J. Microbiol. Biotechnol. (2013), 23(9), 1327–1338
http://dx.doi.org/10.4014/jmb.1306.06007

jmb

Inhibition of Tumor Growth in a Mouse Xenograft Model by the Humanized Anti-HGF Monoclonal Antibody YYB-101 Produced in a Large-Scale CHO Cell Culture

Seong-Won Song[1,2], Song-Jae Lee[2], Chang-Young Kim[2], Jae-Kyung Song[2], Eui-Jung Jung[2], Yong Bock Choi[3], Sung-Won Min[3], and Jong-Won Oh[1,4,5]*

[1]*Graduate Program in Biomaterials Science and Engineering, Yonsei University, Seoul 120-749, Republic of Korea*
[2]*Bioresearch Institute, Yooyoung Pharmaceuticals Co. Ltd., Seoul 152-719, Republic of Korea*
[3]*iBio Inc., Samsung Cancer Research Building, Seoul 110-799, Republic of Korea*
[4]*Department of Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea*
[5]*Translational Research Center for Protein Function Control, Yonsei University, Seoul 120-749, Republic of Korea*

Received: June 7, 2013
Revised: July 6, 2013
Accepted: July 8, 2013

First published online
July 15, 2013

**Corresponding author*
Phone: +82-2-2123-2881;
Fax: +82-2-362-7265;
E-mail: jwoh@yonsei.ac.kr

pISSN 1017-7825, eISSN 1738-8872

The humanized anti-hepatocyte growth factor (HGF) monoclonal antibody (mAb) YYB-101 is a promising therapeutic candidate for treating various cancers. In this study, we developed a bioprocess for large-scale production of YYB-101 and evaluated its therapeutic potential for tumor treatment using a xenograft mouse model. By screening diverse chemically defined basal media formulations and by assessing the effects of various feed supplements and feeding schedules on cell growth and antibody production, we established an optimal medium and feeding method to produce 757 mg/l of YYB-101 in flask cultures, representing a 7.5-fold increase in titer compared with that obtained under non-optimized conditions. The optimal dissolved oxygen concentration for antibody production was 70% pO_2. A pH shift from 7.2 to 7.0, rather than controlled pH of either 7.0 or 7.2, resulted in productivity improvement in 5 L and 200 L bioreactors, yielding 737 and 830 mg/ml of YYB-101, respectively. The YYB-101 mAb highly purified by affinity chromatography using a Protein A column and two-step ion exchange chromatography effectively neutralized HGF in a cell-based assay and showed potent tumor suppression activity in a mouse xenograft model established with human glioblastoma cells.

Keywords: Chinese hamster ovary cells, anti-human hepatocyte growth factor monoclonal antibody, humanized antibody, process development, anti-HGF antibody YYB-101, antitumor activity

Introduction

Hepatocyte growth factor (HGF) was initially discovered as a potent mitogen of rat hepatocytes in primary culture [24, 25]. HGF induces mitogenesis, motogenesis, and morphogenesis in various cell types *via* interaction with its functional receptor, c-Met [2, 12]. Several *in vitro* studies have indicated that HGF also stimulates the scattering and migration of cancer cells [20, 23]. Abnormal signaling *via* HGF/c-Met is responsible for many types of cancers, and elevated levels of circulating HGF have been correlated with tumor metastasis and cancer progression [32, 34]. Considerable effort has been expended searching for specific inhibitors of the HGF/c-Met pathway, in development of novel therapeutic agents for treating various cancers [6, 13]. Inhibitors of c-Met tyrosine kinase have shown promising efficacy in preclinical and clinical trials. In addition to small-molecule inhibitors, antibodies directed against HGF and c-Met are also under development [7, 17, 22, 28].

An early study claimed that a minimum of three antibodies directed against different epitopes are required to completely inhibit c-Met activation [5]. Indeed, the development of novel, high-potency therapeutic antibodies has been a long-standing challenge in the pharmaceutical industry, because each antibody recognizes a specific epitope and thus produces a distinct clinical outcome. Nevertheless, several monoclonal antibodies (mAbs) blocking the HGF/c-Met signaling pathway have been developed, and their tumor-suppression activities have been demonstrated in xenograft mouse models [3, 16, 19], confirming the feasibility of using HGF-neutralizing antibodies to block c-Met activation. Among the HGF-neutralizing antibodies, the recombinant mAb SFN68, generated by immunizing rabbits with the HGF/c-Met complex, is quite unique in that it is more specific to the HGF/c-Met interaction than other antibodies elicited by immunizing with HGF only [15]. The cDNA for SFN68 was constructed to produce an intact antibody composed of rabbit/human chimeric Fab and human IgG_1 Fc fragments. The resulting chimeric SFN68 mAb, recognizing a nonlinear epitope, effectively interfered with HGF/c-Met interaction in a cell-based assay [15]. The SFN68 antibody was further engineered to generate a humanized anti-HGF antibody, named YYB-101 (also known as ABX101), which we sought to produce in large-scale bioreactors for use in toxicity and efficacy studies.

Here, we describe an optimized bioprocess for the production of humanized anti-HGF YYB-101 mAb using recombinant Chinese hamster ovary (CHO) cells. We optimized the bioprocessing medium using diverse chemically defined basal and feed media and established an optimal feeding strategy. In addition, the bioreactor operation conditions were optimized in a 5 L bioreactor, and the established optimal conditions were further verified in a 200 L bioreactor. Finally, the antitumor activity of purified YYB-101 was confirmed using a cell-based HGF-neutralization assay and a xenograft mouse model involving implantation with human glioblastoma cells.

Materials and Methods

Cell Lines and Culture Media

The CHO cell line CHO-YYB-101, which expresses the humanized anti-HGF YYB-101 mAb, was generated by iBio Co., Ltd., Seoul, Korea. Briefly, the CHO-YYB-101 cell line was established by transfecting the serum-free suspension culture-adapted dihydrofolate reductase (DHFR)-deficient CHO cell line DG44 (Invitrogen, Carlsbad, CA, USA) with an expression vector harboring the cDNA encoding YYB-101 under control of the cytomegalovirus (CMV) promoter, the DHFR-coding gene required for gene amplification using methotrexate (MTX), and a neomycin resistance gene as a selection marker (J.H. Chung *et al*., unpublished data; full details will be reported elsewhere). The transfected cells were selected in the presence of 250 mg/ml of G418 for 3–4 weeks, and the transfected gene was amplified by selecting clones adapted to grow in medium containing 1–2 mM MTX (Sigma-Aldrich, St. Louis, MO, USA). Isogenic high-producers were then isolated from among the MTX-resistant clones using the limiting-dilution method. For optimization of the medium, eight different chemically defined basal media were used, including CD OptiCHO (Invitrogen), CDM4CHO (Hyclone, Logan, UT, USA), CDM4mab (Hyclone), PowerCHO (Lonza, Verviers, Belgium), ProCHO5 (Lonza), ExCell 302 (SAFC Biosciences, Lenexa, KS, USA), ExCell DHFR (SAFC Biosciences), and ExCell CD (SAFC Biosciences). The feed media used included CHO CD Efficient Feed A, B, and C (Invitrogen), Cell Boost kit (Hyclone), and Xtreme (Lonza). Each feed medium was added to the individual basal medium to a final concentration of 10% (v/v) on day 0. When two different feed media were supplemented together, they were combined to a final concentration of 5% each. All media used for CHO cell cultivation were supplemented with 8 mM L-glutamine. The human malignant glioblastoma cell line U87 MG was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured as previously described [27]. Mink lung epithelial cells (Mv-1-Lu; ATCC CCL-64) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine at 37°C in a 5% CO_2 atmosphere.

