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Multi-paratopic VEGF decoy receptor have superior anti-tumor effects through anti-EGFRs and targeted anti-angiogenic activities

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ABSTRACT

Limitation of current anti-Vascular Endothelial Growth Factor (VEGF) cancer therapy is transitory responses, inevitable relapses and its insufficient tumor-targeting. Thus, multifaceted approaches, including the development of bispecific antibodies and combination strategies targeting different pathways have been proposed as an alternative. Here, we developed a novel multi-paratopic VEGF decoy receptor, Cetuximab-VEGF-Grab and Trastuzumab-VEGF-Grab, by genetically fusing VEGF decoy receptor (VEGF-Grab) to a single chain Fv of anti-Epidermal Growth Factor Receptor (EGFR) antibody (Cetuximab and Trastuzumab). These multi-paratopic VEGF decoy receptor, which recognize VEGF and EGFR family (EGFR or HER2), effectively suppressed both VEGF and EGFR pathways *in vitro*, to levels similar to those of the parental VEGF-Grab and anti-EGFR antibodies. In addition, the concurrent binding of multi-paratopic VEGF decoy receptor to VEGF and EGFR family enabled their specific localization to EGFR + tumor *in vitro* and *in vivo*. Furthermore, Cetuximab-VEGF-Grab and Trastuzumab-VEGF-Grab exhibited the enhanced anti-tumor activities compared to VEGF-Grab in EGFR + tumor xenograft mouse model via anti-EGFR and the targeted anti-angiogenic activities. These results indicate that multi-paratopic VEGF decoy receptor can be a promising agent, combining tumor-targeted anti-angiogenic therapy with efficient blockade of proliferative signals mediated by EGFR family.

1. Introduction

Cancers are neoplastic diseases with potential for metastatic dissemination [1]. Normal cells gradually develop to a neoplastic state by acquiring a series of characteristics, including sustained proliferative signaling, induction of angiogenesis, resistance to cell death, evasion of growth suppression, limitless replicative potential, activation of invasion and metastasis, avoidance of immune destruction and reprogrammed energy metabolism [1]. Based on their underlying molecular mechanisms, targeted therapies have been developed that block key cellular pathways involving each of these acquired characteristics. In particular, anti- Epidermal Growth Factor Receptor (EGFR) agents that inhibit proliferative signaling and anti-angiogenic drugs targeting the Vascular Endothelial Growth Factor (VEGF) pathway have been widely utilized clinically, in combination with other anti-cancer therapies such as radiation and chemotherapy [2,3]. Some tumors, however, may be primarily resistant, or may develop resistance, to therapies targeting a specific pathway, leading to transitory responses and inevitable relapses [4]. Multifaceted approaches, including the development of bispecific antibodies and combination strategies

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targeting different pathways, may therefore be more effective and durable and help overcome tumor resistance [5,6].

We recently reported an improved soluble decoy VEGF receptor, called VEGF-Grab, consisting of the D2-D3 domain of VEGFR1 and the Fc domain of human IgG1 [7]. VEGF-Grab has shown more potent decoy activity against VEGFA and placental growth factor (PlGF) than aflibercept, with greater anti-angiogenic, anti-tumor and anti-metastatic efficacy. Moreover, optimization of the charge balance of VEGF-Grab, via three mutations in the VEGFR1 D3 domain and an additional O-glycosylation at one of those three mutation sites, has improved the biochemical properties and pharmacokinetic profiles of VEGF-Grab, as well as increasing its long-lasting anti-angiogenic efficacy, compared with aflibercept [8]. Because PlGF may be a potential target for anti-tumor and anti-angiogenic therapy [9], the increased affinity of VEGF-Grab for PlGF may enhance its anti-tumor activity. Although VEGF-Grab showed enhanced anti-angiogenic activity compared to aflibercept in mouse models of lewis lung carcinoma (LLC) tumors and spontaneous breast cancer (MMTV-PyMT), it remains possible that its insufficient tumor-targeting may give rise to adverse effects such as hemorrhage, thrombosis, and impaired wound-healing [10,11]. Therefore, further optimization of VEGF-Grab for tumor-targeting is required for better clinical outcomes.

Selection of appropriate protein targets is crucial in designing multifaceted approaches that enhance clinical anti-cancer efficacy without increasing systemic toxicity. Members of the EGFR family, consisting of EGFR, HER2, HER3, and HER4 (also called ErbB-1, ErbB-2, ErB-3, and ErB-4, respectively), have been recognized as successful clinical targets, not only because EGFR family members are highly expressed on the surfaces of various types of tumors, including gliomas, and lung, breast, colon, head and neck, ovarian and prostate tumors, but because deregulation of these members plays an important role in tumorigenesis and progression [12,13]. Clinical trials of various bispecific and multivalent antibodies have adopted anti-EGFR family antibodies as a paratope in combination with other paratopes targeting various different proteins such as other receptor tyrosine kinases (RTKs) and CD3 [5,14,15]. In this study, we fused a single chain Fv of anti-EGFR therapeutic antibody (cetuximab or trastuzumab) to the N-terminus of VEGF-Grab to yield a novel multi-paratopic-VEGF decoy receptor, called Cetuximab-VEGF-Grab (Cet-Grab) and Trastuzumab-VEGF-Grab (Tras-Grab), respectively. These engineered multi-paratopic-VEGF decoy receptors can bind simultaneously to VEGF Family (VEGFA and PlGF) and EGFR family (EGFR for Cet-Grab and HER2 for Tras-Grab) with a similar binding affinity as parental VEGF-Grab and anti-EGFR antibody (cetuximab and trastuzumab). Moreover, Cetuximab-VEGF-Grab and Trastuzumab-VEGF-Grab effectively suppressed not only VEGF signaling but EGFR family signaling *in vitro* and *in vivo*, and specifically localized to tumor areas, thus enhancing its anti-tumor efficacy in xenograft mouse model compared to VEGF-Grab.

