**Antiangiogenic Activity of a Neutralizing Human Single-Chain Antibody Fragment against Fibroblast Growth Factor Receptor 1**

Roberto Ronca, Patrizia Benzoni, Daria Leali, Chiara Urbinati, Mirella Belleri, Michela Corsini, Patrizia Alessi, Daniela Coltrini, Stefano Calza, Marco Presta, and Patrizia Dell’Era

**Abstract**

Fibroblast growth factor receptor-1 (FGFR-1) transduces proangiogenic and proliferative signals in human cancers. Thus, FGFR-1 may represent a target for the development of antiangiogenic/antineoplastic therapies. We screened a human single-chain fragment variable (scFv) antibody phage display library against the extracellular domain of the FGFR-1-IIIc isoform that harbors the FGF binding site. Several phages were isolated and tested for specificity and sensitivity, and the most promising antibody fragment RR-C2 was characterized for its biochemical and biological properties. ScFv RR-C2 specifically recognizes FGFR-1α and FGFR-1β isoforms in ELISA, Western blotting, and surface plasmon resonance analysis with a $K_d$ value of 300 and 144 nmol/L for the 2 receptor isoforms, respectively. The antibody fragment also recognizes FGFR-1 when the receptor is exposed on the cell surface, thus preventing the formation of the ternary complex among FGFR-1, its ligand FGF2, and cell surface heparan sulfate proteoglycans. Accordingly, scFv RR-C2 specifically inhibits FGF2-mediated mitogenic activity in endothelial cells of human, bovine, and murine origin in a nanomolar range of concentrations. Also, the antibody fragment prevents FGF2-triggered sprouting of both human umbilical vein endothelial cell spheroids and of murine endothelium from aortic rings. Finally, the antibody fragment hampers the angiogenic activity exerted both by FGF2 in the chick embryo chorioallantoic membrane assay and by S115 mouse mammary tumor cells in the Matrigel plug assay. Taken together, the data show that scFv RR-C2 recognizes and neutralizes FGFR-1 activity in different animal species, including humans, thus representing a novel tool for the development of antiangiogenic/antineoplastic therapies.

**Introduction**

Angiogenesis, the growth of new capillary blood vessels from preexisting vasculature, is a fundamental process required for embryogenesis, tissue growth, and repair. Also, neovascularization may contribute to pathologic conditions such as psoriasis, diabetic retinopathy, and cancer when hypoxic, injured, or diseased tissues produce and release angiogenic inducers (1). They include members of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families, as well as several other growth factors and cytokines (2).

Given the importance of neovascularization in tumor growth and metastatic process, massive efforts have been undertaken to develop antiangiogenic strategies and the VEGF/VEGFR receptor (VEGFR) system has been extensively investigated as a target for antineoplastic therapy (3). Even though inhibition of the VEGF/VEGFR system markedly disrupts angiogenic switching and initial tumor growth, phenotypic resistance may emerge in late-stage lesions, as tumors regrow during treatment after an initial period of growth suppression. A mechanism of escaping anti-VEGF therapies is represented by the compensatory upregulation of the FGF/FGFR system, as observed in experimental tumor models (4) and cancer patients (5).

FGFs represent a family of 22 structurally related polypeptide growth factors that promote a wide range of biological effects (6, 7). Among them, FGF1 and FGF2 were the first identified angiogenic factors (8, 9). They promote the proliferation, migration, differentiation, and tubulogenesis of endothelial cells in culture and are potent stimulators of angiogenesis in vivo (10). FGFs mediate their biological responses by binding to cell surface high-affinity tyrosine kinase (TK) FGFRs, designated FGFR-1 to FGFR-4 (11). FGFRs are composed...
of an extracellular portion consisting of 3 immunoglobulin (Ig)-like domains (D1, D2, and D3), a hydrophobic transmembrane region, and a cytoplasmic TK tail. The ligand-binding site for FGFs is located in the Ig-like domains D2–D3 and the linker that connects them (12). Ligand specificity is achieved primarily through splicing events in which the alternative exons IIIb and IIIc encode the carboxyl terminal portion of the third Ig-like loop. Indeed, alternative splicing of FGFR-1 results in isoforms FGFR-1-IIIb and FGFR-1-IIIc with distinct FGF binding characteristics: FGFR-1-IIIb binds efficiently to FGF1, FGF3, and FGF10, whereas FGFR-1-IIIc binds to FGF1, FGF2, FGF4, FGF6, FGF8, and FGF9 (13). A variety of other alternative spliced receptor molecules have been described, including the β isomers that lack the first Ig-like domain whereas the α isoforms identify the full-length receptors (14).

