HB-EGF Inhibition in Combination with Various Anticancer Agents Enhances its Antitumor Effects in Gastric Cancer

AYAKO SANUI[1*], FUSANORI YOTSUMOTO[2*], HIROSHI TSUJIOKA[1], TATSUYA FUKAMI[1], SHINJI HORIUCHI[1], KYOKO SHIROTA[1], TOSHIYUKI YOSHIZATO[1], TATSUHIKO KAWARABAYASHI[1], MASAHIDE KUROKI[2] and SHINGO MIYAMOTO[1*]

[1]*Department of Obstetrics and Gynecology, and*
[2]*Department of Biochemistry, Faculty of Medicine, Fukuoka University, Japan*

Abstract. *Advanced gastric cancer (GC) is one of the most lethal malignancies. Although many anticancer agents exist for the treatment of GC, its prognosis remains extremely poor. Therefore, further development of targeted therapies is required for patients with GC. To assess the role of heparin-binding epidermal growth factor-like growth factor (HB-EGF) as a target for GC therapy, the expression of EGF receptor ligands in GC cell lines, and the antitumor effects of an HB-EGF inhibitor (CRM197) as a single agent and in combination with other anticancer agents was assessed in GC cells. HB-EGF was the predominantly expressed ligand among EGF receptor ligands in all the cells. CRM197 induced significant cell apoptosis. Anticancer agents augmented the secretion of HB-EGF into the medium and simultaneously induced cell apoptosis. Combination of CRM197 with other anticancer agents significantly enhanced cell apoptosis. Additionally, co-administration of CRM197 and paclitaxel resulted in synergistic antitumor effects. These results suggested that HB-EGF is a rational target for GC therapy.*

Gastric cancer is one of the most common types of cancer worldwide, with a particularly high incidence in East Asia, Eastern Europe, Central and South America (1). This disease is also associated with high mortality because many patients present with locally advanced or metastatic disease.

Chemotherapy is the main treatment option for patients with advanced gastric cancer. Fluorouracil (5-FU), cisplatin, docetaxel, paclitaxel, epirubicin and irinotecan are the major constituents of conventional regimens. To date, although many clinical trials have been performed, there is no standard treatment for the advanced disease. A number of different classes of targeted agents, such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2)-targeted monoclonal antibodies (cetuximab and trastuzumab) have shown promising activity in advanced gastric cancer (2, 3). However, it remains unclear how conventional anticancer agents and novel molecularly targeted agents should be optimally combined to effectively treat patients with advanced gastric cancer. On the basis of the existing evidence, rapid development of molecularly targeted therapies is required to improve the prognosis of patients with gastric cancer.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is an EGFR ligand (4). HB-EGF is initially synthesized as a transmembrane protein, similar to other members of the EGF family of growth factors (5). The membrane-anchored form of HB-EGF (proHB-EGF) is cleaved at the cell surface by a protease to yield the soluble form of HB-EGF (sHB-EGF) *via* a mechanism known as ectodomain shedding (6). Members of the A disintegrin and metalloprotease (ADAM) family, such as ADAM9, ADAM10, ADAM12 and ADAM17, and other metalloproteases are involved in this process (7). Ectodomain shedding of proHB-EGF is induced by stimuli including phorbol ester 12-*O*-tetradecanoylphorbor-13-acetate (TPA), calcium ionophore, various growth factors, cytokines and anticancer agents (8-11). We previously reported the validation of HB-EGF as a target for therapy in ovarian, breast, bladder and gastric cancer (12). In addition, it has also been reported that an abundance of HB-EGF expression is involved in resistance developed by cancer cells to conventional chemotherapeutic agents (13).

Cross-reacting material 197 (CRM197) is a non-toxic mutant of diphtheria toxin that shares immunological properties with the native molecule. CRM197 binds to human HB-EGF and blocks its mitogenic activity by prohibiting

*Both authors contributed equally to this work.

Correspondence to: Shingo Miyamoto, Department of Obstetrics and Gynecology, Faculty of Medicine, Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, Fax: +81 928654114, e-mail: smiya@cis.fukuoka-u.ac.jp

Key Words: HB-EGF, HER2, 5-FU, paclitaxel, gastric cancer.