Cell Culture for Antibody Production

Screening for optimal basal and feed media and establishment of an optimal feeding strategy were carried out using parallel cultures in 125 ml shake flasks with a 30 ml working volume. Shake flasks were cultured at 37°C with agitation at 130 rpm in a humidified atmosphere of 8% CO_2 using a Multitron incubator (Infors AG, Bottmingen, Switzerland). Fed-batch culturing was carried out in a 5-L BIOSTAT B Plus bioreactor (Sartorius, AG, Göttingen, Germany) with a 3-L working volume. Gas was provided through a ring sparger, and agitation was provided by a marine impeller with a diameter of 65 mm. For each bioreactor run, an inoculum culture was passed two times in CD OptiCHO medium and then seeded in the bioreactor at a density of 0.3–0.4 × 10^6 cells/ml. The bioreactor was operated at 37°C, with agitation at 75 rpm and aeration at 50 ml/min through the sparger. Aeration was adjusted to 200 ml/min when the cells reached the maximum oxygen-consuming stage. Dissolved oxygen (DO) was maintained at 40% or 70%, depending on the test conditions. The pH was maintained at each set point by controlling the level of incoming CO_2 and by addition of 7.5% sodium bicarbonate. Feed medium was added according to the schedules determined during the medium optimization studies. The feed medium was added to the bioreactor at a rate of 2 ml/min to avoid oxygen depletion. Analytical samples were taken from the bioreactors daily, centrifuged, and stored at 20°C until analysis.

For large-scale culture, an XDR-200 bioreactor (Xcellerex, MA, USA) with a 200 L single-use bioreactor (SUB) bag was used. Gas was supplied through a 20 μm disk with five 0.5 mm drilled holes. Agitation was provided by a bottom-type fixed-angle pitched-blade impeller with four blades. The inoculum was prepared in multiple 2 L flasks and then transferred to a 20 L Wave Bioreactor (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The Wave Bioreactor was rocked at an angle of 6 degrees and rate of 12 rpm and operated at 37°C with a constant flow of 7.5% CO_2 in air at 0.15–0.2 L/min. The cell suspension from the Wave Bioreactor was used to seed the 200 L bioreactor at a working volume of 160 L and a density of 0.4×10^6 cells/ml.

Analytical Methods for Monitoring Cell Culture Profiles

Cell number and viability were determined using an Innovatis Cedex Cell Counter XS (Roche Applied Science, Mannheim, Germany). Glucose, glutamine, and lactate concentrations were measured by enzymatic reactions using a YSI 7100 Multiparameter Bioanalytical System (YSI Life Sciences, Yellow Springs, OH, USA). The specific metabolic rate was determined by plotting measured values against the integral of viable cells, as previously described [30].

Antibody Purification and Protein Analysis

Collected culture broth was clarified using 10SP and 90ZA depth filters (3M Purification Inc., Meriden, CT, USA) to remove cells and debris. The clarified culture broth was loaded onto a Mabselect SuRe Protein A column (GE Healthcare, Uppsala, Sweden) equilibrated with 20 mM sodium phosphate (pH 7.0). After washing with 20 mM sodium phosphate (pH 7.0) containing 1 M NaCl, bound antibodies were eluted with 20 mM citrate (pH 3.5). The pH of the eluted fractions was adjusted to 6.5 with 1 M Tris-HCl buffer (pH 9.0). The eluted fraction was applied to an SP Sepharose HP cation-exchange column (GE Healthcare) equilibrated with 20 mM sodium phosphate (pH 6.5) containing 20 mM NaCl. After washing with 20 mM sodium phosphate (pH 6.5) containing 60 mM NaCl, proteins were eluted with the same buffer containing 150 mM NaCl. The pH of the eluted fraction was adjusted to 7.2 and then applied to a Q Sepharose HP anion exchange column (GE Healthcare) equilibrated with 20 mM sodium phosphate (pH 7.2) containing 150 mM NaCl. The flow-through fraction was collected. A Viresolve Pro membrane (Millipore, Billerica, MA, USA) was used to remove any viruses present in the flow-through fraction. Finally, the bulk antibody product was concentrated using a 30 kDa molecular mass cut-off Pellicon 3 ultrafiltration membrane (Millipore). The buffer was then exchanged by diafiltration using 12 vol of final formulation buffer (20 mM sodium phosphate, pH 7.2, 150 mM NaCl).

The quality of the purified antibodies was assessed using size-exclusion chromatography (SEC) on a Zenix-C SEC-300 column (7.8 × 300 mm, 3 μm; Sepax Technology, Newark, DE, USA) connected to a Waters high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector. Antibodies loaded onto the column were eluted using 100 mM sodium phosphate (pH 7.0) and 100 mM NaCl as the mobile phase. Protein elution was monitored at 280 nm. For cation-exchange HPLC, samples were separated using a ProPac WCX-10 weak cation-exchange column (Dionex, CA, USA) connected to a Waters HPLC system equipped with a PDA detector. Mobile phases A and B consisted of 20 mM HEPES buffer (pH 7.0) and 20 mM HEPES buffer with 1 M NaCl (pH 7.0), respectively. Proteins were eluted with a gradient from 85% to 0% mobile phase A over 35 min, followed by a wash with mobile phase B buffer (100%) at a flow rate of 0.5 ml/min.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Western Blot Analysis, and Enzyme-Linked Immunosorbent Assay

SDS-PAGE was carried out using 4–12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen), and proteins were visualized by Coomassie Brilliant Blue staining. For western blot analysis, proteins were electrotransferred onto a polyvinylidene difluoride membrane (Invitrogen). The membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) and then washed three times with TBST (10 min each). The membrane was then incubated for 1 h in blocking buffer containing goat anti-human IgG (KPL, Gaithersburg, MD, USA). After washing with TBST, the membrane was incubated for 1 h in blocking buffer containing the secondary antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (H+L) (KPL), diluted 1:5,000 with blocking buffer. After washing with TBST, the membrane was incubated with TMB membrane substrate (KPL) to visualize the anti-HGF mAb.

The titer of anti-HGF mAb in the culture supernatant was determined by ELISA. Briefly, 96-well Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of 1.0 mg/ml of goat anti-human IgG Fc (KPL) dissolved in 0.1 M carbonate buffer (pH 9.6). The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h, and incubated with 100 μl of appropriate culture supernatant for 1 h at 37°C. The plates were then washed, and incubated with HRP-conjugated goat anti-human IgG antibody (KPL) for 1 h, after which TMB substrate solution (KPL) was added to allow for color development. After 10 min, the reaction was stopped by adding 2 N H_2SO_4. Absorbance was measured at 450 nm using a microplate reader. Data were analyzed using Gen5 software (BioTek, Winooski, VT, USA).

