Development of Human Single-Chain Antibodies to the Transferrin Receptor that Effectively Antagonize the Growth of Leukemias and Lymphomas

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Abstract

The major route of iron uptake by cells occurs through transferrin receptor (TfR)-mediated endocytosis of diferric-charged plasma transferrin (holo-Tf). In this work, we pursued TfR antibodies as potential cancer therapeutics, characterizing human single-chain variable antibody fragments (scFv) specific for the human TfR isolated from a phage display library. We hypothesized that many of these antibodies would function as ligand mimetics because scFvs from the library were selected for binding and internalization into living cells. In support of this hypothesis, the anti-TfR scFvs identified were antagonists of TfR binding to holo-Tf, particularly two of the most potent antibodies, 3TF12 and 3GH7, which blocked the in vitro proliferation of a number of hematopoietic cancer cell lines. We optimized this activity of 3TF12 and 3GH7 by engineering 55-kDa bivalent antibody formats, namely, F12CH and H7CH, which could block cell proliferation with an IC50 of 0.1 μg/mL. We found that the mechanism of the scFv antibody cytotoxicity was unique compared with cytotoxic anti-TfR monoclonal antibodies that have been described, causing cell surface upregulation of TfR along with the inhibition of holo-Tf cell uptake and induction of cell death. In a nude mouse model of erythroleukemia, administration of F12CH reduced tumor growth. Together, our findings define a new class of fully human anti-TfR antibodies suitable for immunotherapy against tumors whose proliferation relies on high levels of TfR and iron uptake, such as acute lymphoid and myeloid leukemias.

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Introduction

The transferrin receptor (TfR) is a type II transmembrane glycoprotein made of two 90-kDa monomers (1). It plays a crucial role in the cellular uptake of iron and in the regulation of cell growth through its binding to diferric charged plasma transferrin (holo-Tf). Iron is involved in a variety of cellular processes including metabolism, respiration, and DNA synthesis. Regulation of TfR expression is regulated posttranscriptionally through iron-responsive elements sensitive to intracellular iron levels. When low level of iron is in the cells, TfR mRNA is stabilized, and it gets degraded as the concentration increases (2, 3). Because highly proliferating cells frequently overexpress the TfR (4–6), it has long been considered an attractive target for cancer. TfR expression is also found on the blood brain barrier, which makes it interesting for therapeutic or diagnostic approaches in brain diseases. Consequently, in the last 25 years, a number of anti-TfR monoclonal antibodies (mAb) were raised using hybridoma technology and tested for inhibitory effects in vitro and in vivo in mouse models of cancer (7) or for brain targeting (8). A phase I trial with iron-depleting anti-human TfR IgA-42/6 (9) was encouraging because it did not show major side effects in patients with the administration of the mAb and some patients with hematologic cancer showed partial therapeutic responses (10). Indeed, a concern for the use of cytotoxic anti-TfR mAbs for cancer immunotherapy could be their adverse effects toward iron-dependent cells such as hematopoietic lineage cells, specifically maturing erythroid cells.

The anti-TfR mAbs studied to date have revealed various mechanisms of cytotoxicity when used as naked antibodies in vitro. As a general feature, increased antibody valency correlated with higher toxicity, with pentameric IgM, dimeric IgA, or engineered dimeric avidin-fused-IgG3 being more active than monomeric IgG (9, 11, 12). In a study where IgG was converted to monovalent Fab, no cytotoxic activity was recovered (13). Therefore, TfR cross-linking at the cell surface seemed to be required for antibody cytotoxicity. Depending
of the intensity of the cross-link, mAbs induced either receptor blockade at the cell surface (IgM; ref. 11) or receptor internalization (IgA and IgG; refs. 14–16), both likely resulting in inhibition of holo-Tf uptake and iron deprivation of the target cells. Inhibition of holo-Tf binding seems to be not required for antibody cytotoxicity because, whereas IgG-A24 (17) and IgA-42/6 (9) inhibited holo-Tf binding (by a competitive and a noncompetitive mechanism, respectively), avidin-fused IgG3 (12) and IgM-R17208 (11) did not. Notably, IgG-A24 and avidin-fused-IgG3 reduced TfR expression by impairing TfR recycling to the cell surface and routing the internalized TfR to the lysosomal degradation pathway (15, 18).

