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Tumor-penetrating peptide fused EGFR single-domain antibody enhances cancer drug penetration into 3D multicellular spheroids and facilitates effective gastric cancer therapy

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ABSTRACT

Human tumors, including gastric cancer, frequently express high levels of epidermal growth factor receptors (EGFRs), which are associated with a poor prognosis. Targeted delivery of anticancer drugs to cancerous tissues shows potential in sparing unaffected tissues. However, it has been a major challenge for drug penetration in solid tumor tissues due to the complicated tumor microenvironment. We have constructed a recombinant protein named anti-EGFR-iRGD consisting of an anti-EGFR VHH (the variable domain from the heavy chain of the antibody) fused to iRGD, a tumor-specific binding peptide with high permeability. Anti-EGFR-iRGD, which targets EGFR and αvβ3, spreads extensively throughout both the multicellular spheroids and the tumor mass. The recombinant protein anti-EGFR-iRGD also exhibited antitumor activity in tumor cell lines, multicellular spheroids, and mice. Moreover, anti-EGFR-iRGD could improve anticancer drugs, such as doxorubicin (DOX), bevacizumab, nanoparticle permeability and efficacy in multicellular spheroids. This study draws attention to the importance of iRGD peptide in the therapeutic approach of anti-EGFR-iRGD. As a consequence, anti-EGFR-iRGD could be a drug candidate for cancer treatment and a useful adjunct of other anticancer drugs.

1. Introduction

Gastric cancer is one of the world's leading causes of cancer-related death with a high incidence and mortality rate, particularly in Eastern Asia [1,2]. Despite recent advances in cancer therapy, such as chemotherapy, radiotherapy and biological immune therapy, most advanced malignancies still remain incurable. Thus, the research and development of new therapeutics is essential.

With the advent of molecular engineering and phage display technology, more antibodies have been explored. Antibodies have different formats and VHH as a minimal functional format has some advantages, such as: lower immunogenicity, facile genetic manipulation, high physicochemical stability, recognition of hidden antigenic sites and high expression levels. Therefore, there seems to be a trend in therapeutic and diagnostic antibodies towards smaller antigen-binding antibody formats [3,4]. The variable domain from the heavy chain of the antibody (VHH), often called a single domain antibody (sdAb) [5] or nanobody [6] due to its size in the nanometer range, is considered to be the smallest naturally derived antigen-binding fragment [7]. Human tumors frequently express high levels of epidermal growth factor receptor (EGFR), which has been associated with a poor prognosis when overexpressed [8]. EGFR (ErbB1, HER1) is a 170-kDa transmembrane tyrosine kinase receptor, overexpressed in a wide variety of human

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cancers including 27.4% of 511 gastric cancer tissues [9]. Progress in genetic engineering has guided the way for development of various EGFR inhibitors including monoclonal antibodies, (cetuximab, panitumumab, etc.), tyrosine-kinase inhibitors (gefitinib, erlotinib, lapatinib, etc.), antisense oligonucleotides and sdAbs [10].

Although targeted delivery of anticancer drugs to cancerous tissues shows potential in sparing unaffected tissues, it is still a major challenge for the targeted therapeutic to penetrate deep into solid tumor tissues. In solid cancers, the homeostatic regulation of tissues breaks down, cancer cells are in the state of hypoxia, interstitial fluid pressure increases [11–14], and the extracellular matrix (ECM) hinders the movement of drugs and molecules into the tumor tissue [15–17].

It has been reported that the tumor-penetrating and cell-internalizing peptide iRGD (sequence: CRGDKGPDC) contains both a RGD (Arg-Gly-Asp) domain and a CendR motif (R/KXXR/K). It first binds to αvβ3 and αvβ5 integrins, which are expressed highly in tumor vessels and many different types of cells in the tumor [18]. Subsequently, iRGD is proteolytically cleaved to CRGDK/R. The truncated peptide loses affinity for the primary receptor integrin, and binds to neuropilin-1 (NRP-1), triggering a cell internalization and tissue penetration pathway [19,20].

Since Sutherland et al. [21,22] established multicellular spheroids (MCS) in the 1970s, this three-dimensional (3D) MCS in vitro tumor model has been demonstrated as a practical and simple model that reflects many of the properties of natural solid tumors. The 3D culture conditions in MCS can produce an ECM [23,24], which creates a major obstacle for drug penetration into tumor tissues. In addition, large MCS (>200 μm in diameter) have been demonstrated to form three different regions: proliferating periphery cell populations, a viable and quiescent intermediate zone, and a necrotic core from the outside in [25,26]. Furthermore, Minchinton et al. [27] mentioned that, MCS were an ideal platform for studying drug penetration, along with multilayered cell cultures and in vivo methods.

In this study, an anti-EGFR sdAb selected by phage display was used as a ligand to interact with EGFR. To efficiently deliver anti-EGFR sdAbs into the tumor and overcome the difficulty of poor penetration of anticancer drugs in solid tumors [27,28], we introduced a C-end Rule peptide iRGD to an anti-EGFR sdAb. Afterwards, the anticancer activity of the recombinant proteins anti-EGFR and anti-EGFR-iRGD were examined. Penetration of anti-EGFR and anti-EGFR-iRGD through both MCS culture system and in vivo methods was then evaluated. To study the effect of anti-EGFR-iRGD on drug delivery and efficacy, we also administered the protein as a combination therapy with several types of cancer drugs, such as DOX, bevacizumab, nanoparticles in a 3D multicellular spheroid model.