Cell-Based HGF-Neutralizing Activity Assay

The biological activity of the anti-HGF mAb was determined by measuring its ability to reverse the transforming growth factor (TGF)-β–induced growth inhibition mediated by HGF. The assay method was established by slight modification of the previously reported method [1, 29]. A total of 1×10^4 CCL-64 lung epithelial cells were seeded per well in a 96-well plate and treated with 1.25 ng/ml of human TGF-β1 (ProSpec-Tany TechnoGene Ltd.,

Ness-Ziona, Israel). In a separate plate, human HGF (ProSpec-Tany TechnoGene Ltd.) at a concentration of 250 ng/ml was preincubated for 1 h at 37°C with 2-fold serial dilutions of purified antibody (0–10 μg/ml). The mixture containing free HGF not neutralized by the antibody was then added to the TGF-β1-treated CCL-64 cells and incubated for 72 h, after which cell proliferation was measured using a modified MTT assay with CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Antitumor Activity Assay Using a Xenograft Mouse Model

A total of 5 × 10^6 U87 MG cells in 100 μl of PBS were implanted subcutaneously in the right flank of 5- to 6-week-old female BALB/c nude mice obtained from Japan SLC, Inc. (Hamamatsu, Japan). Mice were randomized into four groups (n = 10 per group). When tumors reached a volume of ~100 mm^3, mice were injected intraperitoneally twice per week for up to 28 days with 0.5, 1.5, or 5.0 mg/kg of YYB-101 mAb prepared from a 200 L bioreactor or 5.0 mg/kg of human IgG (Sigma) as a control. The tumor size was measured twice per week using a digital caliper (Mitutoyo, Utsunomiya, Japan), and the tumor volume (in mm^3) was determined using the formula (length × $width^2$)/2, where length represents the longest axis and width represents the measurement at a right angle to the length. Data were expressed as the mean tumor volume ± SE for each treatment group.

Results

Optimization of Basal Medium by Formulation of Chemically Defined Media

To maximize anti-HGF YYB-101 mAb production, we sought to optimize the basal medium by combining CD OptiCHO basal medium, in which the YYB-101-producing

Table 1. Effect of basal medium formulation on cell growth and antibody productivity.

No.	Medium 1	(%)	Medium 2	(%)	Max. cell density (10^5 cells/ml)	Viability at day 10 (%)	mAb Conc. (mg/l)
1	CD OptiCHO	100	-	0	88.93	51.2	102
2	CD OptiCHO	80	CDM4MAb	20	118.35	60.2	158
3		60	CDM4MAb	40	104.0	57.7	184
4		40	CDM4MAb	60	93.57	39.9	204
5		20	CDM4MAb	80	90.34	13.4	221
6		0	CDM4MAb	100	82.40	14.7	245
7	CD OptiCHO	80	CD FortiCHO	20	88.48	42.0	137
8		60	CD FortiCHO	40	97.68	48.7	130
9		40	CD FortiCHO	60	118.72	54.4	141
10		20	CD FortiCHO	80	126.33	37.4	149
11		0	CD FortiCHO	100	132.67	27.7	136
12	CD OptiCHO	80	PowerCHO2	20	88.38	43.1	153
13		60	PowerCHO2	40	81.39	44.7	190
14		40	PowerCHO2	60	74.23	48.7	218
15		20	PowerCHO2	80	56.83	31.9	264
16		0	PowerCHO2	100	42.02	56.7	258
17	CD OptiCHO	80	ProCHO5	20	96.01	44.9	139
18		60	ProCHO5	40	86.47	56.8	152
19		40	ProCHO5	60	82.28	51.8	195
20		20	ProCHO5	80	60.55	67.3	215
21		0	ProCHO5	100	45.21	52.9	193
22	CD OptiCHO	80	CDM4CHO	20	77.37	56.8	143
23		60	CDM4CHO	40	65.25	47.8	155
24		40	CDM4CHO	60	61.72	52.9	145
25		20	CDM4CHO	80	54.63	47.0	136
26		0	CDM4CHO	100	12.81	20.8	8

CHO cell line was initially established, with five different commercially available chemically defined media at different final concentrations of CD OptiCHO (0%, 20%, 40%, 50%, 80%, and 100%). During cultivation of CHO cells in each of the media formulations for 10 days, we monitored cell growth, cell viability, and antibody titer. The results, which are summarized in Table 1, showed that CD OptiCHO medium combined with PowerCHO2 at a ratio of 2:8 yielded the highest antibody titer (264 mg/l), representing approximately a 2.6-fold enhancement in mAb production over that achieved using 100% CD OptiCHO medium. The CDM4Mab (100%) medium enhanced both growth and mAb productivity (245 mg/l), although cell viability decreased rapidly in the late stage of culture compared with CD OptiCHO basal medium (viability of cells in 100% CDM4Mab or 100% CD OptiCHO at day 10, 51.2% vs. 14.7%, respectively). As the CD FortiCHO medium concentration increased in mixtures with CD OptiCHO medium, the maximum cell density gradually increased, and cell viability was highest at the ratio of 4:6. At this ratio, the antibody titer was increased by 40% compared with the titer in 100% CD OptiCHO medium. With the ProCHO5 and CDM4CHO media added to CD OptiCHO at different ratios, we could not attain the high antibody titer or cell density observed with other media formulations. Even at the highest cell viability (67%) obtained with a 2:8 CD OptiCHO:ProCHO5 formulation, the antibody titer did not reach the highest level obtained with other formulations. Furthermore, increasing the CDM4CHO concentration resulted in a gradual reduction in both the maximum cell density and antibody titer. Based on these results, we finally selected the 100% CDM4Mab, CD OptiCHO:PowerCHO2 (2:8), and CD OptiCHO:CD FortiCHO (4:6) formulations for further optimization in fed-batch cultures.

Effects of Feed Media and Feeding Strategy on Antibody Production

Having selected the three different basal media formulations, we tested the effect of feed supplementation on antibody production by adding each of 10 different feed media to each of the optimized basal medium formulations to a final concentration of 10% (v/v). When antibody titers were assessed at day 12 in batch cultures, the highest antibody titer achieved in the feed medium screening experiments was 635 mg/l (Fig. 1). This improvement, which represented a >4-fold increase over the titer achieved with the OptiCHO:CD FortiCHO (4:6) basal medium (155 mg/l), was observed by feeding with CHO CD Efficient Feed B. Feed B also enhanced the titer most effectively

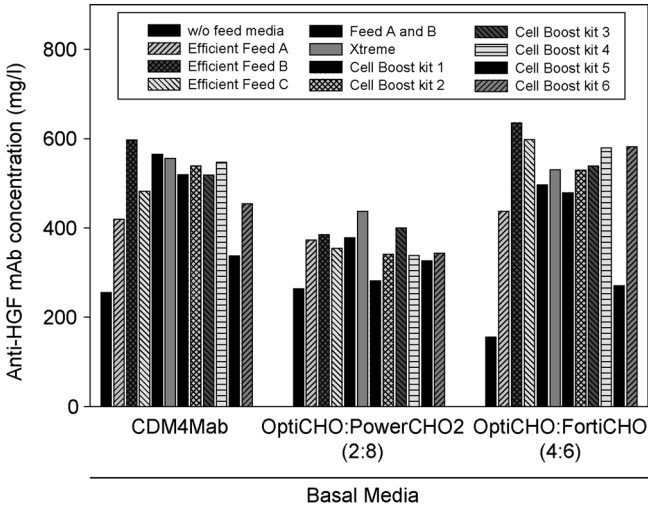

Fig. 1. Effect of feed supplementation of various basal media on production of the humanized anti-HGF YYB-101 mAb.
Each of the feed media shown in the inset was added to a final concentration of 10% (v/v) on day 0 to each of the indicated basal media. YYB-101–producing CHO cells were cultivated for 12 days in 125 ml Erlenmeyer shaker flasks containing the respective formulated basal medium. The titer of YYB-101 was determined using an ELISA.

when added to CDM4MAb medium, yielding an antibody titer of 597 mg/l. Although the CD OptiCHO:PowerCHO2 (2:8) medium yielded the highest antibody titer in basal medium screening (Table 1), antibody productivity was not enhanced as much as with the other two basal medium formulations when feed was added. We thus selected the CD OptiCHO:CD FortiCHO (4:6) formulation with 10% CHO CD Efficient Feed B as a production medium for use in subsequent fed-batch experiments.