FGFR-1 is expressed by endothelial cells in vitro (10) and in vivo (15) whereas FGFR-2 is less frequently expressed by cultured endothelium (16), and the expression of FGFR-3 or FGFR-4 has never been reported in endothelial cells. Transduction of a dominant-negative FGFR-1 form in the retina of Tryp1-Tag mice that develop early vascularized tumors of the retinal pigment epithelium results in a significant decrease in tumor burden and vascularity (17). Also, FGFR-1 vaccination has been shown to inhibit the growth and neovascularization of different experimental tumors (18). Thus, experimental evidence identifies FGFR-1 as an interesting target for the development of antiangiogenic strategies in cancer.

Humanized antibodies in single-chain fragment variable (scFv) format are antibody fragments composed of Vh and VL domains joined with a flexible polypeptide linker that prevents their dissociation. In this configuration, scFv retains the specific, monovalent, antigen-binding affinity of an entire IgG while showing improved pharmacokinetics for tissue penetration (19). Moreover, scFv fragments can be easily conjugated, allowing their usage as cytokine, radionuclide, or drug carriers (20).

Here, we describe the selection from the human antibody phage display library ETH2-Gold (21) of a novel neutralizing recombinant scFv antibody specific for the extracellular domain of the FGFR-1-IIIc isoform. When challenged in different in vitro, ex vitro, and in vivo assays, this anti-FGFR-1 scFv fragment specifically inhibits FGF-induced angiogenesis, thus suggesting its potential use as a novel tool for antiangiogenic therapies.

**Materials and Methods**

**Recombinant proteins and cell lines**

Recombinant proteins FGFR-1β (IIIc)/Fc (FGFR-1β), FGFR-2α (IIIb)/Fc (FGFR-2), FGFR-3 (IIIb)/Fc (FGFR-3), and FGFR-4 (IIIb)/Fc (FGFR-4) were from R&D Systems, Inc. sFGFR-1α(IIIc) /Fc (FGFR-1α), sVEGFR-2/Fc, and sVEGFR-1/Fc (Flt1) were from ReliaTech. Human recombinant FGF2 was provided by Tecnenog. Fetal bovine aortic endothelial GM7373 cells (22) were obtained from and cultured as recommended by the NICOM Human Genetic Mutant Cell Repository. Immortalized Balb/c murine brain endothelial cells (MBECs) were obtained from R. Auerbach (University of Wisconsin, Madison, WI) and grown in Dulbecco’s modified minimal essential medium (DMEM; Gibco Life Technologies) supplemented with 10% fetal calf serum (FCS; Gibco Life Technologies). Human umbilical vein endothelial cells (HUVEC) were grown in M199 medium (Gibco Life Technologies) supplemented with 20% FCS, endothelial cell growth factor (100 μg/mL; Sigma-Aldrich) and porcine heparin (100 μg/mL, Sigma-Aldrich). HUVECs were used at early passages (I–IV) and grown on plastic surface coated with porcine gelatin (Sigma-Aldrich). Wild-type CHO-K1 and A745-CHO/flg-1A cells (23) were grown in Ham’s F-12 medium supplemented with 10% FCS. Tramp C2-R1 cells were grown in DMEM supplemented with 50 mmol/L of β-mercaptoethanol, 2.0 mmol/L of l-glutamine, 5 μg/mL of bovine insulin (Sigma-Aldrich), 10−8 mol/L of dihydrotestosterone (DHT; Sigma-Aldrich), and 10% FCS. They were obtained by transducing murine prostate tumor Tramp C2 cells (24) with the 3 Ig-like loop isoform IIIc of human FGFR-1 cDNA (25). S115 mouse mammary carcinoma cells (26) were kindly provided by M. Jalkanen (Biotie) and maintained in DMEM supplemented with 5% heat-inactivated FCS, 1 mmol/L of sodium pyruvate, 1 mmol/L of glutamine, and 10 mmol/L of DHT. For all the cell lines, no authentication was done by the authors.

