Suppression of proHB-EGF Carboxy-Terminal Fragment Nuclear Translocation: A New Molecular Target Therapy for Gastric Cancer

Takaya Shimura, Hiromi Kataoka, Naotaka Ogasawara, Eiji Kubota, Makoto Sasaki, Satoshi Tanida, and Takashi Joh

Abstract

Purpose: Inactivation of epidermal growth factor (EGF) receptor (EGFR) represents a promising strategy for the development of selective therapies against epithelial cancers and has been extensively studied as a molecular target for cancer therapy. However, little attention has been paid to remnant cell-associated domains created by cleavage of EGFR ligands. The present study focused on recent findings that cleavage of membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF), an EGFR ligand, induces translocation of the carboxyl-terminal fragment (CTF) of HB-EGF from the plasma membrane to the nucleus and regulates cell cycle.

Experimental Design: Two gastric cancer cell lines, MKN28 and NUGC4, were used. KB-R7785, an inhibitor of proHB-EGF shedding, was used to suppress HB-EGF-CTF nuclear translocation with cetuximab, which inhibits EGFR phosphorylation. Cell growth was analyzed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay, apoptosis was evaluated by assay of caspase-3 and caspase-7, and cell cycle was investigated by flow cytometry.

Results: Immunofluorescence study confirmed that KB-R7785 inhibited HB-EGF-CTF nuclear translocation under conditions of proHB-EGF shedding induction by 12-*O*-tetradecanoylphorbol-13-acetate in gastric cancer cells. KB-R7785 inhibited cell growth in a dose-dependent manner and high-dose KB-R7785 induced apoptosis. Moreover, KB-R7785 induced cell cycle arrest and increased sub-G_1 DNA content. KB-R7785 suppressed cyclin A and c-Myc expression. All effects of KB-R7785 were reinforced by combination with cetuximab.

Conclusions: These results suggest that both inhibition of EGFR phosphorylation and inhibition of HB-EGF-CTF nuclear translocation play crucial roles in inhibitory regulation of cancer cell growth. Suppression of HB-EGF-CTF nuclear translocation might offer a new strategy for treating gastric cancer.

Molecular target therapies have recently undergone substantial development, creating effective therapies for various cancers. In particular, epidermal growth factor (EGF) receptor (EGFR) is one of the most attractive targets for cancer therapy. Many studies of EGFR have been reported in experimental of models of epithelial cell neoplasia (1, 2). EGFR belongs to the ErbB receptor tyrosine kinase family, which includes erbB1 (EGFR), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4), and EGFR plays key roles in the regulation of essential normal cellular processes and the pathophysiology of hyperproliferative diseases such as cancer.

EGFR transactivation reportedly requires activation of a disintegrin and metalloprotease (ADAM), which induces ectodomain shedding of EGF ligands to produce soluble factors. Seven ligands have been described thus far for EGFR: EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α, amphiregulin, betacellulin, epiregulin, and epigen. After binding of ligand to the extracellular domain of the receptor, EGFR dimerization occurs, resulting in activation of the intrinsic protein tyrosine kinase and tyrosine autophosphorylation. Subsequently, activation of the EGFR transmits signals to prominent downstream pathways, such as mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathway, and leads to proliferation, cell survival, and angiogenesis (3–5).

Indeed, EGFR-targeting agents such as erlotinib (6), an EGFR tyrosine kinase inhibitor used for advanced or metastatic pancreatic cancer and non-small cell lung cancer, and cetuximab (7, 8), a monoclonal antibody to EGFR used for metastatic colorectal cancer and squamous cell carcinoma in head and neck cancer, have seen clinical application.

The EGF and EGFR gene families have been associated with growth regulation and gastric wall invasion of gastric cancers (9). Moreover, elevated EGFR levels have been reported as an independent indicator of poor prognosis in gastric cancer (10, 11). However, no molecular target agent with clinically sufficient effects on gastric cancer has yet been reported. For

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Received 11/1/07; revised 2/25/08; accepted 3/16/08.

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doi:10.1158/1078-0432.CCR-07-4794

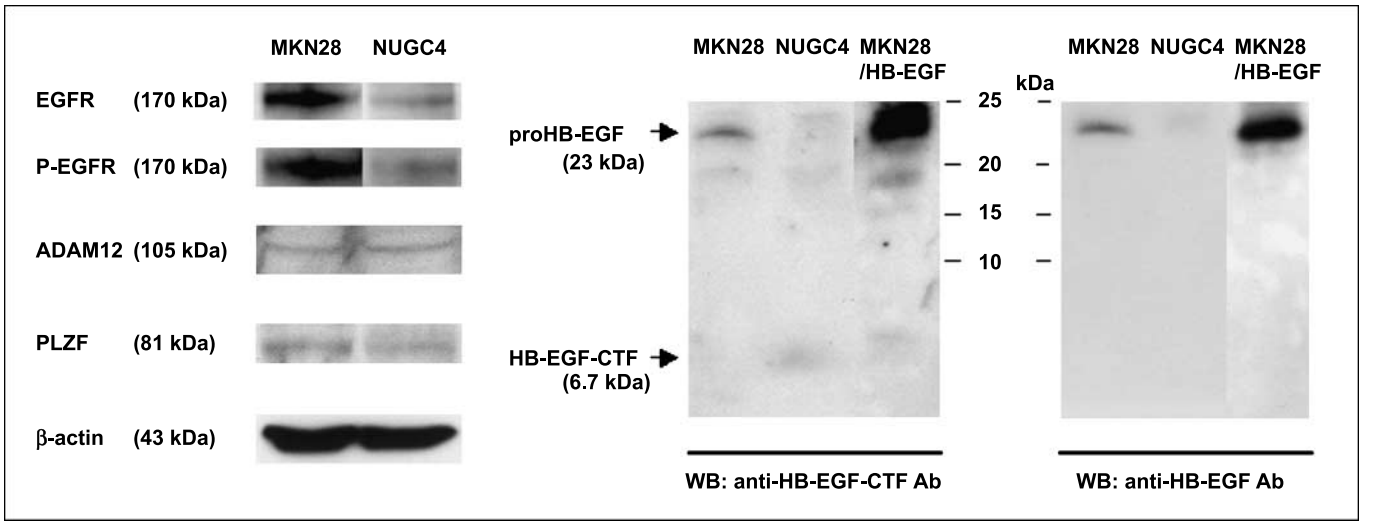

Fig. 1. Western blot analysis of EGFR, phospho-EGFR, ADAM12, PLZF, proHB-EGF, and HB-EGF-CTF expression in MKN-28 and NUGC4 gastric cancer cell lines in 10% FBS. Each lane contains 100 μg protein. We used MKN28 cells overexpressing proHB-EGF (MKN28/HB-EGF) to recognize the size of the forms of proHB-EGF and HB-EGF-CTF as control values. We used anti-HB-EGF-CTF antibody to recognize the cytoplasmic region of proHB-EGF and anti-HB-EGF antibody to recognize the proHB-EGF ectodomain. For bands of increased MKN28/HB-EGF, the 6.7-kDa band detected with only anti-HB-EGF-CTF antibody was considered as HB-EGF-CTF and the 23-kDa band detected with both anti-HB-EGF-CTF antibody and anti-HB-EGF antibody was considered as proHB-EGF. Loading control comprised β-actin.

example, the efficacy of gefitinib was limited in pretreated patients with metastatic gastric cancer (12). These results suggest that controlling gastric cancer cell proliferation using EGFR tyrosine kinase inhibition alone is difficult.