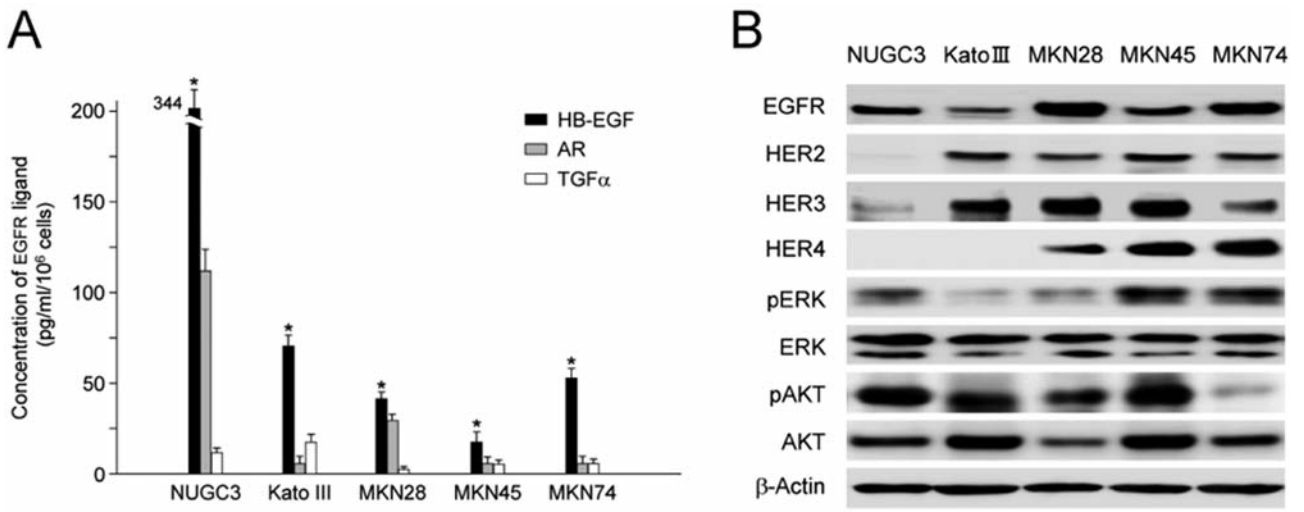

Figure 1. *Cell characteristics in gastric cancer. A and B: Differences in the expressions of EGFR ligands (A) or ErbB family and ErbB-related signaling molecules (B) in gastric cancer cell lines. A: Amount of EGFR ligands in culture media from cancer cells incubated for 48 h. The concentrations of heparin-binding epidermal growth factor-like growth factor (HB-EGF), amphiregulin (AR), transforming growth factor (TGF)-α and EGF are presented as the concentrations per 1×10^6 cells. EGF level was below the limit of detection. Each value represents the mean and SD (n=3). *p<0.05, versus AR, TGF-α or EGF. B: Protein expressions of EGFR, HER2, HER3, HER4, Akt, ERK and β-actin in gastric cancer cells as shown by Western blotting.*

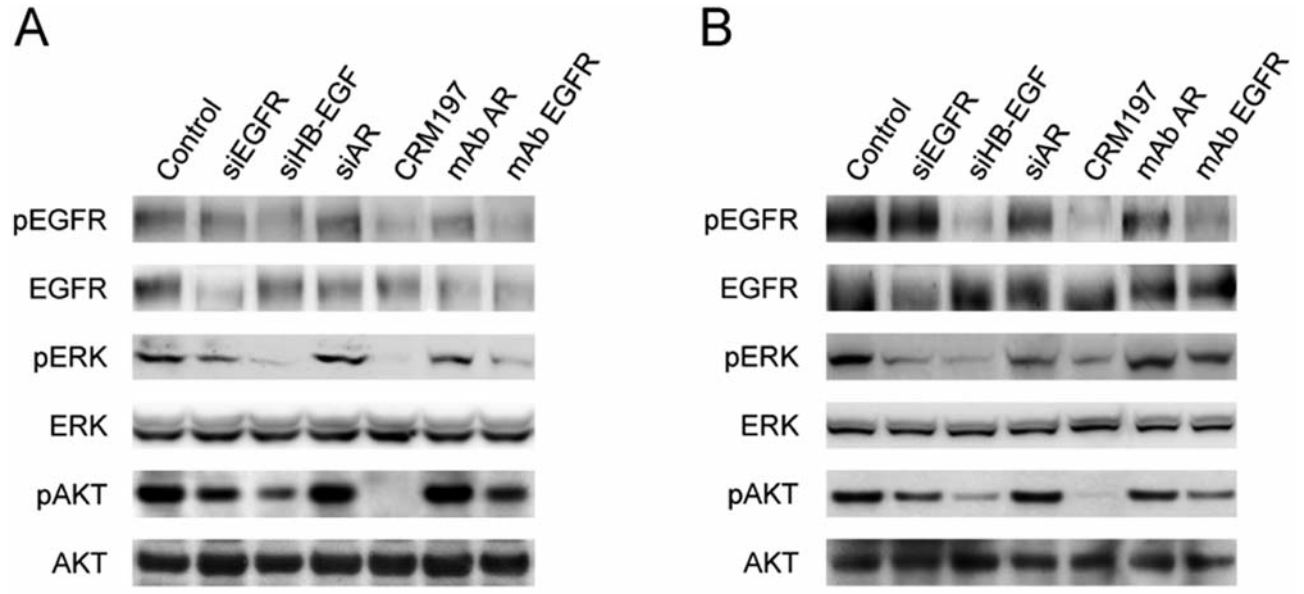

Figure 2. *Alterations in the tyrosine phosphorylation of EGFR, ERK and AKT. siRNA for HB-EGF, amphiregulin and EGFR were individually transfected into NUGC3 (A) and MKN28 (B) cells. In addition, CRM197 (1 μg/ml) or inhibitory antibody against amphiregulin (AR;10 μg/ml) or EGFR (10 μg/ml) was incubated with NUGC3 (A) and MKN28 (B) cells for 72 h.*

