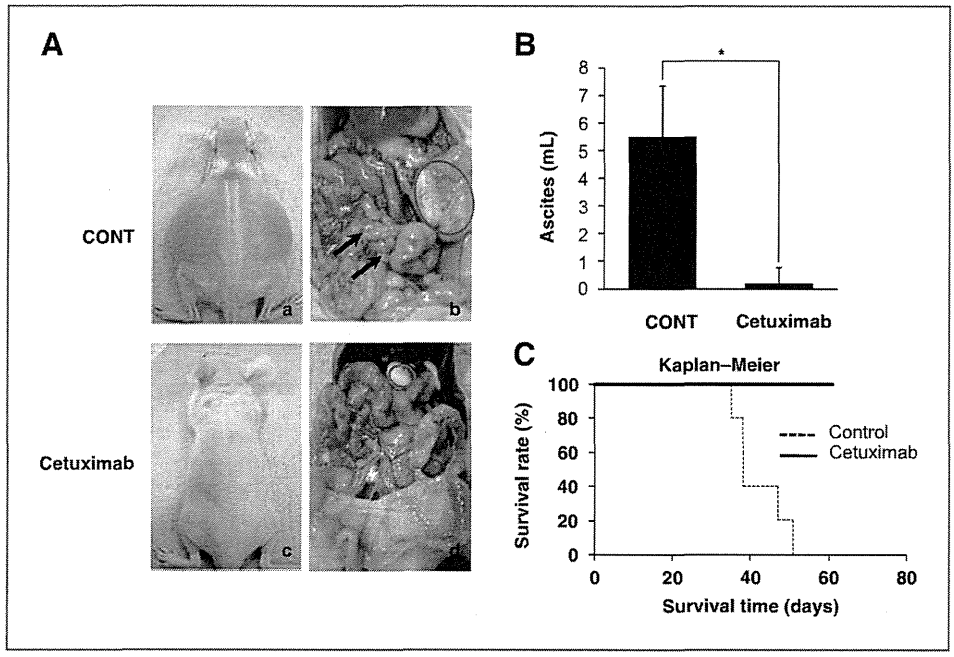

Figure 6. Cetuximab inhibits experimental peritoneal carcinomatosis. A, beginning 3 days after inoculation with CXCR4-expressing NUGC4 cells (1×10^6 per mouse), mice were injected intraperitoneally with normal IgG (a and b; 2 mg/kg) or cetuximab (c and d; 2 mg/kg) twice weekly for 2 weeks, and results were assessed 40 days later. b and d, omental tumors in the abdominal cavity. Arrows indicate disseminated tumors on the mesenterium in the peritoneal cavity. B, reduction of ascitic fluid formation by cetuximab. Representative results of 2 experiments (5 animals per group per experiment) are shown. Bars, SD. *, $P < 0.05$. C, real survival curves according to the Kaplan–Meier method. No cetuximab-treated mouse died for up to 60 days after tumor inoculation, whereas all mice treated with normal IgG were dead within 50 days.

(ADAM-17; refs. 37, 38). Interestingly, we found that HB-EGF and CXCL12 enhanced TACE expression and amphiregulin protein secretion from CXCR4-expressing NUGC4 cells and that amphiregulin itself enhanced TACE expression. These findings suggest that TACE-dependent amphiregulin shedding by NUGC4 cells is regulated in both an autocrine and a paracrine manner.

HB-EGF, which was initially reported to be produced predominantly by macrophages (39), was later found to be expressed in ovarian cancers, with ectodomain shedding of pro–HB-EGF shown to be critical for tumor formation (13). We found that malignant ascites from gastric cancer patients contained high concentrations of the soluble form of HB-EGF. Because HB-EGF was not detected in the conditioned media of any of the human gastric cancer cell lines tested, and pro–HB-EGF was not detected in NUGC4 cells (data not shown), HB-EGF may be produced predominantly by macrophages, especially regulatory macrophages, in the peritoneal cavity (40).

Interestingly, the 2 EGFR ligands, amphiregulin and HB-EGF, have shown different biological activities in target cells, with amphiregulin mainly stimulating cell proliferation (22) and HB-EGF mainly stimulating chemotaxis (23). Similarly, we found that amphiregulin more effectively cell enhanced NUGC4 cell proliferation than HB-EGF, whereas HB-EGF more strongly induced the migration of human normal fibroblasts than amphiregulin. These findings may be due to the preference of these ligands for certain receptors. Amphiregulin binds to ErbB1 (EGFR), whereas HB-EGF binds to both ErbB1 (EGFR) and ErbB4 (24). NUGC4 cells express EGFR, but little ErbB4, whereas normal human fibroblasts express both ErbB4 and EGFR. Furthermore, knockdown of EGFR in NUGC4 cells effectively reduced the amphiregulin-enhanced proliferation compared with ErbB4, whereas knockdown of ErbB4 in fibroblasts effectively reduced the HB-EGF-enhanced proliferation compared with EGFR. Furthermore, knockdown of EGFR, but not ErbB4, effectively reduced the chemotactic effects of amphiregulin and HB-EGF in NUGC4 cells, whereas knockdown of either EGFR or ErbB4 effectively in fibroblasts reduced the chemotactic effects of HB-EGF. These results suggest that the amphiregulin enhanced proliferation of NUGC4 cells is mediated predominantly by EGFR, whereas HB-EGF enhancement of fibroblast chemotaxis is mediated by both EGFR and ErbB4.