**Selection, production, and purification of anti-FGFR-1 scFv antibodies**

ETH2-Gold human antibody phage display library (21) was obtained from Phigenen. The selection protocol was essentially the same as described by Viti and colleagues (27). Briefly, immunosorb wells (ThermoFisher Scientific) were coated with antigen at a concentration of 250 ng per well in PBS, overnight at room temperature. Wells were then rinsed with PBS and blocked for 2 hours at room temperature with 2% (w/v) skimmed milk in PBS (MPBS). After rinsing with PBS, 1012 phage particles in 2% MPBS were added to the wells and incubated for 2 hours at room temperature. Unbound phages were washed away by rinsing the immunowells 10 times with PBS/0.1% Tween 20 and 10 times with PBS. The bound phages were eluted in 1 mL of triethylamine (100 mmol/L), pH 7.4. The eluted phages were used for the infection of exponentially growing Escherichia coli TG1.

The purification of scFv antibody fragments from selected bacterial clones was produced by inoculating a single fresh colony in 20 mL of 2xYT medium, 100 μg/mL of ampicillin, and 5% glucose. This preculture was grown overnight at 37 °C to give an OD600 nm = 0.9; diluted 1:100
in 2xYT medium, 100 µg/mL of ampicillin, and 0.1% glucose; and then regrown at 37°C to give OD600 max = 0.5. The cells were then induced by the addition of Isopropyl-
beta-D-1-thiogalactopyranoside (IPTG) (final concentration 1.0 mmol/L) and grown overnight at 37°C. The scFv fragments were purified from the bacterial supernatant by affinity chromatography using Protein A Sepharose resin (GE Healthcare) according to the manufacturer’s instructions.

For endotoxin removal, Triton X-114 was added to the protein preparation to a final concentration of 1%. The mixture was incubated at 4°C for 30 minutes with constant agitation. The sample was then transferred to 37°C for 10 minutes and centrifuged at 16,000 × g for 30 minutes at room temperature. The upper aqueous phase containing the protein was carefully removed and subjected to Triton X-114 phase separation for 2 more cycles (28). The resulting aqueous phase was assayed for endotoxin content by using PYROGENT Plus Single Test assay (Lonza).

ELISA, immunoprecipitation, and Western blot protocols are described in Supplementary Methods.

**Surface plasmon resonance**

Purified scFv binding properties were analyzed by surface plasmon resonance (SPR) with a Biacore X apparatus (BIAcore; GE Healthcare). Antigen-coated sensor chips were prepared according to manufacturer’s instructions by injecting the different recombinant receptors (FGFR-1α, FGFR-1β, FGFR-2, FGFR-3, and FGFR-4) all at 100 ng/µL onto CM5 sensor chips, allowing the immobilization of approximately 5,000 RU for each molecule. VEGFR-2 was immobilized (approximately 10,000 RU) on the second cell of each sensor chip, allowing its use as a negative control and for blank subtraction.

Sensorgrams for kinetic measurements were generated by the injection of soluble scFv antibodies at concentrations ranging from 20 to 1,200 nmol/L in HBS-EP buffer (BIAcore AB 10 mmol/L of HEPES, 150 mmol/L of NaCl, 3.0 mmol/L of EDTA, and 0.005% Tween-20) on the 2 cells of the sensor chip. Binding parameters were calculated by the nonlinear curve-fitting software package BIAevaluation 3.2 (GE Healthcare), using a single-site model with a drifting baseline.

**Murine aorta ring assay**

The assay was performed as described previously with minor modifications (29). Briefly, aortic rings were obtained by cross-sectioning the thoracic aorta of 2-month-old C57BL/6 female mice at 1-mm intervals. Rings were placed individually on the bottom of 4-well plates, with the luminal axis lying parallel to the bottom of the plate. Next, 30 µL of polymerizing fibrin solution was applied onto each ring. After 5 minutes, wells were filled with 600 µL of serum-free endothelial cell basal medium (Clonetics), 30 ng/mL of FGF2, and 10 µg/mL of aprotinin in the absence or presence of scFv antibody. Medium and stimuli were replaced every even day. After 6 days, the angiogenic response was measured under an inverted microscope by counting the number of neovessels sprouting out of the ring (6–9 rings per experimental point). Endothelial cell sprouts formed by more than a single cell were morphologically distinguishable from scattering fibroblasts/myofibroblasts by their thicker appearance and cohesive pattern of growth. Each branch was scored as an additional sprout (29).