2. Materials and methods

2.1. Cell lines and cell culture

Freestyle 293F cells (R790-07, Gibco®), A431 cells (human cervix epidermoid carcinoma, #21555, KCLB), SKBR3 cells (human breast adenocarcinoma, #30030, KCLB), SKOV3 cells (human ovarian adenocarcinoma, #30077, ATCC), and human umbilical vein endothelial cells (HUVECs, CC-2519, Lonza) were authenticated according to ATCC guidelines and used within 6 months of receipt. Freestyle 293F cells (R790-07, Gibco®) were maintained in suspension culture in Freestyle293F medium (12338018, Gibco®) at 37 °C and 8% CO2 with 125 rpm agitation. A431 cells were cultured in DMEM (LM001-05, Welgene) and SKBR3 and SKOV3 cells were cultured with RPMI1640 (LM011-05, Welgene), supplemented with 10% heat-inactivated FBS (S001-01, Welgene) and 100 μg/ml penicillin/streptomycin. HUVECs were cultured in EBM-2 (CC-3156, Lonza) supplemented with EGM-2 (CC-3162, Lonza) and penicillin/streptomycin on gelatin (G9391, Sigma-Aldrich; 2% in PBS) pre-coated plates. All cells were grown at 37 °C and 5% CO2.

2.2. Antibodies

The antibodies used in this study are listed in Supplement Table 1.

2.3. Expression and purification of recombinant proteins

Genes encoding cetuximab or trastuzumab single chain variable fragment (scFv), in which the variable regions of cetuximab or trastuzumab's heavy and light chains were connected by (G4S) 3 linker [16–18], were cloned into the N-termini of VEGF-Grab3 construct [7] (See also Supplement Methods, Fig. 1A and Supplement Fig. 1). Using polyethylenimine (765090, Sigma-Aldrich), constructs encoding VEGF-Grab, scFv-Cetuximab-VEGF-Grab3 (Cet-Grab), and scFv-Trastuzumab-VEGF-Grab3 (Tras-Grab) were transfected into Freestyle293F cells grown in suspension. The cells were cultured for 3 days in the presence of 5 mM sodium butyrate (303410, Sigma-Aldrich) and harvested by centrifugation. Supernatants containing VEGF-Grab, Cet-Grab, or Tras-Grab were loaded onto Protein A Sepharose (GE Healthcare Life Sciences). VEGF-Grab, Cet-Grab, or Tras-Grab was eluted with 200 mM glycine, pH 2.7, neutralized immediately with 1 M Tris-HCl (pH 8.0) and dialyzed against PBS. Protein identification of the multi-paratopic-VEGF decoy receptor (Cet-Grab or Tras-Grab) was performed by high-resolution mass spectrometry. Briefly, tryptic peptides obtained from the protein with proteolytic enzyme treatment were separated on a C18 column and subsequently analyzed using high resolution TOF MS (MS/MS). The amino acid sequences of fusion proteins were identified by peptide mapping using accurate mass value (<20 ppm) of tryptic peptides.

2.4. Binding affinity analysis

The binding affinities of multi-paratopic-VEGF decoy receptor (Cet-Grab or Tras-Grab) to EGFR family extracellular domain (EGFR for Cet-Grab and HER2 for Tras-Grab), VEGFA, or PlGF were measured using biolayer light interferometry with BLITZ system (ForteBio, Pall Life Sciences). Biotinylated EGFR family ECD (EGFR or HER2), VEGFA, or PlGF was loaded onto hydrated streptavidin (SA) biosensors (1805020, Forte Bio) for 2 min, followed by washing of the loaded biosensors for 2-min with PBS to remove any unbound protein. These biosensor tips were immersed in 4 μl drops containing 25–50 nM indicated proteins (VEGF-Grab, Cet-Grab, cetuximab, Tras-Grab or trastuzumab), and association (on) rates (kon) were measured over a 2-min interval. The sensors were subsequently immersed in PBS buffer for 2 min to measure dissociation (off) rates (koff). K_D was calculated as the ratio of off-rate to on-rate (koff/kon). The sensorgrams were fit with the BLITZ Pro 1.1 software using a 1:1 binding model with the global fitting function (grouped by color, Rmax). For simultaneous binding analysis, biotinylated VEGF family (VEGFA or PlGF) was loaded onto SA biosensors for 90 s, and the VEGF pre-loaded biosensors were immersed in 4 μl drops containing 100 nM multi-paratopic-VEGF decoy receptor (Cet-Grab or Tras-Grab) for 90 s and subsequently in 25–50 nM EGFR family ECD (EGFR for Cet-Grab and HER2 for Tras-Grab) for 120 s to measure association (on) rates (kon), followed by a 2-min washing interval with PBS to measure