Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel rabbit anti-hepatocyte growth factor monoclonal neutralizing antibody inhibits tumor growth in prostate cancer cells and mouse xenografts

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ARTICLE INFO

Article history:
Received 10 June 2015
Accepted 15 June 2015
Available online xxx

Keywords:
Hepatocyte growth factor (HGF)
C-Met
Prostate cancer
Rabbit monoclonal antibody

ABSTRACT

The hepatocyte growth factor and its receptor c-Met are correlated with castration-resistance in prostate cancer. Although HGF has been considered as an attractive target for therapeutic antibodies, the lack of cross-reactivity of monoclonal antibodies with human/mouse HGFs is a major obstacle in preclinical developments. We generated a panel of anti-HGF RabMAbs either blocking HGF/c-Met interaction or inhibiting c-Met phosphorylation. We selected one RabMAb with mouse cross-reactivity and demonstrated that it blocked HGF-stimulated downstream activation in PC-3 and DU145 cells. Anti-HGF RabMAb inhibited not only the growth of PC-3 cells but also HGF-dependent proliferation in HUVECs. We further demonstrated the efficacy and potency of the anti-HGF RabMAb in tumor xenograft mice models. Through these *in vitro* and *in vivo* experiments, we explored a novel therapeutic antibody for advanced prostate cancer.

1. Introduction

Prostate cancer is the most common malignancy among men and the second-leading cause of cancer deaths in the United States. It is estimated that there will be more than 220,800 new cases and 27,540 deaths from prostate cancer in 2015 in the United States [1]. In developing countries, its incidence is also on the rise. Androgen deprivation therapy is the first-line treatment for advanced prostate cancer. Unfortunately, nearly all patients develop castration-resistant prostate cancer (CRPC) within 2–3 years [2]. Molecular mechanisms of the growth of CRPC including: ①androgen receptor aberrations; ②the maintenance of tumor-derived androgens; ③activation of other growth factors or signaling pathways, such as PTEN mutation [3,4]. Although a number of novel agents have sprung up for the treatment of CRPC over the last decade, interest remains in targeted therapies [5].

The hepatocyte growth factor/scatter factor (HGF/SF) is a growth factor that plays a critical role in the regulation of cell proliferation, malignant progression and angiogenesis in a wide variety of human carcinomas [6]. It is believed that HGF and its receptor are correlated with castration-resistance in prostate cancer [7–10]. HGF derived from prostate stroma significantly increases the proliferation, motility, and invasion of malignant epithelial cells. C-Met is preferentially expressed in the androgen-insensitive prostate cancer cell lines, such as PC-3 and DU-145, but not the androgen-sensitive LNCaP cell line [11,12]. HGF or c-Met expression was increased after castration or hormone therapy [7] and androgen ablation can induce c-Met expression which may contribute to the castration-resistant tumor growth [9,10]. Additionally, serum HGF levels are highly expressed in late stage and metastatic prostate cancer and HGF is related to the development of bone metastasis in prostate cancer [8]. Therefore, HGF/c-Met becomes an attractive target for the treatment of CRPC.

To date, several antagonists are being exploited to target HGF/c-Met signaling pathway, such as neutralizing antibodies to HGF [13], or small-molecule c-Met kinase inhibitors [14]. Compared to small-molecule c-Met kinase inhibitors, the development of therapeutics antibodies has been accelerated in recent years because of its higher specificity and better safety. In preclinical studies, human tumor xenograft mouse models are the major testing system to evaluate efficacy of these potential therapeutic antibodies.

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http://dx.doi.org/10.1016/j.bbrc.2015.06.107
0006-291X/

Typically, HGF is produced by stromal cells and functions as a paracrine growth factor in prostate cancer [15]. However, the conventional mouse derived antibodies are negatively selected against epitopes displayed by the mouse antigen [16,17]. The lack of cross-reactivity with the mouse antigen is a major obstacle in the applications of monoclonal antibodies (mAbs) in the preclinical development, such as rilotumumab (AMG102), a monoclonal antibody against HGF, can be only tested in autocrine HGF/c-Met-dependent human tumor xenograft models during preclinical stages [13,18], but not prostate cancer models. In the previous study, we have successfully a novel platform for efficiently generating rabbit monoclonal antibodies (RabMAbs) recognizing both human and mouse antigens and proved their better efficacy *in vivo* and *in vitro* than currently available drug in the market [19,20]. In this study, using the same technology, we generated and characterized a panel of anti-HGF RabMAbs neutralizing HGF/c-Met interaction. We selected one of the anti-HGF RabMAbs with mouse cross-reactivity and then evaluated its efficacy and potency in tumor xenograft mice models. Through these *in vitro* and *in vivo* experiments, we explored a novel therapeutic antibody for advanced prostate cancer.

2. Materials and methods

2.1. Cell lines and proteins

Human prostate cancer cell line PC-3 and DU145 were obtained from ATCC and maintained in RPMI1640 supplemented with 10% FBS at 37 °C in 5% CO_2. Human umbilical vein endothelial cells (HUVECs) from Lonza (Walkersville, MD) were maintained in Endothelial Basal Medium-2 (EGMH-2, Lonza) supplemented with 2% fetal bovine serum and growth factors (BulletKitH, Lonza). The fusion protein rabbit IgG Fc-hHGF and Fc-c-Met were provided by Yikang Bio-tech Co. (Hangzhou, China) and human HGF (hHGF) and mouse HGF (mHGF) were perchased from R&D System (Mineapolis, MN).