EGFR binding (14). In addition, CRM197 and paclitaxel exhibited synergistic antitumor effects in ovarian cancer cells harboring HB-EGF expression (11). As such, combining chemotherapy with an HB-EGF inhibitor would potentially be efficacious in the treatment of patients with gastric cancer.

The objective of this study was to investigate the *in vitro* synergistic antitumor effect between CRM197 and conventional chemotherapeutic agents for the treatment of gastric cancer. We examined HB-EGF expression and apoptosis of gastric cancer cells incubated with anticancer agents.

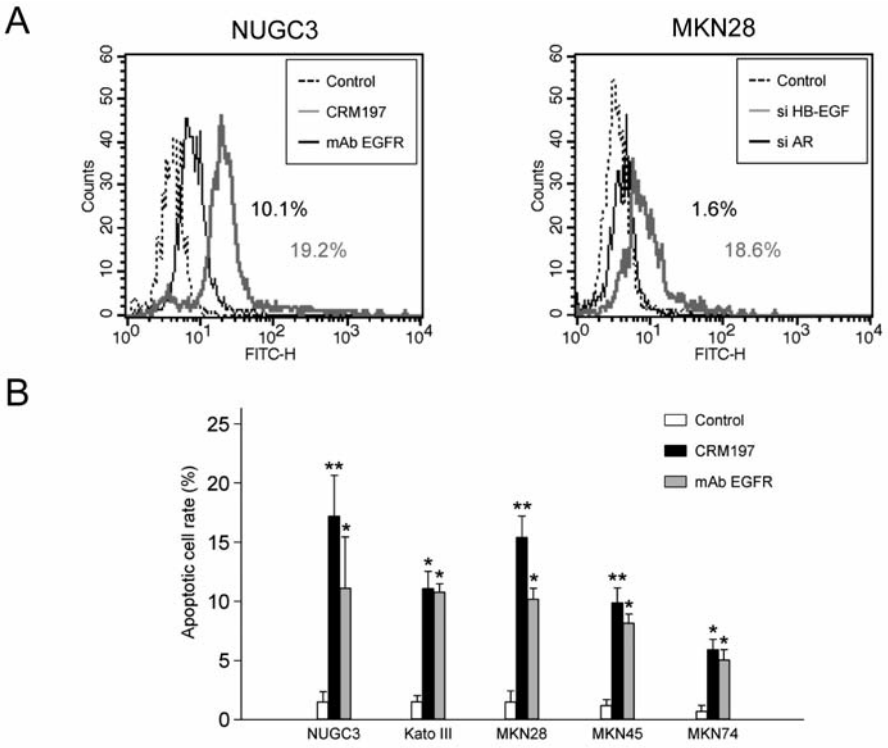

Figure 3. *Flow cytometric analysis of apoptotic cells. A: Apoptotic gastric cancer cells. Left panel, NUGC3 cells, mAb EGFR: inhibitory antibody against EGFR. Each histogram is representative of three independent experiments. Right panel, MKN28 cells. Each histogram is representative of three independent experiments. B: Differences in the cell apoptotic rate after treatment with control IgG (10 μg/ml), CRM197 (1 μg/ml) or inhibitory antibody against EGFR (10 μg/ml) in gastric cancer cell lines. Each value represents the mean and SD (n=4). * and **p<0.05 versus the value for the cell apoptotic rate after treatment with control IgG and an inhibitory antibody against EGFR (10 μg/ml), respectively.*

Materials and Methods

Reagents and antibodies. CRM197 and trastuzumab was kindly provided by Professor Eisuke Mekada of the Department of Cell Biology, Osaka University, Osaka, Japan, and by Chugai Pharmaceutical Co. (Tokyo, Japan), respectively. 5-FU, cisplatin and paclitaxel were obtained from Calbiochem (San Diego, CA, USA). EGFR, HB-EGF and amphiregulin (AR) siRNAs were purchased from TaKaRa Bio Inc. (Shiga, Japan). Primary antibodies utilized included rabbit polyclonal anti-EGFR (sc-03), mouse monoclonal anti-ErbB-4 (sc-8050) and anti-Neu (sc-284) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-HER3, rabbit polyclonal anti-ERK, mouse monoclonal anti-phosphotyrosine (Millipore-Upstate Biotechnology Inc., Lake Placid, NY, USA), rabbit polyclonal anti-Akt, mouse monoclonal anti-phospho-Akt (Ser473), rabbit monoclonal phospho-p44/42 mitogen-activated protein kinase (MAPK) (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology Inc., Beverly, MA, USA) and mouse monoclonal anti-β-actin (Sigma, St. Louis, MO, USA). Peroxidase-conjugated anti-mouse IgG and peroxidase-conjugated anti-rabbit IgG were obtained from Amersham Corp. (Arlington Heights, IL, USA) and Zymed (San Francisco, CA, USA), respectively. FITC-conjugated anti-goat IgG was obtained from Sigma. Anti-EGFR neutralizing antibody was purchased from Millipore-Upstate Biotechnology Inc. Anti-human AR neutralizing antibody was purchased from R&D Systems Inc. (Minneapolis, MN, USA).