A recent study using human primary keratinocytes showed that cleavage of membrane-anchored HB-EGF (proHB-EGF) induced translocation of the carboxyl-terminal fragment of proHB-EGF (HB-EGF-CTF) from the plasma membrane to the nucleus and regulated cell cycle by abrogation of repressors such as promyelocytic leukemia zinc finger (PLZF; refs. 13, 14). Conventional EGFR-targeting agents mainly aim for EGFR inactivation through inhibition of ligand binding to receptors and inactivation of EGFR tyrosine kinase. However, such approaches may be insufficient to inhibit cancer cell proliferation, as cell proliferation signals remain active through HB-EGF-CTF nuclear translocation, even if EGFR is completely inactivated.

We therefore studied the effect of suppressing HB-EGF-CTF nuclear translocation using KB-R7785, an inhibitor of HB-EGF shedding, with EGFR inactivation by cetuximab in gastric cancer cell lines. We report herein that suppression of HB-EGF-CTF nuclear translocation might represent a crucial target for gastric cancer therapy.

Materials and Methods

Materials. Cetuximab (IMC-C225, Erbitux), a monoclonal antibody to EGFR, was used to inhibit EGFR phosphorylation. KB-R7785 was selected as one of the most potent inhibitors for HB-EGF shedding by ADAM12 from over 2,000 metalloproteinase inhibitors (15). KB-R7785 directly binds to ADAM12, inhibiting shedding of HB-EGF by ADAM12 and suppressing HB-EGF-CTF nuclear translocation (13). Cetuximab was purchased from Merck KGaA, and KB-R7785 was donated by Dr. Shigeki Higashiyama (Ehime University).

Cell culture and transfection. The present investigation used the MKN28 and NUGC4 gastric cancer cell lines (Japan Health Science Research Resources Bank). Cell lines were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin and incubated at 37°C in an atmosphere of 95% air and 5% CO_2.

For the establishment of gastric cancer cells expressing human proHB-EGF (MKN28/HB-EGF), MKN28 cells were transiently transfected with a plasmid encoding proHB-EGF (donated by Dr. Shigeki Higashiyama) using LipofectAMINE 2000 (Invitrogen).

Immunofluorescence microscopy. Samples were fixed with ethanol and acetone. Incubation with primary antibodies against HB-EGF-CTF (donated by Dr. Shigeki Higashiyama) or PLZF (Calbiochem) was generally done in a solution of PBS containing 0.1% milk. Secondary antibodies were Cy3-conjuncted and AffiniPure $F(ab)_2$ Fragment Goat Anti-Rat IgG (H + L; Jackson Immuno Research Laboratories) or Alexa Fluor 488 goat anti-mouse IgG (H + L; Invitrogen). All sections were counterstained with 4′,6-diamidino-2-phenylindole (Kirkegaard and Perry Laboratories). Images were obtained using an Eclipse 80i fluorescence microscope (Nikon).

Human gastric cancer cells (MKN28 and NUGC4) in a subconfluent state were switched to serum-free medium for 24 h and then incubated for another 30 min in the absence (controls) or presence of 100 μmol/L KB-R7785 alone, 10 μg/mL cetuximab alone, or 100 μmol/L KB-R7785 with 10 μg/mL cetuximab and stimulated with 200 nmol/L 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Cell Signaling Technology) for 60 min. Cells were stimulated by TPA, an activator of PKC, to investigate localization of HB-EGF-CTF by shedding proHB-EGF (16), and subcellular localization of HB-EGF-CTF was then analyzed by immunofluorescence study.

Western blotting. Cells were washed with PBS(-) and subsequently dissolved in 1× cell lysis buffer (Cell Signaling Technology) containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, and 1 μg/mL leupeptin with the addition of 1 mmol/L phenylmethylsulfonyl fluoride. After disruption in an ice bath using a Bio-ruptor sonicator (Cosmo Bio) for 15 s, lysates were centrifuged at 15,000 rpm for 10 min at 4°C. Each sample was normalized on an equal protein concentration using a protein assay kit (Bio-Rad Laboratories). A equal quantity of 2× SDS-PAGE sample buffer [0.5 mol/L Tris-HCl (pH 7.2), 1% SDS, 100 mmol/L β-mercaptoethanol, and 0.01% bromophenol blue] was added to each sample and boiled for 5 min at 100°C. Aliquots of

sample were fractioned on 8% to 15% SDS-PAGE and then electroblotted to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk in PBS(-) for 1 h at room temperature. The membrane was incubated with primary antibodies, EGFR, phospho-EGFR (Upstate Biotechnology), ADAM12 (Santa Cruz Biotechnology), PLZF, HB-EGF-CTF, or HB-EGF (R&D Systems) overnight at 4°C and then washed with 0.05% Tween 20 in PBS(-) three times at 5-min intervals. The membrane was incubated with secondary antibody for 1 h at room temperature followed by three washes with 0.05% Tween 20 in PBS(-) three times at 5-min intervals. The membrane was treated with enhanced chemiluminescence detection reagents (Amersham) for 1 min at room temperature and exposed to scientific imaging films (Eastman Kodak), and proteins were visualized as the bands. Filters were stripped and reprobed with monoclonal β-actin antibody (Abcam plc) as an internal control.

To assess inhibition of EGFR phosphorylation by KB-R7785 and/or cetuximab, aliquots of gastric cancer cell lines (MKN28 and NUGC4) were plated in RPMI 1640-10% FBS in six-well culture plates. After 24 h of plating, cells were serum starved for 48 h and subsequently incubated for another 60 min in the absence (controls) or presence of 100 μmol/L KB-R7785 alone, 10 μg/mL cetuximab alone, or 100 μmol/L KB-R7785 with 10 μg/mL cetuximab and then stimulated with 10 ng/mL EGF (Upstate Biotechnology), a ligand of EGFR, for 10 min to phosphorylate EGFR. Using the proteins of each sample, activation of EGFR was analyzed by Western blotting.

Growth inhibition assay. Inhibition of cell growth in response to cetuximab and/or KB-R7785 was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay as described previously (17). Briefly, cells (10^4 per well) in RPMI 1640-10% FBS were plated in a 96-well culture plate with three replicates per treatment. After 24 h of plating, medium was replaced with medium containing 3% FBS to minimize the contribution of serum-derived growth factors, and incubation for 48 h at 37°C continued in the presence of cetuximab (0, 10, and 100 μg/mL) with 0, 10, 50, or 100 μmol/L KB-R7785. All incubations were terminated by adding 20 μL CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) to each well. After reaction for 2 h at 37°C, intensity of color was measured at 490 nm.