2. Materials and methods

2.1. Reagents, cell lines, and tumors

Doxorubicin hydrochloride (DOX) was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen China). Paclitaxel liposome was obtained from Nanjing Si Ke Pharmaceutical Co., Ltd. (Nanjing, China), bevacizumab and cetuximab were purchased from Roche (Basel, Switzerland) and Merck (Darmstadt, Germany). siRNA targeting human EGFR mRNA (GenBank Accession No. NM_005228), integrin αv mRNA (GenBank Accession No. NM_001144999), and NRP-1 mRNA (GenBank Accession No. NM_001024628) were designed and synthesized by Guangzhou RiboBio Company (Guangzhou, China). Antibodies were purchased from Cell Signaling Technology (USA) (for human EGFR antibody), Signalway Antibody (USA) (for p-EGFR Tyr1172), Novus biologicals (USA) (for human αvβ3 antibody), and Abcam (USA) (for human NRP-1 antibody). Mouse monoclonal anti-human CD31 and rat monoclonal anti-mouse CD31 were purchased from Gene Tech Company Limited (Shanghai, China) and BD Pharmingen (San Jose, California). An Alexa 594 conjugated donkey anti-mouse secondary antibody and donkey anti-rat secondary antibody were purchased from Abcam (USA) and Molecular Probes (Eugene, Oregon). Full length DNA for anti-EGFR in the vector pSJF2 was a gift from Dr. Shenghua Li at Tianjin International Joint Academy of Biotechnology and Medicine (Tianjin, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for the cell viability assay was obtained from Sigma-Aldrich (USA). Fluorescein isothiocyanate (FITC) and coumarin-6, were obtained from Sigma Chemical Co (USA). All other chemicals were used as received without further treatment.

Human gastric adenocarcinoma cell line BGC-823, human epithelial colorectal adenocarcinoma cell line Caco-2, human breast adenocarcinoma cell line MCF-7 and murine hepatic cancer cell line H22, were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology. BGC-823 and H22 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, while other cells were cultured in DMEM-medium (for MCF-7 cells) or MEM-medium (for Caco-2 cells), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C and 5% CO_2 (all available from Invitrogen, Grand Island, NY, USA). All animal procedures were carried out in compliance with guidelines set by the Animal Care Committee at Drum Tower Hospital (Nanjing, China). Five million BGC-823 gastric cancer cells in 0.1 mL PBS were subcutaneously injected in the lower right axilla of athymic nude BALB/c mice (5–6 weeks, male, 18–22 g), while 5 million H22 cells were subcutaneously injected in the right axilla of ICR mice (4–5 weeks, male, 18–22 g) to produce xenograft tumors. Tumor volumes were calculated from 2 diameter measurements using a digital vernier caliper and the formula: tumor volume = (length × width^2) / 2, where length is the longest dimension and width is the widest dimension.

2.2. Preparation and characterization of recombinant proteins anti-EGFR and anti-EGFR-iRGD

Recombinant proteins anti-EGFR and anti-EGFR-iRGD were prepared as follows. The anti-EGFR sequence was cloned into the bacterial expression vector pET28a with a hexahistidine (His) tag placed at the N-terminus of anti-EGFR. To enhance cell- and tissue-penetration of anti-EGFR, oligonucleotides encoding iRGD were synthesized and ligated downstream of oligonucleotides encoding the anti-EGFR, with a glycine–serine linker (Asp) 4 Lys, i.e., G4S tag, placed in between. Both of the recombinant plasmids were confirmed by DNA sequencing. Proteins were expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) after induction by isopropyl β-D-1-thiogalactopyranoside. The cells were then harvested by centrifugation, and suspended and disrupted by sonication. The supernatant of the cell lysate was further purified using nickel–nitrilotriacetic acid affinity chromatography under native conditions by the ÄKTA fast protein liquid chromatography system according to the manufacturer's instructions. The eluted fractions were analyzed by 4% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by XIMA MALDI-TOF (Shimadzu Kratos, Manchester, UK). Labeled recombinant proteins were prepared by conjugating with FITC at the amine groups of lysine. The labeled protein was dialyzed and filtered (0.22 μm).

2.3. Initial model building and molecular dynamics

To exhibit recombinant proteins intuitively, 3D structures of anti-EGFR and anti-EGFR-iRGD were modeled using the Accelrys Discovery Studio 4.0, based on the template of B39 VHH (PDB: 4NC2_B) [29]. Molecular dynamics simulations were conducted to refine the modeled 3D structures with AMBER 12.0. Energy minimization was performed in order to remove possible poor contacts between the solute and solvent (5000 steps for the water molecules followed by 5000 steps for the whole system). Molecular dynamics simulations were then conducted at constant temperature (300 K) and pressure (1.0 atm) with a time