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Targeting hepatocyte growth factor receptor (Met) positive tumor cells using internalizing nanobody-decorated albumin nanoparticles

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ABSTRACT

The hepatocyte growth factor receptor (HGFR, c-Met or Met) is a receptor tyrosine kinase that is involved in embryogenesis, tissue regeneration and wound healing. Abnormal activation of this proto-oncogene product is implicated in the development, progression and metastasis of many cancers. Current therapies directed against Met, such as ligand- or, dimerization-blocking antibodies or kinase inhibitors, reduce tumor growth but hardly eradicate the tumor. In order to improve anti-Met therapy, we have designed a drug delivery system consisting of crosslinked albumin nanoparticles decorated with newly selected anti-Met nanobodies (anti-Met-NANAPs). The anti-Met NANAPs bound specifically to and were specifically taken up by Met-expressing cells and transported to lysosomes for degradation. Treatment of tumor cells with anti-Met NANAPs also resulted in downregulation of the total Met protein. This study shows that anti-Met NANAPs offer a potential system for lysosomal delivery of drugs into Met-positive tumor cells.

1. Introduction

The hepatocyte growth factor (HGF) receptor (HGFR, c-Met or Met) is a receptor tyrosine kinase (RTK) that is primarily expressed on epithelial cells. HGF is the only known, high affinity ligand for this receptor [1]. Binding of HGF to Met activates the receptor, resulting in tyrosine phosphorylation of the receptor and the activation of several downstream signal transduction pathways such as the MAPK-, STAT- and PI3kinase/Akt-pathway [1–4]. Met/HGF signaling is important for embryogenesis and also for tissue regeneration and wound healing in the adult life [5–7]. Deregulated Met signaling is implicated in the development, progression and metastasis of a wide variety of human cancers [8,9]. This can be due to mutational activation, receptor/ligand overexpression, autocrine activation or ligand-independent activation of Met. Met is therefore an attractive target for anti-cancer therapy and several agents interfering with the Met/HGF pathway are under development such as Met/HGF signaling small molecule inhibitors, antibodies and decoy receptors [10,11]. Anti-Met therapies can be further augmented by combining them with chemotherapeutic drugs, especially when these are combined in a targeted drug delivery system [12].

Like most RTKs, ligand binding and activation of the Met tyrosine kinase initiates internalization of the receptor–ligand complexes, which is followed by intracellular trafficking and degradation of both receptor and ligand in lysosomes [11–15]. This internalization and lysosomal trafficking can be used for targeted delivery and release of drugs inside tumor cells. Previously, we designed anti-EGFR Nanobody-Albumin Nanoparticles (NANAPs), which are albumin-based nanoparticles decorated with nanobodies that target the epidermal growth factor receptor (EGFR). NANAPs were shown to bind to and internalize into EGFR-expressing cells and were found to be suitable for targeted delivery of anti-cancer drugs into cells [16].

A nanobody (or VHH) is the antigen-binding domain of heavy-chain-only antibodies found in members of the *cameilidae* family. Despite their small (~15 kDa) size, their binding affinity is similar

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to that of monoclonal antibodies [17]. Due to their small size and less complex structure nanobodies can be easily produced in prokaryotic systems giving them advantage over monoclonal antibodies or antibody fragments [18]. Nanobody-coupled liposomal and polymeric nanoparticles have already been shown to induce tumor regression in mice bearing head and neck squamous carcinoma tumors [19,20].

In this study we aimed to develop albumin nanoparticles that specifically target the Met receptor. We hypothesized that the specific uptake and lysosomal trafficking of anti-Met albumin nanoparticles would stimulate Met downregulation and allow intracellular delivery of nanoparticles into Met expressing cancer cells.