Cells and cell culture. The following cell lines were obtained commercially: MKN28 cells from the Japanese Collection of Research Bioresources (Osaka, Japan); MKN45, MKN74, NUGC3 and KatoIII cells from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (ICN Biomedicals, Irvine, CA, USA), 100 U/ml of penicillin G and 100 μg/ml of streptomycin (Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37˚C.

Soluble HB-EGF, AR and transforming growth factor (TGF)-α in cell culture medium. Cells were incubated for 48 h. The levels of HB-EGF, AR, TGF-α and EGF in culture medium (CM) were determined using a commercially available sandwich ELISA (DuoSet Kit; R&D Systems Inc.) according to the manufacturer's instructions. All samples were assayed in triplicate. Each mean value was considered to be representative of the CM.

Transfections of siRNAs for EGFR ligands and EGFR. Cells (4×10^5) were seeded on poly-lysine-coated 10-cm dishes (50-60% confluence). The siRNAs for the EGFR ligands and EGFR were individually transfected into cells using TransIT-TKO (Mirus, Madison, WI, USA) according to the manufacturer's instructions. The final concentration of each siRNA for transfection was 25 nM. After incubation for 72 h, the cells were subjected to apoptosis assays and immunoblot analyses.

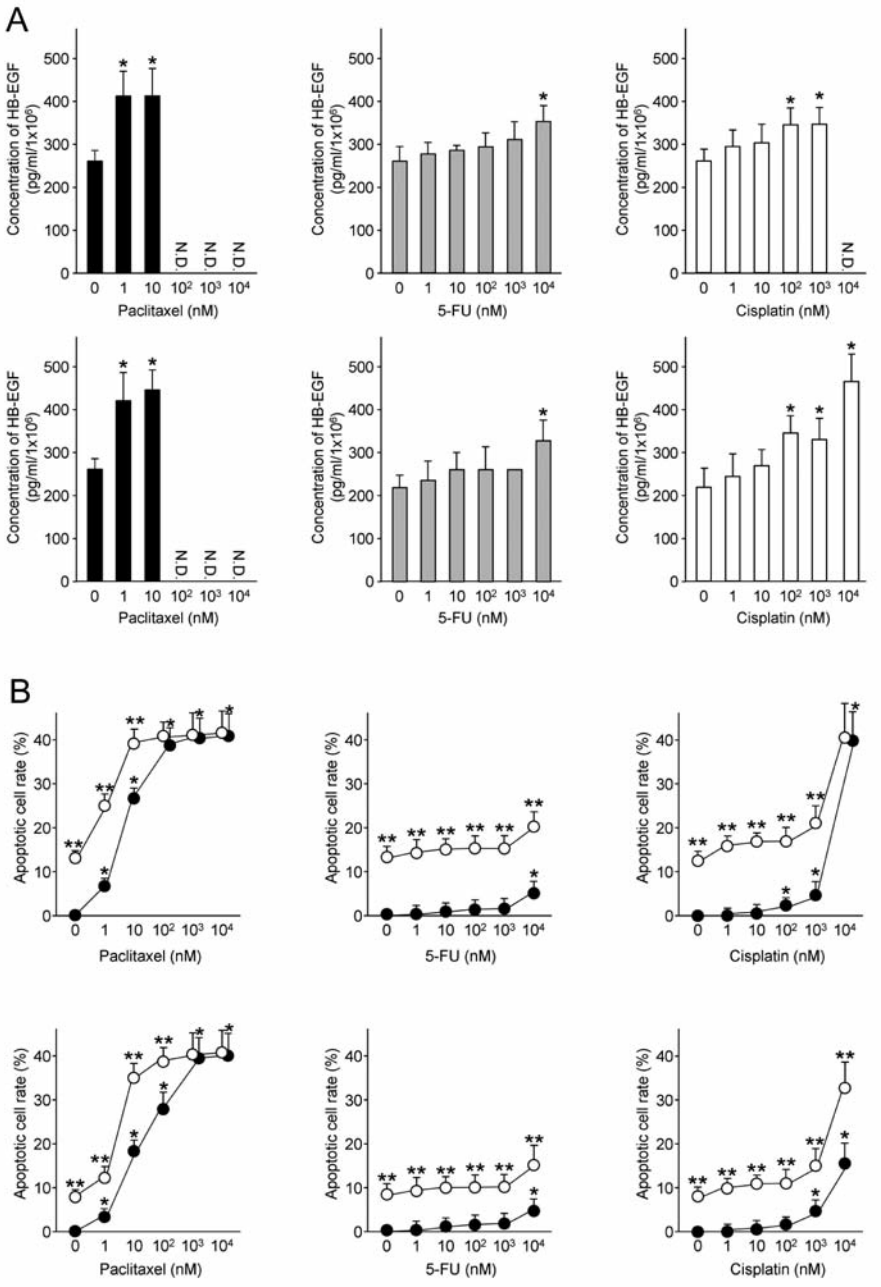

Figure 4. *Alterations in the concentration of HB-EGF (A) and cell apoptotic rate (B) after treatment of CRM197 with 5-fluorouracil (5-FU), cisplatin, or paclitaxel. A: Concentration of HB-EGF in the culture medium after treatment with different concentrations (0-10 μM) of 5-FU, cisplatin or paclitaxel in NUGC3 (upper panel) and MKN28 (lower panel) cells. *p<0.05 versus control (0 nM). ND: Below the limit of detection. B: Cell apoptotic rate after treatment of cells with CRM197 (1 μg/ml) with different concentration (0-10 μM) of 5-FU, cisplatin or paclitaxel in NUGC3 (upper panel) and MKN28 (lower panel) cells. Each value represents the mean and SD (n=4). * and **p<0.05 versus the value for cell apoptotic rate after treatment with control (0 nM) and with 5-FU, cisplatin or paclitaxel alone at the concentration shown, respectively.*

