A Novel Anti-Human HB-EGF Monoclonal Antibody with Multiple Antitumor Mechanisms against Ovarian Cancer Cells

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Abstract

Purpose: Heparin-binding epidermal growth factor (EGF)–like growth factor (HB-EGF) is a member of the EGF family and plays a pivotal role in tumor progression in ovarian cancer. We developed an anti-HB-EGF monoclonal antibody (mAb) and investigated its antitumor activities in vitro and in vivo to evaluate its potential as a therapeutic antibody against ovarian cancer.

Experimental Design: We prepared mAbs from HB-EGF null mice immunized with recombinant human soluble HB-EGF and evaluated their binding and neutralizing activity against HB-EGF. Next, we generated a mouse–human chimeric antibody and examined its in vitro and in vivo antitumor activities.

Results: Two murine anti-HB-EGF mAbs were developed, and one of them, KM3566, was revealed to have a high binding reactivity for membrane-anchored HB-EGF (pro-HB-EGF) expressed on the cell surface, as well as neutralizing activity against growth promoting activity of soluble HB-EGF. The mouse–human chimeric counterpart for KM3566 (cKM3566) induced dose-dependent antibody-dependent cellular cytotoxicity (ADCC) against cancer cells expressing HB-EGF in vitro, and significantly inhibited tumor growth in severe combined immunodeficient mice inoculated with MCAS or ES-2 human ovarian cancer cells.

Conclusions: A novel anti-HB-EGF chimeric antibody, cKM3566, with two antitumor mechanisms, neutralization and ADCC, exhibits potent in vivo antitumor activity. These results indicate that cKM3566 is a promising antiovarian cancer therapeutic antibody. Clin Cancer Res; 17(21); 1–9. ©2011 AACR.

Introduction

Ovarian cancer causes more deaths than any other cancer of the female reproductive system. In 2010, an estimated 21,880 new cases and 13,850 deaths of ovarian cancer were reported in the United States (1). Ovarian cancer usually has a poor prognosis because many cases are diagnosed in advanced stages. Standard treatment involves surgery followed by chemotherapy, and most patients achieve a complete response after debulking surgery and platinum-based chemotherapy. However, 50% of these patients relapse and die of the disease. Therefore, the development of novel treatments which can increase the survival of the patients with ovarian cancer is needed.

Heparin-binding epidermal growth factor (EGF)–like growth factor (HB-EGF) is a growth factor which belongs to the EGF family (2). HB-EGF is synthesized as a type 1 transmembrane protein called pro-HB-EGF and is released by proteolytic ectodomain shedding, which yields a soluble form of HB-EGF (sHB-EGF; ref. 3). Studies of knock-in mice expressing an uncleavable mutant form of HB-EGF indicated that the major functions of HB-EGF were mediated by sHB-EGF in vivo (4). Moreover, several lines of evidence indicate that sHB-EGF and pro-HB-EGF have distinct biological activity (5). Thus, ectodomain shedding is critical for HB-EGF functions. The elevated expression of HB-EGF has been reported in several kinds of cancers, such as ovarian (6, 7), gastric (8, 9), and breast cancer (10–12). Especially, increasing evidence has suggested a critical role for HB-EGF in ovarian cancer progression. Tumor formation of ovarian...
cancer cells was completely blocked by RNA interference targeting pro-HB-EGF or by CRM197, which is a nontoxic mutant of diphtheria toxin and a specific HB-EGF inhibitor (6). Ovarian cancer patients with high HB-EGF expression had significantly worse prognosis than those with low HB-EGF expression (7). In addition, HB-EGF is suggested to be involved in tumor resistance against chemotherapeutic agent, paclitaxel (13). These results indicate that inhibition of HB-EGF function is a promising strategy for ovarian cancer therapy. In fact, CRM197 is currently being evaluated in a phase I clinical trial for ovarian cancer in United States.

In this study, we generated new anti-HB-EGF monoclonal antibodies (mAbs). Over the last decade, mAb therapy has been established as an effective treatment against cancer. As a therapeutic antibody against cancer, chimeric or humanized antibody with human immunoglobulin G1 (IgG1) isotype is commonly used, because it can mediate multiple effector functions, including antibody-dependent cellular cytotoxicity (ADCC). Recent studies have revealed that ADCC is one of the important therapeutic mechanisms of clinically effective antibodies (14, 15).

