Published in final edited form as:

Clin Cancer Res. 2010 December 1; 16(23): 5750–5758. doi:10.1158/1078-0432.CCR-10-0531.

Monoclonal Antibodies to Fibroblast Growth Factor Receptor 2 Effectively Inhibit Growth of Gastric Tumor Xenografts

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Abstract

Purpose—Overexpression of Fibroblast Growth Factor Receptor 2 (FGFR2) may be a causative factor of a number of human tumors, especially gastric tumors of the poorly differentiated type. We investigated whether monoclonal antibodies (mAbs) directed against FGFR2 can inhibit the growth of tumors in xenograft models.

Experimental Design—We generated and characterized three mAbs that recognize different epitopes on FGFR2: GAL-FR21, GAL-FR22 and GAL-FR23. The ability of the mAbs to recognize the FGFR2IIIb and FGFR2IIIc isoforms of FGFR2 was determined, as was their ability to block binding of FGF ligands to FGFR2. The capability of the mAbs to inhibit FGF-induced FGFR2 phosphorylation and to down-modulate FGFR2 expression was also investigated. Finally, the ability of the anti-FGFR2 mAbs to inhibit tumor growth was determined by establishing xenografts of SNU-16 and OCUM-2M human gastric tumor cell lines in nude mice, treating with each mAb (0.5 – 5 mg/kg i.p. twice weekly), and monitoring tumor size.

Results—Of the three mAbs, GAL-FR21 binds only the FGFR2IIIb isoform, whereas GAL-FR22 and GAL-FR23 bind to both the FGFR2IIIb and FGFR2IIIc forms, with binding regions respectively in the D3, D2-D3 and D1 domains of FGFR2. GAL-FR21 and GAL-FR22 blocked the binding of FGF2, FGF7 and FGF10 to FGFR2IIIb. GAL-FR21 inhibited FGF2 and FGF7 induced phosphorylation of FGFR2, and both mAbs down-modulated FGFR2 expression on SNU-16 cells. These mAbs effectively inhibited growth of established SNU-16 and OCUM-2M xenografts in mice.

Conclusions—Anti-FGFR2 mAbs GAL-FR21 and GAL-FR22 have potential for the treatment of gastric and other tumors.

Keywords

FGFR2; FGF2; FGF7; gastric cancer; xenograft

The Fibroblast Growth Factor (FGF) family plays important roles in embryonic development, tissue repair, angiogenesis and the growth of certain tumors (1,2). While the family has 22 known members in humans, there are only four cellular FGF receptors, designated FGFR1 – FGFR4, with the various FGFs binding the different FGFRs to varying

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extents (3,4). The FGF receptors are structurally related transmembrane tyrosine kinases: each FGFR consists of an extracellular domain (ECD) comprising three immunoglobulin (Ig)-like domains (D1, D2 and D3), a single transmembrane helix, and an intracellular kinase domain (5). The FGFRs are characterized by multiple alternative splicing of their mRNAs, leading to a variety of isoforms (3). Notably, there are forms containing all three Ig domains (α isoforms) or only the two Ig domains D2 and D3 without D1 (β isoforms). Of special importance in FGFR2, all forms contain the first half of D3 denoted IIIa, while two alternative exons can be utilized for the second half of D3, leading to IIIb and IIIc forms. These are respectively denoted FGFR2IIIb and FGFR2IIIc; the corresponding beta forms are denoted as FGFR2(β)IIIb and FGFR2(β)IIIc. Importantly, the FGFR2IIIb form is a high affinity receptor for FGF1 and FGF2 as well as for FGF7 (Keratinocyte Growth Factor; KGF) and FGF10, whereas FGFR2IIIc binds both FGF1 and FGF2 well but does not bind KGF (6). Indeed, FGFR2IIIb is the main receptor for KGF (3) and is thus also designated as KGFR.

The FGFRs and their isoforms are differentially expressed in various tissues. Generally, FGFR2IIIb (and the IIIb forms of FGFR1 and FGFR3) are expressed in epithelial tissues, while FGFR2IIIc is expressed in mesenchymal tissues (7), although this correlation is not strict (8). Certain FGF ligands of these receptors have an opposite pattern of expression. Thus, FGF3 and FGF7 are expressed in mesenchymal tissues so may be paracrine effectors of epithelial cells expressing FGFR2IIIb, while the FGF4 subfamily members FGF4-6 bind to FGFR2IIIc and are expressed in both epithelial and mesenchymal lineages so may have either autocrine or paracrine functions (3). Because of the expression patterns of the isoforms of FGFR2 and their ligands, FGFR2 plays a role in epithelial-mesenchymal interactions (9), so it is not surprising that knock-out of FGFR2IIIb in mice leads to embryonic defects and lethality (10).