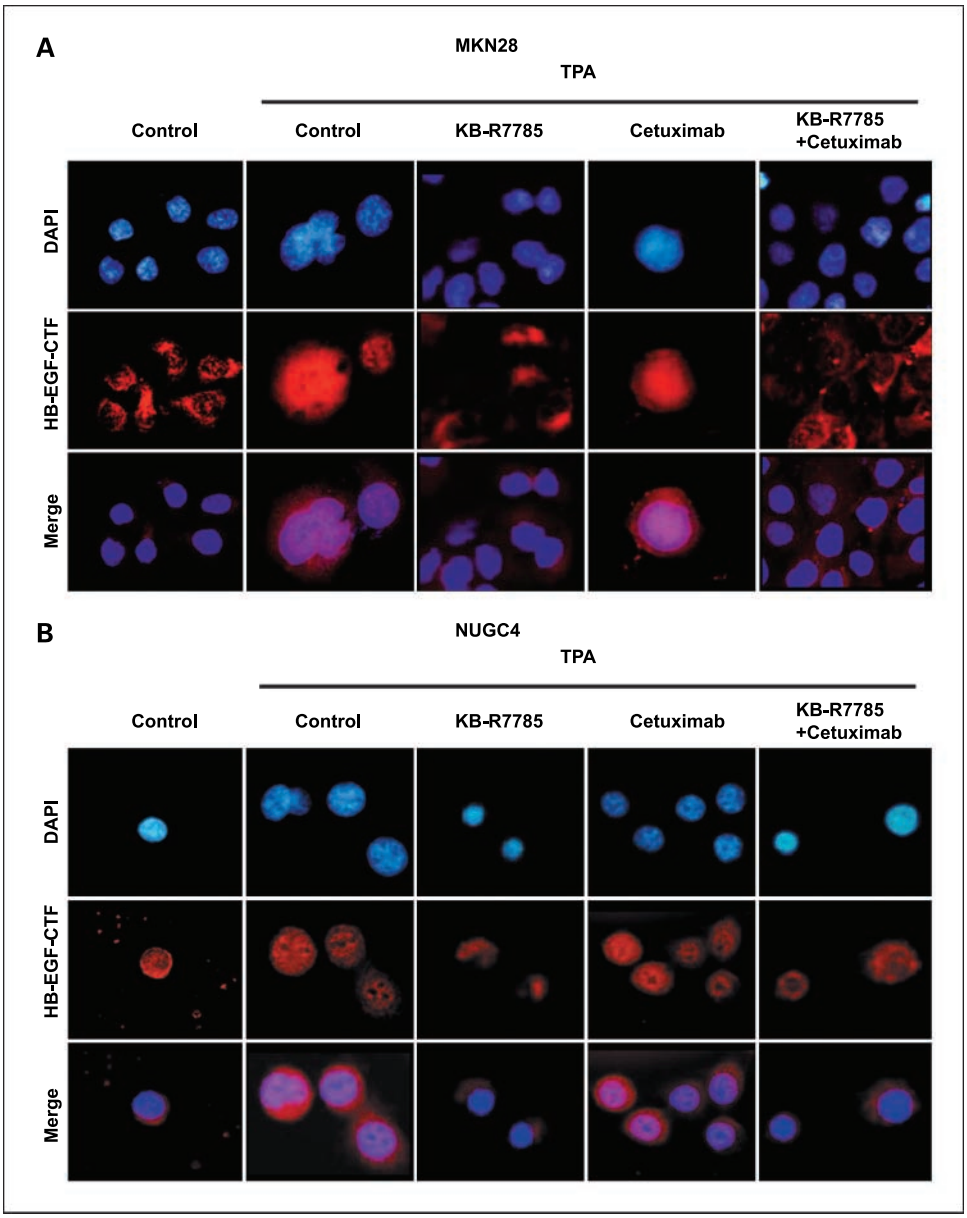

Fig. 2. Nuclear translocation of HB-EGF-CTF after TPA-inducible processing of proHB-EGF in gastric cancer cells by immunofluorescence microscopy. *A*, MKN28; *B*, NUGC4. Cells were treated with KB-R7785 (100 μmol/L), cetuximab (10 μg/mL), and TPA (200 nmol/L). Nuclei stained blue with 4′,6-diamidino-2-phenylindole (DAPI) and HB-EGF-CTF stained red with antibody against HB-EGF-CTF.

Assessment of apoptosis. Apoptosis was assessed by analysis of activation of caspase-3 and caspase-7 using the substrate DEVD-aminoluciferin from the Caspase-Glo 3/7 Assay kit (Promega) according to the instructions of the manufacturer. Briefly, cells (10^4 per well) in RPMI 1640-10% FBS were plated in a 96-well culture plate with three replicates per treatment. After 24 h of plating, medium was replaced with medium containing 3% FBS, and incubation for 48 h at 37°C continued in the presence of cetuximab (0, 10, and 100 μg/mL) with 0, 10, 50, or 100 μmol/L KB-R7785. Caspase-Glo reagent was added to each well and subsequently incubated for 1 h, and luminescence was measured using a LUMAT LB 9507 luminometer (Berthold Technologies).

Cell cycle analysis. Cell lines were seeded in 6-cm dishes and cultured in RPMI 1640-10% FBS. In a subconfluent state, medium was replaced with medium containing 3% FBS and cells were treated with nothing (control), 100 μmol/L KB-R7785 alone, 10 μg/mL cetuximab alone, or 100 μmol/L KB-R7785 with 10 μg/mL cetuximab and then incubated for 48 h at 37°C. Cells in each sample were subsequently collected and washed with PBS(-).

For DNA staining, cells were fixed in 70% ethanol for 4 h at 4°C and incubated with 0.25 mg/mL RNase for 30 min at 37°C. After washing, cells were stained using 0.05 mg/mL propidium iodide. Data acquisition was done using a FACScan flow cytometer (Becton Dickinson). Cell cycle distribution was analyzed with ModFit software (Verity Software House).

Assessment of cyclin A and c-Myc by Western blotting. Aliquots of MKN28 and NUGC4 gastric cancer cell lines were plated in RPMI 1640-10% FBS in six-well culture plates. In a subconfluent state, medium was replaced with medium containing 3% FBS, and incubation for 24 h at 37°C continued in the absence (controls) or presence of 100 μmol/L KB-R7785 alone, 10 μg/mL cetuximab alone, or 100 μmol/L KB-R7785 with 10 μg/mL cetuximab. Using antibodies against cyclin A and c-Myc (Santa Cruz Biotechnology), expression of cyclin A and c-Myc proteins in each sample was analyzed by Western blotting.

Statistics. Values are expressed as mean ± SE. Data were analyzed using one-factor ANOVA and Tukey-Kramer's multiple comparison procedure, as appropriate. P values < 0.05 were considered statistically significant.

Results

EGFR, phospho-EGFR, ADAM12, PLZF, proHB-EGF, and HB-EGF-CTF expression in MKN28 and NUGC4. We first evaluated expression of EGFR and phospho-EGFR in human gastric cancer cell lines by Western blotting. Furthermore, ADAM12 (a major sheddase for proHB-EGF; refs. 15, 18), PLZF (a repressor interacting with HB-EGF-CTF; ref. 13), proHB-EGF (19), and HB-EGF-CTF were measured.