Tumor-stroma interactions play a significant role in tumor development and progression (41), and stromal fibroblasts are frequently associated with cancer progression at both primary and metastatic sites. In particular, scirrhous gastric cancers, diffusely infiltrating cancers, Borrmann type 4, also known as linitis plastica-type cancer, are characterized by rapid cancer cell infiltration and proliferation, accompanied by extensive stromal fibrosis (42). Peritoneal carcinomatosis occurs frequently in patients with scirrhous gastric cancer. To our knowledge, this study was the first to show that HB-EGF stimulated fibroblasts

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present in the stomach enhanced cell migration and proliferation, accompanied by increased phosphorylation of ERK and Akt. Therefore, HB-EGF produced in the peritoneal cavity may facilitate the accumulation and proliferation of fibroblasts and may induce fibrosis associated with peritoneal carcinomatosis in patients with scirrhous gastric cancer. It will therefore be important to clarify the mechanisms that regulate the expression of the soluble form of HB-EGF and the biological significance of stromal fibroblasts in gastric cancer.

We previously reported that the CXCR4/CXCL12 axis is involved in the chemotactic response, proliferation, and survival of gastric cancers in the peritoneal cavity (5). The results presented here extend our knowledge, showing that the EGFR/EGFR ligand axis is also important in these events and that these 2 axes interact with each other. CXCL12 stimulated the expression of TACE and enhanced the secretion of amphiregulin by CXCR4-expressing NUGC4 cells, especially in combination with HB-EGF. In NUGC4 cells, the EGF ligands amphiregulin and HB-EGF more potently stimulated proliferation than did CXCL12, whereas CXCL12 more potently enhanced motility. Because all 3 proteins, amphiregulin, HB-EGF, and CXCL12, are present in malignant ascites, further studies exploring the interaction between the CXCR4/CXCL12 and the EGFR/EGFR ligand axes may provide additional insights into the pathogenesis of peritoneal carcinomatosis of gastric cancer.

Cetuximab is a specific anti-EGFR monoclonal antibody, which is widely used to treat patients with several types of solid tumors, including colon, head and neck, and non–small cell lung cancers (43–45). We confirmed that cetuximab efficiently blocked the proliferation and migration of NUGC4 cells stimulated by amphiregulin and HB-EGF, indicating its anti-EGFR activity. Although cetuximab did not inhibit HB-EGF-induced fibroblast proliferation, this was not unexpected, because HB-EGF stimulated fibroblast proliferation predominantly through ErbB4. Importantly, cetuximab showed dramatic therapeutic efficacy in an experimental peritoneal carcinomatosis model in nude mice, as shown by the reduction in volume of ascitic fluids, the inhibition of disseminated tumor growth, and the prolongation of survival. These results suggest that cetuximab may be therapeutically useful for controlling peritoneal carcinomatosis of gastric cancer patients if amphiregulin and HB-EGF are highly expressed in their malignant ascites.

In conclusion, we have shown here that the EGFR ligands amphiregulin and HB-EGF collaborate with the CXCR4/CXCL12 axis in promoting peritoneal carcinomatosis by stimulating gastric cancer cell migration, proliferation, and survival. EGFR activation mediated by these EGFR ligands is a novel mechanism underlying the development of peritoneal carcinomatosis from gastric cancer. Therefore, inhibition of the EGFR/EGFR ligand axis may be a useful strategy for treating patients with gastric cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

Preclinical Development

E7080 Suppresses Hematogenous Multiple Organ Metastases of Lung Cancer Cells with Nonmutated Epidermal Growth Factor Receptor

Hirokazu Ogino[1], Masaki Hanibuchi[1], Soji Kakiuchi[1,2], Van The Trung[1], Hisatsugu Goto[1], Kenji Ikuta[1], Tadaaki Yamada[1,4], Hisanori Uehara[3], Akihiko Tsuruoka[5], Toshimitsu Uenaka[5], Wei Wang[4], Qi Li[4], Shinji Takeuchi[4], Seiji Yano[4], Yasuhiko Nishioka[1], and Saburo Sone[1,2]