Several FGFs are believed to play a role in cancer, both by stimulating angiogenesis and tumor cells directly (2,11). Correspondingly, FGFR2IIIb is expressed or overexpressed in many types of tumors (12,13). For example, FGF7 and FGFR2IIIb are overexpressed in many pancreatic cancers (14), and their coexpression correlates with poor prognosis (15). Somatic mutations of the FGFR2 gene were found in 10-12% of large panels of endometrial carcinomas, and in several tested cases were required for tumor cell survival (16,17). One of the most common mutations found, a Ser-252 $\rightarrow$ Trp substitution, is known to also be associated with the congenital skeletal disorder Apert syndrome (18) and increases ligand binding and receptor activation (19). FGFR2IIIb RNA was expressed in 16 of 20 epithelial ovarian cancers (EOCs) but not in normal ovarian surface epithelium (20); and the FGFR2IIIb ligands FGF1, FGF7 and FGF10 induced proliferation, motility and protection from cell death in EOC cell lines (21), suggesting that FGFR2IIIb may contribute to the malignant phenotype in ovarian cancer. The FGFR2 gene is also amplified in a subset of breast cancers (22), and genome-wide association studies of breast cancer patients have demonstrated the FGFR2 locus to be strongly associated with susceptibility to breast cancer (23,24). Of particular interest, amplification and overexpression of FGFR2 is strongly associated with the poorly differentiated, diffuse type of gastric cancer, which has an especially unfavorable prognosis (25).

The association of FGFR2 overexpression or mutation with many types of cancer suggests that FGFR2 may be an excellent therapeutic target. Several small molecule antagonists of FGFR2 have been reported (26,27,28), but these agents also have inhibitory activity toward other tyrosine kinase receptors, increasing their potential for toxicity if used to treat human patients. On the other hand, highly specific monoclonal antibodies (mAbs) against various growth factors or their receptors including VEGF, EGF receptor, and HER2 are now being used to treat various types of cancer with considerable success, suggesting a mAb approach

to targeting FGFR2. However, only a few anti-FGFR2 mAbs have been reported (29,30), perhaps because the very high sequence homology between mouse and human FGFR2 makes development of such mAbs difficult; moreover, the ability of the reported mAbs to inhibit tumor growth was not established. In this study, we have therefore generated and characterized several anti-FGFR2 mAbs and shown that they block the functional activity of FGFR2 in vitro and inhibit growth of FGFR2-overexpressing gastric tumor xenografts in vivo.

Materials and Methods

Cell lines

SNU-16 (ATCC CRL 5974) and KATO-III (HTB-103) were obtained from ATCC. OCUM-2M was from Osaka City University (Osaka, Japan). Upon receipt, the cell lines were expanded and frozen in vials, and a new vial thawed for each series of experiments.

Reagents

The α isoform (D1, D2 and D3 domains) and β isoform (D2 and D3 domains) of human FGFR2IIIb and FGFR2IIIc were expressed as immunoadhesin molecules by respectively fusing the extracellular domain residues 1-378 (α isoform) and 152-378 (β isoform) of FGFR2IIIb or 1-377 (α isoform) and 152-377 (β isoform) of FGFR2IIIc to the human Ig Fc region (residues 216 to 446) in the pDisplay vector (Invitrogen). Each FGFR2-Fc protein was expressed by transfecting human 293F cells (Invitrogen), and the FGFR2-Fc was purified from culture medium using a protein A/G column. Fc fusion proteins of the other FGFRs as well as FGFR2IIIb(S252W)-Fc with the S252W mutation were similarly generated and produced. Mouse FGFR2IIIb-Fc was purchased (R&D Systems).