Western blotting. To examine the ErbB expression-related signaling molecules, cells were rinsed in phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate, then lysed in immunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris (pH 8.0), 0.2 unit/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate). Extracts and immunoprecipitants were then subjected to SDS-PAGE and immunoblotting analysis as described previously (11).

Cell apoptosis assay. Cells were harvested, pooled, and fixed with 4% paraformaldehyde at 4˚C for 30 min and re-suspended in 70% ethanol at 20˚C for 30 min. After washing in PBS, the cells were

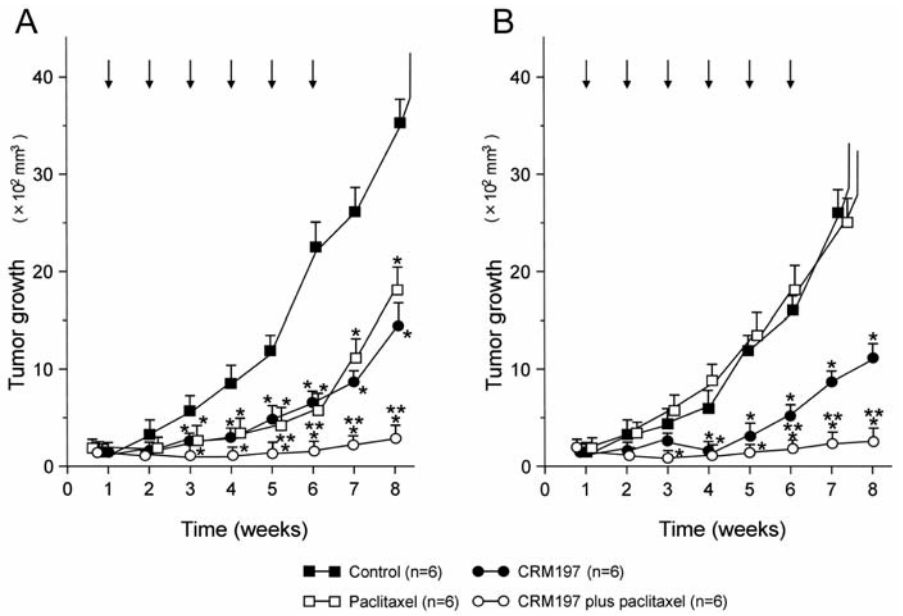

Figure 5. *Synergistic antitumor effect of CRM197 with paclitaxel in NUGC3 and MKN28 cells. Ten or seven days after injection of NUGC3 (A) or MKN28 (B) cells, control saline, CRM197 or paclitaxel, or their combination was injected intraperitoneally each week for 6 weeks into mice bearing NUGC3 or MKN28 tumors (arrows). Tumor volume was calculated as described in the Materials and Methods section. Each tumor volume represents the mean±SD (N=6). Statistical analysis was performed using Tukey HSD test: *p<0.05 versus the value for tumor-bearing control mice injected with saline and **p<0.05 versus the values for tumor-bearing mice administered CRM197 alone.*

incubated with TdT reaction reagent (MEBSTAIN Apoptosis Kit Direct; MBL Co. Ltd., Nagoya, Japan) for 1 h at 37˚C, according to the manufacturer's instructions. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells were quantified as apoptotic cells by flow cytometric analysis using a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ, USA).