MKN28 expressed higher levels of EGFR and phospho-EGFR than NUGC4. With respect to ADAM12 and PLZF, both gastric cancer cell lines expressed the same levels. MKN28 expressed higher levels of proHB-EGF than NUGC4 but lower levels of HB-EGF-CTF (Fig. 1).

Nuclear translocation of HB-EGF-CTF by TPA-inducible processing. Under serum-starved culture conditions, immunostaining of HB-EGF-CTF was mainly observed in cytoplasm. When TPA was added to activate the processing of proHB-EGF, nuclear translocation of HB-EGF-CTF was induced. Similarly, nuclear accumulation of HB-EGF-CTF was observed after TPA and cetuximab treatment. However, when KB-R7785 with or without cetuximab was added to cells, nuclear export of HB-EGF-CTF was inhibited and HB-EGF-CTF immunostaining was observed at the plasma membrane. Inhibition of HB-EGF-CTF nuclear translocation by KB-R7785 was observed in both MKN28 and NUGC4 (Fig. 2A and B).

Inhibition of EGF-induced EGFR phosphorylation in the presence of KB-R7785 and/or cetuximab. We compared EGFR phosphorylation by EGF in the presence of KB-R7785 and/or cetuximab. EGF induced EGFR phosphorylation and KB-R7785 did not inhibit EGFR phosphorylation, but cetuximab with or without KB-R7785 suppressed EGFR phosphorylation in gastric cancer cells (Fig. 2C). KB-R7785 showed no additional suppressive effects for EGFR phosphorylation.

KB-R7785 inhibitory cancer cell growth regulation. In the next step, we investigated cell growth regulation by KB-R7785 and/or cetuximab. Cell growth inhibition of MKN28 and NUGC4 was barely changed by cetuximab in concentrations between 10 and 100 μg/mL.

In contrast, gastric cancer cell growth was significantly inhibited by KB-R7785 in a dose-dependent manner at all concentrations of cetuximab and was more inhibited by combination of KB-R7785 with cetuximab. Inhibitory effects of KB-R7785 and cetuximab on cell growth were stronger in NUGC4 than in MKN28 (Fig. 3A).

KB-R7785-induced apoptosis. We next examined whether inhibition of gastric cancer cell growth by KB-R7785 and/or cetuximab involves apoptosis. We first confirmed as a positive control for induction of apoptosis that 3 μg/mL cisplatin (Randa; Nihon Kayaku), an anti-cancer drug, induced apoptosis at 9-fold greater levels than control in MKN28 and 8-fold greater levels than control in NUGC4 (data not shown). KB-R7785 tended to induce apoptosis in a dose-dependent manner, and 100 μmol/L KB-R7785 significantly induced apoptosis regardless of cetuximab concentration in both NUGC4 and MKN28 cells (Fig. 3B).

Moreover, we examined localization of HB-EGF-CTF during MTS and apoptosis assays. The percentage of cells displaying nuclear staining for HB-EGF-CTF was decreased significantly in

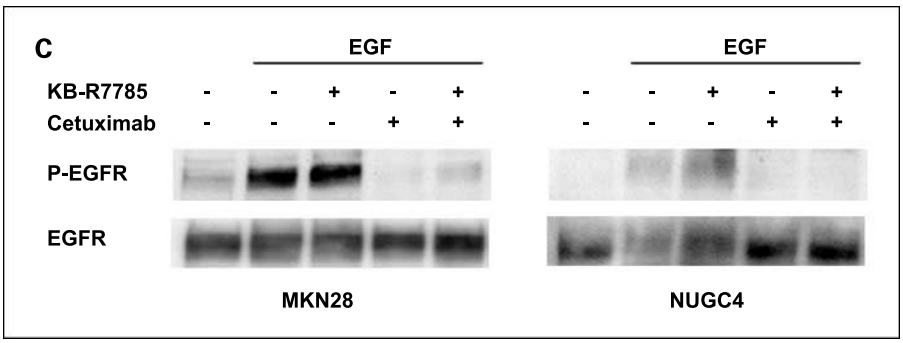

Fig. 2 *Continued*. *C,* Western blot analysis of EGFR phosphorylation induced by EGF (10 ng/mL), KB-R7785 (100 μmol/L), and cetuximab (10 μg/mL) in gastric cancer cells (MKN28 and NUGC4). Loading control comprised EGFR.