Generation of mAbs to FGFR2

Female 6-week old Balb/c mice (Charles River Laboratories) were immunized with human FGFR2IIIb-Fc or FGFR2IIIc-Fc in monophosphoryl lipid A/ trehalose dicorynomycolate (MPL/TDM, Sigma) in their rear footpads about 20 times, once per week. Sera titers were determined by an FGFR ELISA described below. Three days after the final injection, popliteal lymphoid cells were fused with P3/X63-Ag8U1 mouse myeloma cells using an electrofusion device according to the manufacturer's instructions (Cyto Pulse Science). Two weeks after the fusion, hybridoma culture supernatants were screened first for binding to FGFR2IIIb-Fc binding by the ELISA described below. Positive hybridomas were further screened for their ability to bind to FGFR2 positive tumor cell lines. Selected hybridomas were cloned twice by limiting dilution. The isotype of the mAbs produced by these selected clones was determined using isotype specific antibodies (BioRad).

ELISAs

Each step of each assay was performed by room temperature incubation with the appropriate reagent for 1 hour, except the initial plate coating step was done overnight at 4°C. Between each step, plates were washed 3 times in PBS containing 0.05% Tween 20. Data points were generally in triplicate. To determine binding of the mAbs to FGFR2 isoforms, ELISA plates were coated with goat anti-human IgG-Fc (2 μ g/ml; Jackson ImmunoResearch Labs) first, followed by blocking with 2% BSA. The plates were then incubated with the α and β isoforms of FGFR2IIIb-Fc and FGFR2IIIc-Fc or control human IgG, followed by incubation with various concentrations of mAbs. The bound murine Ab was detected by the addition of HRP-goat anti-mouse IgG and TMB substrate (Sigma-Aldrich). To determine the ability of the mAbs to block ligand binding to FGFR2, plates were coated with 50 μ g/ml heparin (Sigma), to which FGFs are known to bind (1). The plates were then incubated with 0.2 μ g/

ml of human FGF2, FGF7 or FGF10 (R&D Systems) and blocked with 2% BSA. The plates were next incubated with FGFR2IIIb-Fc (0.5 µg/ml) in the presence of various concentrations of mAbs, and the bound FGFR2IIIb-Fc was detected with HRP-goat anti-human IgG-Fc and substrate.

Flow cytometry

Appropriate cells (2×10^5) were washed twice in cell sorting buffer (CSB: PBS /1% FBS/ 0.02% NaN_3), resuspended in 50 µl of CSB and incubated with 1 µg/50 µl of an anti-FGFR2 mAb for 1 hr at 4°C on a shaker. Cells were then washed twice in CSB and the bound antibodies were detected by incubation with ALEXA 488-goat anti-mouse IgG (Invitrogen) for 1 hr on ice. After washing twice in CSB, cells were analyzed on a FACScan.

FGFR2 phosphorylation assay

SNU-16 cells were grown in RPMI medium with 10% FCS and then in serum-free RPMI/ 0.1% BSA for 24 hr. The cells were resuspended at 3×10^5 cells/well in RPMI/0.1% BSA and incubated with mAb for 30 min, followed by addition of FGF2 (100 ng/ml) or FGF7 (10 ng/ml) for 10 min. Cells were then placed on ice and washed with ice-cold PBS. Cell lysates were prepared and the level of phosphorylated FGFR2 determined using a Phospho-FGF R2α DuoSet IC kit (R&D Systems, DY684-2) for ELISA according to the manufacturer's instructions.

Down-modulation of FGFR2 expression

SNU-16 cells (5×10^5 /well) were treated with mAbs (10 µg/ml) or FGF2 (100 ng/ml) in RPMI with 10% FCS for 20 hr and the level of cell surface FGFR2 determined by flow cytometry as described above, using biotinylated GAL-FR23 mAb followed by streptavidin-R-phycoerythrin for detection. In addition, total cell lysate was prepared after various periods of treatment with the mAbs, separated by SDS gel electrophoresis and processed for western blotting. The FGFR2IIIb band was detected by incubation with 0.2 µg/ml of GAL-FR21 mAb, followed by HRP-goat anti-mouse IgG.

Xenograft models

Animal experiments were conducted in accordance with U.S. Public Health Service policy. SNU-16 or a subclone of OCUM-2M cells grown in complete DMEM medium were harvested in PBS. Female 5- to 6-week-old athymic nude mice were injected s.c with 5×10^6 cells in 0.1 ml of PBS containing 50 µl of matrigel (BD Biosciences) in the dorsal area. When the tumor sizes reached $\sim 150 \text{ mm}^3$, mice were grouped randomly ($n = 5-7$ /group) and appropriate doses of mAbs (0.5 - 5 mg/kg) in a volume of 0.1 ml were administered i. p. twice per week. Tumor volumes were determined twice weekly by measuring in two dimensions, length (a) and width (b), and calculating volume as $V = ab^2/2$. Statistical analysis was performed by Student's t test applied to the final timepoint.