Tumor growth in nude mice. Using subconfluent cell cultures, a total volume of 250 μl containing 5×10^6 cells in serum-free RPMI1640 was injected subcutaneously into female BALB/c *nu/nu* mice at 5 weeks of age (Charles River Laboratories, Japan). Injected mice were examined weekly for tumor. Weekly intraperitoneal injection was administered to tumor-bearing mice. Tumor volume was calculated as described previously (15). All experimental use of animals complied with the animal care guidelines published by Kyushu and Fukuoka Universities.

Statistical analysis. Data obtained in the two experiments were analyzed using the Mann-Whitney *U*-test; a value of $p<0.05$ was considered statistically significant. Data obtained from the experiments were analyzed using the Tukey HSD test; a value of $p<0.05$ was considered to be statistically significant.

Results

Characterization of gastric cancer cells. The protein expression of HB-EGF was highly elevated, compared with other EGFR ligands in the five gastric cancer cell lines studied (Figure 1A). Reportedly, mRNA of HB-EGF was also identified as the predominant EGFR ligand (12). The expression of EGFR, HER3, phosphorylated ERK and phosphorylated AKT was found in all cells (Figure 1B). Distinct expression of HER2 was detected in KatoIII, MKN28, MKN45 and MKN74 cells. Accordingly, NUGC3 and MKN28 cells were recognized as representatives of cells harboring overexpression of EGFR and HB-EGF, and overexpression of EGFR, HER2 and HB-EGF, respectively. These cell lines were studied further.

In vitro antitumor effects of CRM197. To elucidate whether HB-EGF plays a pivotal role in EGFR signaling, alterations in EGFR, ERK and AKT activation were examined after transfection of siRNA for HB-EGF, AR and EGFR, and after incubation with CRM197 and inhibitory antibody against AR and EGFR. The introduction of a siRNA for EGFR and HB-EGF, but not for AR, attenuated the activation of EGFR, ERK and AKT in NUGC3 and MKN28 cells (Figure 2A and 2B). The presence of CRM197 and inhibitory anti-EGFR antibody also reduced phosphorylation of EGFR, ERK and AKT, whereas inhibitory anti- AR antibody did not influence the activation of EGFR, ERK and AKT (Figure 2A and 2B). To validate HB-EGF as a rational target for gastric cancer therapy, the *in vitro* antitumor effects were examined by suppressing HB-EGF and EGFR. The presence of CRM197 and inhibitory anti-EGFR antibody significantly augmented

the number of apoptotic cells in all five cell lines, compared with the presence of control IgG (Figure 3A and 3B). These results suggest that HB-EGF and EGFR are rational targets for gastric cancer therapy.

Alterations in HB-EGF expression and cell apoptosis mediated by anticancer agents. To investigate the relationship between HB-EGF expression and cell apoptosis in the presence of anticancer agents, we examined the soluble form of HB-EGF in the medium, and cell apoptosis after incubation of cells with 5-FU, cisplatin and paclitaxel. At concentrations of 1 nM and 10 nM of paclitaxel, a marked increase of HB-EGF expression in the medium was found (Figure 4A), while significant cell apoptosis was detected in both cell lines (Figure 4B). At greater than 100 nM of paclitaxel, most cells were detached from the plate, and cell apoptosis and HB-EGF expression were not measurable in either cell line (Figure 4A and 4B). No significant increase in cell apoptotic rate or HB-EGF expression was found with incubation of either cell line with less than 1 μM of 5-FU or less than 10 nM of cisplatin (Figure 4A and 4B). Incubation of cells with 10 μM of 5-FU or 10-100 nM of cisplatin caused a minor increase in cell apoptotic rate and HB-EGF expression in both cell lines (Figure 4A and 4B). At 10 μM of cisplatin, most cells were detached from the plate; cell apoptosis but not HB-EGF expression was measurable in NUGC3 cells (Figure 4A and 4B). These results suggest the presence of synergistic *in vitro* antitumor effects between CRM197 and conventional chemotherapeutic agents, accompanied by mediation of HB-EGF expression by conventional chemotherapeutic agents. Specifically, paclitaxel evokes abundant secretion of HB-EGF compared with 5-FU and cisplatin.

Synergistic antitumor effects of CRM197 and paclitaxel in xenografted mice. To elucidate the *in vivo* antitumor effects of paclitaxel and CRM197, tumor formation in xenografted mice was continuously monitored following injection of NUGC3 and MKN28 cells. When 50 mg/kg of CRM197 were administered alone, tumor growth from NUGC3 and MKN28 cells in mice was significantly suppressed in (Figure 5A and 5B). Paclitaxel (10 mg/kg) administration induced partial suppression of NUGC3 cell tumor formation (Figure 5A), whereas MKN28 cell tumor formation was not suppressed by paclitaxel (10 mg/kg) administration (Figure 5B). Co-administration of CRM197 (50 mg/kg) and paclitaxel (10 mg/kg) completely suppressed both NUGC3 and MKN28 cell tumor formation. These results indicate a synergistic *in vivo* antitumor effect induced by CRM197 in combination with paclitaxel.