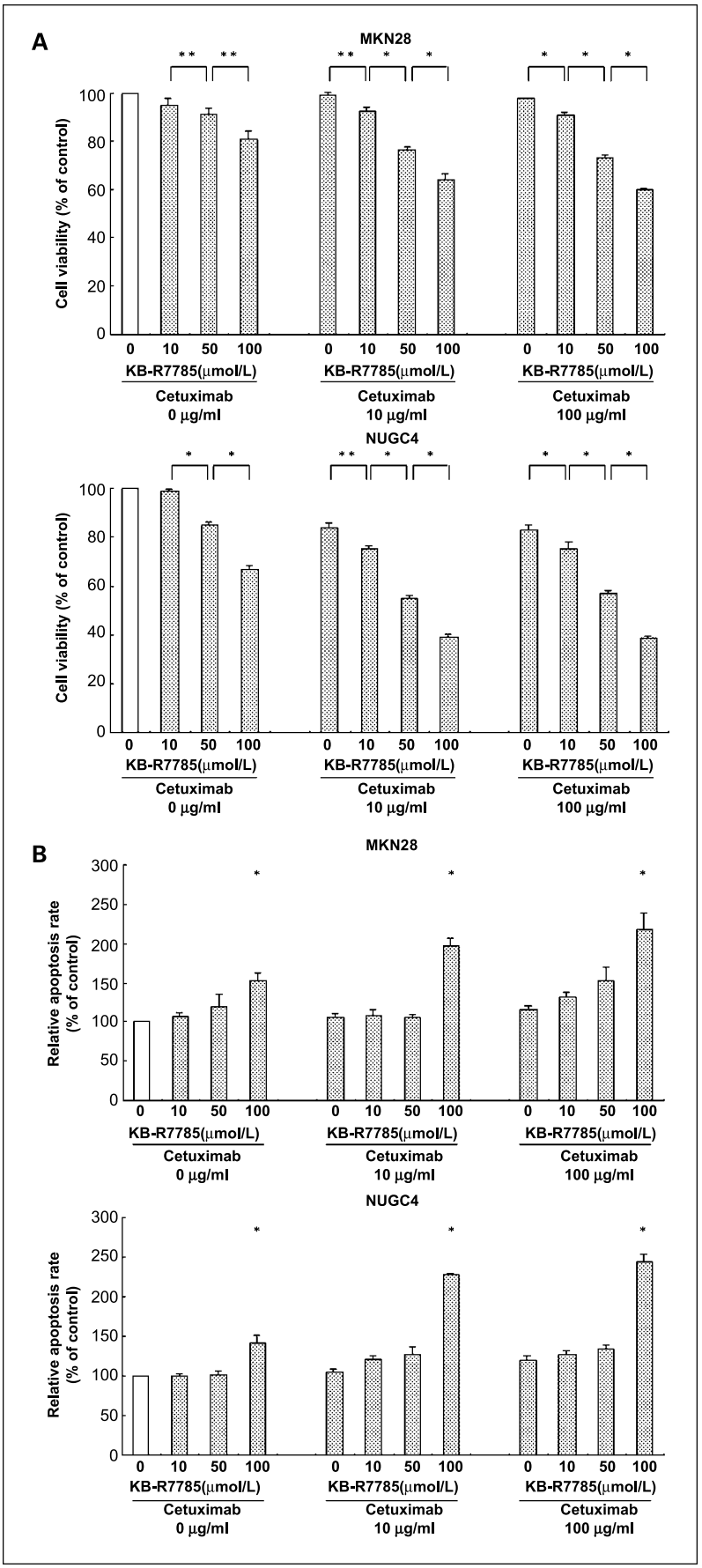

Fig. 3. *A,* MTS assays showed growth-inhibitory effects on gastric cancer cells (MKN28 and NUGC4) induced by cetuximab (0, 10, or 100 μg/mL) in combination with KB-R7785 (0, 10, 50, or 100 μmol/L). Mean of five independent experiments in triplicate; *bars,* SE. Values of cells in FBS alone (without KB-R7785 and cetuximab) were used as control (*white box*). *, $P < 0.01$; **, $P < 0.05$. *B,* apoptosis assay revealed induced apoptosis of gastric cancer cells (MKN28 and NUGC4) by cetuximab (0, 10, or 100 μg/mL) in combination with KB-R7785 (0, 10, 50, or 100 μmol/L). Apoptosis was assessed by analyzing activation of caspase-3 and caspase-7. Mean of three independent experiments in triplicate; *bars,* SE. Values of apoptosis of cells in FBS alone (without KB-R7785 and cetuximab) were used as control (*white box*). *, $P < 0.01$, treatment by cetuximab with 100 μmol/L KB-R7785 versus treatment by cetuximab with 0, 10, or 50 μmol/L KB-R7785.

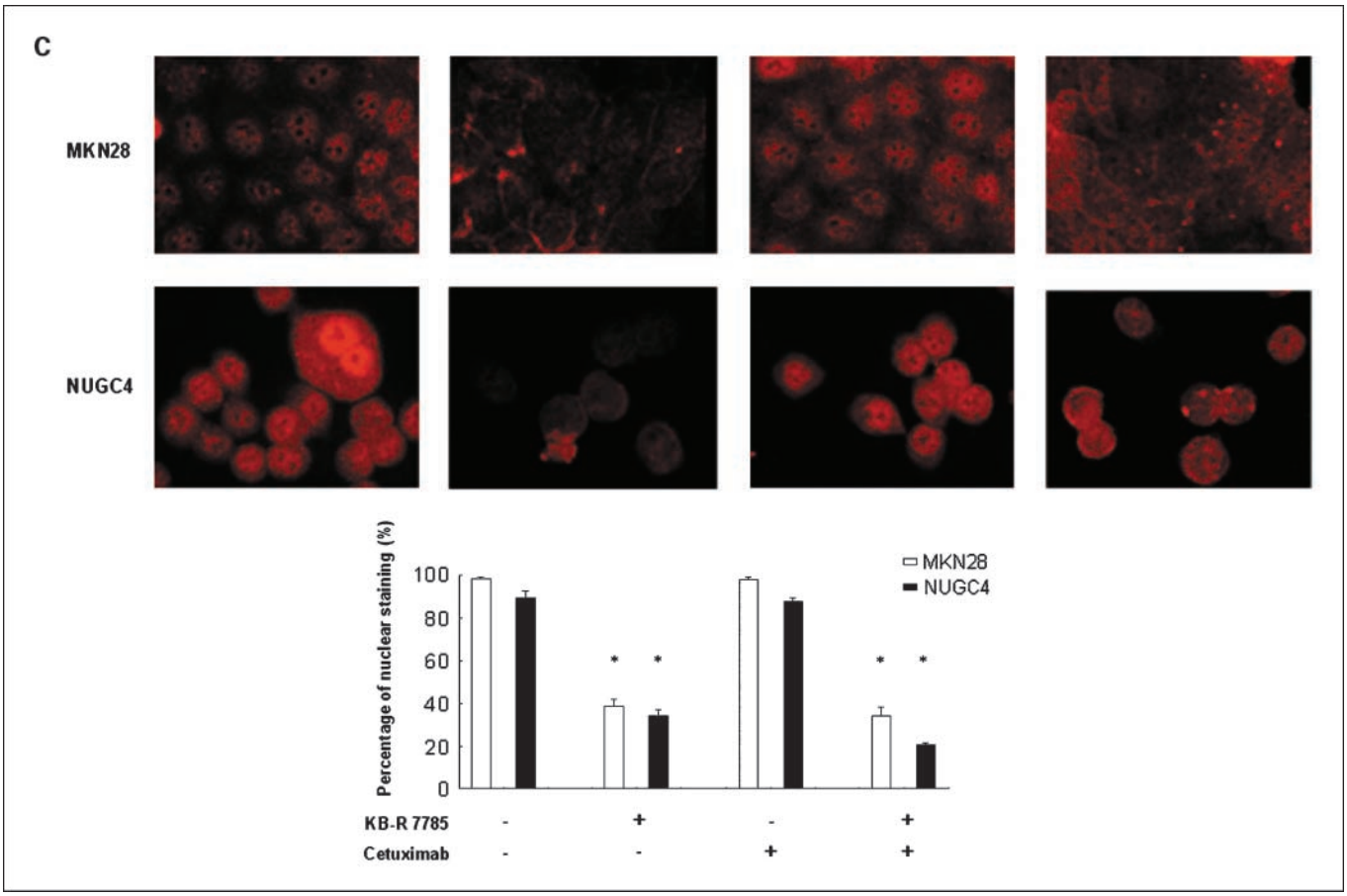

Fig. 3 *Continued*. *C,* intracellular localization of HB-EGF-CTF under conditions of MTS and apoptosis assay in gastric cancer cells (MKN28 and NUGC4) were observed by immunofluorescence microscopy. Three staining regions were randomly selected under a ×400 field and the total for three regions was calculated as the positive rate of cells displaying nuclear staining. Percentage of cells showing nuclear staining is shown for 100 μmol/L KB-R7785 and/or 10 μg/mL cetuximab. Mean of three independent experiments in triplicate; *bars,* SE. *, $P < 0.01$, treatment by KB-R7785 alone or KB-R7785 with cetuximab versus treatment by cetuximab alone or no treatment.

gastric cancer cells treated by KB-R7785 with or without cetuximab (Fig. 3C). These results suggest that cell growth inhibition and apoptosis by KB-R7785 might be mainly due to the suppression of HB-EGF-CTF nuclear translocation.

Cell cycle regulation by cetuximab and KB-R7785. Cell cycle regulation by KB-R7785 and/or cetuximab was studied in MKN28 and NUGC4 using FACScan. Addition of 100 μmol/L KB-R7785 or 10 μg/mL cetuximab induced G_1-phase increase and S-phase decrease and 100 μmol/L KB-R7785 with 10 μg/mL cetuximab induced more G_1-phase accumulation and a S-phase decrease in MKN28 and NUGC4. KB-R7785 induced sub-G_1-phase accumulation, corresponding to the apoptotic cell fraction, and more sub-G_1-phase accumulation was detected for KB-R7785 with cetuximab. These findings correlate with the data in Fig. 3C, suggesting that 100 μmol/L KB-R7785 induced apoptosis (Fig. 4). These findings suggest that the inhibitory regulation of nuclear translocation of HB-EGF-CTF by KB-R7785 induced apoptosis and G_1 arrest, and the effects of KB-R7785 increased in combination with cetuximab.