Results

Generation and binding properties of anti-FGFR2 mAbs

After screening several thousand hybridomas from 5 fusions in an FGFR2IIIb-Fc binding ELISA followed by flow cytometry on SNU-16 cells (31), we selected three mAbs (GAL-FR21, GAL-FR22 and GAL-FR23) for further analysis. GAL-FR21 is of the IgG1 isotype while GAL-FR22 and GAL-FR23 are of the IgG2b isotype.

GAL-FR21 bound to only FGFR2IIIb-Fc while GAL-FR22 and GAL-FR23 bound to both FGFR2IIIb-Fc and FGFR2IIIC-Fc in ELISA (Fig. 1A, B). GAL-FR21 bound to denatured

FGFR2IIIb-Fc in a western blot (data not shown), suggesting that GAL-FR21 recognizes a linear epitope. While the binding affinities of the mAbs have not been determined precisely by BioCore or similar methods, the low EC₅₀s for binding in the ELISA (approximately 20 pM, 5 pM and 10 pM for GAL-FR21, GAL-FR22 and GAL-FR23 respectively) suggest the affinities are very high. In the same ELISA format, none of the mAbs displayed detectable binding affinity to FGFR1, FGFR3 or FGFR4. Since the mAbs were generated against soluble forms of FGFR2, we needed to verify that they bind to FGFR2 in its native, membrane-bound form. In flow cytometry, all three mAbs strongly stained SNU-16 gastric tumor cells overexpressing FGFR2IIIb (Fig. 2A) while only GAL-FR22 and GAL-FR23 stained 293F cells transfected with FGFR2IIIc (Fig. 2B); none of the mAbs stained the host 293F cells (not shown). The mAbs stained OCUM-2M gastric tumor cells (32) and another gastric tumor line KATO-III very similarly to SNU-16 cells (not shown). Finally, when FGFR2IIIb containing the S252W mutation associated with Apert syndrome and certain tumors was expressed on 293F cells by transfection, all the mAbs bound the mutant FGFR2IIIb as well as normal FGFR2IIIb (Fig. 2C).

In order to help interpret the results of the xenograft experiments described below, we determined the ability of GAL-FR21 and GAL-FR22 to bind to mouse FGFR2IIIb. In an ELISA assay, GAL-FR21 bound to mouse FGFR2IIIb within several-fold as well as human FGFR2IIIb (Fig. 1C), while GAL-FR22 exhibited weak binding to mouse FGFR2IIIb (Fig. 1D). The cross-reactivity of GAL-FR21 with mouse FGFR2IIIb may seem surprising because the mAb was generated by immunization with human FGFR2IIIb. However, human and mouse FGFR2 have high sequence homology, and the very low level expression of FGFR2 in tissues of the adult animal may make it relatively easy to break tolerance. In any case, the good reactivity of GAL-FR21 with FGFR2IIIb from mouse and probably other species will facilitate animal studies of this mAb.

To further define the binding region of the mAbs, we compared their binding to the β isoforms (ECD domains 2-3) with binding to the α isoforms (ECD domains 1-3) of FGFR2IIIb and FGFR2IIIc. Results shown in Fig. 3A demonstrate that GAL-FR21 bound to both α and β isoforms of FGFR2IIIb but not FGFR2IIIc. This suggests that the GAL-FR21 binding region does not involve D1 but may be in the D3 domain, where the sequence difference between FGFR2IIIb and FGFR2IIIc is located. GAL-FR22 bound to α and β forms of both FGFR2IIIb and FGFR2IIIc, indicating that the binding region of GAL-FR22 does not involve D1 but is contained in D2-D3IIIa, where FGFR2IIIb and FGFR2IIIc share the same sequences. In contrast, GAL-FR23 bound only to the α but not the β forms of both receptors, implying the binding region is located in the D1 domain. Hence GAL-FR21, GAL-FR22 and GAL-FR23 must bind three different epitopes of FGFR2. This conclusion is supported by the fact that in a competitive binding ELISA, none of the mAbs competed with each other for binding to FGFR2 (Fig. 3B).