2.2. Generation of rabbit anti-HGF monoclonal antibodies

Anti-Human HGF RabMAbs were generated by the method described previously [19]. Briefly, New Zealand white rabbits were immunized subcutaneously with 0.2 mg Fc-hHGF. Splenocytes were harvested and fused with rabbit plasmacytoma cells 240E-W2. Hybridoma supernatants were screened by antigen binding and neutralization of HGF/c-Met interaction. Positive hybridomas cells were lysed and the total RNA was extracted by using the Qiagen TurboCapture mRNA kits (Qiagen, Inc,Vaencia, CA). L chain and the variable region of H (VH) chain were amplified by PCR with rabbit H and L chain primers and cloned into pTT5 expression vector. The L and H chain plasmids were co-transfected into 293-6E cells to express recombinant RabMAbs.

2.3. Anti-HGF RabMAbs binding to human HGF and mouse HGF ELISA

96-well plates coated with 2 μg/ml hHGF or mHGF in coating buffer overnight at 4 °C. The plates were then blocked with PBS containing 1% BSA and 0.05% Tween-20 1 h at room temperature. RabMAbs were added to wells for 1.5 h at room temperature (RT). Plates were washed twice in TBST (PBS with 0.05% Tween-20), and alkaline phosphatase-coupled goat anti-rabbit IgG (Pierce, Rockford, IL) was added for another 1.5 h. After washing three times, phosphatase substrate p-nitrophenyl phosphate substrate was added, and absorbance was measured at 405 nm.

2.4. Receptor-ligand binding assay

96-well plates coated with 5 μg/ml of Fc-c-Met overnight at 4 °C. Serial dilutions of anti-HGF RabMAbs or control rabbit IgG were pre-incubated with hHGF for 1.5 h. After 1 h of incubation, the mixture was transferred to the plates and incubated for another 1 h. HGF bound to immobilized c-Met was detected by a mouse anti-hHGF monoclonal antibody (Abcam, Cambridge, UK), followed by the addition of goat anti-mouse IgG alkaline phosphatase conjugated antibody (Fisher Scientific/Pierce Biotechnology, Rockford, IL). The data were evaluated by calculating the percentage inhibition compared with the maximal signal (control antibody) and the IC50 values were calculated.

2.5. Quantitative real time PCR (qPCR)

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcription was conducted following the instructions of the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA). For qPCR, 1 μl gene primers with SYBR Green PCR Master Mix (Applied Biosystems) in 20 μl reaction volume were performed. Primers were designed as: HGF, forward, 5′-CTCA-CACCCGCTGGGAGTAC-3′, reverse, 5′-TCCTTGACCTTGGATGCATTC-3′; c-Met, forward, 5′- CTGCCTGCAATCTACAAGGT-3′, reverse, 5′-ATGGTCAGCCTTGTCCCTC-3′; actin, forward, 5′-CTCCTCCTGAGCG-CAAGTACTC-3′, reverse, 5′-TCCTGCTTGCTGATCCACATC-3′. mRNA levels of tested genes were normalized to Actin according to the following formula: $2^{-(CT\ test - CT\ Actin)}$, where CT is the threshold cycle. Fold of gene expression of PC-3 cells was defined as '1'.

2.6. Inhibition of HGF-stimulated c-Met phosphorylation in prostate cancer cell lines

PC3 and DU145 cells (1×10^6 cells/well) were plated in 6-well plates and starved overnight. 10 μg/ml Anti-HGF RabMAbs were pre-mixed with 50 ng/ml hHGF for 1 h and then added to the cells for 10 min. Cells were washed with cold PBS and lysed in RIPA buffer (Cell Signaling, Danvers, MA). Protein samples boiled in SDS-sample buffer were resolved on a 6% SDS-PAGE, transferred onto a nitrocellulose membrane, and then blotted with rabbit monoclonal anti-human phosphorylated c-Met (diluted in the ratio 1:1000), rabbit anti-human phosphorylated Akt1, rabbit anti-human phosphorylated ERK1/2, rabbit anti-human c-Met rabbit or rabbit anti-human GAPDH antibody (diluted in the ratio 1:1000, respectively) (Abcam, Cambridge, UK). Blots were detected using the ECL kit (Pierce, Rockford, IL).

2.7. Cell proliferation assays

Two thousand PC-3 cells or 4×10^3cells DU145 were seeded in 96-well plates and cultured in RPMI1640 supplemented with 10% FBS at 37 °C in 5% CO_2 overnight. Following this incubation, the cells were washed once with serum-free RPMI1640 and replaced with a medium containing 5% FBS and treated with or without 50 ng/ml human HGF and 10 μg/ml, 20 μg/ml, or 30 μg/ml of RabMAbs. After 72 h, cell proliferation assays were carried out using the MTS tetrazolium kit (Promega, Madison, WI). For endothelial cell proliferation assay, HUVEC (4×10^3cells/well) were cultured for 3 days in medium containing HGF (50 ng/ml) or VEGF (15 ng/ml).

2.8. PC-3 cells xenograft experiments

All animals performed in this study conformed to the recommendations in the guide for the Care and Use of Laboratory Animals

Please cite this article in press as: Y. Yu, et al., A novel rabbit anti-hepatocyte growth factor monoclonal neutralizing antibody inhibits tumor growth in prostate cancer cells and mouse xenografts, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.06.107