KB-R7785 suppressed cyclin A and c-Myc expression. HB-EGF-CTF generated by ectodomain shedding of proHB-EGF reportedly exports PLZF, a transcriptional repressor of cyclin A (20, 21), from nucleus to cytoplasm, thus increasing expression of cyclin A and promoting entry to S phase in the cell cycle (13). Conversely, c-Myc is widely known as a crucial regulator of normal and neoplastic cells (22, 23). Activation of growth factor receptors such as EGFR by ligand binding leads to increased expression of c-Myc, a transcriptional regulator for cell proliferation (24). proHB-EGF and subsequent HB-EGF-CTF signaling can modulate c-Myc expression and cell cycle progression (25). We thus investigated the intracellular localization of PLZF using immunofluorescence microscopy and expressions of cyclin A and c-Myc by Western blotting in gastric cancer cells treated with KB-R7785 and/or cetuximab. PLZF was observed in the cytoplasm under normal conditions and treatment with cetuximab alone did not result in any changes to the localization of PLZF. However, PLZF tended to localize in nuclei following KB-R7785 treatment with or without cetuximab (Fig. 5A).

MKN28 showed high levels of cyclin A and NUGC4 displayed moderate levels. Cetuximab alone barely changed the expression of cyclin A, but KB-R7785 decreased cyclin A expression. Cyclin A expression was clearly decreased by KB-R7785 in NUGC4 but was only slightly decreased in MKN28. Expression level of c-Myc was moderate in MKN28 and high in NUGC4 and was decreased by KB-R7785 alone or cetuximab alone. Moreover, KB-R7785 with cetuximab prominently attenuated c-Myc expression (Fig. 5B). These results suggest that cyclin A is mainly regulated by HB-EGF-CTF nuclear

translocation, and EGFR does not seem to play a crucial role. Conversely, regulation by nuclear translocation of HB-EGF-CTF is as important as EGFR for c-Myc expression.

Discussion

EGFR and EGFR ligands have been well studied as molecular targets for cancer therapy (26–28). In contrast, studies targeting HB-EGF-CTF produced by proHB-EGF shedding as a cancer therapy have not been reported previously. The present work thus focused on HB-EGF-CTF as a new molecular target for gastric cancer therapy.

ADAMs are a family of cell surface membrane glycoproteins, with >30 ADAMs currently identified, and the multidomain structure enables diverse roles in a wide range of cellular processes. Accumulating evidence associates increased expression of individual ADAM family members with various cancers (18). A recent report indicated that ADAM9, ADAM12, and ADAM15 are up-regulated and play important roles in gastric cancer (29). The proHB-EGF is proteolytically cleaved by ADAM9, ADAM10, ADAM12, and ADAM17 (15, 30–33), a process called ectodomain shedding, resulting in the release of HB-EGF into the extracellular space and the production of a plasma membrane-associated remnant (HB-EGF-CTF).

In completing this study, we confirmed expression of EGFR, ADAM12, PLZF, proHB-EGF, and HB-EGF-CTF by Western blotting and HB-EGF-CTF translocation from the plasma membrane to the nucleus after TPA stimulation by immunofluorescence microscopy in two gastric cancer cell lines. KB-R7785 was used because this agent can inhibit HB-EGF-CTF nuclear translocation by blocking the shedding of HB-EGF through inhibition of ADAM12. Suppression of HB-EGF-CTF nuclear translocation by KB-R7785 was confirmed using an immunofluorescence study in gastric cancer cells. Furthermore, KB-R7785 showed no additional suppressive effects for EGFR phosphorylation. Therefore, by comparing the reactions of cells treated in KB-R7785 or cetuximab alone with cells treated in KB-R7785 and cetuximab, we investigated the effect of inhibition of HB-EGF-CTF nuclear translocation without signal transduction from EGFR activation.

Cell growth inhibition in MKN28 and NUGC4 was barely detected even with increased concentrations of cetuximab. However, gastric cancer cell growth was significantly inhibited by addition KB-R7785 to cetuximab regardless of cetuximab concentration. These results suggest that not only the inhibition of EGFR phosphorylation but also inhibition of HB-EGF-CTF nuclear translocation by KB-R7785 play crucial roles in inhibitory regulation of cancer cell growth.

Conversely, high-dose KB-R7785 induced apoptosis using the agent alone and more prominent apoptosis in combination with cetuximab. Moreover, KB-R7785 or cetuximab increased G_1-phase arrest and the combination of KB-R7785 and cetuximab achieved a greater increase. These results suggest that suppression of HB-EGF-CTF nuclear translocation and EGFR phosphorylation inhibits gastric cancer cell proliferation by inducing cell cycle arrest and apoptosis. Combination therapy using KB-R7785 with existing anticancer drugs might thus represent a promising new option for antigastric cancer therapies.

PLZF has been reported as a transcriptional repressor of genes such as cyclin A and c-Myc (21, 34). HB-EGF-CTF reportedly increases the expression of cyclin A by export from the nucleus of PLZF and promotes S-phase entry (13). Cyclin A is a member of the cyclin family involved in the progression of cells through the cell cycle and seems to be required for both DNA synthesis and mitosis (35). Cyclin A has been implicated in the control of mitosis, regulation of S phase, and/or G_1-S transition (36). In contrast, the c-Myc oncogene is frequently associated with human malignancies and plays a crucial role in regulating cell proliferation and differentiation (22, 23). Overexpression of c-Myc can cause malignant transformation, and sustained tumor growth depends on continued c-Myc expression (37–40). However, how c-Myc promotes malignant transformation remains unclear. A recent study has shown that single transduction