For this work, we investigated the growth inhibitory potential of six human anti-TfR single-chain variable antibody fragments (scFv) selected by phage display for their ability to internalize rapidly into living cancer cells via receptor-mediated endocytosis (19, 20). We reasoned that the stringency of a functional cell-based phage display selection would allow the isolation of functional ligand-like antibodies with agonistic or antagonistic activities. We showed that the anti-TfR scFvs obtained in this selection compete with holo-Tf binding to TfR and that for the best competitors, this inhibition translated into a pronounced in vitro antiproliferative activity on hematopoietic tumor cell lines of various lineages. Indeed, engineered bivalent 55-kDa scFv formats inhibited ERY-1 erythroleukemia cell growth (21) and, with an IC50 of 0.1 μg/mL in in vitro experiments, cells were preincubated with various concentrations of human holo-Tf (Sigma) or 10 μg/mL scFv before adding phage-scFvs. Holo-Tf binding was tested using either human holo-Tf conjugated to fluorescein (FITC; Invitrogen; 500 nmol/L) or biotinylated mouse Tf (Rockland; 1 μmol/L) and streptavidin coupled to PE (BD). For competition experiments, cells were preincubated for 1 hour with competitors before adding Tf. Direct antibody fragment binding to cells was detected using 50 μg/mL of antibody followed by an anti-(His)6 tag mAb (Qiagen) and PE-conjugated goat anti-mouse immunoglobulins. Cells were analyzed with a FACScalibur (BD) using CellQuest Pro Software for mean fluorescence intensity (MFI) calculation.

Detection of phage-scFv, holo-Tf, and scFv binding to cells

Cell binding was tested by flow cytometry using 2 × 10^5 cells resuspended in 100 μL of PBS, 1% FBS or PBS, 1% bovine serum albumin [fluorescence-activated cell (FACS) buffer]. All incubations were done at 4°C. Phage-scFv binding (10^11 cfu/mL) was detected with an anti-M13 mouse mAb (GE Healthcare) followed by goat anti-mouse immunoglobulins conjugated to phycoerythrin (PE; BD). For competition experiments, cells were preincubated with various concentrations of human holo-Tf (Sigma) or 10 μg/mL scFv before adding phage-scFvs. Holo-Tf binding was tested using either human holo-Tf conjugated to fluorescein (FITC; Invitrogen; 500 nmol/L) or biotinylated mouse Tf (Rockland; 1 μmol/L) and streptavidin coupled to PE (BD). For competition experiments, cells were preincubated for 1 hour with competitors before adding Tf. Direct antibody fragment binding to cells was detected using 50 μg/mL of antibody followed by an anti-(His)6 tag mAb (Qiagen) and PE-conjugated goat anti-mouse immunoglobulins. Cells were analyzed with a FACScalibur (BD) using CellQuest Pro Software for mean fluorescence intensity (MFI) calculation.

Determination of cell holo-Tf uptake

Holo-Tf-FITC uptake by Raji cells (2 × 10^5 in 100 μL) was evaluated by incubation of the cells with 500 nmol/L holo-Tf-FITC for 3 hours at 37°C. Fluorescence was measured by FACS. Alternatively, to prevent endocytosis, cells were fixed with 4% paraformaldehyde before holo-Tf-FITC addition and/or incubated at 4°C. As expected, fixation or incubation at 4°C strongly reduced the MFI, showing that, in this assay, part of the signal represented the accumulation of intracellular holo-Tf (see Results). For competition experiments, cells were preincubated for 1 hour at 37°C with various concentrations of scFvs before adding holo-Tf-FITC.

Analysis of TfR cell surface levels

Cells (2 × 10^5) were saturated at 4°C for 15 minutes in FACS buffer containing goat serum. Surface TfR was stained with rabbit anti-TfR immunoglobulins (Santa Cruz) followed by goat anti-rabbit immunoglobulins conjugated to FITC (Pierce). Alternatively, cells were washed in cold PBS and then incubated for 10 minutes (4°C) with glycine buffer [50 mmol/L glycine (pH 2.8), 500 mmol/L NaCl] to strip cell surface, washed again with PBS, and labeled using holo-Tf-FITC as described above. Fluorescence was measured by FACS.

