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High Affinity Promotes Internalization of Engineered Antibodies Targeting FGFR1

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Abstract: Fibroblast growth factor receptor 1 (FGFR1) is a plasma membrane protein that transmits signals from the extracellular environment, regulating cell homeostasis and function. Dysregulation of FGFR1 leads to the development of human cancers and noncancerous diseases. Numerous tumors overproduce FGFR1, making this receptor a perspective target for cancer therapies. Antibody-drug conjugates (ADCs) are highly potent and selective anticancer agents. ADCs are composed of antibodies (targeting factors) fused to highly cytotoxic drugs (warheads). The efficiency of ADC strategy largely depends on the internalization of cytotoxic conjugate into cancer cells. Here, we have studied an interplay between affinity of anti-FGFR1 antibodies and efficiency of their cellular uptake. We have developed a unique set of engineered anti-FGFR1 antibodies that bind the same epitope in the extracellular part of FGFR1, but with different affinities. We have demonstrated that these antibodies are effectively taken up by cancer cells in the FGFR1-dependent manner. Interestingly, we have found that efficiency, defined as rate and level of antibody internalization, largely depends on the affinity of engineered antibodies towards FGFR1, as high affinity antibody displays fastest internalization kinetics. Our data may facilitate design of therapeutically relevant targeting molecules for selective treatment of FGFR1 overproducing cancers.

Keywords: affinity; cancer therapy; engineered antibodies; FGFR1; internalization

1. Introduction

Cancer is one of the top causes of mortality worldwide. Currently, nearly one in six deaths is due to cancer and it is expected that the number of new cases will rise by 70% in the coming two decades [1]. Traditional anti-cancer therapies usually aim for inhibition of high proliferative capacity of cancer cells. However, most of the targeted pathways are also critical for maintenance of normal cells, thus giving rise to numerous side effects of conventional anti-cancer drugs. In recent years, engineered monoclonal antibodies and antibody fragments have attracted attention as molecules that may ensure specificity of the cancer treatment [2]. Such antibodies inactivate the specific receptor on cancer cells, resulting in induction of apoptosis, or lead to cancer cell death by stimulation of the immune system of the patient [2]. Alternatively, engineered antibodies may be physically linked to highly potent cytotoxic drugs in the antibody-drug conjugates (ADCs). In the ADC approach, antigen-positive cancer cells are recognized by the antibody part of the ADCs. Next, ADCs bound to the cell surface antigen are internalized, utilizing one of cellular endocytic routes. Subsequently, ADCs traffic via cellular vesicular

compartments to their final lysosomal destination, where proteolytic degradation releases cytotoxic drugs from the ADCs. Drug moiety diffuses out from lysosomes and binds its intracellular target causing cell death [3,4]. Therefore, the effectiveness of ADC therapy depends on the selectivity and strength of antigen binding, tumor penetration and on the efficiency of ADCs internalization from the cell surface [5–9].

The fibroblast growth factor receptors comprise a group of four conserved receptor tyrosine kinases (FGFR1-FGFR4) that, in conjunction with extracellular fibroblast growth factors (FGFs), transmit signals across the plasma membrane. Binding of FGFs to FGFRs (fibroblast growth factor receptors) leads to the activation of the receptor cytoplasmic tyrosine kinase domain that recruits numerous signaling molecules further propagating the signal [10,11]. The FGFR-dependent signaling cascades govern cell metabolism, proliferation, and apoptosis and are critical for angiogenesis, organogenesis, and wound healing [12]. The aberrations in the FGFRs such as gene amplification, rearrangements, and somatic mutations are often observed in cancer and can be found in over 7% of all tumors [13].

FGFR1 is an attractive target for selective chemotherapy in ADC, as it is localized on the cell surface, thus being easily accessible to extracellular targeting molecules [10,11]. In numerous cancer cell types, the level of FGFR1 is elevated in comparison to the normal cells that may ensure selectivity of drug targeting [14]. Moreover, FGFR1 is rapidly internalized, mainly via clathrin-mediated endocytosis, providing the intracellular release of the drug after lysosomal degradation of ADC inside cancer cells [15]. The requirements for the design of highly internalizing antibodies against FGFR1 suitable as an ADC's carrier are still largely undefined. We have recently developed novel antibody fragment scFvD2-Fc and have demonstrated that bivalency of scFvD2-Fc promotes internalization of this anti-FGFR1 engineered antibody by inducing receptor dimerization [16,17]. Here, we have assessed the importance of engineered antibodies affinities towards FGFR1 for their internalization. Using the phage display approach, we have selected two novel FGFR1-specific antibodies that bind to the same epitope within extracellular part of FGFR1 as scFvD2-Fc, but with different affinities. We have demonstrated that all these engineered antibodies are efficiently internalized via receptor-mediated endocytosis and are delivered through endosomes to lysosomes. Interestingly, our data show that an antibody with the highest affinity to FGFR1 displays the fastest internalization rate. Taken together, our data may facilitate the effective design of highly internalizing engineered antibodies suitable for ADC strategy of cancer treatment.