Abstract

While epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors improve the prognosis of patients with *EGFR* mutant lung cancer, the prognosis of patients with nonmutant *EGFR* lung cancer, especially those with metastases, is still extremely poor. We have assessed the therapeutic efficacy of E7080, an orally available inhibitor of multiple tyrosine kinases including VEGF receptor 2 (VEGFR-2) and VEGFR-3, in experimental multiple organ metastasis of lung cancer cell lines without *EGFR* mutations. E7080 markedly inhibited the *in vitro* proliferation of VEGF-stimulated microvascular endothelial cells. Intravenous inoculation into natural killer cell–depleted severe combined immunodeficient mice of the small cell lung cancer cell lines H1048 (producing low amounts of VEGF) and SBC-5 (producing intermediate amounts of VEGF) resulted in hematogenous metastases into multiple organs, including the liver, lungs, kidneys, and bones, whereas intravenous inoculation of PC14PE6, a non–small cell lung cancer cell line producing high amounts of VEGF, resulted in lung metastases followed by massive pleural effusion. Daily treatment with E7080 started after the establishment of micrometastases significantly reduced the number of large (>2 mm) metastatic nodules and the amount of pleural effusion, and prolonged mouse survival. Histologically, E7080 treatment reduced the numbers of endothelial and lymph endothelial cells and proliferating tumor cells and increased the number of apoptotic cells in metastatic nodules. These results suggest that E7080 has antiangiogenic and antilymphangiogenic activity and may be of potential therapeutic value in patients with nonmutant *EGFR* lung cancer and multiple organ metastases. *Mol Cancer Ther; 10(7); 1218–28. ©2011 AACR.*

Introduction

Lung cancer is one of the most prevalent malignancies and the leading cause of cancer-related deaths worldwide (1). The high mortality of this disease is predominantly due to the high metastatic potential of lung cancer. Although platinum-based cytotoxic chemotherapy is standard treatment for both small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC) with metastases (2), the median survival of these patients is only about 12 to 14 months. Recently, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors have been shown to prolong progression-free survival of lung cancer patients with tumors containing mutated *EGFR*, extending the median survival of these patients to 22 to 30 months (3, 4). Nevertheless, the prognosis of patients with metastatic SCLC and NSCLC with nonmutant *EGFR* lung cancer has not yet been improved (5).

Angiogenesis is essential for tumor enlargement and metastasis and is regulated by proangiogenic and antiangiogenic molecules (6). VEGF-related molecules (VEGF, VEGF-B, VEGF-C, VEGF-D, and placental growth factor) and receptors (VEGFR-1, VEGFR-2, and VEGFR-3) play pivotal roles in angiogenesis and lymphangiogenesis (7). Binding of VEGF to VEGFR-2 is the critical signal for tumor angiogenesis, as well as inducing vascular hyperpermeability and promoting the production of pleural effusion and ascites (8, 9). VEGF-C and VEGF-D activate VEGFR-3 and are considered lymphangiogenic factors, although the fully processed form of VEGF-C also

Authors' Affiliations: Departments of [1]Respiratory Medicine and Rheumatology, [2]Medical Oncology, and [3]Molecular and Environmental Pathology, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima; [4]Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Ishikawa; and [5]Discovery Department, Oncology PCU, Eisai Product Creation Systems, Research Laboratories, Eisai Co. Ltd., Ibaraki, Japan

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Corresponding Author: Saburo Sone, Department of Respiratory Medicine and Rheumatology, Institute of Health Biosciences, University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Phone: 81-88-633-7127; Fax: 81-88-633-2134; E-mail: ssone@clin.med.tokushima-u.ac.jp

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activates VEGFR-2 (10). In addition, the binding of VEGF-C to VEGFR-3 has been found to promote intratumoral lymphangiogenesis and lymph node metastasis in a preclinical study (11).

The anti-VEGF monoclonal antibody, bevacizumab, has been used successfully to treat patients with several malignant diseases including nonsquamous NSCLC (12, 13). However, the combination of bevacizumab and cytotoxic chemotherapy has been found to have marginal effects on patient survival (12, 14), suggesting the need for novel therapeutic modalities.

E7080 is an orally active inhibitor of VEGFR-2 and VEGFR-3, with additional activity against other receptor tyrosine kinases, including fibroblast growth factor receptors (FGFR), platelet-derived growth factor receptors (PDGFR), and c-Kit (15). E7080 shows potent antitumor effects in xenograft models of various types of tumors by inhibiting angiogenesis, especially through VEGFR-2/3 suppression (16, 17). We have tested the therapeutic efficacy of E7080 against lung cancer cell lines expressing wild-type EGFR, using *in vivo* experimental metastasis models.

Materials and Methods

Cell lines

The human SCLC cell line SBC-5 and a human NSCLC cell line PC14PE6 were kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan; ref. 18) in 2006 and Dr. I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX; ref. 9) in 1999, respectively. The human SCLC cell line H1048 and human NSCLC cell line PC-9 were purchased from American Type Culture Collection in 2004 and Immuno-Biological Laboratories Co.; ref. 19) in 2007, respectively. Flow cytometric analyses revealed that H1048, PC14PE6, and SBC-5 cells showed high, intermediate, and very low wild-type EGFR protein expression, respectively (data not shown). SBC-5 cells were maintained in Eagle's Minimum Essential Media (MEM) and H1048, PC14PE6, and PC-9 cells were maintained in RPMI 1640 medium, each supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (50 μg/mL). Human dermal microvascular endothelial cells (HMVEC) were purchased from KURABO in 2007 and maintained in HuMedia-MvG with growth supplements (KURABO). All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for *Mycoplasma* with the use of a MycoAlert Mycoplasma Detection Kit (Lonza). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagents

4-[3-Chloro-4-(*N'*-cyclopropylureido)phenoxy]-7-methoxyquinoline-6-carboxamide (E7080) was synthesized by Eisai Co. Ltd., as described (15, 16). For *in vitro* experiments, a stock solution of E7080 (10 mmol/L) was prepared in dimethyl sulfoxide, stored at −20°C, and diluted with culture media before use. For *in vivo* experiments, E7080 was dissolved in distilled water and stored until use at 4°C. Recombinant human VEGF165 and recombinant human basic fibroblast growth factor (bFGF) were purchased from R&D Systems. An anti-mouse interleukin 2 receptor β-chain monoclonal antibody, TM-β1 (IgG2b), was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan; ref. 20).