Quantitative analysis of cell viability

Cells (5 × 10^5–10^6) were plated in six replicate in 96-well plates. Antibody solutions diluted in culture medium were added to the wells. When nontreated cells reached confluence (3–9 d), cell viability was estimated using the CellTiter

**Materials and Methods**

**Cell culture**

Adherent and suspension cells (obtained from American Type Culture Collection, except ERY-1 cells, which were a gift from Prof. Michel Arock, Laboratoire de biologie et Pharmacologie Appliquée, École Normale Supérieure de Cachan, Cachan, France; ref. 21) were grown respectively in DMEM and RPMI containing 10% fetal bovine serum (BD Biosciences, San Jose, CA; ref. 22). Phage-scFvs and soluble antibody fragment expression using pSyn-Cys-His6 (a generous gift of Bin Liu, University of California, San Francisco, San Francisco, CA; ref. 23) plasmids. Antibody fragments were expressed and purified from E. coli periplasm by Ni-NTA affinity chromatography and gel filtration as described elsewhere (24). Phage-scFv F5 (24), specific for ErbB2, was used as a control. For the expression of soluble antibody fragments, scFv cDNA was subcloned from phagemid pHEN into pUC119mycHis (25) or pSyn-Cys-His6 (ge for ErbB2, was used as a control. For the expression of soluble antibody fragments, scFv cDNA was subcloned from phagemid pHEN into pUC119mycHis (25) or pSyn-Cys-His6 (26) precipitated using polyethylene glycol-NaCl precipitation and filtered (0.22 μm) just before use. A scFv binding botulinum neurotoxin (scFv Bot; ref. 27), produced in the same conditions, was used as an irrelevant antibody fragment.

**Phage-scFvs and soluble antibody fragment preparation**

Phage-scFvs, expressed from phHEN-1 phagemids (22), were prepared using polyethylene glycol-NaCl precipitation and titrated as described elsewhere (23). Phage-scFv F5 (24), specific for ErbB2, was used as a control. For the expression of soluble antibody fragments, scFv cDNA was subcloned from phagemid pHEN into pUC119mycHis (25) or pSyn-Cys-His6 (a generous gift of Bin Liu, University of California, San Francisco, San Francisco, CA; ref. 26) plasmids. Antibody fragments were expressed and purified from E. coli periplasm by Ni-NTA affinity chromatography and gel filtration as described elsewhere (25). Monomer (expression using pUC119mycHis) and homodimer (scFv-CH2)2 (expression using pSyn-Cys-His6) solutions were kept at 4°C and sterile filtered (0.22 μm) just before use. A scFv binding botulinum neurotoxin (scFv Bot; ref. 27), produced in the same conditions, was used as an irrelevant antibody fragment.

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96 AQueous cell proliferation assay (Promega). Data are represented using antibody mass concentration for direct comparison of monovalent versus bivalent format activity, as identical mass concentration corresponds to the same paratope molar-ity (e.g., 10 μg/mL for 400 nmol/L) for both formats. In some assays, wells were supplemented with 25 μmol/L of ferric ammonium citrate (FAC) as a source of soluble iron or ZnSO4 as a nonspecific metal cation control (Sigma).

**Western blot analysis**

Cells were washed with cold PBS and lysed in a hypertonic lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, 10 mmol/L EDTA (pH 8), 1 mmol/L DTT, 1% NP40] supplemented with protease inhibitor cocktail (Roche Diagnostics). Lysates were incubated for 20 minutes on ice and cleared by centrifugation at 14,000 × g for 10 minutes at 4°C. Fresh lysates were quantified with BCA Assay Protein Quantitation Kit (Interchim). Twenty micrograms of proteins were combined in reducing loading buffer [2 mol/L Tris (pH 6.7), 4% SDS, 20% β-mercaptoethanol, 40% glycerol, bromophenol blue], resolved by SDS-PAGE, and transferred onto a nitrocellulose membrane (Amersham Bioscience). TfR was detected with an anti-human TfR mAb (Zymed), revealed with horseradish peroxidase–conjugated secondary antibodies, and visualized by enhanced chemiluminescence (GE Healthcare). For normalization, membranes were reprobed with goat anti-actin immunoglobulins or mouse anti-tubulin mAb (Santa Cruz).