Ligand blocking activities of the anti-FGFR2 mAb in vitro

X-ray crystallography shows that FGF ligands interact extensively with the D2-D3 region of the FGF receptors (5). Thus GAL-FR21 and GAL-FR22, which have binding regions in the D2 and D3 domains, have the potential to block ligand/receptor binding. Since all three mAbs bind to FGFR2IIIb, we investigated the blocking activities of these mAbs toward the binding of FGF2, FGF7 and FGF10 to FGFR2IIIb. For this purpose, the FGF ligands bound to heparin-coated ELISA plates were incubated with soluble FGFR2IIIb-Fc with or without the respective mAbs, and the bound FGFR2IIIb-Fc was detected by the addition of HRP-goat anti-human IgG, taking advantage of the Fc tag in the fusion protein. As shown in Fig. 4, GAL-FR21 and GAL-FR22 effectively blocked the binding of FGFR2IIIb to each of FGF2, FGF7 and FGF10 with IC₅₀s of approximately 1.5 - 3 μ g/ml. In contrast, as expected

from the location of its epitope in D1, GAL-FR23 did not inhibit binding of the tested FGF10 to FGFR2IIIb.

The anti-FGFR2 mAbs inhibit ligand-induced FGFR2IIIb phosphorylation

We further investigated the blocking activities of the anti-FGFR2 mAbs using a functional in vitro assay, FGF-induced FGFR2IIIb phosphorylation. In this experiment, SNU-16 cells were serum starved overnight and treated with 10 μ g/ml of mAb for 30 min, followed by the addition of FGF2 (100 ng/ml) or FGF7 (10 ng/ml) in the presence of heparin for 10 min. Phosphorylated FGFR2 in the cell lysates was detected using an ELISA specific for p-FGFR2. GAL-FR21 and GAL-FR22 by themselves demonstrated weak agonist activity in this assay (compare the level of p-FGFR2 in the absence of ligand when cells were treated with GAL-FR21 or GAL-FR22 versus control mAb in Fig. 5A). Thus we were not able to demonstrate complete inhibition of FGF-induced FGFR2 phosphorylation by these mAbs. The level of phosphorylation was the same in the presence of GAL-FR21 whether or not FGF7 was added (Fig. 5A), indicating effective blocking of FGF7-induced phosphorylation by this mAb. In contrast, GAL-FR22 did not block FGF7-induced phosphorylation in the conditions used (Fig. 5A). It is possible that low residual binding of FGF7 to FGFR2IIIb in the presence of GAL-FR22 is sufficient to induce phosphorylation of FGFR2IIIb, while this is evidently not true for GAL-FR21. However, both GAL-FR21 and GAL-FR22 significantly inhibited FGF2-induced phosphorylation of FGFR2IIIb (Fig. 5B), with GAL-FR21 more effective than GAL-FR22 and with GAL-FR23 having no effect as expected. Despite their inhibitory effect on ligand-induced phosphorylation, none of the mAbs inhibited proliferation of SNU-16 (or OCUM-2M) cells in vitro (data not shown).

The anti-FGFR2 mAbs down-modulate FGFR2 expression

To further define the functional activity of GAL-FR21 and GAL-FR22, we investigated whether binding of the mAbs to FGFR2 reduces membrane FGFR2 expression. SNU-16 cells treated overnight with the mAbs were collected and the level of FGFR2 on the cell membrane was analyzed by flow cytometry using the GAL-FR23 mAb (Fig. 5C), which does not compete with GAL-FR21 or GAL-FR22 for binding (Fig. 3B). We also determined the level of total cellular FGFR2 by immunoblotting of cell lysates with GAL-FR21 (Fig. 5D), which binds to denatured FGFR2IIIb. Treatment with GAL-FR21 or GAL-FR22 down-modulated membrane expression of FGFR2 by approximately 50% (Fig. 5C). The total level of FGFR2 determined in the immunoblot analysis was also substantially reduced compared to the control (Fig. 5D), in the case of GAL-FR21 by 8 but not 2 hours of treatment. As a positive control we also treated cells with FGF2, which reduced membrane and total FGFR2 even more strongly than the mAbs. Down-modulation of receptors by their ligands is of course a well-known phenomenon.