2. Materials and methods

2.1. Cell culture and cell lines

The human ovarian carcinoma cell line TOV-112D (cat nr. CRL-11731), human lung carcinoma cell line A549 (cat.nr. CCL-185) and the human epidermoid squamous carcinoma cell line A431 (cat. nr. CRL-1555) were all obtained from American Tissue Culture Collection (ATCC, LGC Standards GmbH, Wesel, Germany) and the human gastric cancer cell line MKN45 (cat. nr. ACC-409) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). TOV-112D and A431 cells were cultured in Dulbecco's Modification of Eagle Medium (DMEM: Gibco, Invitrogen, Breda, The Netherlands) supplemented with 100 units/ml streptomycin, 0.1 mg/ml penicillin, 2 mM L-glutamine and 10% fetal calf serum (FCS) at 37°C and 5% CO_2. MKN45 cells were cultured in RPMI 1640 (Life Technologies Inc., Invitrogen) supplemented with 100 units/ml streptomycin, 0.1 mg/ml penicillin, 2 mM L-glutamine and 20% FCS. A549 cells were cultured in Ham's F12 (Life Technologies, Inc., Invitrogen) supplemented with streptomycin, penicillin, L-glutamine and 10% FCS under the previously mentioned conditions. Met expression of cells seeded for one or two days was determined by western blotting using mouse-anti-HGFR (MAB3581, R&D Systems Europe Ltd, Abingdon, UK) followed by goat-anti-mouse^{Alexa488} (Invitrogen) incubation. For the generation of the stable TOV + Met cell line, human Met-encoding cDNA (kindly provided by Dr. Morag Park, McGill University, Montreal, Canada) was first transferred from the pXM vector into a pMX-IRES-Zeo vector (generated from the pMX-SupF vector kindly provided by prof. Garry P. Nolan, Stanford University School of Medicine, Stanford, California, USA) via XhoI-XhoI digestion (Thermo Scientific, Breda, The Netherlands) and ligation (T4 ligase, Promega, Madison, USA). TOV-112D cells were transfected with this pMX-Met-IRES-Zeo using Fugene HD (Roche, Mannheim, Germany) and grown under selection pressure using 200 μg/ml of Zeocin (Invitrogen) for 8 weeks. Single clones were tested for Met-expression by western blotting.

2.2. Nanobody selection

Nanobodies directed against Met were selected using phage display technology from a phage antibody library that was made from *Llama Glama* immunized with A431 membrane vesicles, as previously described [21]. For selections, Maxisorp 96-wells plates (Nunc, Roskilde, Denmark) were coated overnight with rabbit-anti-hIgG in PBS (1:2000, Dako, Glostrup, Denmark) at 4°C. Next day, non-specific binding was blocked with 2% skimmed milk (Marvel) in PBS (MPBS) for an hour at RT. Subsequently, 1 μg of Met-Fc fusion protein diluted in MPBS (R&D systems) per well was captured for 1 h at room temperature (RT). After three washes with PBS, captured antigen was incubated with phages (blocked in 2% MPBS) for 2 h at RT. After washing, bound phages were detached by 100 mM triethylamine (TEA) elution. For phage ELISA, Maxisorp plates were coated with rabbit-anti-hIgG as described above and 50 ng/well of Met ectodomain-Fc fusion protein (R&D Systems) was captured for 1 h at RT in 2% MPBS. Single clone phages were incubated for 2 h at RT, washed extensively and bound phage was detected with mouse-anti-M13^{HRP} (1:10,000), followed by o-phenylenediamine (OPD) development. The reaction was stopped by the addition of 3M H_2SO_4 and OD was read at 450 nm. The cDNAs of the selected clones were sequenced and then re-cloned into a pET28a vector containing C-terminal Myc-6-His tags and which allows the purification of nanobodies from the periplasmic space of *E.coli*.

2.3. Production and purification of nanobodies

Nanobodies were produced as described before [16], but with minor modifications. Briefly, *E. coli BL*-21 Codon Plus (DE3)-RIL (Agilent Technologies Inc., Santa Clara, CA, USA) cells were transformed with pET28-derived nanobody-encoding plasmid and a single antibiotic-resistant colony was picked. Production was performed by growing these bacteria in 2xTY medium containing 2% (w/v) glucose and 100 μg/ml ampicillin at 37°C overnight. Four hundred milliliters of 2xTY medium (supplemented with 100 μg/ml ampicillin and 0.2% (w/v) glucose) was inoculated with the bacteria from the overnight culture with an OD_{600} of 0.1. This bacterial culture was subsequently incubated at 37 °C at 250 rpm until it reached an OD_{600} of 0.6. Subsequently, 0.5 mM IPTG was added to the bacterial culture to induce nanobody expression and the culture was further incubated at 37°C for 3.5 h. After 3.5 h, cells were spun down (5000 rpm, 15 min, 4°C) and the obtained pellet was stored over night at −20°C. The following day, periplasmic fractions were made by incubating the pellet in 6.4 ml ice cold TES (200 mM Tris–HCl, 0.5 mM EDTA, 500 mM sucrose, pH 8.0). To this suspension, 10 ml of diluted ice cold TES (1:3 in water) was added and incubated on ice for 30 min. The bacteria were spun down at 4 °C for 15 min at 5000 rpm and the supernatant was collected. The pellet was resuspended in 10 ml ice cold TES, 120 μl 1 M $MgSO_4$ was added and the mixture was incubated on ice for 30 min. The suspension was spun down at 4 °C for 15 min at 5000 rpm and the supernatant containing the nanobody was added to the previously collected supernatant. The his-tagged nanobodies were purified by means of immobilized metal ion affinity chromatography (IMAC) on TALON resin (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol (Batch/Gravity-Flow), except 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0 was used for all washing steps and elution was performed with the same wash buffer but containing 300 mM imidazole, pH 8.0 buffer. Imidazole was removed by means of dialysis against PBS overnight at 4°C. Finally, protein fractions and purity were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were determined using a Micro BCA assay (ThermoScientific).