**Apoptosis assays**

Cell death–associated changes were analyzed by FACS to follow changes in cell granularity and cell size by measuring forward scatter and side scatter parameters, respectively. Annexin V-FITC (Immunotools) was used for the assessment of phosphatidylserine (PS) exposure combined with propidium iodide (PI; 1 μg/mL) for the determination of cell viability. Apoptosis induction through mitochondrial membrane depolarization was also investigated with the lipophilic cation 3,3-dihexyloxacarbocyanine iodide [DiOC6(3); 40 nmol/L].

**In vivo experiments**

Athymic nu/nu female mice, 8 weeks old, from the facilities of the Institut Gustave Roussy, were irradiated at 4 Gy and injected s.c. with ERY-1 (2 × 10⁶) diluted in 100 μL of PBS. Tumor volume was determined with the formula.
vol = (π/6) × [(length + width)/2]^3. When tumors reached a mean diameter <300 mm^3, mice were randomized five per group before injecting antibody (200 μg) or PBS i.p., twice a week, on the opposite side of the tumor. Tumor growth was measured with calipers and monitored biweekly. At day 25, tumors were excised and fixed before hematein-eosin-safran (HES) staining. Data were analyzed with a Mann-Whitney test. Differences were considered significant when P < 0.05.

**Results**

We previously reported the isolation of tumor-specific human antibodies generated by selecting a nonimmune human phage antibody library for internalization into live SKBR3 breast carcinoma cells (19, 24). Following this process, we obtained a panel of six distinct TfR specific scFvs, namely, 3TF2, 3TF12, 3TG9, 3GH9, 3GH7, and C3.2, which were characterized as TfR specific based on their ability to immunoprecipitate human TfR (20).

**Anti-TfR antibodies and holo-Tf bind overlapping sites on TfR**

Because these antibodies had been isolated on the basis of their rapid endocytosis by cancer cells, we hypothesized that some of them were holo-Tf mimetics, binding epitopes identical to, or overlapping with, the natural TfR ligand holo-Tf. The colon carcinoma ErbB2-positive cell line LS174T, which expresses high levels of TfR (20), was used to design a competitive binding assay between phage-scFvs and human holo-Tf (Fig. 1A). Indeed, all six anti-TfR phage-scFvs binding to LS174T cells were inhibited in a concentration-dependent manner by cell preincubation with increasing concentrations of holo-Tf (IC_{50} <10 nmol/L). As a control, the binding of anti-ErbB2 phage-scFv F5 was not affected by holo-Tf. For further analysis, (His)_6-tagged soluble scFvs were expressed in *E. coli* and affinity purified by Ni-NTA chromatography, followed by gel filtration. The gel filtration elution profile showed that the scFvs essentially exist as 28-kDa molecular species that correspond to scFv monomers (Fig. 1B and data not shown). Each of the monomeric soluble scFvs inhibited the binding of all six anti-TfR phage-scFvs (but not of control phage F5; Fig. 1C) and of human holo-Tf (Fig. 1D) to LS174T cells. Thus, selection of anti-TfR scFvs based on rapid internalization into cells generated six distinct scFvs with overlapping paratopes competing for natural ligand holo-Tf binding to TfR. Based on this inhibition, we hypothesized that the scFvs would also inhibit holo-Tf entry into cells and, therefore, cell iron supply and proliferation of cancer cells.