The anti-FGFR2 mAbs demonstrated potent anti-tumor activity in vivo

As noted previously, GAL-FR21 and GAL-FR22 bind very well to the gastric cancer cell lines SNU-16 and OCUM-2M, which overexpress FGFR2 (33). Thus, we investigated the anti-tumor activities of the anti-FGFR2 mAbs on xenografts of these cell lines. Antibody was administered i.p. twice weekly at dose levels of 0.5 - 5 mg/kg after tumor size had reached ~ 150 mm³. The GAL-FR21 and GAL-FR22 mAbs inhibited growth of the SNU-16 xenografts essentially completely at a dose level of 1 mg/kg and in some cases tumor regression was observed (Fig. 6A; $p = 0.0014$ for GAL-FR21 vs mIgG and $p = 0.0005$ for GAL-FR22 vs mIgG). The inhibitory effect of GAL-FR21 on the xenografts was essentially independent of dose level from 5 mg/kg down to the quite low dose level of 0.5 mg/kg (10 μ g/injection) (Fig. 6B). The mAbs GAL-FR21 and GAL-FR22 tested at 2.5 mg/kg also inhibited growth of OCUM-2M xenografts (Fig. 6C; $p = 0.003$ for GAL-FR21 or GAL-FR22 vs mIgG). Since the anti-tumor activities of these mAbs were so potent, we also

measured total body weight at the end of the experiment as an indication of potential toxicity. After subtracting the tumor weight, there were no appreciable differences between the average body weights of the mice treated with any of the mAbs or with PBS. Nor did the mAb treated mice exhibit any other obvious signs of toxicity such as lethargy or failure to feed, although histopathology was not conducted. These results are especially meaningful for GAL-FR21, which binds well to mouse FGFR2.

Discussion

Gastric cancer is a fairly common type of malignancy in Western countries, with about 21,000 new cases in the United States in 2009, very comparable to the incidence of ovarian cancer or liver cancer (34). For reasons that are not well understood, gastric cancer is much more common in Asian countries, making it the second leading cause of cancer death worldwide (35). The prognosis for gastric cancer is poor unless it is detected at an early stage, so the 5-year survival rate in the U.S. is only about 25% (35). Surgery is a common treatment for gastric cancer but is rarely curative when the cancer is advanced, and chemotherapy, often platinum-based, is also not very effective. No targeted therapies have been approved for gastric cancer, although several small-molecule tyrosine kinase inhibitors including sunitinib are being tested for this indication, according to the clinicaltrials.gov web site. Indeed, in a recent Phase III trial in gastric cancer, the anti-VEGF mAb bevacizumab (Avastin) did not prolong survival³. Hence, drugs directed against other molecular targets in gastric tumors are certainly needed.

Gastric cancer is classified into two major types (36): well-differentiated and poorly differentiated. The poorly differentiated form, which is also called diffuse or infiltrative, itself has several subtypes (36): scirrhous, signet ring cell carcinoma and mucinous adenocarcinoma. FGFR2 overexpression is preferentially associated with the poorly differentiated type of gastric carcinoma: in a series of gastric cancer patients, 20 of 38 tumors of the poorly differentiated type overexpressed FGFR2, but none of 11 tumors of the well-differentiated type did, suggesting that the overall incidence of FGFR2 overexpression in gastric cancer may be as high as 40% (25). Although some tumors of each of the poorly differentiated subtypes overexpressed FGFR2, not enough data was presented to determine if the incidence of FGFR2 overexpression differs between the subtypes (25). The FGFR2-overexpressing cell lines SNU-16 and OCUM-2M used here for xenograft studies originated from poorly differentiated gastric tumors (31,32), with OCUM-2M known to come from the scirrhous subtype. The poorly differentiated type of gastric cancer has a worse prognosis than the well differentiated type (36), so the existence of the well-defined target FGFR2 in this type is fortunate.