Next, we sought to establish the optimal feeding schedules to improve antibody productivity. As summarized in Table 2 and Fig. 1, addition of either CHO CD Efficient Feed B or C medium to the production medium described above had the greatest effect on enhancing the antibody titer. The maximum feed concentration was limited to 20% (v/v), because both Feed B and Feed C inhibited cell growth at concentrations over 25% in the production medium (data not shown). The antibody titers obtained after cultivation for 14 days under different feeding schedules are summarized in Table 2. Overall, CHO CD Efficient Feed B yielded higher antibody titers than CHO CD Efficient Feed C under the same feeding schedule. By feeding CHO CD Efficient Feed B at 5% (v/v) each time on days 0, 2, 4, and 6, we obtained the highest antibody titer (757 mg/l). We thus used this feeding schedule in the subsequent bioprocess optimization experiments.

Table 2. Effects of feed medium and feeding schedule on production of humanized anti-HGF YYB-101 mAb by recombinant CHO cells.

No.	Feed medium	Total feed concentration (%)	Feeding time and concentration (%)					mAb Conc. (mg/l)
			Day 0	Day 2	Day 4	Day 6	Day 8	
1	CHO CD Efficient feed C	5	5					600.19
2	CHO CD Efficient feed B	5	5					622.85
3	CHO CD Efficient feed C	5		5				627.93
4	CHO CD Efficient feed B	5		5				594.81
5	CHO CD Efficient feed C	10	5	5				642.12
6	CHO CD Efficient feed B	10	5	5				671.11
7	CHO CD Efficient feed C	10		5	5			632.16
8	CHO CD Efficient feed B	10		5	5			665.53
9	CHO CD Efficient feed C	15	5	5	5			660.18
10	CHO CD Efficient feed B	15	5	5	5			708.75
11	CHO CD Efficient feed C	15		5	5	5		625.34
12	CHO CD Efficient feed B	15		5	5	5		697.8
13	CHO CD Efficient feed C	20	5	5	5	5		636.74
14	CHO CD Efficient feed B	20	5	5	5	5		757.08
15	CHO CD Efficient feed C	20		5	5	5	5	638.23
16	CHO CD Efficient feed B	20		5	5	5	5	711.12

Bioprocess Optimization for Large-Scale Production of Humanized Anti-HGF YYB-101 mAb

Using the optimized production medium and feeding schedule, we tested the effect of DO on the growth of YYB-101–producing CHO cells and antibody yield in a 5 L stirred bioreactor. During fed-batch operation of this reactor, glucose was supplemented, if necessary, to prevent the concentration from falling below 10 mM. The DO concentration was controlled at either 40% or 70% of air saturation, and the pH was maintained at 7.0. As shown in Figs. 2A and 2B, at both DO levels, the cell growth and viability profiles were similar, whereas the antibody titer increased by 17.6% at a DO of 70% of air saturation at day 13 (408 and 480 mg/l at 40% and 70% DO, respectively).

To determine the optimal pH, fed-batch cultures were operated at a DO of 70% of air saturation. The pH of the culture medium was controlled at 6.8, 7.0, or 7.2. Alternatively, the pH was shifted from 7.2 to 7.0 at day 2. As shown in Fig. 3, under the pH shift condition, we obtained the highest antibody titer (737 mg/l), which was a 44% increase over the titers achieved at pH 7.0 or 7.2. This greater volumetric productivity was associated with an increase in viable cell number beginning at day 7. However, it should be noted that productivity was not improved at pH 7.0, even though the viable cell number was always higher than in the pH-shifted cultures. Our time-course data suggested that shifting the pH from 7.2 to 7.0 during the early stage of the culture is important for accelerating cell growth and maintaining cell viability longer in order to control overgrowth and improve antibody production.

For testing the *in vivo* efficacy of YYB-101, we produced the antibody in a 200 L single-use bioreactor operated at 37°C, a DO of 70% of air saturation, and with pH shifting (pH shift from 7.2 to 7.0 on day 2). As in the case of the 5 L bioreactor, the same feed was added four times, on days 0, 2, 4, and 6. Under these conditions, which were optimized with a 5 L bioreactor, we obtained 830 mg/l of antibody after cultivation for 14 days, with an increased maximal viable cell number compared with that of the 5 L reactor (1.02×10^7 cells/ml *vs.* 1.25×10^7 cells/ml), although the specific growth rates were similar in both reactors (Table 3). In addition, similar specific productivities obtained with both reactors (9.454 and 9.444 pg/cell/day in the 5 L and 200 L reactors, respectively). Of note, despite the similar growth rates in these reactors, the specific glucose consumption, lactate production, and ammonia production rates were higher in the 200 L reactor, likely due to the higher cell density and antibody titer in the 200 L bioreactor.

***In Vitro* and *In Vivo* HGF-Neutralization Activity of Humanized Anti-HGF YYB-101 mAb**

To investigate the quality of the anti-HGF antibody produced in 5 L and 200 L bioreactors, the antibody was purified by affinity chromatography using a Protein A column.

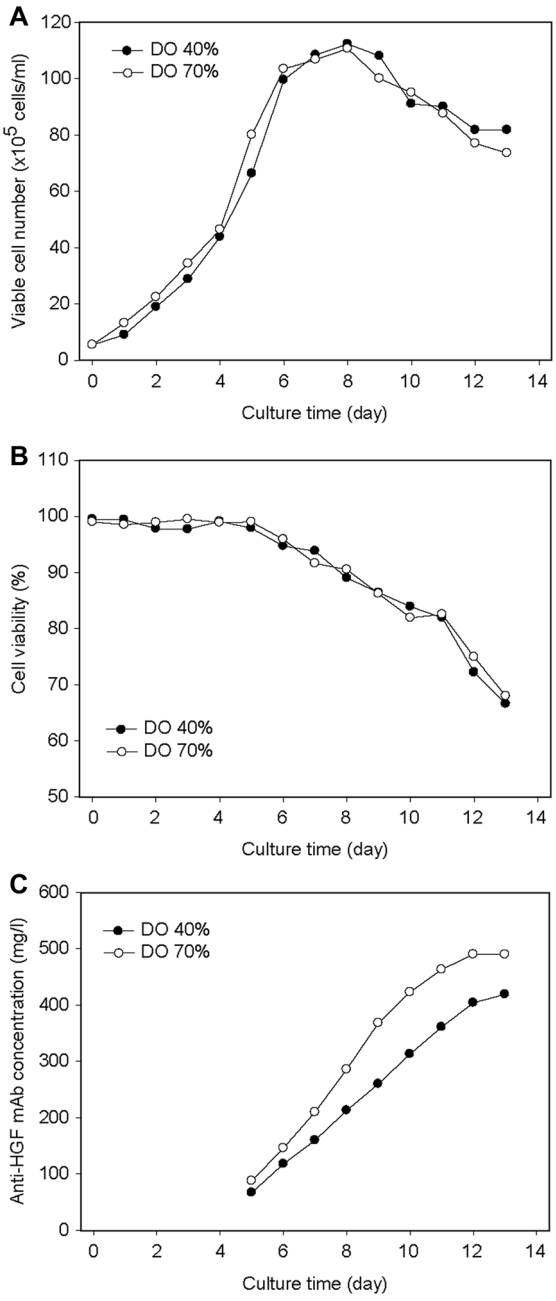

Fig. 2. Growth and antibody production profile of the anti-HGF YYB-101 mAb-producing CHO cell line in a 5 L bioreactor.
YYB-101–producing CHO cells were cultivated at two different DO levels (40% and 70% of air saturation) in a 5 L bioreactor. Viable cell density (**A**), viability (**B**), and YYB-101 titer in the culture medium (**C**) were measured during cultivation in the CD OptiCHO:CD FortiCHO (4:6) basal medium with feeding of CHO CD Efficient Feed B on days 0, 2, 4, and 6 (5% (v/v) each time). Glucose was supplemented to maintain a level of 10 mM.