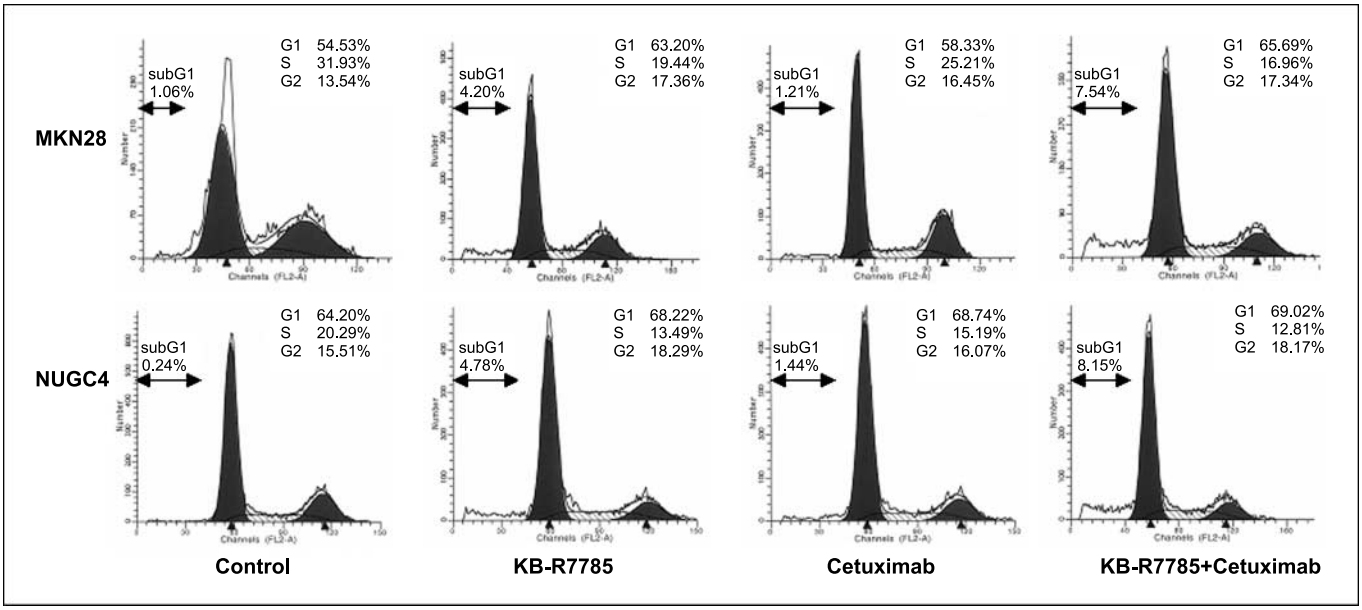

Fig. 4. Flow cytometry of gastric cancer cell lines (MKN28 and NUGC4) treated with KB-R7785 (100 μmol/L) and/or cetuximab (10 μg/mL) for 48 h.

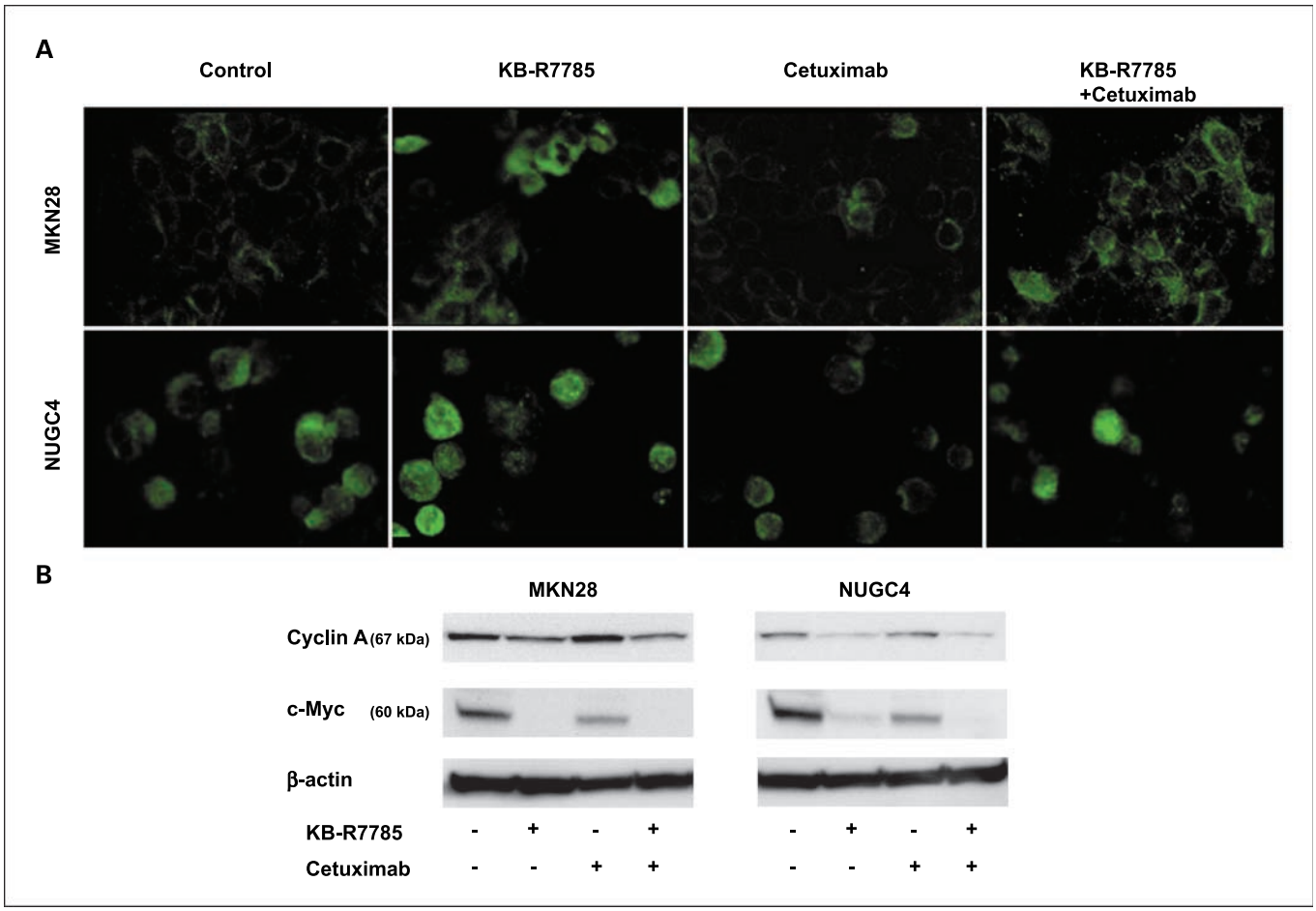

Fig. 5. *A,* intracellular localization of PLZF under conditions of MTS and apoptosis assay in gastric cancer cells (MKN28 and NUGC4) was observed by immunofluorescence microscopy. PLZF shows green fluorescence with antibody against PLZF. *B,* Western blot analysis for cyclin A and c-Myc expression in gastric cancer cells (MKN28 and NUGC4) treated with KB-R7785 (100 μmol/L) and/or cetuximab (10 μg/mL). Each lane contained 100 μg protein. Loading control comprised β-actin.

initiated by generation of HB-EGF-CTF in the shedding event plays an important intermediary role between growth factor receptor activation and c-Myc gene induction (25). We therefore investigated the localization of PLZF and expression of cyclin A and c-Myc to determine if these proteins receive inhibitory regulation by KB-R7785 and/or cetuximab. KB-R7785 suppressed export from nucleus of PLZF in gastric cancer cells, but cetuximab did not. Gastric cancer cells treated with cetuximab alone displayed barely any changes in cyclin A expression, whereas cells treated with KB-R7785 showed decreased cyclin A expression. These findings suggest that cell cycle arrest through the suppression of HB-EGF-CTF nuclear translocation partially depends on cyclin A in gastric cancer cells as reported previously in keratinocytes (13). Cetuximab decreased c-Myc expression, but more attenuation of c-Myc expression was observed by addition of KB-R7785. These results support the previous finding that full induction of c-Myc by growth factor receptors such as EGFR requires HB-EGF-CTF signaling (25). In other words, inhibition of both EGFR and HB-EGF-CTF is important to inhibit c-Myc expression in gastric cancer cells. Moreover, these results suggest that c-Myc and cyclin A might be important factors for cell growth by HB-EGF-CTF nuclear translocation.