Importantly, FGFR2 overexpression appears to be an oncogenic driver in the gastric tumors where it appears, rather than a mere marker, as shown by the effect of FGFR2 antagonists. The overexpression of FGFR2 in SNU-16, OCUM-2M and KATO-III cells is associated with high basal levels of FGFR2 activation site phosphorylation (33). The small molecule FGFR2 inhibitor PD173074 potently inhibited this phosphorylation and the growth of the SNU-16, OCUM-2M and KATO-III cell lines *in vitro* and induced apoptosis of SNU-16 and OCUM-2M cells (33). Similarly, the small molecule FGFR2 inhibitor Ki23057 suppressed proliferation of the scirrhous gastric carcinoma cell lines OCUM-2MD3 and OCUM-8 overexpressing FGFR2, but not proliferation of three nonscirrhous gastric cancer lines, and strongly inhibited growth of OCUM-2MD3 xenografts in mice (27). And the FGFR2 inhibitor AZD2171 potently inhibited FGFR2 phosphorylation, cell growth *in vitro*, and xenograft growth *in vivo* of the OCUM-2M and KATO-III cell lines (28). However, all these

³Genentech, Inc. press release dated February 22, 2010.

agents inhibit other tyrosine kinase receptors in addition to FGFR2, including FGFR1, FGFR3, VEGFR-1, VEGFR-2, VEGFR-3 and/or PDGF-R β . Besides increasing the potential for toxicity of these agents when used in human patients, this lack of specificity somewhat weakens the evidence they provide for FGFR2 as the cause of the cancerous phenotype of the tested cell lines. Partly filling this gap, FGFR2-specific siRNA also strongly inhibited growth of the OCUM-2M and KATO-III cell lines (28,33). Importantly, the ability of the GAL-FR21 and GAL-FR22 mAbs, which are highly specific for FGFR2, to almost completely inhibit the growth of SNU-16 and OCUM-2M xenografts provides decisive additional evidence for the causative role of FGFR2.

We have not fully established the mechanism of action of the anti-FGFR2 mAbs against SNU-16 and OCUM-2M tumor xenografts. Both mAbs down-modulate FGFR2 expression on the SNU-16 cells, which may reduce cell-signaling from the activated FGFR2 that is driving cell proliferation. It is also possible that cross-linking of the FGFR2 by the mAbs directly transmits a pro-apoptotic signal. However, the mAbs do not inhibit cell proliferation in vitro, in contrast with their potent growth-inhibitory effects in vivo. This phenomenon has also been seen with mAbs to other growth factors or their receptors. For example, the L2G7 mAb to hepatocyte growth factor (37) strongly inhibits growth of xenografts from several tumor cell lines against which it has little direct effect in vitro (our unpublished data). The greater vulnerability of the cells to anti-FGFR2 mAbs when growing in vivo than in vitro is probably a result of the increased stresses they are subjected to as tumors, for example hypoxia, but this question should be the subject of further investigation.

Several other potential modes of action probably do not make a major contribution to the anti-tumor effects of the mAbs. ADCC is unlikely to be an important factor because GAL-FR21, which is of the IgG1 isotype that mediates ADCC poorly (38), inhibits tumor growth as well as GAL-FR22, which is of the IgG2b isotype known to mediate ADCC well. Anti-angiogenic effects are also probably not important: while certain FGFs such as FGF2 are potent angiogenic factors (2), FGFR2 is only one of the receptors for these factors. Moreover, GAL-FR22 only weakly binds the mouse FGFR2 expressed on endothelial cells of growing blood vessels in the xenografts, but inhibits tumor growth as well as GAL-FR21, which binds mouse FGFR2 strongly. In addition, although tested in different xenograft models, the anti-tumor effects of GAL-FR21 and GAL-FR22 are stronger than those generally seen with the murine precursor antibody of bevacizumab (39), an anti-angiogenic mAb that inhibits VEGF. Finally, although the mAbs effectively block binding of the FGF2, FGF7 and FGF10 ligands to FGFR2, this is also unlikely to contribute to their anti-tumor effect in the models used here. Indeed, FGFR2 is already maximally activated in OCUM-2M cells in the absence of ligand, and while FGF7 does stimulate FGFR2 phosphorylation in SNU-16 cells, this does not further stimulate downstream signaling pathways (33). Hence, blocking of FGF7 or other FGF ligands should have little effect on proliferation of these gastric tumor cell lines. However, the ability of the mAbs to inhibit ligand binding may be more important in treatment of other types of tumors, for example pancreatic or prostate, where FGF7 and/or FGF10 are believed to play a role in tumorigenicity (17,40,41).

The findings reported here are very analogous to the recent demonstration that a mAb directed to another of the FGF receptors, FGFR3, blocks ligand binding and inhibits growth of bladder tumor xenografts (42). The ability of the GAL-FR21 and GAL-FR22 mAbs to inhibit growth of xenografts from two gastric tumor cell lines without apparent toxicity suggests that it will be worthwhile to humanize one of these mAbs, and then test the humanized mAb in clinical trials for the treatment of the poorly differentiated form of gastric carcinoma and potentially other types of cancer.