Expression of VEGF, VEGF-C, VEGF-D, and VEGFR mRNAs

Expression of VEGF, VEGF-C, VEGF-D, and VEGFR mRNAs was measured by reverse transcriptase (RT) PCR. Total cellular RNA was isolated using RNeasy Mini kits and RNase-free DNase kits (Qiagen) according to the manufacturer's protocols. Total RNAs were reversely transcribed using an Omniscript RT kit (Qiagen). PCRs were carried out using Ex Taq Hot Start Version (Takara) and the primers are shown in Supplementary Table S1. RT-PCR products were electrophoresed on agarose gels, and the bands were visualized by ethidium bromide staining.

VEGF production

Tumor cells (1×10^6) were cultured in RPMI 1640 medium with 10% FBS for 24 hours. The cells were washed with PBS and incubated for 48 hours in RPMI 1640 medium with 10% FBS. The culture medium was harvested and centrifuged, and the supernatants were stored at −70°C until analysis. VEGF concentration was assayed by ELISA as described by the manufacturer (R&D Systems). All samples were run in triplicate, and each assay was conducted 3 times independently.

Flow cytometric analysis

HMVECs and tumor cells were harvested and resuspended in PBS. After 2 washes with PBS, the cells were incubated for 45 minutes at 4°C with phycoerythrin (PE)-labeled anti VEGFR-1, VEGFR-2, and VEGFR-3 antibodies or with PE-labeled mouse IgG1 antibody. The intensity of fluorescence was measured by flow cytometric analysis using FACScan (Becton Dickinson).

Cell proliferation assay

Cell proliferation was measured using the MTT dye reduction method (21). Briefly, tumor cells (2×10^3 cells per 100 μL per well), plated in triplicate in 96-well plates, were incubated in medium containing 10% FBS for 24 hours. HMVECs (5×10^3 cells per 100 μL per well), plated in triplicate in 96-well plates precoated with 1.5% gelatin, were incubated in Eagle's MEM containing 5% FBS for 24 hours. Tumor cells were incubated with several concentrations of E7080 for a further 72 hours. HMVECs were incubated for 72 hours with E7080 in the presence or absence of VEGF or bFGF. To each well was added 50 μL of MTT solution (2 mg/mL; Sigma), followed by incubation for a further 2 hours. The media containing

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MTT solution were removed, and the dark blue crystals in each well were dissolved in 100 μL dimethyl sulfoxide. The absorbance was measured at test and reference wavelengths of 550 and 630 nm, respectively.

To determine the bioactivity of VEGF produced by the tumor cells, WST assays were conducted using Cell Counting Kit-8 (Dojindo). Briefly, HMVECs (5×10^3 cells per 100 μL per well) were plated in triplicate in 96-well plates precoated with 1.5% gelatin. After 24 hours, HMVECs were incubated with cell culture supernatants of tumor cells in the presence or absence of E7080 (10 nmol/L) for 72 hours. Following the addition of 10 μL of WST-8 reagents, the cells were incubated for a further 2 hours, and absorbance was measured at 450 nm and 630 nm.

Western blot analysis

HMVECs were cultured until confluence in EBM medium containing 0.5% FBS for 24 hours. Cells were treated with E7080 at indicated concentrations for 120 minutes, stimulated with VEGF165 (20 ng/mL) for 10 minutes, and collected in Cell Lysis Buffer (Cell Signaling Technology) containing 1 mmol/L phenylmethylsulfonylfluoride. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology). For Western blot analysis, 20 μg of total protein was resolved by SDS-PAGE (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Atto). After 3 washes, the membranes were incubated with Blocking One (Nacalai Tesque Inc.) for 1 hour at room temperature and incubated overnight at 4°C with 1:1,000 dilutions of primary antibodies to p-VEGFR-2 (Tyr 996; Cell Signaling Technology), VEGFR-2 (C-1158; Santa Cruz Biotechnology), phospho-p44/p42 MAPK (Thr202/Tyr204; Cell Signaling Technology), and p44/p42 MAPK (Cell Signaling Technology). After washing, the membranes were incubated for 2 hours at room temperature with species-specific horseradish peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescent substrate (Pierce Biotechnology).