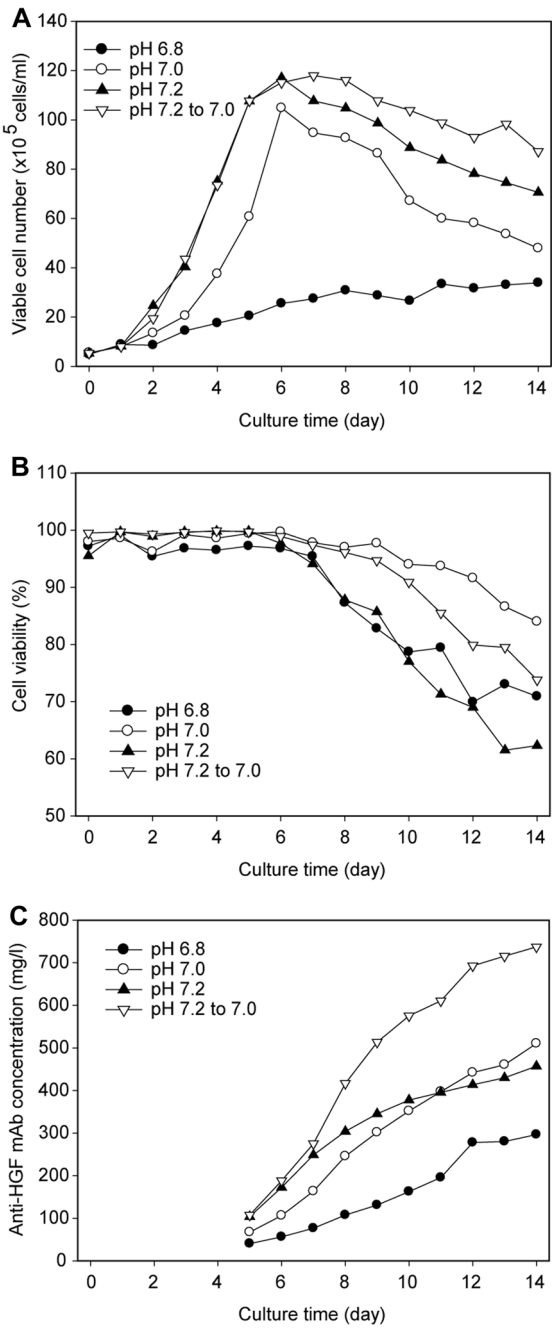

Fig. 3. Optimization of pH for the production of YYB-101 mAb.
YYB-101–producing CHO cells were cultivated in a 5 L bioreactor operated at the indicated pH or under a pH-shifting condition (shift from pH 7.2 to 7.0 on day 2). Viable cell density (**A**), viability (**B**), and YYB-101 titer in the culture medium (**C**) were measured during cultivation under the same conditions described in the legend for Fig. 2.

The affinity purified YYB-101 was further purified by sequential cation-exchange and anion-exchange chromatographies, with the flow-through fraction containing highly purified YYB-101 collected at each step. The flow-through fraction was subjected to a viral removal step, after which the

Table 3. Kinetic parameters of YYB-101–producing CHO cells cultivated in bioreactors.

	Culture duration (h)	Max. viable cell density, $X_{max.}$ ($\times 10^7$ cells/ml)	Specific growth rate, μ (h^{-1})	Specific growth rate, μ (day^{-1})	Specific glucose consumption rate, (μmol/10^6 cells/h)	Specific glutamine consumption rate, (μmol/10^6 cells/h)	Specific lactate production rate, (μmol/10^6 cells/h)	Specific ammonia production rate, (μmol/10^6 cells/h)	Max. Ab conc., (mg/l)	Volumetric productivity (mg/l/day)	Specific productivity (pg/cell/day)
5 L	328	1.02	0.0270	0.648	0.036	0.022	0.036	0.017	737.3	67.03	9.454
200 L	308	1.25	0.0280	0.672	0.087	0.023	0.110	0.023	830.1	64.68	9.444

Table 4. Summary of humanized anti-HGF YYB-101 mAb purification.

Step	Volume (ml)	Concentration (mg/ml)	Total mAb (mg)	Recovery (%)	Total yield (%)
Cell supernatant	175,000	0.634	110,892.3	100	100
Clarification	218,800	0.352	77,066.8	69.5	69.5
Protein A	14,650	4.990	73,102.2	94.9	65.9
SP Sepharose	8,740	7.716	67,436.8	94.5	64.4
Q Sepharose	10,040	6.670	66,966.0	99.3	60.8
Virus removal and ultrafiltration	3,150	21.121	66,530.1	99.9	60.4

antibody was concentrated by ultrafiltration. The purification and recovery yields in each step of the purification process are summarized in Table 4. The purified YYB-101 mAb was homogeneous in terms of the molecular mass of both the heavy and light chains. Furthermore, there were no detectable levels of cleaved forms of YYB-101, even though as little as 0.3 μg of the purified antibody could be visualized by Coomassie Blue staining (Fig. 4A) or western blot analysis with an anti-human IgG antibody (Fig. 4B).

Because soluble aggregates and charge variants are commonly found in recombinant mAb preparations, we performed SEC and cation-exchange HPLC to evaluate the product quality. The chromatograms of SEC (Fig. 4C) and cation-exchange HPLC (Fig. 4D) showed that there were no apparent size or charge differences between the antibodies produced in the 5 L and 200 L bioreactors. More importantly, the purified YYB-101 mAb showed HGF-neutralizing activity in a cell-based assay in which the growth rescue effect of HGF in cultures of TGF-β–treated CCL-64 (Mv-1-Lu) mink lung epithelial cells was measured in the presence of increasing concentrations (0.01–10 μg/ml) of YYB-101. Serially diluted purified YYB-101 was preincubated with HGF for neutralization prior to adding the mixture to the TGF-β–treated CCL-64 cells. As shown in Fig. 5, the anti-HGF YYB-101 mAbs produced in both the 5 L and 200 L bioreactors neutralized the HGF, and cell proliferation was inhibited almost to the level observed in the slower-growing control cultures because the inhibitory effect of TGF-β on cell proliferation could not be rescued by HGF in the presence of the neutralizing antibody.