Statement of Translational Relevance

Due to the well-known limitations of conventional chemotherapy, contemporary approaches to cancer treatment are focused on targeting specific molecular pathways that contribute to tumorigenicity. The accompanying article provides validation of Fibroblast Growth Factor Receptor 2 (FGFR2) as such a cancer target. While small-molecule antagonists of FGFR2 have previously been shown to inhibit certain tumors, especially gastric tumors overexpressing FGFR2, the lack of specificity of the inhibitors made it difficult to draw definitive conclusions regarding the role of FGFR2. In contrast, we have developed several monoclonal antibodies that are highly specific for FGFR2 and shown that they strongly inhibit growth of xenografts from two gastric tumor cell lines, thus isolating the role of FGFR2 as a causative factor in some gastric tumors. In addition, after humanization to reduce immunogenicity, one of the anti-FGFR2 antibodies will itself be a viable clinical candidate for the treatment of gastric and possibly other cancers.

Acknowledgments

NIH grants 5R44CA101283-03 and 1R43CA141748-01

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Fig. 1.

ELISA of binding of mAbs GAL-FR21, GAL-FR22, GAL-FR23 and negative control mAb mIgG to human FGFR2IIIb-Fc (A) and human FGFR2IIIC-Fc (B), and binding of GAL-FR21 (C) and GAL-FR22 (D) to mouse (m) versus human (h) FGFR2IIIb. In B, the curves for GAL-FR21 and mIgG binding superimpose and cannot be distinguished.

Fig. 2.

Flow cytometry of binding of mAbs GAL-FR21, GAL-FR22, GAL-FR23 and negative control mAb mIgG to SNU-16 cells (A), 293F-FGFR2IIIc transfectants (B), and 293F-FGFR2IIIb(S252W) transfectants (C).

Fig 3.

A, ELISA of mAbs GAL-FR21, GAL-FR22, GAL-FR23 and negative control mAb mIgG binding to each of FGFR2IIIb-Fc, FGFR2(β)IIIb-Fc, FGFR2IIIc-Fc and FGFR2(β)IIIc-Fc. B, Competitive binding ELISA to FGFR2IIIb-Fc of each of the mAbs GAL-FR21, GAL-FR22, GAL-FR23 and mIgG against the mAbs in biotinylated form (labeled on the horizontal axis). A 100:1 ratio of unlabeled to biotinylated mAb was used.

Fig. 4. ELISA measuring inhibition of binding of FGF2 (A), FGF7 (B) and FGF10 (C) to FGFR2IIIb by mAbs GAL-FR21, GAL-FR22, GAL-FR23 (FGF10 only) and negative control mAb mIgG.

Fig. 5. Inhibition of FGFR2 phosphorylation induced by FGF7 (A) or FGF2 (B) by mAbs GAL-FR21, GAL-FR22, GAL-FR23 and mIgG, measured by p-FGFR2 specific ELISA. C, Flow cytometry of SNU-16 cells treated with mAbs GAL-FR21, GAL-FR22, negative control mAb mIgG or FGF2 using GAL-FR23 as staining reagent. D, Immunoblot of lysates of SNU-16 cells treated for various times with 10 µg/ml of GAL-FR21, GAL-FR22 or negative control mAb mIgG or with FGF2, separated by SDS-PAGE and stained with GAL-FR21. Error bars are S.D. In A with FGF7, $p = 0.001$ for GAL-FR21 vs mIgG. In B, $p = 0.004$ for GAL-FR21 vs mIgG and $p = 0.0006$ for GAL-FR22 vs mIgG. Statistics are by Student's t test.

Fig. 6. Inhibition of growth of SNU-16 (*A, B*) or OCUM-2M (*C*) gastric tumor xenografts by respectively 1 mg/kg or 2.5 mg/kg of the indicated mAb or as labeled in the legend (*B*), administered i.p. twice weekly. The means of groups of 5-7 mice are shown; the error bars are S.E.M. The 0.5 mg/kg curve in *B* is largely superimposed with the 5 mg/kg curve, and the GAL-FR21 curve in *C* is largely superimposed with the GAL-FR22 curve.