**Chick embryo chorioallantoic membrane assay**

The basic procedures of this method, the angiogenic response observation and blood vessel counting, were performed as described in Ribatti and colleagues (30). Gelatin sponges (Gelfoam; Pfizer) containing vehicle or FGF2 (150 ng per embryo) in the absence or presence of 3.0 µg of RR-C2 scFv were placed on the chick embryo chorioallantoic membrane (CAM) of fertilized white leghorn chicken eggs at day 11 of incubation (31). Antibody treatment was repeated 30 hours later. At day 14, newly formed blood microvessels converging toward the implant were counted at magnification ×5, using a STEMI SR stereomicroscope equipped with an objective f equal to 100 mm with adapter ring 475070 (Zeiss Inc.), by 2 observers in a double-blind fashion, as described in detail by Ribatti and colleagues (30).

**Treatment with scFv RR-C2 and in vivo Matrigel plug assay**

The Matrigel plug assay was performed as described (32). Briefly, C57BL/6 male mice (8–10 weeks) were injected intravenously with scFv RR-C2 (100 µg per mouse) or vehicle. After 24 hours, unpolymerized Matrigel (Trevigen) was mixed with 0.3 × 10⁶ S115 cells alone or in combination with scFv RR-C2 (25 µg/mL) or PBS to a final volume of 400 µL and injected s.c. into each flank of 4 animals per experimental group. Antibody intravenous administration was repeated on day 3, and animals were sacrificed on day 7. Then, Matrigel plugs were collected and divided into 2 parts. One half was embedded in Tissue Tec OCT (Sigma-Aldrich), snap-frozen by immersion in liquid nitrogen–cooled isopentane, and analyzed by immunofluorescence. The other half was processed for RNA extraction and quantitative real-time PCR (qPCR) analysis as described in Supplementary Methods.

**Immunofluorescence analysis of Matrigel plugs**

Frozen sections (8 µm) were fixed in cooled acetone (10 minutes), air dried, and washed 3 times with Tris-buffered saline (TBS). After blocking with 10% goat serum in TBS, sections were incubated with a rat IgG2a anti-mouse CD31 monoclonal antibody (1:100 dilution; BD Pharmingen), followed by incubation with a biotinylated mouse anti-rat IgG1/2a antibody (1:100 dilution; BD Pharmingen) and Texas red avidin (1:800 dilution; Vector Laboratories, Inc.). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were taken using a fluorescence microscope.
microscope (Zeiss Inc.) equipped with an Olympus N547 digital camera (Olympus) at magnification ×200.

**Results**

**Selection of an FGFR-1–specific scFv antibody (RR-C2)**

To isolate a neutralizing humanized scFv antibody directed against the FGF binding site of FGFR-1, the phage display library ETH2-Gold (21) was incubated for the first 2 rounds of selection with the immobilized extracellular portion of the human 3 Ig-like loop FGFR-1 protein, isofrom IIIC, linked to the Fc fragment of human IgG (FGFR-1α). To avoid the isolation of antibodies that recognize the Fc portion of the recombinant protein, all the incubations were carried out in the presence of an excess of human IgG. The third round of selection was instead performed against the 2 Ig-like loop FGFR-1β isoform to enrich the phage population in antibodies that recognize the FGF binding site of the receptor. As anticipated, phage titers increased through the 3 selection steps, up to 1.5 × 10⁸ colony-forming units. Phages eluted from the final challenge were used to infect *E. coli* TG1 bacteria that were then plated to form single colonies. Next, 192 independent colonies were randomly picked, grown, and induced with IPTG. Colony supernatants containing soluble scFv fragments were tested for their capacity to recognize immobilized FGFR-1β in ELISA. Four independent clones were selected for their significant FGFR-1β binding capacity, and scFv fragments were subjected to a small-scale purification. Then, equal amounts of the 4 purified scFv fragments were assayed for their capacity to specifically recognize both FGFR-1α and FGFR-1β in ELISA (Fig. 1A). Because of its higher specific reactivity, the scFv RR-C2 antibody was chosen among the 4 clones for further characterization experiments. Production was scaled up and the scFv RR-C2 antibody was purified on Protein A Sepharose column (molecular weight = 27 kDa, purity >90%). The yield of scFv RR-C2 was equal to approximately 900 μg/L of bacterial culture with 80% or greater of the purified antibody being in a monomeric form, as assessed by gel filtration chromatography (data not shown).