**Anti-TfR scFvs inhibit the growth of human hematopoietic cancer cell lines**

The proliferation of various cancer cell lines in the presence of antibodies (10 μg/mL) was compared with non-treated cells using an MTT assay. None of the scFvs had antiproliferative activity on SKBR3 (the cell line initially used to isolate these antibodies), LS174T, and MCF7 adenocarci-
noma cell lines (not shown). Nevertheless, anti-TfR scFvs 3TF12 and 3GH7 induced slight but reproducible inhibition on the T-lymphoma cell line Jurkat whereas the other anti-TfR scFvs and an irrelevant scFv Bot had no effect (Fig. 2A). This antiproliferative effect could be related to the better inhibition of holo-Tf binding by 3TF12 and 3GH7 compared with the other scFvs (Fig. 1D). Because anti-TfR antibodies display a greater antigrowth activity on tumor cells of hematopoietic origin than on carcinoma cells (3, 28), 3TF12 and 3GH7 scFvs were further tested on other lymphoma or...
leukemia cell lines including U937 (histiocytic lymphoma), Raji (Burkit lymphoma), HL60 (promyelocytic leukemia), and K562 and ERY-1 (erythroleukemia). The greatest antiproliferative activity (75% inhibition) was obtained on Raji and ERY-1 cell lines (Fig. 2B). The degree of inhibition was independent of TfR surface levels (Fig. 2C) because, for instance, HL60 and U937 cells display similar TfR levels as Raji cells, with lower inhibition by the antibodies.

Conversion into a bivalent format improves the antiproliferative effects of 3TF12 and 3GH7

We next tested whether the inhibitory potential of the monomeric scFvs could be improved by conversion into homodimeric bivalent formats with increased avidity for TfR. Monovalent scFv 3TF12 and 3GH7 were engineered into 55-kDa bivalent formats (named F12CH and H7CH, respectively) by adding a COOH-terminal cysteine (29). SDS-PAGE analysis of purified 55-kDa molecular species (Fig. 3A) showed that they corresponded mainly to covalently linked homodimers. As expected, F12CH and H7CH showed a stronger ability to inhibit holo-Tf binding to TfR than their monovalent counterparts (Fig. 3B). We tested next if the increase of valency would influence the antiproliferative effect of the antibodies on different hematopoietic cancer cell lines. Similar to the monomeric scFvs (Fig. 2B), the strongest and fastest effect was obtained on ERY-1 and Raji cells, with all cell lines showing increased sensitivity with bivalent formats (Fig. 3C). Then, to compare quantitatively the antiproliferative activity of bivalent and monovalent formats, dose-dependent growth inhibition curves were established on ERY-1 cells (Fig. 3D). IC\textsubscript{50} values of 0.33 and 4.86 μg/mL were obtained for 3TF12 or 3GH7, and IC\textsubscript{50} values of 0.1 and 0.09 μg/mL for F12CH or H7CH. Thus, a 3- to 50-fold reduction in IC\textsubscript{50} value was achieved by changing antibody format.

F12CH and H7CH bivalent scFvs induce Raji and ERY-1 cell death

The mechanisms responsible for the decrease in cell viability induced by F12CH and H7CH antibodies were investigated on the most sensitive cell lines, Raji and ERY-1, by exploring membrane modifications. For Raji cells (Fig. 4A), antibodies induced detectable cell death with externalization of PS (>33% of Annexin V\textsuperscript{+} cells), but mitochondrial membrane potential did not collapse. Notably, cell granularity increased significantly without a decrease in cell size. Large vacuoles, a hallmark of autophagy, were visualized by light microscopy (not shown), suggesting possibly an autophagy-related cell death. For ERY-1 cells, antibodies induced cell size decrease, externalization of PS (>75% of Annexin V\textsuperscript{+} cells), and mitochondrial membrane depolarization (Fig. 4B) consistent with an apoptotic cell death.