2. Results

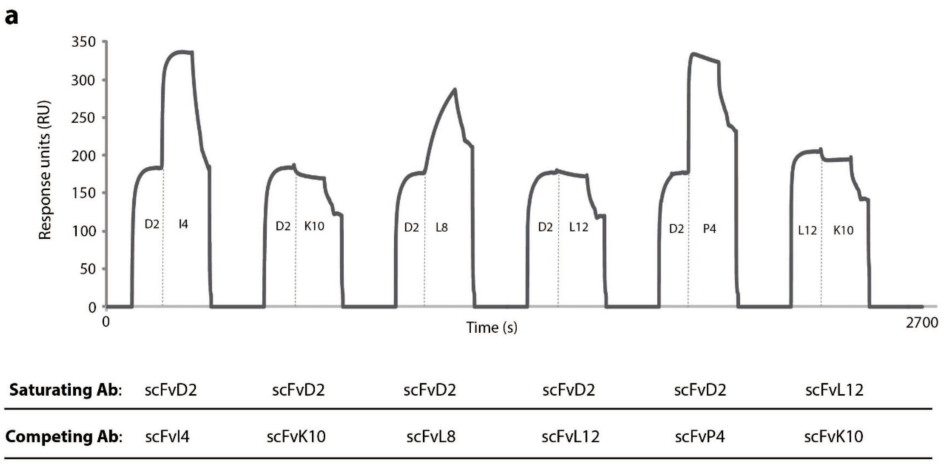
2.1. Engineered Antibody Fragments Recognize the Same Epitope within D1 Domain of the FGFR1

To select the panel of antibody fragments that specifically recognize FGFR1, we employed the phage display technique using Tomlinson I and Tomlinson J libraries and the extracellular part of FGFR1 (composed of D1, D2, and D3 domains) fused to the Fc fragment (FGFR1.D1-D2-D2-Fc) as an antigen [18]. For each library, we performed three consecutive rounds of selection. We obtained four novel scFv proteins that interact with the extracellular region of FGFR1: scFvK10, scFvL8, scFvL12 and scFvP4.

Our group has recently selected and characterized scFvD2 as a high affinity FGFR1 interactor [16]. To obtain panel of scFv fragments that bind to the same region of the extracellular part of FGFR1 as scFvD2 we employed epitope binning using surface plasmon resonance (SPR). We intended to select scFv proteins whose interaction with FGFR1-D1-D2-D3-Fc was blocked by the saturating concentrations of scFvD2. To this end, sensors with immobilized FGFR1-D1-D2-D3-Fc were incubated first with high concentration (1 μM) of scFvD2 and then with novel anti-FGFR1 scFv proteins at the same concentration. We observed that binding of scFvD2 to the extracellular part of FGFR1 inhibited receptor interaction with scFvK10 and scFvL12 (Figure 1a). Moreover, the saturation of FGFR1-D1-D2-D3-Fc with scFvL12 blocked subsequent binding of scFvK10, suggesting that these three

antibody fragments compete for the same binding site on FGFR1 (Figure 1a). To identify the epitope recognized by these three scFv proteins, we performed SPR binding studies with sensor-immobilized full length extracellular part of FGFR1 (FGFR1.D1-D2-D3-Fc) and truncated receptor variant lacking D1 domain (FGFR1.D2-D3-Fc). The lack of the D1 domain abolished the interaction of scFvD2, scFvK10, and scFvL12 with immobilized FGFR1 fragment, indicating that these proteins recognize epitope within the D1 domain of the receptor (Figure 1b). We confirmed this finding, showing that all three engineered antibodies bind to sensor-immobilized purified D1 domain fused to GST (GST-FGFR1.D1$_{25–124}$) (Figure 1b).

To validate our experiments, we used FGF1, a natural FGFR1 ligand that recognizes binding site formed by D2 and D3 domains of the receptor. As expected, FGF1 interacted with FGFR1.D1-D2-D3-Fc and FGFR1.D2-D3-Fc, but did not bind to GST-FGFR1.D1$_{25–124}$ (Figure 1b). To identify the precise epitope within the D1 domain recognized by scFv proteins, we prepared a set of C-terminal truncations of D1 domain fused to glutathione S-transferase GST (Figure 1c). Next, we performed pull down experiments with purified GST-tagged D1 domain truncations and GST as a control. We found that scFvD2, scFvK10, and scFvL12 bound to the full length D1 domain (GST-FGFR1.D1$_{25–124}$) and to the D1 truncation composed of residues 25–76 (GST-FGFR1.D1$_{25–76}$). The interaction was not observed when only N-terminal peptide, containing residues 25–40, was applied (GST-FGFR1.D1$_{25–40}$) (Figure 1c). These data demonstrate that engineered anti-FGFR1 antibodies recognize epitope that is located within residues 41–76 of the D1 domain of FGFR1.