Animals

Male severe combined immunodeficient mice (SCID) mice and athymic BALB/c nude mice, 5 to 6 weeks old, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout this study. All experiments were carried out in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

In vivo metastasis models

To facilitate metastasis formation, SCID mice were pretreated with anti-mouse interleukin 2 receptor β-chain antibody to deplete natural killer (NK) cells (20). Two days later, the mice were inoculated with SBC-5 or H1048 cells (1.0×10^6 per mouse) into the tail vein. Nude mice were intravenously inoculated via the tail vein with PC14PE6 cells (1.0×10^6 per mouse). As we reported previously, micrometastases were detected as early as 14 days after tumor cell inoculation (9). To evaluate the therapeutic efficacy of E7080 against established metastatic nodules, the mice were treated once daily with 1, 3, and 10 mg/kg/d E7080 by oral gavage, beginning 2 (SBC-5 and PC14PE6 cells) or 4 weeks (H1048 cells) after inoculation. Five (SBC-5 and PC14PE6 cells) or 8 weeks (H1048 cells) after tumor cell inoculation, the mice were anesthetized by intraperitoneal injection of pentobarbital, and radiographs were taken to determine bone metastases. The mice were killed humanely under anesthesia and the major organs were removed and weighed. The lungs were fixed in Bouin's solution (Sigma) for 24 hours. The number of metastatic colonies on the surface of the organs and the number of osteolytic lesions evident in radiograms were counted by 2 investigators independently (H. Ogino and M. Hanibuchi). To determine the effect of E7080 on survival, mice (10 per group) were treated once daily with 10 mg/kg/d E7080 from day 14 until they became moribund.

Immunohistochemical-immunofluorescent determination of endothelial cells, proliferating and apoptotic tumor cells, and VEGF production

Major organs containing metastases were fixed in 10% formalin and embedded in paraffin, or TissueTek optimum cutting temperature medium (Sakura) and frozen immediately. The paraffin-embedded tissues were used to quantitate *in vivo* cell proliferation using mouse anti-human Ki-67 mAb (MIBI; 1:50 dilution; DAKO), apoptosis using the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method with the Apoptosis Detection System (Promega), and VEGF production using mouse anti-human VEGF mAb (1:100 dilution; Pharmingen). To detect endothelial cells, frozen tissue sections (10 μm thick) were fixed with cold acetone and incubated with rat anti-mouse CD31/PECAM-1 monoclonal antibody (1:100 dilution; Pharmingen). To detect lymph endothelial cells, frozen sections were fixed with midl form and incubated with rabbit anti-mouse LYVE-1 monoclonal antibody (1:100 dilution; Abcam). Appropriate secondary antibodies conjugated with peroxidase and the 3,3′-diaminobenzidine tetrahydrochloride (DAB) Liquid System (DakoCytomation) was used to detect immunostaining. To detect CD31 and LYVE-1 simultaneously, frozen sections were fixed with midl form for 1 minute and incubated with rat anti-mouse CD31 and rabbit anti-mouse LYVE-1 antibodies, followed by incubation with anti-rat IgG conjugated to Alexa488 (green) and anti-rabbit IgG conjugated to Alexa594 (red; 1:100 dilution; Molecular Probes). In each analysis, the 5 areas containing the highest number of stained cells within a section were selected for histologic quantification under light microscopy or fluorescent microscopy with a 200-fold magnification. The results were independently evaluated by 2 investigators (H. Ogino and M. Hanibuchi).

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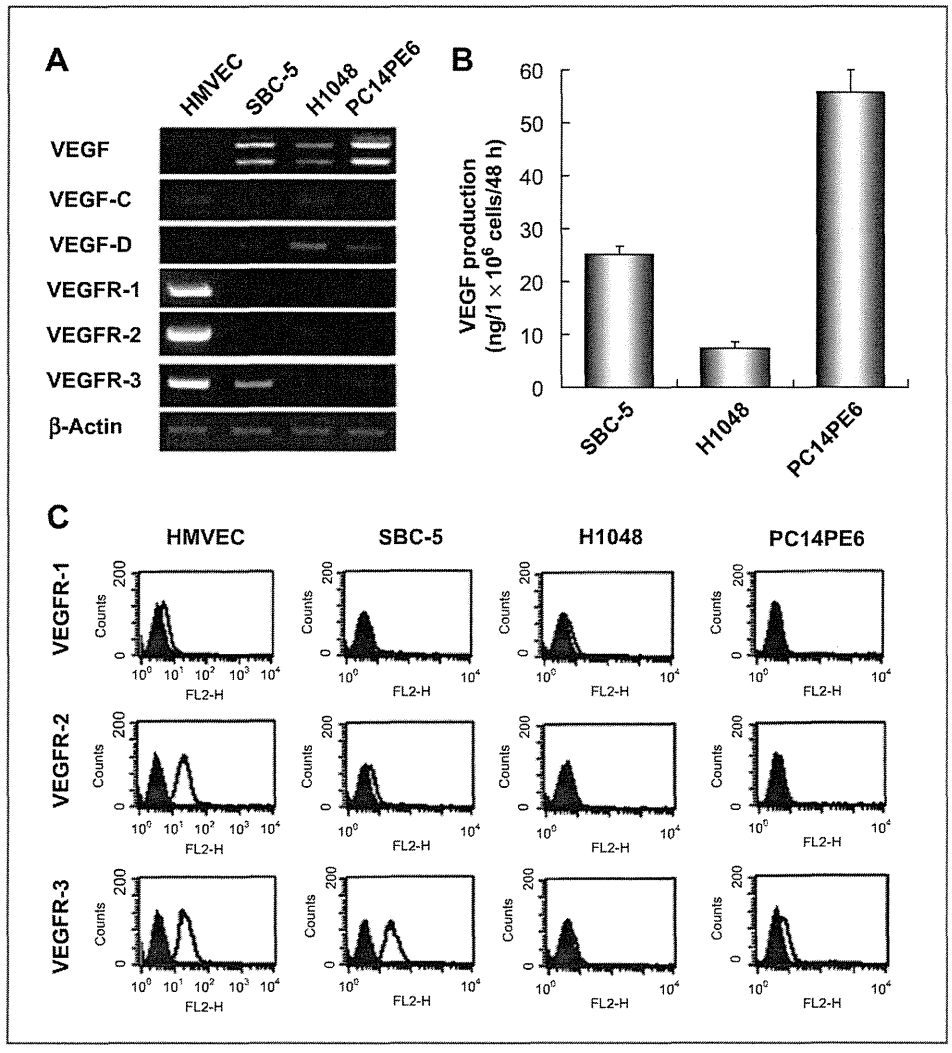