We evaluated the *in vivo* biological activity of the anti-HGF YYB-101 mAb using a xenograft mouse model in which cells of the human glioblastoma-derived line U87 MG, which is known to contain an HGF/c-Met autocrine loop [31], were implanted subcutaneously. When the tumor size reached 100 mm^3, anti-HGF mAb was administered to each mouse (n = 10 per group) intraperitoneally twice per week at a dose of 0.5, 1.5, or 5.0 mg/kg. As shown in Fig. 6, at a dose of either 1.5 or 5.0 mg/kg, the anti-HGF mAb dramatically inhibited tumor growth, whereas with a lower dose of anti-HGF mAb (0.5 mg/kg) or with human IgG used as a control (5.0 mg/kg), there was no noticeable inhibitory effect (Fig. 6). This result is the first clear demonstration of the *in vivo* antitumor activity of humanized anti-HGF YYB-101 mAb.

Discussion

The clinical applicability of mAbs has been limited because mouse-origin antibodies are immunogenic and therefore have low effector functions in humans. Recent developments in antibody engineering technologies enabled us to develop a humanized anti-HGF antibody, providing a satisfactory solution for this problem. We expressed the humanized anti-HGF mAb, designated YYB-101, using a serum-free suspension culture-adapted CHO cell line and

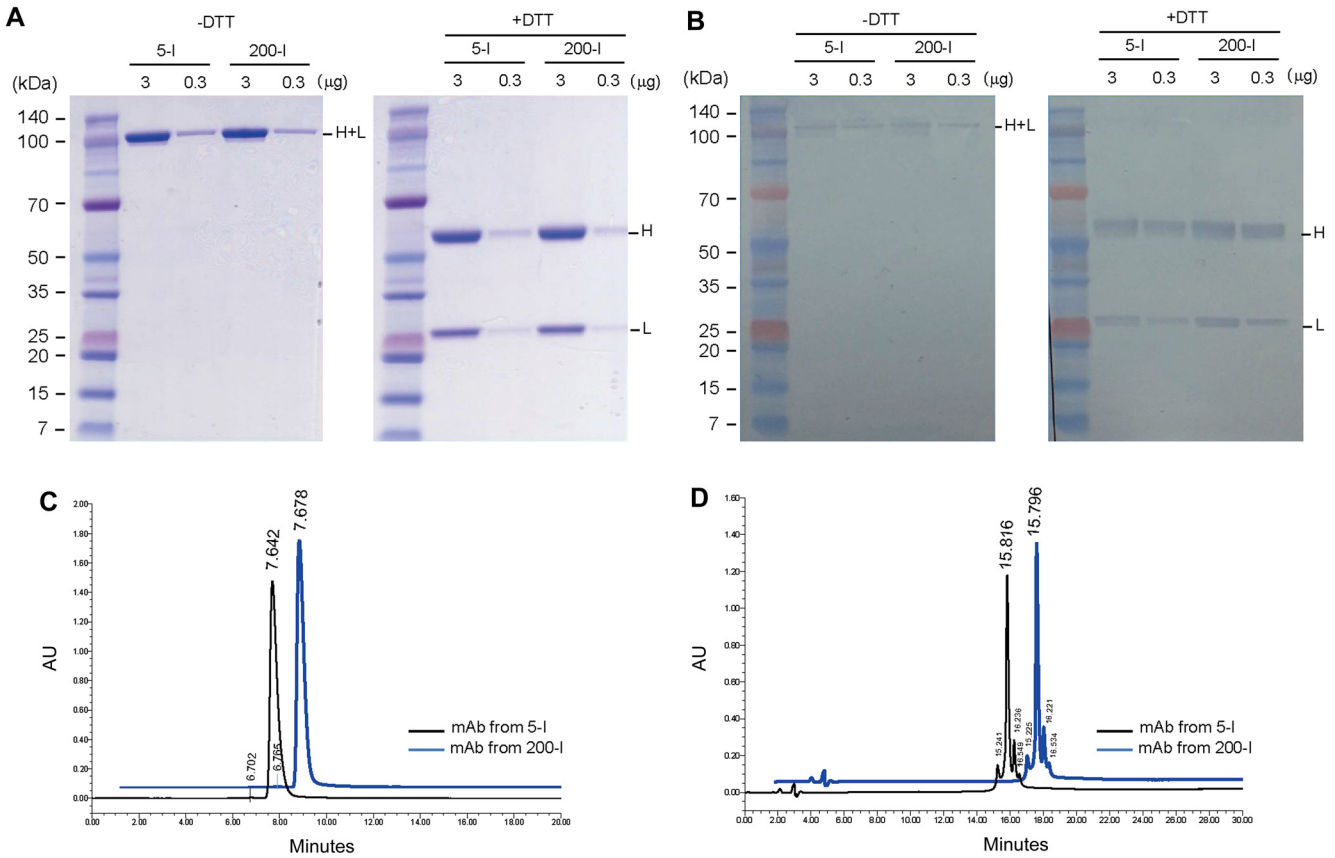

Fig. 4. Analysis of purified anti-HGF YYB-101 mAbs produced in 5 L and 200 L bioreactors.
(**A**) Indicated amounts of purified anti-HGF YYB-101 mAb produced in the indicated bioreactors were subjected to SDS-PAGE under reducing (+DTT) or nonreducing (DTT) conditions and visualized by Coomassie Blue staining. Protein molecular mass markers are shown on the left (kDa). L, light chain; H, heavy chain. (**B**) The purified antibodies resolved as described in (**A**) were analyzed by western blotting using an anti-human IgG antibody. (**C**-**D**) SEC (**C**) and cation exchange HPLC (**D**) chromatograms of purified YYB-101 mAbs produced in the indicated bioreactors. The number on each peak represents the retention time in minutes for the purified antibody.

showed its HGF-neutralizing activity using both a cell-based assay and a xenograft mouse model in which mice were subcutaneously implanted with human glioblastoma cells. The results demonstrated the ability of the YYB-101 mAb to block HGF/c-Met signaling. Despite considerable effort to develop therapeutic mAbs that block the HGF/c-MET interaction, to date only four antibodies have been developed for which their efficacy has been evaluated in preclinical and clinical trials. This includes three anti-human HGF mAbs, two of which (SCH900105/AV-299 and TAK-701) are humanized and are in clinical trial phases 2 and 1, respectively, and one fully human antibody (AMG102) currently in a phase 3 trial [10]. The remaining antibody is the anti-human c-MET mAb, designated MetMAb [14]. These mAbs, which inhibit activation of the HGF/c-MET pathway, showed promising clinical efficacy in c-MET–driven tumor patients. The humanized anti-HGF antibody YYB-101 is the first in this class of mAbs, in that it was generated by immunizing rabbits with the HGF/c-Met complex and its cDNA was further modified by phage display technology followed by gene manipulation. Currently, the YYB-101 mAb is undergoing preclinical studies.