Discussion

In this study, HB-EGF was the predominant EGFR ligand in the gastric cancer cell lines studied. The inhibition of HB-EGF attenuated the activation of ERK as a cell proliferating signal as well as AKT as a cell survival signal, and induced significant gastric cancer cell apoptosis. Conventional chemotherapeutic agents evoked HB-EGF expression, probably due to escape from cell death. CRM197 and conventional chemotherapeutic agents, especially paclitaxel, promoted both *in vitro* and *in vivo* antitumor effects.

Except for cetuximab and trastuzumab, molecularly targeted agents, including vascular endothelial growth factor (VEGF)-targeted bevacizumab, proteasome inhibitor bortezomib, everolimus (an inhibitor of the mammalian target of rapamycin) and COX2 inhibitor etodolac have been investigated in patients with advanced gastric cancer (16-19). Clinical trials involving the use of these agents have been performed. A phase II study of bevacizumab in combination with irinotecan and cisplatin produced some efficacy (20). Promising efficacy in treatment of gastric cancer with some of the above agents is expected (16-19). A phase I study on the use of CRM197 has been performed in patients with advanced ovarian cancer at Fukuoka University. Combining chemotherapy with conventional chemotherapeutic agents and cetuximab, trastuzumab and bevacizumab has been shown to produce a statistically significant improvement in survival, compared with conventional chemotherapy alone (2, 3, 20). However, the efficacy and safety profiles of these agents appear to be limited in the treatment of gastric cancer (21-23). Therefore, further clinical development of molecular targeted therapies is required to improve the prognosis in patients with advanced gastric cancer.

In conclusion, HB-EGF is recognized as a key molecule involved in the pathogenesis and growth of gastric cancer and a rational target for gastric cancer therapy. CRM197 in combination with conventional chemotherapeutic agents may lead to improved clinical outcomes in patients with advanced gastric cancer.

Acknowledgements

This work was supported in part by funds from the Central Research Institute of Fukuoka University (Fukuoka, Japan), a Grant-in-Aid from the Kakihara Science and Technology Foundation (Fukuoka, Japan) and Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan) to S. Miyamoto.

References

1 Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol *24*: 2137-2150, 2006.

2 Pinto C, Di Fabio F, Siena S, Cascinu S, Rojas Llimpe FL, Ceccarelli C, Mutri V, Giannetta L, Giaquinta S, Funaioli C, Berardi R, Longobardi C, Piana E and Martoni AA: Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). Ann Oncol *18*: 510-517, 2007.