Because HB-EGF exists in 2 forms, pro-HB-EGF and sHB-EGF, an anti-HB-EGF mAb of human IgG1 isotype which has both a high binding reactivity for pro-HB-EGF expressed on the cell surface and a neutralizing activity against sHB-EGF growth factor activity is expected to exert potent antitumor activities through 2 mechanisms, ADCC and neutralization. To date, no anti-HB-EGF antibody with multiple antitumor mechanisms has yet been reported.

In this study, we generated new anti-HB-EGF mAbs and investigated their in vitro and in vivo antitumor activities and evaluated their potential as a therapeutic antibody against ovarian cancer.
peroxidase (HRP)-labeled anti-mouse immunoglobulin (Dako Corp.) was added to each well. To compare the binding activity of various isotypes of antibodies, HRP-labeled anti-mouse immunoglobulin kappa chain (Beckman Coulter, Inc.) was used as a labeled antibody. After 1 hour of incubation, plates were washed and 2,2'-azino-bis (3-ethylbenz-thiazoline)-6-sulfonic acid (Wako) was added as a substrate. Absorbance at 415 nm with a reference wavelength of 490 nm was measured using an E-max microplate reader (Molecular Devices Corp.).

In the inhibition assay, the inhibitory activity of the anti-HB-EGF antibody against binding of rhHB-EGF to epidermal growth factor receptor (EGFR) was measured by a cell-based assay. DER cells were seeded at 2.0 x 10^4 cells per well into 96-well plates overnight. Hybridoma culture supernatant was added into each well and then biotinylated rhHB-EGF (20 ng/mL) and Streptavidin–Alexa 647 (Invitrogen Corp.) were sequentially added into each well and mixed. After 4 hours of incubation, mean fluorescence intensity (MFI) was measured by an 8200 cellular detection system (Applied Biosystems).

Dual positive hybridoma cells in the above 2 assays were cloned twice by limiting dilution, and 2 hybridoma clones were established (KM3566, KM3579). The immunoglobulin subclass of KM3566 (IgG1) or KM3579 (IgG2b) was determined with anti-mouse isotype-specific antibodies (Zymed).

Neutralization assay for HB-EGF growth factor activity
HB-EGF growth factor activity assay was done using human ovarian cancer MCAS cells. MCAS cells were seeded in 96-well plates for cell suspension culture (Sumitomo Bakelite Co., Ltd.) and cultured overnight. Six ng/mL of rhHB-EGF was preincubated with serially diluted anti-HB-EGF mAbs for 2 hours at 4°C and incubated with the preincubated MCAS cells (final rhHB-EGF concentration: 3 ng/mL) and incubated for 48 hours at 37°C, 5% CO₂. The number of living cells was then measured by Cell Count Reagent SF (Nacalai Tesque Inc.) according to the manufacturer's instructions. Statistical significance between the experimental groups was determined by a 2-tailed unpaired t test.

Cell binding assay
Vero cells expressing C-terminally HA-tagged HB-EGF, HA-tagged TGF-α, HA-tagged epiregulin, HA-tagged betacellulin, and HA-tagged amphiregulin were prepared as previously described (20). The expression of all constructs was analyzed by immunoblotting using an anti-HA tag antibody as a primary antibody. Cell binding assays were done using these cells as described previously (20). Briefly, these cells were seeded in 48-well plates and incubated with anti-HB-EGF mAbs at 1 µg/mL for 2 hours at 4°C. The cells were then washed and fixed with 1.75% formaldehyde in PBS (+) for 20 minutes at 4°C. After washing, the fixed cells were blocked with blocking solution (0.2 mol/L glycine, 0.1 mol/L Tris-HCl, pH 8.1) for 30 minutes at 4°C. Then, the cells were incubated with HRP-conjugated anti-mouse IgG antibody (Millipore). After washing, color development detection was carried out by peroxidase substrate (Nacalai Tesque) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (Thermo Electron Corp.).