**Characterization of the scFv RR-C2 antibody**

Purified scFv RR-C2 was tested in different biochemical assays to assess its capacity to specifically recognize the II and III Ig-like loop regions of FGFR-1 and to discriminate among the different FGFRs. As shown in Fig. 1B, RR-C2 scFv efficiently recognizes both FGFR-1α and FGFR-1β isoforms in a Western blot. At variance, no cross-reactivity was observed for the 3 Ig-like loop IIIc isofrom of the other FGFR family members R2, R3, and R4 and the unrelated receptor VEGFR1/FH1, all of them sharing the same human Fc fragment.

Similar results were obtained when the native recombinant receptor proteins were tested by the SPR analysis. In keeping with the Western blotting data, scFv RR-C2 specifically recognized sensor chip-immobilized FGFR-1α and FGFR-1β isoforms in SPR (Fig. 1C) whereas no significant binding was observed for immobilized VEGFR2 (Fig. 1C) or for the other FGFRs (data not shown). Also, the binding of the antibody to immobilized FGFR-1β was competed by both free FGFR-1α and FGFR-1β (Fig. 1D). Finally, SPR was exploited to measure the affinity of the interaction between the 2 FGFR-1 isoforms and scFv RR-C2. To this purpose, dose–response curves were generated by the injection of soluble scFv at concentrations ranging from 20 to 1,200 nmol/L on the FGFR-1α and FGFR-1β sensor chips (Supplementary Fig. S1). Dissociation constants ($K_{\text{d}}$) for FGFR-1α and FGFR-1β were equal to 300 nmol/L ($K_{\text{on}} = 3.09 \times 10^4$; $K_{\text{doff}} = 9.35 \times 10^{-7}$) and 144 nmol/L ($K_{\text{on}} = 3.35 \times 10^4$; $K_{\text{doff}} = 4.81 \times 10^{-5}$), respectively.

Next, we tested the capacity of scFv RR-C2 antibody to recognize the unmodified FGFR-1 in a cellular context. To this purpose, TRAMP-C2 cells overexpressing human FGFR-1α were lysed and immunoprecipitated with scFv RR-C2 or with an irrelevant scFv antibody (a-scFv). Next, immunoprecipitates were subjected to Western blot analysis by using a commercial rabbit polyclonal antibody directed against the intracellular domain of FGFR-1. As anticipated, scFv RR-C2 specifically immunoprecipitates the transduced 130-kDa FGFR-1 recognized by the commercial antibody in the Western blot (Fig. 1E).

FGFs exert their biological activity by leading to the formation of a productive ternary complex by binding both FGFRs and heparan sulfate proteoglycans (HSPG) (33). RR-C2 scFv antibody has been isolated against a region of FGFR-1 that comprises the FGF-binding site, raising the possibility that this antibody may effectively neutralize FGF/FGFR-1 interaction. On this basis, scFv RR-C2 was evaluated for the capacity to prevent the formation of HSPG/FGF2/FGFR-1 ternary complexes. To this purpose, we used a cell–cell adhesion model in which FGF2 mediates the interaction of HSPG-deficient CHO cells stably transfected with the murine 2 Ig-like loop FGFR-1/flg (34), which shows 98% amino acid identity with human FGFR-1β, to a monolayer of CHO-K1 cells expressing HSPGs but not FGFRs (35). The results shown in Fig. 1F show the capacity of scFv RR-C2 to inhibit the formation of this HSPG/FGF2/FGFR-1 ternary complex whereas a-scFv was ineffective. In conclusion, the scFv RR-C2 antibody specifically recognizes the recombinant extracellular domain of FGFR-1, isofrom IIIC, *in vitro* and on the cell surface, thus preventing the interaction of the receptor with its own ligand.

**In vitro antiangiogenic activity of scFv RR-C2**

To assess the capacity of scFv RR-C2 antibody to inhibit the angiogenic response triggered by the FGFR-1 ligand FG2 on endothelial cells in culture, fetal bovine aortic endothelial GM7373 cells were stimulated with the growth factor in the presence of increasing concentrations of scFv RR-C2. Bovine FGFR-1, like murine FGFR-1, has a...
98% amino acid identity with the human counterpart, reaching 100% homology in the D2–D3 domain. As shown in Fig. 2A, scFv RR-C2 neutralizes FGF2-induced cell proliferation in a dose-dependent manner whereas the heat-denatured antibody or control a-scFv was ineffective. Accordingly, scFv RR-C2 exerted a similar inhibitory activity on FGF2-stimulated endothelial cells of murine (MBEC) and human (HUVEC) origin (Fig. 2B). No inhibition of cell proliferation was instead exerted by the antibody fragment when GM7373 cells (Fig. 2B) or MBECs (Supplementary Fig. S2) were stimulated with VEGF-A, EGF, the phorbol-12-myristate-13-acetate (PMA), or 10% FCS, thus confirming the specificity of scFv RR-C2.