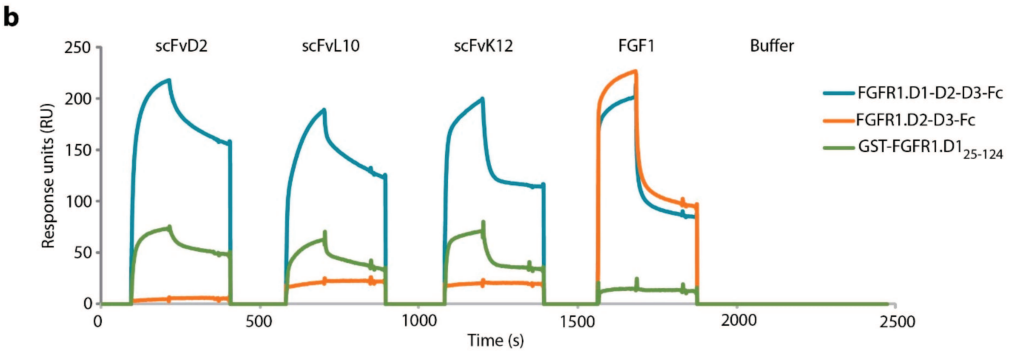


Figure 1. *Cont.*

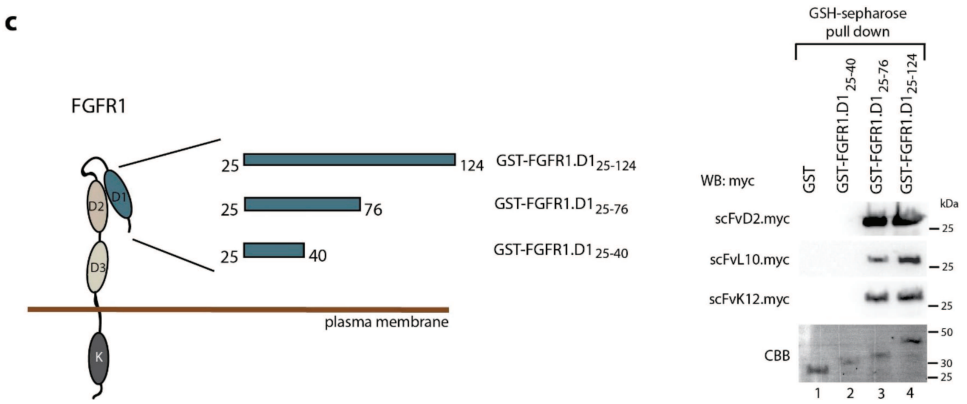


Figure 1. Engineered antibodies recognize the same epitope within the extracellular region of FGFR1. (**a**) SPR-based epitope binning. FGFR1.D1-D2-D3-Fc was immobilized on sensors and incubated with high concentrations of saturating antibodies. Next, competing antibodies were injected to assess if their binding to the extracellular region of FGFR1 is blocked by saturating antibodies. (**b**) SPR analysis of the interaction of scFv proteins with FGFR1 truncations. CM5 sensors were coated with FGFR1.D1-D2-D3-Fc, FGFR1.D2-D3-Fc, or GST-FGFR1-D1$_{25–124}$, and the association and dissociation were monitored for 240 s after injection of scFv proteins (1 μM), or FGF1 (1 μM). (**c**) Pull down experiments with scFv proteins and truncated variants of the D1 domain of FGFR1. GST and GST-tagged truncated versions of the D1 domain were bound to Glutathione Sepharose and incubated with scFv proteins. After extensive washing, bound proteins were eluted and analyzed by Western blotting. Membranes were first stained with CBB to visualize eluted proteins and then detected with anti-c-myc antibodies (for visualization of scFv proteins containing C-teminal c-myc).

Next, we assessed specificity of scFvD2, scFvK10, and scFvL12 towards FGFR1 using SPR. Using extracellular regions of all four FGF receptors (FGFR1-4) immobilized on CM5 sensors, we observed binding of scFvD2, scFvK10, and scFvL12 only to FGFR1 (Figure 2). The high selectivity of scFv-Fc proteins towards FGFR1 can be attributed to the very low homology of FGF receptors within residues 41–76 (13.9% identity, while sequence identity in the full extracellular region of FGF receptor is 36.7%) (Figure S1). To confirm that immobilized fragments of FGF receptors were functional, we employed developed in our group scFvF7 that recognizes FGFR2 [19] as well as commercial anti-FGFR3 and anti-FGFR4 antibodies as controls (Figure 2 and Figure S2).