3 Van Cutsem E, Kang Y, Chung H, Shen L, Sawaki A, Lordick F, Hill J, Lehle M, Feyereislova A and Bang Y: Efficacy results from the ToGA trial: A phase III study of trastuzumab added to standard chemotherapy (CT) in first-line human epidermal growth factor receptor 2 (HER2)-positive advanced gastric cancer (GC). J Clin Oncol *27*: 18s (suppl; abstr LBA4509), 2009.
4 Riese DJ and Stern DF: Specificity within the EGF family/ErbB receptor family signaling network. Bioessays *20*: 41-48, 1998.
5 Higashiyama S, Abraham JA, Miller J, Fiddes JC and Klagsbrun M: A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science *251*: 936-939, 1991.
6 Izumi Y, Hirata M, Hasuwa H, Iwamoto R, Umata T, Miyado K, Tamai Y, Kurisaki T, Sehara-Fujisawa A, Ohno S and Mekada E: A metalloprotease-disintegrin, MDC9/meltrin-gamma/ADAM9 and PKCdelta are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. EMBO J *17*: 7260-7272, 1998.
7 Seals DF and Courtneidge SA: The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev *17*: 7-30, 2003.
8 Goishi K, Higashiyama S, Klagsbrun M, Nakano N, Umata T, Ishikawa M, Mekada E and Taniguchi N: Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. Mol Biol Cell *6*: 967-980, 1995.
9 Dethlefsen SM, Raab G, Moses MA, Adam RM, Klagsbrun M and Freeman MR: Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. J Cell Biochem *69*: 143-153, 1998.
10 Tanida S, Joh T, Itoh K, Kataoka H, Sasaki M, Ohara H, Nakazawa T, Nomura T, Kinugasa Y, Ohmoto H, Ishiguro H, Yoshino K, Higashiyama S and Itoh M: The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. Gastroenterology *127*: 559-569, 2004.
11 Yagi H, Yotsumoto F, Sonoda K, Kuroki M, Mekada E and Miyamoto S: Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer. Int J Cancer *124*: 1429-1439, 2009.
12 Yotsumoto F, Yagi H, Suzuki SO, Oki E, Tsujioka H, Hachisuga T, Sonoda K, Kawarabayashi T, Mekada E and Miyamoto S: Validation of HB-EGF and amphiregulin as targets for human cancer therapy. Biochem Biophys Res Commun *365*: 555-561, 2008.
13 Wang F, Liu R, Lee SW, Sloss CM, Couget J and Cusack JC: Heparin-binding EGF-like growth factor is an early response gene to chemotherapy and contributes to chemotherapy resistance. Oncogene *26*: 2006-2016, 2007.
14 Mitamura T, Higashiyama S, Taniguchi N, Klagsbrun M and Mekada E: Diphtheria toxin binds to the epidermal growth factor (EGF)-like domain of human heparin-binding EGF-like growth factor/diphtheria toxin receptor and inhibits specifically its mitogenic activity. J Biol Chem *270*: 1015-1019, 1995.
15 Miyamoto S, Hirata M, Yamazaki A, Kageyama T, Hasuwa H, Mizushima H, Tanaka Y, Yagi H, Sonoda K, Kai M, Kanoh H, Nakano H and Mekada E: Heparin-binding EGF-like growth factor is a promising target for ovarian cancer therapy. Cancer Res *64*: 5720-5727, 2004.
16 Iwata C, Kano MR, Komuro A, Oka M, Kiyono K, Johansson E, Morishita Y, Yashiro M, Hirakawa K, Kaminishi M and Miyazono K: Inhibition of cyclooxygenase-2 suppresses lymph node metastasis *via* reduction of lymphangiogenesis. Cancer Res *67*: 10181-10189, 2007.
17 Jatoi A, Dakhil SR, Foster NR, Ma C, Rowland KM Jr., Moore DF Jr., Jaslowski AJ, Thomas SP, Hauge MD, Flynn PJ, Stella PJ and Alberts SR: Bortezomib, paclitaxel, and carboplatin as a first-line regimen for patients with metastatic esophageal, gastric, and gastroesophageal cancer: phase II results from the North Central Cancer Treatment Group (N044B). J Thorac Oncol *3*: 516-520, 2008.
18 Yamada Y, Doi T, Muro K, Boku N, Nishina T, Takiuchi H, Komatsu Y, Hamamoto Y, Ohno N, Fujita Y and Ohtsu Y: Multicenter phase II study of everolimus in patients with previously treated metastatic gastric cancer: main results. Presented at the American Society of Clinical Oncology Gastrointestinal Cancers Symposium; 2009 January 15-19; San Francisco, USA.
19 Moehler M, Hartmann JT, Lordick F, Al-Batran S, Reimer P, Trarbach T, Ebert M, Daum S, Weihrauch M and Galle PR: AIO Upper GI Group: Sunitinib in patients with chemorefractory metastatic gastric cancer. Preliminary results of an open-label, prospective nonrandomized multicenter AIO phase II trial. Pesented at the American Society of Clinical Oncology Gastrointestinal Cancers Symposium; 2009 January 15-19; San Francisco, USA.
20 Shah MA, Ramanathan RK, Ilson DH, Levnor A, D'Adamo D, O'Reilly E, Tse A, Trocola R, Schwartz L, Capanu M, Schwartz GK and Kelsen DP: Multicenter phase II study of irinotecan, cisplatin, and bevacizumab in patients with metastatic gastric or gastroesophageal junction adenocarcinoma. J Clin Oncol *24*: 5201-5206, 2006.
21 Han SW, Oh DY, Im SA, Park SR, Lee KW, Song HS, Lee NS, Lee KH, Choi IS, Lee MH, Kim MA, Kim WH, Bang YJ and Kim TY: Phase II study and biomarker analysis of cetuximab combined with modified FOLFOX6 in advanced gastric cancer. Brit J Cancer *100*: 298-304, 2009.
22 Barros-Silva JD, Leitão D, Afonso L, Vieira J, Dinis-Ribeiro M, Fragoso M, Bento MJ, Santos L, Ferreira P, Rêgo S, Brandão C, Carneiro F, Lopes C, Schmitt F and Teixeira MR: Association of *ERBB2* gene status with histopathological parameters and disease-specific survival in gastric carcinoma patients. Br J Cancer *10*: 100: 487-493, 2009.
23 EI-Rayes BF, Patel B, Zalupski M, Hammad N, Shields A, Heibrun L, Venkatramanamoorthy R and Philip P: A phase II study of bevacizumab, docetaxel, and oxaliplatin in gastric and GEJ cancer. Presented at the American Society of Clinical Oncology Meeting Abstracts *27*: 4563, 2009.

Received June 8, 2010
Revised July 14, 2010
Accepted July 16, 2010