F12CH and H7CH induce intracellular iron depletion responsible for cell death

We next studied whether F12CH- and H7CH-induced Raji cell death was related to iron deprivation. We first assessed, by FACS analysis, the effect of both anti-TfR antibodies on holo-Tf cell uptake (Fig. 5A). Interestingly, for both antibodies, an almost complete inhibition of holo-Tf uptake was obtained with a 2 μg/mL dose, with a significant reduction being observed with a 0.2 μg/mL dose. We concluded that inhibition of holo-Tf binding to TfR (seen Fig. 3B) was accompanied by inhibition of holo-Tf uptake, and consequently, the antibodies may block the physiologic ferric iron delivery into the cells. Because intracellular iron concentration regulates TfR expression, we next investigated TfR expression levels in antibody-treated cells. Both surface and total TfR levels were increased in cells treated with F12CH or H7CH for 4 days compared with untreated cells or irrelevant
scFv–treated cells (Fig. 5B). Thus, as iron chelator desferrioxamine does (30, 31), F12CH and H7CH decrease intracellular iron levels leading to increased TfR expression. Interestingly, F12CH-induced TfR overexpression was reversed when the culture medium was supplemented with FAC as a source for soluble iron (Fig. 5C, left). Finally, to show unequivocally the role of iron depletion in cell death, cells were treated with the antibodies in the presence of metal supplements. Figure 5C (right) showed that FAC, but not ZnSO4, reversed F12CH and H7CH cytotoxicity. Altogether, these data show that in Raji cells, F12CH and H7CH anti-TfR bivalent antibodies are cytotoxic through intracellular iron deprivation.

F12CH inhibits tumor growth in an erythroleukemia xenograft mouse model

To evaluate the efficacy of one of the anti-TfR antibodies in vivo, a xenograft mouse model was developed by s.c. injection of the ERY-1 cell line into nude mice. When the tumor reached about 200 mm³, mice received twice-per-week i.p. injections of F12CH antibody, irrelevant scFv Bot, or vehicle (PBS) on the opposite side of the tumor. Figure 6A showed that F12CH induced a significant inhibition of tumor growth at days 18 and 22 after treatment (P < 0.01 and P < 0.005, respectively). No weight loss was observed in the mice during the experiment. HES staining done on tumors sampled on day 25 showed fibrosis and prenecrotic cells in F12CH-treated mice as opposed to vehicle- or scFv Bot–treated mice (Fig. 6B). The effect observed on tumor growth in this model is physiologically relevant as we showed that mouse Tf cross-reacts with human TfR (Fig. 6C), allowing iron supply to implanted tumor cells, and that antibody F12CH cross-reacts with mouse TfR (Fig. 6D) expressed on some host cells. Therefore, this xenograft model recapitulates, in part, interactions that would occur following F12CH antibody administration in humans.

Discussion

In this work, we characterized six human scFvs specific for the human TfR, previously isolated from a phage display library (20). Because the library had been selected for binding
and internalization into living cells, we hypothesized that many of these scFvs would function as ligand mimetics. All six antibodies, in a scFv monovalent format, interfered with natural ligand holo-Tf binding to TfR with varying efficiencies. The two scFvs with the strongest inhibition of holo-Tf binding to TfR also showed the greatest growth inhibition in vitro (Fig. 2A). Interestingly, these two scFvs, 3TF12 and 3GH7, represented respectively 20% and 18% of the scFvs recovered in the phage antibody library selection. The other four (3TF2, 3TG9, 3GH9, and C3.2) represented only 2% each. This highlights the effectiveness of functional cell-based antibody phage display selection for the isolation of highly functional binders compared with more conventional existing methods to obtain mAbs, particularly hybridoma technology using recombinant antigens.

As mentioned before, previous cytotoxic mAbs binding TfR were all multivalent. Interestingly, here, in vitro inhibition of proliferation was obtained with monovalent antibody formats. Monovalent 3TF12 and 3GH7 IC_{50}s for growth inhibition on ERY-1 cells were estimated to be 0.35 and 4.86 μg/mL, respectively. Previous reports of IC_{50}s for TfR binding antibodies include dimeric IgA-42/6 (4 paratopes) IC_{50} on colony forming unit-granulocytes-macrophages of 5 μg/mL (32); monoclonal avidin-fused IgG3 (4 paratopes) IC_{50} on K562 cells of 20 μg/mL (12); and IgG-A24 (2 paratopes) IC_{50} on activated peripheral blood mononuclear cells of 0.5 μg/mL (17). When monovalent scFvs were converted to bivalent antibody formats, in vitro growth inhibition activity was increased, with IC_{50}s on ERY-1 cells reaching 0.1 μg/mL for both F12CH and H7CH. Therefore, F12CH and H7CH cytotoxicity compares similarly with murine IgG-A24, the most active anti-TfR antibody described to date, which has cytotoxic activity on samples of acute and chronic forms of adult T-cell leukemia ex vivo (17) and on a human xenograft model of mantle lymphoma in mice (15) as a naked antibody.