Figure 1. *In vitro* production of VEGF by lung cancer cell lines bearing nonmutant EGFR. A, expression of mRNAs encoding members of the VEGF and VEGFR families in human lung cancer cell lines and endothelial cells (HMVEC) bearing nonmutant EGFR as determined by RT-PCR. B, VEGF production by lung cancer cells bearing nonmutant EGFR as determined by ELISA. C, expression of VEGFRs protein by HMVECs and lung cancer cell lines bearing nonmutant EGFR as determined by flow cytometry.

Statistical analysis

Differences were analyzed by 1-way ANOVA, followed when appropriate by Newman–Keuls multiple comparison tests. Values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using the GraphPad Prism Program Ver. 4.01.

Results

Expression of VEGF, VEGF-C, VEGF-D, and their receptors in human endothelial cells and lung cancer cell lines *in vitro*

We determined the expression of VEGF, VEGF-C, VEGF-D, and their receptors in HMVECs and 3 lung cancer cell lines at both the mRNA and protein levels. PC14PE6, SBC-5, and H1048 cells showed high, intermediate, and low VEGF expression, respectively (Fig. 1A and B). Although VEGFR-1 and VEGFR-2 were expressed only in HMVECs, both SBC-5 cells and HMVECs expressed VEGFR-3 mRNA and protein (Fig. 1A and C). However, we could not detect the production of its ligands, VEGF-C and VEGF-D, in the 3 lung cancer cell lines by ELISA (data not shown). Although these cell lines expressed mRNA of several receptors, including FGFR-1, FGFR-2, PDGFR-α, PDGFR-β, and c-kit, the level was generally very low (Supplementary Fig. S1). We also examined the production of ligands for FGFRs (acidic FGF and bFGF), PDGFRs (PDGF-AA, PDGF-AB, and PDGF-BB), and c-Kit (stem cell factor). The levels of these ligands were much lower than that of VEGF (data not shown).

E7080 inhibition of VEGF-induced proliferation and VEGFR-2 phosphorylation of endothelial cells, but not tumor cells, *in vitro*

Chemical structure of E7080 was shown in Fig. 2A. When we assayed the effect of the multiple tyrosine kinase inhibitor, E7080, on the proliferation of tumor cells and endothelial cells *in vitro*, we found that E7080 did not inhibit the proliferation of the 3 human lung cancer cells ($IC_{50} > 1{,}000$ nmol/L; Fig. 2B). In contrast, low-dose E7080 suppressed the proliferation of VEGF-stimulated HMVECs