Flow cytometry
Binding analysis of anti-HB-EGF mAbs for pro-HB-EGF expressed on the cell surface was carried out by flow cytometry (FCM). Cells were detached with 0.02% EDTA solution (Nacalai Tesque) and blocked with 1 mg/mL of human IgG (Sigma-Aldrich Co. LLC.) and stained with various concentrations of anti-HB-EGF mAbs or control antibodies diluted with staining buffer (1% BSA, 0.02% EDTA, and 0.05% sodium azide in PBS) for 60 minutes on ice. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (H+L) (KPL, Inc.) diluted with staining buffer for 60 minutes on ice. To compare the binding reactivity of various isotypes of antibodies, FITC-labeled anti-mouse kappa chain (Novus Biologicals) was used as a labeled antibody. Stained cells were then analyzed using an EPICS XL-MCL FCM instrument (Beckman Coulter).

Production and purification of mouse–human chimeric anti-HB-EGF antibody with IgG1 isotype
The heavy- and light-chain variable region cDNAs from hybridoma cells producing KM3566 or anti-DNP mAb were isolated by PCR and cloned into the expression vector (21) for production of recombinant antibody with human IgG Fc domain. The vector was then introduced into FLTV (-/-) CHO cells via electroporation, and the transfectant cells were grown in serum-free EX-CELL302 (JRH Bioscience). The chimeric KM3566 antibody (cKM3566) and anti-DNP mAb was then purified from the supernatant using a Prosep-A column (Nihon Millipore).

ADCC
Peripheral blood mononuclear cells (PBMC) from healthy donors were prepared using Lymphoprep (Axis- Shield) and used as effector cells. Target cells (1 x 10⁴ cells per well) and effector cells (2.5 x 10⁵ cells per well, effector to target ratio is 25:1) were put into 96-well plates and incubated with various concentrations of KM3566 and cKM3566 for 4 hours at 37°C, 5% CO₂. After centrifugation, the released lactate dehydrogenase (LDH) in the supernatant was detected by a CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega). Percentage of target cell lysis was calculated according to the following formula:

\[
\% \text{Cytotoxicity} = 100 \times (E-S)/(M-S)
\]

where E is experimental release (values in the supernatant from target cells incubated with antibody and effector cells), S is the spontaneous release (values in the supernatant from target cells incubated with effector cells without antibody), S' is the spontaneous release (values in the supernatant from target cells incubated with medium alone), and M is the maximum release (values in the supernatant from target
cells lysed with a lysis solution provided in the kit). Blood donors were randomly selected from healthy volunteers registered at Kyowa Hakko Kirin Co., Ltd. All donors gave written informed consent prior to participation.

**In vivo antitumor activity**

All of the *in vivo* experiments were done in conformity with institutional guidelines in compliance with national laws and policies. Antitumor activity of KM3566 and cKM3566 was evaluated in a mouse xenograft model. MCAS cells (1 × 10⁶) or ES-2 cells (1 × 10⁶) were injected s.c. into 6- to 8-week-old female SCID mice. From the day of the inoculation of cancer cells, KM3566 (10 mg/kg) or cKM3566 (10 mg/kg) or saline solution was injected i.v. twice a week for 3 weeks. For the therapeutic model, MCAS cells (1 × 10⁶) or ES-2 cells (1 × 10⁶) were injected s.c. into 6- to 8-week-old female SCID mice. Seven days later, tumors reached about 100 mm³ in size, and the mice were randomized into groups of 5 animals each. Mice were treated with cKM3566 (10 mg/kg) or saline solution by i.v. injection twice a week for 2 or 3 weeks. The tumor volume was calculated according to the following formula:

\[
\text{Tumor volume (mm}^3\text{)} = 0.5 \times (\text{major diameter}) \times (\text{minor diameter})^2
\]

**Statistical analysis**

Statistical significance between the experimental groups was determined by a 2-tailed unpaired *t* test.