These results demonstrate that three independently selected scFv proteins display high specificity towards FGFR1 and recognize the same epitope (residues 40–76) within the D1 domain of FGFR1.

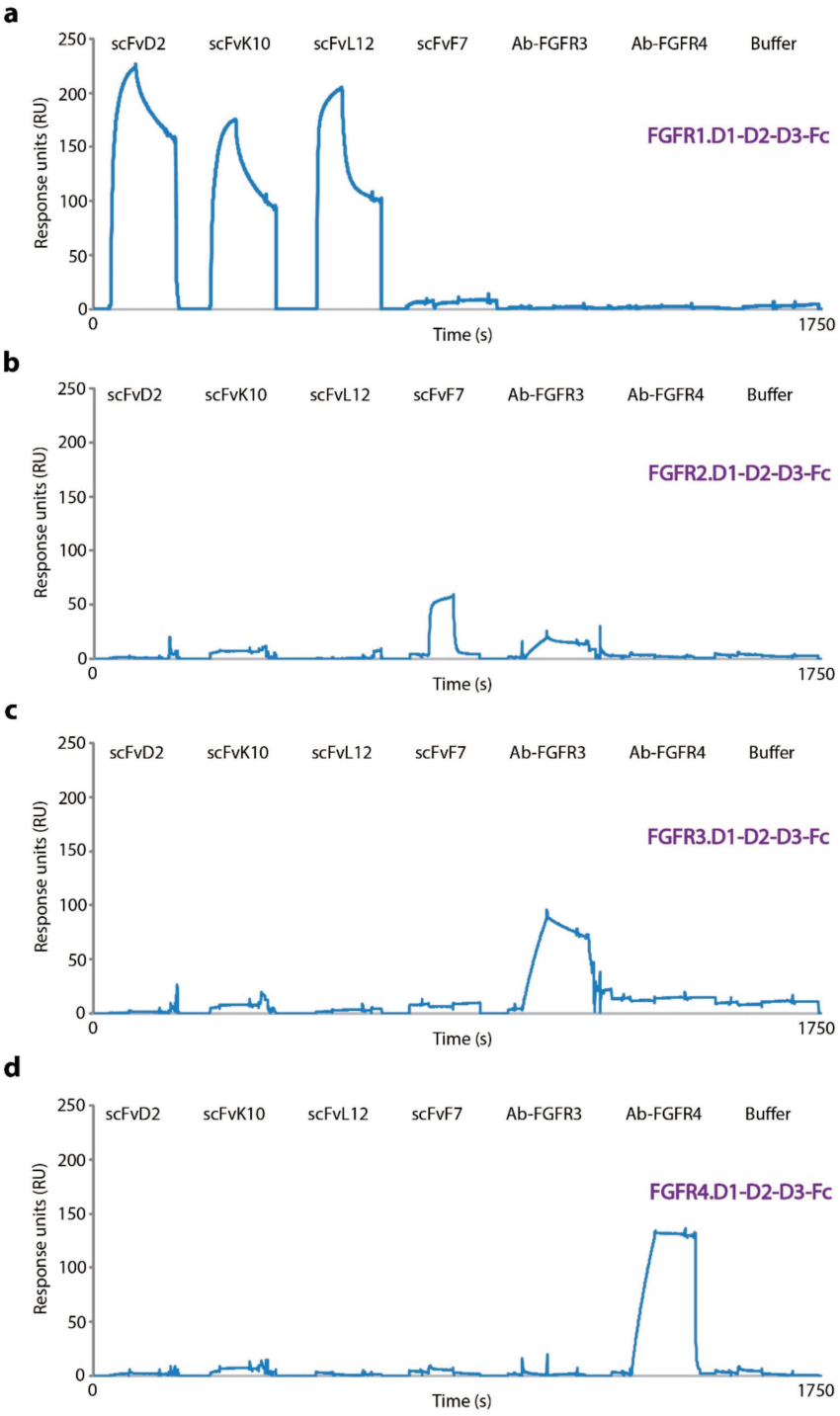


Figure 2. Specificity of scFv proteins towards FGFR1. Extracellular regions of FGFR1 (FGFR1.D1-D2-D3-Fc (**a**); FGFR2 (FGFR2.D1-D2-D3-Fc (**b**); FGFR3 (FGFR3.D1-D2-D3-Fc (**c**); and FGFR4 (FGFR4.D1-D2-D3-Fc (**d**); were immobilized on CM5 sensors and incubated with scFvD2, scFvL10, scFvK12, scFvF7 (specific towards FGFR2), and commercial anti-FGFR3 and anti-FGFR4 antibodies.

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