2.4. FACS analysis

For FACS analysis of binding of the anti-Met nanobodies, Met-expressing A431 cells were trypsinized, blocked and incubated with anti-Met VHHs (developed in house) or EGF-^{Alexa488} (Invitrogen) on ice for 2 h. The cells were washed three times with PBS and fixed in 4% paraformaldehyde (PFA). Auto-fluorescence was quenched with 100 mM glycine in PBS for 15 min and VHHs were detected with Prot-G purified rabbit-anti-VHH (developed in house), followed by goat-anti-rabbit^{Alexa488} (Invitrogen) incubation. FACS analysis was performed on a BD analyzer (BD Biosciences, Breda, The Netherlands).

2.5. Binding and affinity determination of nanobodies

TOV-112D, TOV + Met (1.5×10^4 per well) or MKN45 cells (6×10^4 per well) were seeded in a 96-wells plates (Nunc) one day before the assay. The cells were pre-incubated with binding medium (2% BSA in CO_2-independent medium (Gibco) for 10 min on ice after which the cells were incubated in binding medium supplemented with or without nanobodies (0.005–1000 nM) for 2 h on ice. After three washes with ice cold PBS, cells were fixed in 4% PFA and fixative was subsequently quenched with 100 mM glycine in PBS for 15 min. Bound nanobody was detected with rabbit-anti-VHH (1:1000) in PBS containing 2% BSA (PBA) followed by goat-anti-rabbit IRDye800CW (Li-COR Biosciences, UK, 1:500 in PBA) incubation for 1 h at RT each. Finally, cells were washed twice with PBA and fluorescence was measured using the Odyssey Infrared Imager. Binding affinity (K_D) for one site specific binding was determined by curve fitting using GraphPad Prism 5.02 for Windows (GraphPad Software, San Diego, CA).

2.6. HGF competition

HGF was labeled with ^{125}I (Perkin Elmer, USA) according to the IODO-GEN (Sigma–Aldrich) method as described previously [22]. The specific activity of I^{125}-HGF was measured at ~30,000 CPM/μg. Maxisorp 96-well ELISA plates (Nunc) were coated with rabbit anti-human IgG (Dako, Glostrup, Denmark; 4 μg/mL in PBS). The next day, wells were washed with PBS, blocked with 1% MPBS and subsequently incubated with 0.1 μg/mL of Met-ECD-Fc. After washing with PBS, the wells were incubated with 1 nM of ^{125}I-HGF in the presence or absence of 1000 nM of nanobodies for 1 h. Wells were washed four times with PBS and bound I^{125}-HGF was collected after 5 min incubation with 1 M NaOH; radioactivity was measured using a gamma counter (Wallac Wizard, Perkin Elmer). Results were analyzed using GraphPad Prism version 5.02 for Windows, GraphPad Software (San Diego, CA).

2.7. Preparation of albumin nanoparticles

Nanoparticles were prepared by the ethanolic desolvation technique as described previously [16,23] with minor modifications. Briefly, human serum albumin (HSA, 50 mg/ml) was dissolved in deionized water after which the pH was adjusted to 8.3 by addition of 0.1 M NaOH and the solution was filtered through a 0.2 μm filter (Minisart® syringe filters, Sartorius-stedim, Germany). Nanoparticles were formed by adding 92% ethanol drop wise under constant stirring. The formed particles were crosslinked with 8% glutaraldehyde (GA) overnight at RT. The nanoparticles were purified by centrifugation first at 1000 g for 5 min (pellet discarded, supernatant collected) and three times at 45,000 g for 1 h at 4°C in deionized water. Nanoparticles were stored in deionized water at 4°C. Nanoparticle yield was determined by measuring the dry weight by thermogravimetric analysis. The absence of soluble albumin and multimers was checked by SDS-PAGE (4–12% crosslinked, Invitrogen) under reducing conditions followed by Coomassie blue staining. For enabling detection of nanoparticles in fluorescence microscopy or quantification on the Odyssey Infrared Imager, nanoparticles were labeled with either Alexa488-NHS (Invitrogen) or IRDye800CW-NHS (Li-COR) as described before [16].