Media optimization is a critical step for large-scale production of antibodies. Owing to safety concerns related to transmissible spongiform encephalopathy and other contaminants, chemically defined media are usually chosen for large-scale mAb production. To increase cell density, culture viability, and productivity, animal-component–free hydrolysates are often added to chemically defined media [8, 11]. The hydrolysates, however, can be a significant source of medium variability owing to their complex composition and lot-to-lot variation and can thus cause problems in the downstream purification process. Hydrolysates were excluded from our optimized production medium,

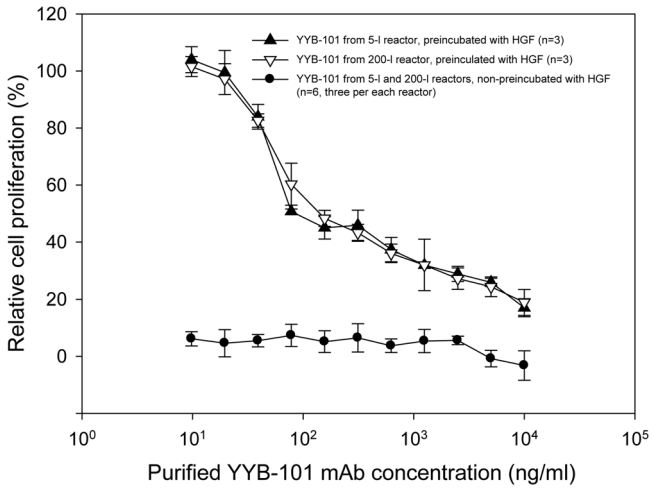

Fig. 5. *In vitro* HGF-neutralizing activity of purified YYB-101 mAb.

Mink lung epithelial cells (CCL-64) were treated with TGF-β (1.25 ng/ml) and cultured with serial dilutions of anti-HGF mAb in the presence of HGF (125 ng/ml). Serially diluted purified YYB-101 mAb produced in a 5 L or 200 L bioreactor was preincubated with HGF prior to adding the mixture to the cells (n = 3 per reactor) or was added directly to the cells (n = 6, three per 5 L and 200 L reactor). After further cultivation of the treated cells for 72 h, an MTT cell proliferation assay was conducted. Relative cell proliferation was calculated as the ratio of absorbance of samples treated with the antibody preincubated with or without HGF to that of control cells treated with HGF. The data are expressed as the mean of triplicate analyses and the bars represent the SD. Results are representative of three independent experiments.

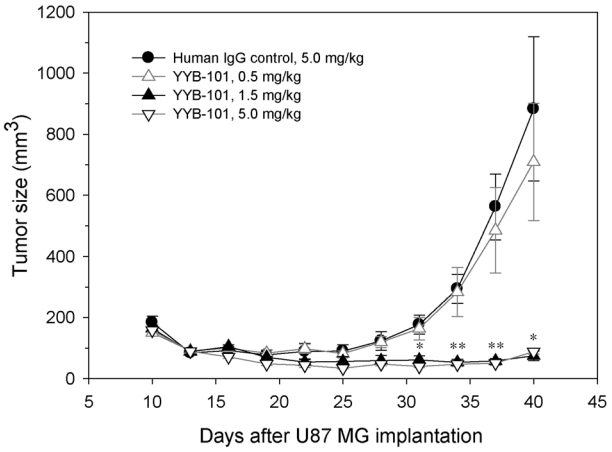

Fig. 6. Inhibition of tumor growth by the humanized anti-HGF YYB-101 mAb in mice subcutaneously xenografted with glioblastoma cells.

U87 MG glioblastoma cells were implanted subcutaneously in the right flank of BALB/c nude mice (n = 10 mice per group). Mice were treated intraperitoneally twice weekly for up to 28 days with 10, 30, or 100 μg of YYB-101 mAb, or 100 μg of human IgG as a control. The treatment started on day 13 when the tumor size reached ~100 mm^3. Data shown are the mean tumor volume ± SE for each treatment group. Asterisks indicate statistically significant differences between YYB-101-administered (1.5 mg/kg or 5 mg/kg) and control groups (*p < .01, **p < .001; Student's t-test).

and instead we used a diverse array of fully chemically defined basal media and feed media. By optimizing the medium using diverse commercially available basal and feed media and establishing an optimal feeding strategy, we could increase antibody productivity by up to 7.5-fold compared with that achieved under non-optimized conditions.

Optimization of culture operating parameters, including physical factors such as temperature, gas flow rate, and agitation speed, and chemical factors such as DO, carbon dioxide, pH, and osmolality, is necessary for bioreactor operation. In this work, we focused on two parameters, DO and pH, which are known to be critical for antibody production. We found that shifting the pH from 7.2 to 7.0 during the early stage of culture was more effective at improving antibody productivity than maintaining a fixed pH of either 7.2 or 7.0. Higher pH levels (≥7.0) typically preferred for rapid cell growth usually lead to concomitant lactate accumulation [33], which causes a decrease in pH and necessitates the addition of base to the reactor, resulting in an increase in osmolality and consequent detrimental effects on cell growth and viability. As shown in Fig. 3, cell growth at pH 7.2 yielded a higher cell density, but cell viability decreased more rapidly compared with reactors operated at pH 7.0. However, induction of rapid cell growth by starting the culture at pH 7.2 and shifting the pH to 7.0 on day 2 resulted in an even higher cell density with a higher, longer-lasting viability, which appeared to be responsible for the dramatic increase in antibody productivity under the pH-shifting condition. We also found that the yield of YYB-101 mAb was higher with the 70% DO condition than with the lower 40% DO condition, although similar maximum cell numbers were obtained with both DO levels in the 5 L bioreactor.

We successfully scaled up the YYB-101 production process in a 200 L bioreactor based on the optimized medium, feeding strategy, and operating conditions, including pH shift and DO concentration. Maintenance of antibody quality during process scale-up is important in the production of therapeutic antibodies. Changes in the biochemical properties of antibodies can affect the therapeutic efficacy and pharmacokinetic behavior of the product. The HGF-neutralization activity of the YYB-101 mAb produced in a 200 L bioreactor was similar to that of the antibody

recovered from a 5 L reactor operated under the 70% DO condition, suggesting that functional antibodies were produced in both bioreactors under the optimized running conditions. Previous studies showed that the DO level significantly affects antibody quality; although cell growth is relatively insensitive to DO in the range of 20–100% of air saturation, decreased glycosylation of antibody N-glycan chains was observed as the DO concentration was reduced [18, 21, 26]. In contrast to the 5 L bioreactor, the gas supply in the 200 L SUB was regulated with four mass-flow controllers, and DO was controlled during the early culture phase with pure O_2. Under such DO-controlled conditions, both maximum cell density and volumetric productivity were increased compared with the 5 L bioreactor. Because the cell culture process can significantly alter product quality in terms of glycosylation, other posttranslational modifications, and impurity profiles, product quality, and potency must be confirmed during the course of process development and scale-up for the production of large amounts of therapeutic mAbs [4, 9, 35]. Using SEC and cation-exchange HPLC, we analyzed the YYB-101 mAb purified from both the 5 L and 200 L bioreactors and found that they displayed similar chromatograms as well as *in vitro* and *in vivo* biological activities, suggesting that a similar posttranslational modification of the antibody occurred in both reactors. However, there may have been microheterogeneities in the glycosylation patterns. Thus, glycan profiling using liquid chromatography-mass spectrometry should be carried out to test whether different degrees of glycosylation affect the therapeutic efficacy of YYB-101 by altering its HGF-binding activity and/or stability.

In summary, we produced a humanized anti-HGF YYB-101 mAb under optimized medium and culture conditions and demonstrated for the first time that the antibody purified to near-homogeneity is capable of inhibiting tumor growth in a xenograft mouse model. Further studies are required to evaluate the toxicity and anticancer potency of the antibody in orthotropic tumor mouse models prior to its application in clinical trials. Nevertheless, our proof-of-concept study shows that YYB-101 is a promising anticancer agent. Currently, YYB-101 is being evaluated in toxicity studies for upcoming clinical trials.