The F12CH and H7CH mechanism of growth inhibition was investigated extensively in vitro. The heterogeneity of cell

Figure 6. F12CH-mediated inhibition of tumor xenografts. A, nude mice were injected s.c. with 2 × 10^6 ERY-1 cells. When tumors reached a volume of about 200 mm^3 (day 0), mice were injected i.p. twice a week (days of injection are indicated by black arrows) with either 200 μg of anti-TfR (F12CH) or control (scFv Bot) antibody, or with vehicle (PBS). One of two experiments is shown; mean (n = 5 mice) ± SD. *, P < 0.01; **, P < 0.005, versus PBS-injected mice. B, representative HES staining of tumors at day 25 of treatment (magnifications, ×50 and ×200). C, cross-reactivity of mouse Tf to human TfR. Mouse FMA3 (a) and human ERY-1 (b) cells were incubated with biotinylated mouse Tf (1 μmol/L) for 1 h at 4°C and bound holo-Tf was detected with streptavidin-PE (thick line). Control experiments were done with only streptavidin-PE (shaded) or with preincubation with 0.1 μmol/L human Tf (dashed line). Fluorescence was measured by FACS. D, cross-reactivity of F12CH to mouse TfR. FMA3 cells were incubated with F12CH (50 μg/mL) for an hour at 4°C (thick line). Antibody binding was detected with an anti-(His)₆ tag mAb followed by PE-conjugated anti-mouse immunoglobulins. Control experiment was done with irrelevant scFv (Bot; dark). Fluorescence was measured by FACS.
line sensitivity was not related to TR levels and therefore probably due to different iron level requirements and/or iron uptake mechanisms. Antibodies induced cell death by a cell line-specific mechanism with clear mitochondrial-dependent apoptosis for ERY-1 and possibly autophagy-related cell death for Raji cells, as large granular vacuoles could be observed on treated cells. For both cell types, the antibodies inhibited holo-Tf cell uptake, and cell death was directly linked to iron deprivation because soluble iron supplementation reversed cytotoxicity (Fig. 5A and C).6

These fully human antibodies represent therefore a promising novel type of anti-TR cytotoxic antibodies for therapy of tumors that are iron dependent for sustained proliferation because, unlike a number of inhibitory anti-TR antibodies previously described (9, 15, 18), they induce an increase (as opposed to a decrease) of cell surface TR and they are cytotoxic in a monovalent format (Fig. 2B; as opposed to bivalent or multivalent format). Their inhibitory mechanism seems to be solely due to their competitive antagonism with the natural ligand. Antibody binding induces rapid antibody-TR internalization,9 and antibody/TR complexes may then be recycled to the cell surface following the same route as with apo-Tf/TR complexes while preventing de novo binding of plasma holo-Tf. Because concentrations of 0.2 μg/mL of the antibodies (which corresponds to about 4 nmol/L) are able to inhibit 50% of holo-Tf binding when holo-Tf is present at 500 nmol/L in vitro (Fig. 3B), it should be possible to obtain efficient inhibition of holo-Tf tumor uptake in vivo with plasma antibody concentrations compatible with clinical applications (about 10 μg/mL) despite the high physiologic plasma concentrations of endogenous holo-Tf (about 10 μmol/L). Indeed, we obtained an antitumor effect with antibody F12CH in a xenograft mouse model. Considering the cross-reactivities of F12CH for host TR and of host Tf for human TR on human transplanted ERY-1 cells, this animal model should be suitable for studying the toxicity and side effects of the antibody. The biodistribution of different mAb formats in various xenograft models shows that bivalent 55-kDa formats have a faster blood clearance than complete 150-kDa IgG (33, 34). By changing the antibody format into complete IgG, the combination of serum half-life increase and recovery of antibody effector functions could improve the antitumor response (35). Finally, 3TF12 and 3GH7 could be readily translated to the clinic because the antibody variable regions are fully human, and we anticipate that it might be an advantage to have two antibodies available with the same functional effect to avoid resistance mechanism consecutive to TR epitope point mutations (11).

Disclosure of Potential Conflicts of Interest

The authors disclosed no potential conflicts of interest.

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