**Results**

**Isolation of anti-HB-EGF mAb**

Considering the difficulty in obtaining mAbs against human HB-EGF because of a high degree of amino acid sequence conservation among species, we used HB-EGF null mice for immunization. The HB-EGF null mice were immunized with rhHB-EGF, and hybridoma cells were made by fusion of murine splenocytes and myeloma cells. To efficiently screen for the hybridoma cells that produce anti-HB-EGF antibodies with neutralizing activity against cell growth promoting activity of sHB-EGF, we conducted an inhibition assay, in which inhibitory activity of the antibodies against binding of rhHB-EGF to EGFR was measured. We also carried out the binding ELISA using plates coated with rhHB-EGF to evaluate the specificity of the antibodies for rhHB-EGF. As a result, we succeeded in isolating 2 anti-HB-EGF mAbs, KM3566 and KM3579. In this study, MAB259, a commercially available anti-HB-EGF mAb, was used as a standard mAb for the evaluation of our newly prepared mAbs. All of these 3 mAbs bound to rhHB-EGF (Fig. 1A) and did not bind to BSA (data not shown). As shown in Fig. 1B, KM3566 and MAB259 showed comparable inhibitory activity against binding of rhHB-EGF to EGFR. KM3579 showed lower inhibitory activity than KM3566 and MAB259, although these 3 antibodies showed equivalent binding activity in the binding ELISA.

**Neutralization activity against HB-EGF growth factor activity**

We evaluated neutralization activity of anti-HB-EGF mAbs against sHB-EGF growth factor activity. We used MCAS cells, which show HB-EGF–dependent growth when cultured in a plate for cell suspension culture, in this assay. As shown in Fig. 2, KM3566 and MAB259 showed comparable neutralization activity against the proliferation of MCAS cells induced by exogenous rhHB-EGF. They inhibited HB-EGF–dependent growth of MCAS cells in a dose-dependent manner. In contrast, KM3579 showed slight neutralization activity only at the highest concentration.

![Figure 1. Reactivity of anti-HB-EGF mAbs against rhHB-EGF. A, the binding activity of anti-HB-EGF mAbs to rhHB-EGF was measured by an ELISA assay. The various concentrations of anti-HB-EGF mAbs, KM3566 (black square), KM3579 (black triangle), MAB259 (black circle), and negative control antibody, KM511 (white square), mouse IgG2b control mAb (white triangle), mouse IgG2a control mAb (white circle) were reacted to plated rhHB-EGF. The binding was detected by HRP-labeled anti-mouse immunoglobulin kappa chain. Points indicate the mean ± SD (n = 3) for the absorbance at 415 nm (reference: 490 nm). B, the inhibitory activity of anti-HB-EGF mAbs against the binding of rhHB-EGF to EGFR was measured in a cell-based assay. Biotinylated rhHB-EGF (20 ng/mL) and Streptavidin–Alexa 647 was incubated with DER cells, 32D cells that stably express the EGFR, in the presence or absence of various concentrations of anti-HB-EGF mAbs, KM3566 (black square), KM3579 (black triangle), and MAB259 (black circle), and negative control antibody, KM511 (white square), mouse IgG2b control mAb (white triangle), mouse IgG2a control mAb (white circle) and the MFI was measured. The MFI at when 20 or 0 ng/mL of biotinylated rhHB-EGF was added with no antibody is indicated by the dotted line (+) or (–), respectively. Points indicate the mean ± SD (n = 3) for the MFI.](http://clincancerres.aacrjournals.org)
Binding specificity

To further evaluate the binding specificity of the anti-HB-EGF mAb, KM3566, we conducted a cell binding assay using Vero cells and Vero cells expressing HA-tagged HB-EGF, HA-tagged TGF-α, HA-tagged epiregulin (EPR), HA-tagged betacellulin (BTC), and HA-tagged amphiregulin (ARG). The expression of all constructs was confirmed by the immunoblotting analysis using an anti-HA tag antibody, although the expression of HA-tagged ARG was low (Fig. 3A). KM3566 specifically bound to HB-EGF, but not to other members of the EGF family of growth factors (Fig. 3B).