Acknowledgments

This work was supported in part by grants from the National Research Foundation of Korea funded by the Korea Government (MSIP) (NRF 2009-0092959, 2012-047807, and 2012-0008693).

References

1. Borset M, Waage A, Sundan A. 1996. Hepatocyte growth factor reverses the TGF-beta-induced growth inhibition of CCL-64 cells. A novel bioassay for HGF and implications for the TGF-beta bioassay. *J. Immunol. Methods* **189:** 59-64.
2. Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, *et al.* 1991. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251:** 802-804.
3. Burgess T, Coxon A, Meyer S, Sun J, Rex K, Tsuruda T, *et al.* 2006. Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors. *Cancer Res.* **66:** 1721-1729.
4. Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl. Microbiol. Biotechnol.* **68:** 283-291.
5. Cao B, Su Y, Oskarsson M, Zhao P, Kort EJ, Fisher RJ, *et al.* 2001. Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. *Proc. Natl. Acad. Sci. USA* **98:** 7443-7448.
6. Cherrington JM, Strawn LM, Shawver LK. 2000. New paradigms for the treatment of cancer: the role of anti-angiogenesis agents. *Adv. Cancer Res.* **79:** 1-38.
7. Comoglio PM, Giordano S, Trusolino L. 2008. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat. Rev. Drug Discov.* **7:** 504-516.
8. Franek F, Hohenwarter O, Katinger H. 2000. Plant protein hydrolysates: preparation of defined peptide fractions promoting growth and production in animal cells cultures. *Biotechnol. Prog.* **16:** 688-692.
9. Gawlitzek M, Estacio M, Furch T, Kiss R. 2009. Identification of cell culture conditions to control *N*-glycosylation site-occupancy of recombinant glycoproteins expressed in CHO cells. *Biotechnol. Bioeng.* **103:** 1164-1175.
10. Giordano S. 2009. Rilotumumab, a mAb against human hepatocyte growth factor for the treatment of cancer. *Curr. Opin. Mol. Ther.* **11:** 448-455.
11. Heidemann R, Zhang C, Qi H, Larrick Rule J, Rozales C, Park S, *et al.* 2000. The use of peptones as medium additives for the production of a recombinant therapeutic protein in high density perfusion cultures of mammalian cells. *Cytotechnology* **32:** 157-167.
12. Higuchi O, Mizuno K, Vande Woude GF, Nakamura T. 1992. Expression of c-met proto-oncogene in COS cells induces the signal transducing high-affinity receptor for hepatocyte growth factor. *FEBS Lett.* **301:** 282-286.
13. Jiang WG, Martin TA, Parr C, Davies G, Matsumoto K, Nakamura T. 2005. Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. *Crit. Rev. Oncol. Hematol.* **53:** 35-69.

14. Jin H, Yang R, Zheng Z, Romero M, Ross J, Bou-Reslan H, *et al.* 2008. MetMAb, the one-armed 5D5 anti-c-Met antibody, inhibits orthotopic pancreatic tumor growth and improves survival. *Cancer Res.* **68:** 4360-4368.
15. Kim K, Hur Y, Ryu EK, Rhim JH, Choi CY, Baek CM, *et al.* 2007. A neutralizable epitope is induced on HGF upon its interaction with its receptor cMet. *Biochem. Biophys. Res. Commun.* **354:** 115-121.
16. Kim KJ, Wang L, Su YC, Gillespie GY, Salhotra A, Lal B, *et al.* 2006. Systemic anti-hepatocyte growth factor monoclonal antibody therapy induces the regression of intracranial glioma xenografts. *Clin. Cancer Res.* **12:** 1292-1298.
17. Kuba K, Matsumoto K, Date K, Shimura H, Tanaka M, Nakamura T. 2000. HGF/NK4, a four-kringle antagonist of hepatocyte growth factor, is an angiogenesis inhibitor that suppresses tumor growth and metastasis in mice. *Cancer Res.* **60:** 6737-6743.
18. Kunkel JP, Jan DC, Jamieson JC, Butler M. 1998. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. *J. Biotechnol.* **62:** 55-71.
19. Martens T, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, *et al.* 2006. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth *in vivo*. *Clin. Cancer Res.* **12:** 6144-6152.
20. Matsumoto K, Nakamura T, Kramer RH. 1994. Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. *J. Biol. Chem.* **269:** 31807-31813.
21. Miller WM, Wilke CR, Blanch HW. 1987. Effects of dissolved oxygen concentration on hybridoma growth and metabolism in continuous culture. *J. Cell. Physiol.* **132:** 524-530.
22. Munshi N, Jeay S, Li Y, Chen CR, France DS, Ashwell MA, *et al.* 2010. ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity. *Mol. Cancer Ther.* **9:** 1544-1553.
23. Nakamura T, Matsumoto K, Kiritoshi A, Tano Y. 1997. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: *in vitro* analysis of tumor-stromal interactions. *Cancer Res.* **57:** 3305-3313.
24. Nakamura T, Nawa K, Ichihara A. 1984. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **122:** 1450-1459.
25. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, *et al.* 1989. Molecular cloning and expression of human hepatocyte growth factor. *Nature* **342:** 440-443.
26. Ozturk SS and Palsson BO. 1990. Effects of dissolved oxygen on hybridoma cell growth, metabolism, and antibody production kinetics in continuous culture. *Biotechnol. Prog.* **6:** 437-446.
27. Pinski J, Schally AV, Halmos G, Szepeshazi K, Groot K. 1994. Somatostatin analogues and bombesin/gastrin-releasing peptide antagonist RC-3095 inhibit the growth of human glioblastomas *in vitro* and *in vivo*. *Cancer Res.* **54:** 5895-5901.
28. Qian F, Engst S, Yamaguchi K, Yu P, Won KA, Mock L, *et al.* 2009. Inhibition of tumor cell growth, invasion, and metastasis by EXEL-2880 (XL880, GSK1363089), a novel inhibitor of HGF and VEGF receptor tyrosine kinases. *Cancer Res.* **69:** 8009-8016.
29. Rafferty B, Maile P, Rigsby P, Gaines Das RE, Robinson CJ. 2001. International standards for hepatocyte growth factor/scatter factor: initial assessment of candidate materials and their evaluation by multicentre collaborative study. *J. Immunol. Methods* **258:** 1-11.
30. Renard JM, Spagnoli R, Mazier C, Salles MF, Mandine E. 1988. Evidence that monoclonal antibody production kinetics is related to the integral of the viable cells in batch systems. *Biotechnol. Lett.* **10:** 91-96.
31. Rosen EM, Laterra J, Joseph A, Jin L, Fuchs A, Way D, *et al.* 1996. Scatter factor expression and regulation in human glial tumors. *Int. J. Cancer* **67:** 248-255.
32. Rubin JS, Bottaro DP, Aaronson SA. 1993. Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. *Biochim. Biophys. Acta* **1155:** 357-371.
33. Schmid G, Blanch HW. Wilke CR. 1990. Hybridoma growth, metabolism, and product formation in HEPES-buffered medium: II. Effect of pH. *Biotechnol. Lett.* **12:** 633-638.
34. Tsarfaty I, Rong S, Resau JH, Rulong S, da Silva PP, Vande Woude GF. 1994. The Met proto-oncogene mesenchymal to epithelial cell conversion. *Science* **263:** 98-101.
35. Walsh G, Jefferis R. 2006. Post-translational modifications in the context of therapeutic proteins. *Nat. Biotechnol.* **24:** 1241-1252.