By binding to their cognate receptors, angiogenic growth factors trigger a complex angiogenic response in endothelial cells that includes an increase in cell proliferation, protease production, and motility (36). This capacity is reflected by the ability of angiogenic growth factors to stimulate endothelium to invade a 3-dimensional gel in an in vitro endothelial cell spheroid-sprouting assay (37). On this basis, HUVEC spheroids were embedded in type I collagen gel and stimulated with FGF2 in the absence or presence of scFv RR-C2 or control a-scFv. As shown in Fig. 2D and in Supplementary Fig. S3, scFv RR-C2 specifically inhibits HUVEC sprouting triggered by FGF2. No effect was instead exerted by the antibody fragment when endothelial cell sprouting was
induced by VEGF-A under the same experimental conditions (Supplementary Fig. S3).

Taken together, these data indicate that scFv RR-C2 specifically neutralizes the biological responses triggered by the FGFR-1 ligand FGF2 in cultured endothelial cells.

Ex vivo and in vivo antiangiogenic activity of scFv RR-C2

On the basis of the in vitro observations, scFv RR-C2 was assessed for its antiangiogenic potential ex vivo in a murine aorta ring assay (38) and in vivo in the chick embryo CAM assay (39). In the first set of experiments, murine thoracic aorta rings were embedded in fibrin gel and stimulated with FGF2 in the absence or presence of native or heat-denatured scFv RR-C2. After 6 days, the angiogenic response was measured by counting the number of neovessels sprouting out of the rings. As shown in Fig. 3A and Supplementary Fig. S4, FGF2 stimulation induces microvessel outgrowth from the aorta rings that is significantly suppressed by the native but not by the heat-denatured antibody.

In a second set of experiments, FGF2 and scFv RR-C2 were adsorbed on gelatin sponges and implanted on developing CAMs at day 11. After 3 days, the angiogenic response was measured by counting the number of newly formed microvessels converging toward the implant. Again, scFv RR-C2 exerts a significant inhibitory effect on blood vessel formation triggered by FGF2 that was fully abolished following heat denaturation of the antibody (Fig. 3B and C).

ScFv RR-C2 inhibits tumor cell proliferation and angiogenesis

Androgen-regulated Shionogi 115 (S115) mouse breast cancer cells represent a prototypic example of hormone-dependent tumors in which testosterone enhances the tumorigenic activity by inducing FGF8b upregulation, thus activating an FGFR-dependent autocrine loop of stimulation (40).

As shown in Fig. 4A, scFv RR-C2 specifically inhibits the proliferation of S115 cells when stimulated by DHT or FGF8b but not by FCS, whereas an irrelevant scFv was ineffective, thus implicating FGFR-1 in the androgen-induced response of S115 cells.

When implanted in the flank of sexually mature male mice via a Matrigel plug, S115 cells induce the appearance of newly formed blood vessels within the plug, as assessed by both qPCR analysis and immunostaining of the endothelial marker CD31 (Fig. 4B and C). In vivo administration of scFv RR-C2 results in a significant
decrease of CD31 mRNA levels and of CD31+ infiltrating vessels in the plugs of antibody-treated animals with respect to the control group (Fig. 4B–D).

Discussion

Angiogenesis is a key step in pathologic conditions in which the uncontrolled release of angiogenic growth factors and/or alterations of the production of natural angiogenic inhibitors are responsible for the activation of the endothelial cell population. The prominent proangiogenic signaling circuit that involves VEGF-A/VEGFR2 represents an elective target for antiangiogenic therapies in cancer patients (41). However, when the activity of VEGF is suppressed for a long period of time, the expression of other angiogenic factors, including FGFs, might emerge, leading to tumor regrowth after an initial period of regression (42). Indeed, an intimate cross-talk has been shown between VEGF and FGF2 (43, 44).