Binding reactivity for pro-HB-EGF expressed on the cell surface

Next, to analyze binding of anti-HB-EGF mAbs to pro-HB-EGF expressed on the cell surface of cancer cells, we conducted FCM using 2 human ovarian cancer cell lines, MCAS and ES-2, and 2 gastric cancer cells, MKN-28 and NUGC-3, as target cells. These cancer cells were stained with pro-HB-EGF or purified pro-HB-EGF and incubated with varying concentrations of rituximab (a control antibody) or rituximab preincubated with serially diluted anti-HB-EGF mAbs, as shown in Fig. 4A–D, respectively. KM3566 showed high binding to all cancer cells tested. In contrast, KM3579 showed variable levels of binding depending on the target cancer cell line, high binding to MCAS and NUGC-3, and low binding to ES-2 and MKN-28. MAB259 showed low binding to all of the cancer cells tested. These results showed that KM3566 has a high binding reactivity for pro-HB-EGF expressed on the cell surface.

To further evaluate the binding of KM3566 to various cancer cell lines, 4 ovarian cancer cell lines, 6 breast cancer cell lines, and 6 stomach cancer cell lines were stained with 20 µg/mL of KM3566 or an isotype-matched control antibody, KM511. The relative MFI values (MFI_{KM3566}/MFI_{KM511}) are shown in Fig. 4E. KM3566 bound to all cancer cells tested, although the binding levels varied among cancer cell lines.

Evaluation of the in vitro antitumor effects of cKM3566

To examine the antitumor effects of KM3566, we generated a mouse-human chimeric antibody with human IgG1 Fc domain (cKM3566). KM3566 and cKM3566 showed equivalent inhibitory activity against binding of rhHB-EGF to EGFR (Fig. 5A). We evaluated human PBMC-mediated ADCC of KM3566 and cKM3566 against MCAS and ES-2 cells. As shown in Fig. 5B, ADCC activity of cKM3566 was detected against both cancer cells in a dose-dependent manner. The cytototoxicity of cKM3566 was observed from 0.01 µg/mL and higher, and the activity saturated at 1 µg/mL. KM3566 is a murine antibody of IgG1 subclass which is...
Studies have shown that KM3566, only one mechanism, neutralization, was expected. In prevention models, anti-HB-EGF mAbs or saline solution was administered from the day of inoculation of cancer cells. As shown in Fig. 6A, in the MCAS model, KM3566 treatment significantly inhibited tumor growth (P < 0.01) and cKM3566 exhibited a more potent antitumor activity than KM3566 (P < 0.01), although tumor formation and growth were observed in all 6 mice in all experimental groups. In the ES-2 model, KM3566 did not show any antitumor activity, and tumor formation and growth were observed in 5 of 6 mice, whereas in the control treatment group, it was observed in all 6 mice. In contrast, cKM3566 completely inhibited tumor growth and tumor formation was observed in only 1 mouse.

Next, we tried to examine the in vivo antitumor activity of cKM3566 in MCAS or ES-2 xenograft tumor therapeutic models. In therapeutic models, cKM3566 or saline solution was administered 7 days after inoculation of cancer cells. As shown in Fig. 6B, cKM3566 significantly inhibited growth of tumor in both models (P < 0.01). The minimum treatment/control ratio (T/C min) in the MCAS and ES-2 xenograft model was 0.65 on day 10 and 0.55 on day 28.
Discussion

Growth factors of the EGF family are generally not considered to be promising targets for cancer therapy because there are many growth factors that can provide growth-promoting signals redundantly, and when a sole factor is neutralized, other ligands are suspected to compensate for it. However, increasing evidence has suggested that HB-EGF is expressed predominantly among all of the growth factors of the EGF family in ovarian cancer and has a pivotal role in its progression (6, 7, 22). In addition, Yagi and colleagues recently reported that a chemotherapeutic agent, paclitaxel, induced transient ERK and Akt activation through elevated expression and ectodomain shedding of HB-EGF, resulting in the escape from apoptosis. CRM197, a nontoxic mutant of diphtheria toxin and a specific inhibitor of HB-EGF, showed synergistic antitumor activities with paclitaxel by restoring the sensitivity to paclitaxel in ovarian cancer SKOV-3 cells (13). These studies suggest that an anti-HB-EGF agent is expected to be effective and exert synergistic antitumor activities with chemotherapeutic agents such as paclitaxel against ovarian cancer.

In this study, we succeeded in isolating a novel anti-HB-EGF mAb KM3566 which has both a high binding reactivity for pro-HB-EGF expressed on the cell surface and a neutralizing activity against sHB-EGF growth factor activity.