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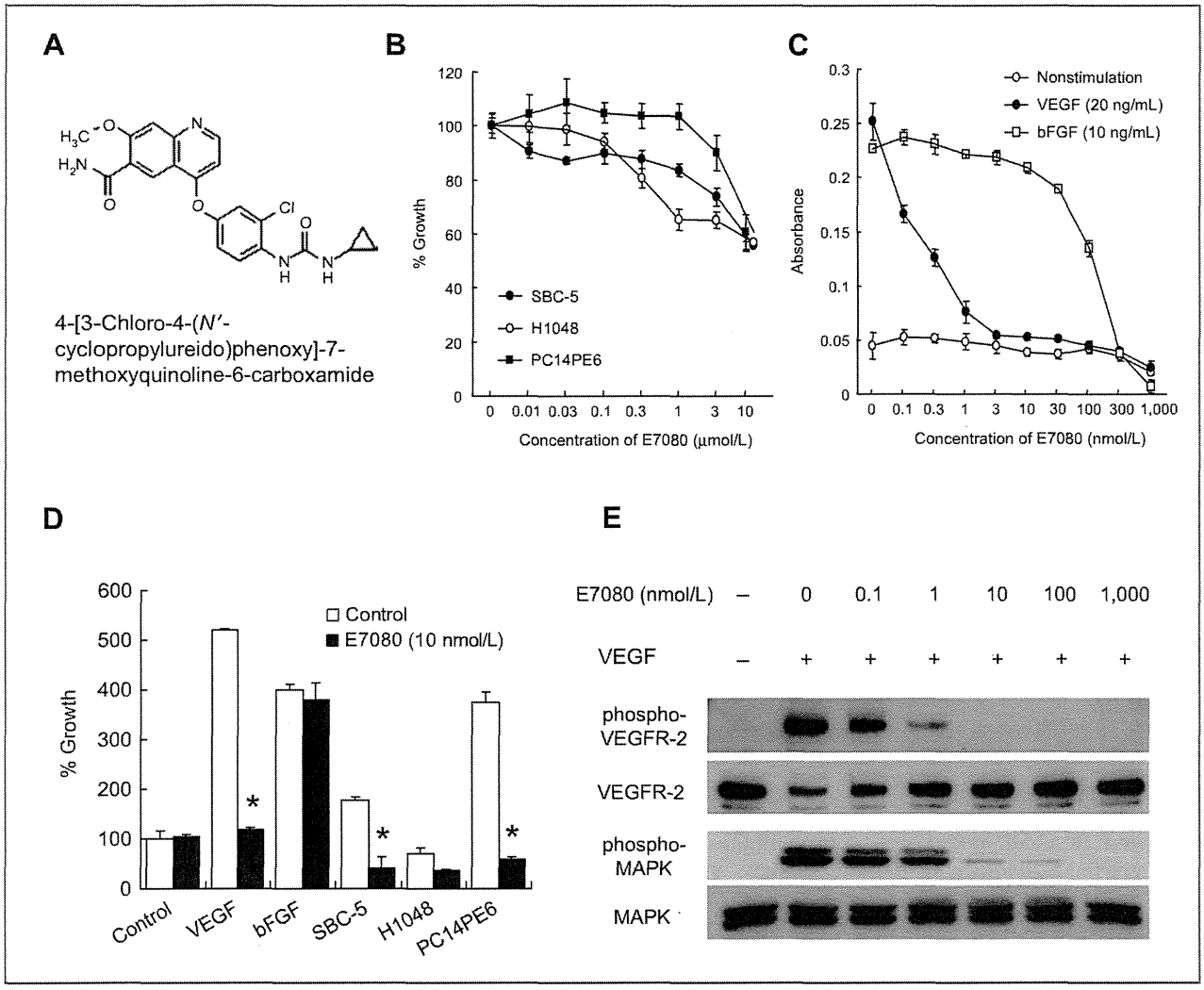

Figure 2. E7080 inhibition of VEGF-induced endothelial proliferation via inhibition of VEGFR-2 phosphorylation. A, chemical structure of E7080. B–D, effect of E7080 on the proliferation of lung cancer cell lines bearing nonmutant EGFR (B), human endothelial cells (HMVEC) stimulated with recombinant VEGF and bFGF (C), and HMVECs stimulated with culture medium from lung cancer cell lines bearing nonmutant EGFR (D) as determined by MTT assays. E, effect of E7080 on VEGFR-2 phosphorylation as determined by Western blot analysis.

(IC_{50} = 0.3 nmol/L), whereas high-dose E7080 suppressed the proliferation of bFGF-stimulated HMVECs (IC_{50} = 100 nmol/L; Fig. 2C). The culture supernatants of SBC-5 and PC14PE6 cells stimulated HMVEC proliferation, effects abrogated by 10 nmol/L E7080, a dose sufficient to inhibit the proliferation of HMVECs induced by VEGF but not by bFGF (Fig. 2D). These results suggest that VEGF but not bFGF may be the predominant tumor cell–derived growth factor for HMVEC produced by these lung cancer cell lines.

E7080 also dose dependently suppressed the VEGF-induced VEGFR-2 phosphorylation and activation of downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, in HMVECs (Fig. 2E), suggesting that E7080 has potent activity against VEGF-induced angiogenesis. We also confirmed that E7080 inhibited VEGF-C–induced phosphorylation of VEGFR-3 (Supplementary Fig. S2), in agreement with previous results (16).

E7080 suppression of enlargement of metastases of lung cancer cells expressing nonmutant EGFR in immunodeficient mice

We next examined the effect of E7080 on metastasis induced by lung cancer cells expressing nonmutant *EGFR*. Intravenous inoculation of the SCLC cell line, SBC-5, into NK cell–depleted SCID mice has been shown to produce experimental metastases within 35 days (22–24). These metastatic lesions were located primarily in the liver where some were greater than 2 mm in diameter. In addition, SBC-5 cells produce lung metastases (<2 mm in diameter) and osteolytic bone metastases detectable by radiography (Fig. 3 and Table 1). Treatment with E7080