Here, we show the isolation, purification, and the inhibitory activity of an scFv antibody directed against FGFR-1, the predominant FGFR expressed by endothelial cells that transduces proangiogenic signals triggered by FGF2 and other members of the FGF family (36). The scFv RR-C2 fragment detects FGFR-1α and FGFR-1β isoforms in both ELISA and Western blotting, thus suggesting that its reactivity is directed against a linear epitope exposed on the surface of the native receptor molecule. As expected, binding of the antibody to immobilized FGFR-1β can be competed by soluble FGFR-1α and, conversely, soluble FGFR-1β inhibits binding to immobilized FGFR-1α (data not shown). Because the 2 isoforms share identical amino acid sequences, scFv RR-C2 should recognize the same epitope on both molecules. However, the antibody fragment shows different binding affinities for the 2 FGFR-1 isoforms. Indeed, the $K_d$ value for scFv RR-C2/FGFR-1α interaction is twice the value of the $K_d$ for FGFR-1β interaction, indicating that the presence of the Ig-like loop D1 affects epitope recognition and scFv/FGFR-1 complex formation. Nevertheless, at variance with commercially available monoclonal antibodies recognizing the extracellular domain of FGFR-1 (data not shown), scFv RR-C2 could immunoprecipitate FGFR-1 in the cellular context of TRAMP-C2 cells overexpressing human FGFR-1α.

Evidence from various experiments has shown the ability of scFv RR-C2 to neutralize the activity of FGFR-1 and, consequently, to impair FGF2-mediated angiogenesis. Indeed, the antibody fragment neutralizes the formation of a productive HSPG/FGF2/FGFR-1 ternary complex in an FGF2-dependent cell–cell adhesion assay (35). Also, in keeping with the high amino acid sequence identity (98%) of the FGFR-1 extracellular domain in the different animal species [see FGFR-1 multiple alignment in HomoloGene database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/homologene)], the antibody specifically inhibits the activity exerted by FGF2 on endothelial cells of human, bovine, murine, and avian origin in various in vitro, ex vivo, and in vivo angiogenesis assays.

The importance of FGFR-1 in cancer is not limited to the endothelial cell response to angiogenic FGFs. Indeed, cancer cells can express this receptor. For instance, FGFR-1 expression in prostate epithelial cells has been tightly correlated with cancer progression (45). Also, FGFR-1 deregulation occurs in numerous other tumor types, including lobular carcinoma of the breast (46), 8p11 myeloproliferative syndrome (47), and astrocytoma (48). Thus, a forthcoming anti–FGFR-1 therapy would have the dual effect of targeting not only tumor endothelium but also cancer cells. Indeed, scFv RR-C2 inhibits both DHT- and FGF8-induced proliferation of S115 mouse...
mammary tumor cells, an androgen-dependent tumor cell line in which DHT triggers an FGF8/FGFR-1 autocrine loop of stimulation (49, 50). Also, in vivo administration of scFv RR-C2 results in a significant decrease of the angiogenic activity exerted by these cells in a Matrigel plug assay. These data indicate that the scFv RR-C2 antibody may act as a valuable antitumor and antiangiogenic FGFR-1 inhibitor.

scFv RR-C2 inhibits endothelial cell proliferation with an ID50 of approximately 100 nmol/L, in keeping with Kd values for FGFR-1α/β isoform interactions. SPR data indicate that the scFv RR-C2/FGFR-1 complex is characterized by a slow off-rate, thus suggesting that once associated, the 2 molecules will form a stable complex. Affinity maturation of scFv RR-C2 will be required to increase its antigen recognition capacity in order to guarantee an in vivo efficient binding (51).

Several human or humanized antibodies against molecules implicated in angiogenesis and/or cancer progression are currently used in clinics (52) because they provide high specificity and a better safety profile than small chemical molecules such as TK inhibitors. When compared with commercially available murine anti-FGFR-1 IgGs, the humanized neutralizing scFv RR-C2 may present several advantages in a variety of uses, ranging from a simple research tool as diagnostic reagent to a highly refined biopharmaceutical (19). Also, the small format and the different pharmacokinetic properties of the antibody may result in a faster clearance and a concomitant strong reduction in secondary effects.

In conclusion, we have isolated a novel neutralizing human scFv antibody directed against the extracellular domain of FGFR-1 that may represent a promising tool for antiangiogenic and/or antineoplastic therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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