Figure 6. In vivo antitumor activities of cKM3566. A, MCAS (1 x 10^6) or ES-2 (1 x 10^6) cells were injected s.c. into SCID mice (day 0). KM3566 (10 mg/kg: black triangle), cKM3566 (10 mg/kg: black circle) or saline solution (control treatment, white circle) was injected i.v. twice a week from day 0 for 3 weeks, and the tumor sizes (mm^3) were measured (n = 6). Arrows below the transverse axis indicate days of antibody treatment. Statistical significance between the cKM3566 versus control treatment and cKM3566 versus KM3566 treatment is indicated as P < 0.05 (‘) or P < 0.01 (‘’), respectively. B, MCAS (1 x 10^6) or ES-2 (1 x 10^6) cells were injected s.c. into SCID mice (day 0). cKM3566 (10 mg/kg: black circle) or saline solution (control treatment, white circle) was injected i.v. twice a week from day 7 for 2 or 3 weeks and the tumor sizes (mm^3) were measured (n = 5). Arrows below the transverse axis indicate days of antibody treatment. Statistical significance between the cKM3566 versus control treatment is indicated as P < 0.05 (‘) or P < 0.01 (‘’).
KM3566 showed high binding to all cancer cells tested in this study. Thus, the mouse-human chimeric antibody of KM3566 with human IgG1 Fc domain was expected to mediate ADCC against cancer cells expressing HB-EGF. In fact, we successfully showed ADCC activity of the mouse-human chimeric antibody, cKM3566, against cancer cells. These results suggest that cKM3566 can exert antitumor activities against cancer cells expressing HB-EGF through 2 mechanisms: neutralization and ADCC.

In the ovarian cancer MCAS prevention model, we showed that KM3566, a murine antibody of IgG1 subclass that does not exhibit any ADCC activity against MCAS cells, significantly inhibited tumor growth. This means that the neutralization activity against HB-EGF growth factor activity contributes to the in vivo efficacy of the antibody, as preceding studies have suggested (6, 23). cKM3566, which can exert ADCC as well as neutralization, exhibited significantly more potent in vivo antitumor activities than KM3566. These results suggest that both mechanisms, neutralization and ADCC, might contribute to the in vivo antitumor efficacy of the antibody, although precise mechanisms need to be solved in future.

On the other hand, in the ovarian cancer ES-2 prevention model, KM3566 did not show any antitumor activity, although cKM3566 dramatically inhibited tumor growth. ES-2 cells inoculated in SCID mice may proliferate independently of HB-EGF, unlike MCAS cells. This likely reflects the clinical situation, in which the extent of dependency on HB-EGF probably varies among cancer cells. The cKM3566 antibody is expected to exert a potent antitumor activity against not only cancer cells with high dependency on HB-EGF but also ones with low dependency on the molecule.

Interestingly, the binding reactivity for pro-HB-EGF varied among the anti-HB-EGF mAbs. KM3566 bound strongly to all of the cancer cells tested, but KM3579 and MAB259 did not. Pro-HB-EGF is known to be associated with other molecules such as CD9, integrins, and heparan sulfate proteoglycans (5, 24–26) on the cell surface. Therefore, the epitope recognized by KM3579 and MAB259 may be masked by pro-HB-EGF–associated molecules in some cases. As for MAB259, it is also possible that it has low binding reactivity for natural HB-EGF molecules produced in human cells, although it has high binding reactivity for rHB-EGF produced in insect cells.

The in vitro antitumor activities of cKM3566 were examined in xenograft tumor therapeutic models using MCAS or ES-2 ovarian cancer cells. cKM3566 showed significant antitumor activities in both cell models ($P < 0.01$). These results indicate that cKM3566 is a promising antiovarian cancer therapeutic antibody.

HB-EGF is widely involved in physiologic and pathologic processes in the body. Analysis of HB-EGF KO mice indicated that HB-EGF is required for the maintenance of heart function and other functions (5, 17). Therefore, we should pay an attention to possible adverse effects of systemic inhibition of HB-EGF signaling, as well as possible adverse effects of inducing systemic ADCC against HB-EGF expressing cells when cKM3566 or its humanized derivative is used for clinical purpose.

Discourse of Potential Conflicts of Interest


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