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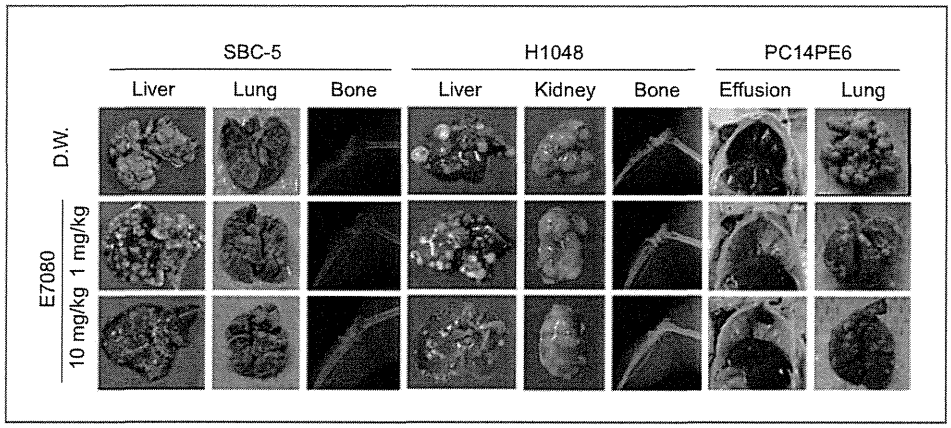

Figure 3. E7080 inhibition of the enlargement of hematogenous multiple organ metastases of lung cancer cells bearing nonmutant *EGFR* in immunodeficient mice. SBC-5 and H1048 cells were inoculated intravenously into NK cell–depleted SCID mice, whereas PC14PE6 cells were intravenously inoculated into nude mice. Mice received oral E7080 on days 14 to 35 after inoculation with SBC-5 or PC14PE6 cells and on days 28 to 56 after inoculation with H1048 cells. Representative pictures of 5 to 8 mice per group are shown. D.W., distilled water.

reduced significantly the number of large liver metastases (>2 mm in diameter) and total liver weight even at low doses (1 mg/kg/d) but did not reduce the total number of liver metastases (Fig. 3 and Table 1). At higher doses (3 and 10 mg/kg/d), E7080 also significantly reduced the number of osteolytic bone lesions but, interestingly, did not affect the number of lung metastases. E7080 treatment did not cause apparent adverse events, such as loss of body weight (data not shown).

We found that a second SCLC cell line, H1048, also produced metastatic nodules in multiple organs of NK cell–depleted SCID mice, although its pattern of metastases differed from that of SBC-5 cells. H1048 cells produced nodules in the liver, kidneys, and bones (Fig. 3). Once daily treatment with high dose (10 mg/kg/d), but not low dose (1 mg/kg/d), E7080 suppressed the number of large metastases in the liver and kidneys, as well as reducing the total weight of the liver and kidney and the number of metastatic nodules in bone (Fig. 3 and Table 1).

In contrast, human lung adenocarcinoma cells, PC14PE6, produced large colonies only in the lung and induced large volumes of pleural effusion in nude mice (Fig. 3 and Table 1; refs. 9, 25). Treatment with E7080 reduced significantly the number of large lung metastases and total lung weight even at low doses (1 mg/kg/d), as well as completely suppressing the production of pleural effusion (Fig. 3 and Table 1). These results suggest that E7080 may prevent the enlargement of metastatic colonies in multiple organs including the lungs.

E7080 inhibition of the growth of macroscopically detectable metastatic nodules

We previously reported that intravenously inoculated SBC-5 cells produce micrometastases and macroscopically detectable metastatic nodules within 14 and 21 days, respectively (22). We therefore determined whether treatment with E7080 could inhibit the growth of macroscopically detectable metastases. Treatment of E7080, started on day 14 or 21, inhibited the numbers of bone and liver metastases larger than 2 mm in diameter (Supplementary Table S2), indicating that E7080 has therapeutic activity against not only micrometastases but macroscopically detectable metastases.

E7080 inhibition of angiogenesis and lymphangiogenesis in metastatic nodules

Histologic analysis showed that treatment with E7080 caused necrosis in liver metastases produced by SBC-5 cells (Fig. 4A). Because E7080 has activity against VEGFR-2 and VEGFR-3, which are crucial for angiogenesis and lymphangiogenesis, we evaluated the effects of E7080 on angiogenesis and lymphangiogenesis, as well as on proliferating and apoptotic cells in the metastases. CD31-positive cells, presumably representing endothelial cells, were detected diffusely in metastatic nodules, whereas LYVE-1–positive cells, presumably representing lymph endothelial cells, were detected predominantly in the periphery of the nodules (Fig. 4A). Double staining for CD31 and LYVE-1 showed that a small population of cells in the periphery was doubly positive for both (Fig. 4B), consistent with previous findings (26, 27). Importantly, E7080 treatment dramatically inhibited the numbers of CD31- and LYVE-1–positive cells (Fig. 4A and C). Moreover, E7080 treatment decreased the number of Ki-67–positive proliferating tumor cells and increased the number of TUNEL-positive apoptotic cells in liver metastases (Fig. 4A and C). There was no discernible difference in VEGF production between control and E7080-treated tumors (Supplementary Fig. S3). These results indicate that E7080 can inhibit both angiogenesis and lymphangiogenesis and therefore suppress the growth of metastases.

E7080 prolongation of the survival of mice bearing metastases of nonmutant EGFR lung cancer cells

We finally determined whether E7080 could prolong the survival of mice bearing metastases. SBC-5 and