In the present study, although expression of EGFR and proHB-EGF was lower in NUGC4 than in MKN28 and expression of ADAM12 and PLZF was almost identical, cell growth of NUGC4 was more inhibited by cetuximab with KB-R7785 than that of MKN28. In other words, the effect by KB-R7785 is independent of the quantity of EGFR and EGFR ligand expressions. Similarly, no relationship between EGFR expression and tumor response to cetuximab has been identified in clinical trials (8, 41). Cell growth inhibitory effects by KB-R7785 with cetuximab in other gastric cancer cell lines such as MKN45, KATOIII, AGS, and GCIY were almost the same as seen in MKN28 (data not shown). We have not been able to properly elucidate the reasons why growth of NUGC4 was strongly inhibited by KB-R7785, but we can suggest three reasons based on the present results. First, HB-EGF-CTF may affect cell growth more deeply in NUGC4 than MKN28 because expression of HB-EGF-CTF is higher in NUGC4 than in MKN28. Second, HB-EGF-CTF nuclear translocation may be more suppressed by KB-R7785 in NUGC4 than in MKN28. Third, expression of cyclin A may be more decreased by KB-R7785 and expression of c-Myc may be higher in NUGC4 than in MKN28. These factors might be related to the strong inhibition of cell growth in NUGC4 by KB-R7785. We considered that further investigation remains

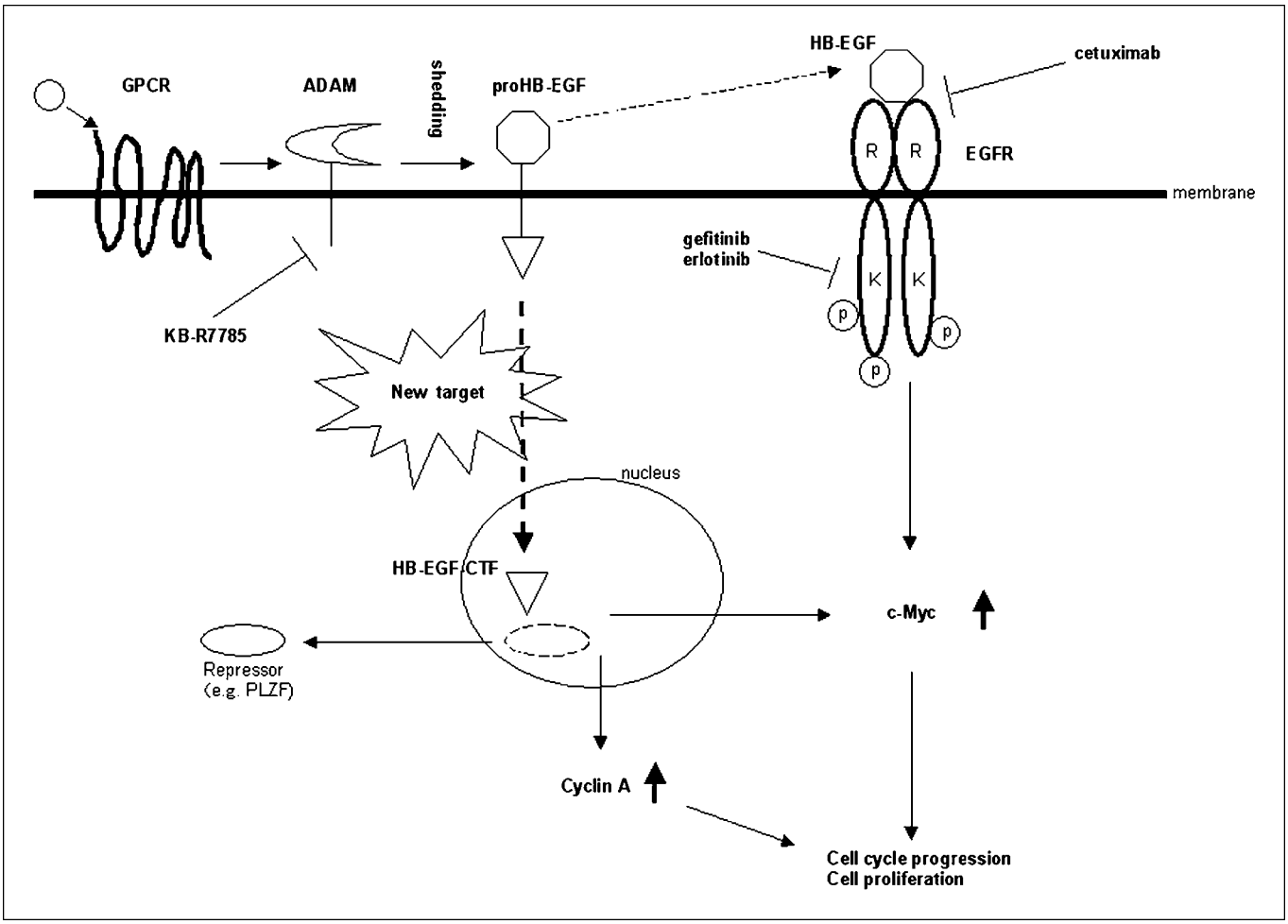

Fig. 6. G-protein-coupled receptor stimulation leads to activation of ADAM, resulting in proHB-EGF shedding. Subsequently released HB-EGF activates EGFR and downstream signaling cascades and finally induces cell proliferation, angiogenesis, migration, and antiapoptosis. Conventional EGFR-targeted agents such as cetuximab (anti-EGFR monoclonal antibody) and gefitinib and erlotinib (EGFR-tyrosine kinase inhibitors) inhibit EGFR phosphorylation. Cleavage of proHB-EGF induces translocation of HB-EGF-CTF from the plasma membrane to the nucleus and binds to the repressor. This translocation induces cell cycle progression and cell proliferation by promotion of cyclin A and c-Myc by abrogation of repressors such as PLZF. KB-R7785 blocks proHB-EGF shedding by inhibiting ADAM and results in suppression of HB-EGF-CTF nuclear export. As a result, functions as an intracellular signal of HB-EGF-CTF are suppressed.

necessary, as the detailed mechanisms are not yet fully understood.

In summary, we show ADAM-mediated EGFR signal transactivation and a biological role for HB-EGF-CTF nuclear translocation (Fig. 6). Our data show that suppression of HB-EGF-CTF nuclear translocation with inactivation of EGFR inhibits cell growth and induces apoptosis and cell cycle arrest in gastric cancer cell lines. However, we have some problems for using KB-R7785 as an anticancer drug, as inhibition of the shedding of EGFR ligands results in strong inhibition of wound healing *in vivo* and *vitro* (42). Suppression of HB-EGF-CTF nuclear translocation appears effective as a new molecular targeting therapy for gastric cancer, and we thus expect the development of new molecular target agents to inhibit HB-EGF-CTF nuclear translocation alone.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Shigeki Higashiyama and Miki Hieda for providing KB-R7785, antibody against HB-EGF-CTF, and the plasmid encoding proHB-EGF and for helpful comments and Yukimi Itoh for technical assistance.

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Clin Cancer Res 2008;14:3956-3965.