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ORIGINAL ARTICLE

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Production and validation of the pharmacokinetics of a single-chain Fv fragment of the MGR6 antibody for targeting of tumors expressing HER-2

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Abstract The HER-2 antigen, which is overexpressed in many breast carcinomas, is an ideal target for monoclonal antibodies due to its low expression in normal tissue and its homogeneous distribution in the tumor mass. We have developed and characterized the murine MAb MGR6 against HER-2, which is able to inhibit proliferation of tumor cells overexpressing HER-2. On the basis of these preclinical results, phase I studies in breast carcinoma patients were conducted and radiolocalization data indicated an antibody half life which directly paralleled that of other whole antibodies and thus resulting in a limited in vivo diagnostic capacity. To obtain a smaller reagent with possibly improved in vivo properties, a single chain variable fragment (scFv) of the original MGR6-producing hybridoma was generated by phage display technology. Biologically active MGR6 scFv was purified rapidly and at high yield by metal affinity chromatography. Competition FACS and ELISA analyses identified an epitope on the HER-2 extracellular domain that was shared by the scFv and the parental MAb. BIAcore analysis indicated a K_{off} of 9.3×10^{-4} s^{-1}, similar to that of the intact MGR6 MAb. Distribution and elimination half-lives of MGR6 scFv, calculated from in vivo preclinical evaluations, were much faster (13 min and 6.2 h, respectively) than previously published results for the intact MAb (mean t1/2β of 46 h). This represents a theoretical improvement in pharmacokinetics with respect to the parental murine MAb and points to the potential for utilizing this fragment in redirecting therapeutic agents, such as radioisotopes, to different human carcinomas overexpressing HER-2.

Key words Single-chain Fv · HER-2 · Phage display

Abbreviations *MAb* Monoclonal antibody · *HAMA* human anti-mouse antibody · *RIT* radioimmunotargeting · *ECD* extracellular domain · *scFv* single-chain variable fragment · *RU* resonance units

Introduction

HER-2, the phospho-glycoprotein product of the c-erbB-2 gene, is a 185-kDa transmembrane tyrosine kinase of the epidermal growth factor receptor family [10, 18]. It is overexpressed in approximately 30% of human adenocarcinomas and correlated with poor prognosis. To date, no specific ligand has been clearly identified, yet this tyrosine kinase evidently appears to be associated with both the enhancement of malignancy and the metastatic phenotype. HER-2 is expressed homogeneously throughout the tumor mass, whereas expression in both normal tissue and in soluble form is very low. These characteristics make HER-2 an ideal target for monoclonal antibody (MAb)-based therapy and diagnosis.

The use of MAbs in tumor therapy and diagnostics as a means to target tumor-associated antigens has rarely lived up to expectations, and attempts to improve the diagnostic and therapeutic capacities of antibodies have met with variable success [9, 14, 30, 40]. A major limiting factor is the accessibility of macromolecules to tumor cells at advanced stages of cancer, i.e., MAbs are generally too large (M_r 150,000) to penetrate sizable tumor masses. Moreover, most MAbs are of murine origin, so

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that repeated administration can induce human anti-mouse antibodies (HAMA), which can severely limit the efficacy of antibody therapy [20]. A large percentage of the HAMA response is directed against the Fc domain, which harbors the more immunogenic region of the immunoglobulin and can also contribute to a heightened induction of anti-idiotypic responses.

Increasing interest has focused on the in vivo pharmacokinetics, biodistribution and clearance rates of smaller antibody fragments such as scFv, in which the Fc portion is removed yet affinity is retained. The main advantage of scFv over intact antibody or Fab fragments is their small size (M_r 30,000), which correlates directly with their increased capacity to penetrate a solid tumor mass rapidly and evenly, even in areas far from blood vessels [7]. In addition, the lack of Fc domains in scFv make them intrinsically less immunogenic and less likely to induce anti-idiotypic responses than intact MAbs, Fab′ and $F(ab')_2$. scFv have proven superior as guiding agents for radioimmunotherapy (RIT), at least in xenograft preclinical models [8, 29].

We have developed and characterized the murine MAb MGR6 against HER-2, which partially cross-reacts with the well-known MAb 4D5, from which the therapeutic agent Herceptin was derived [1]. Like Herceptin, MGR6 inhibits the proliferation of HER-2-overexpressing tumor cells in preclinical models both in vitro and in vivo. On the basis of these preclinical results, phase I studies of toxicity and localization were conducted in breast cancer patients. Data concerning toxicity and immunogenicity have been reported elsewhere [38], while radiolocalization indicated delayed blood clearance with a mean distribution half-life of 46 h (range 13–62 h). Due to this elevated blood activity, the efficacy of tumor imaging was limited; in fact, only peripheral lesions (i.e., iliac wings and limbs) were clearly identified in positive patients, while lesions in the chest (i.e., ribs and vertebrae) were not detected. Furthermore, accumulation of radiolabeled MAb was observed in some lesions of patients with HER-2-negative tumors (manuscript in preparation).

In order to obtain a smaller reagent with improved in vivo properties, we generated a scFv by phage display technology using the original MGR6-producing hybridoma. Like the parental MAb, the MGR6 scFv recognized an epitope on the extracellular domain (ECD) of the human tumor-associated antigen HER-2. MGR6 scFv was readily purified at relevant yields, and its K_{off} closely paralleled that of the parental MAb.

Materials and methods

Reagents

The following murine MAbs were used: MGR6 (IgG2a) directed against the ECD of HER-2 [4], IdM6.4 (IgG1) directed against the idiotype of MGR6 MAb [38], and 9E10 (IgG1) directed against the C-terminal myc tag peptide [25]. MAbs were affinity-purified from hybridoma supernatants on protein A–Sepharose Cl-4B columns (Pharmacia Biotech). The 9E10 MAb was insolubilized on a CNBr Sepharose Cl-4B column as described [13]. The anti-lysozyme scFv D1.3, produced and purified as described [11], was used as an unrelated negative control.

HER-2 ECD, produced in baculovirus as an 81-kDa recombinant molecule, was kindly provided in purified form by Dr. M. Jeschke (Friedrich Miescher Institute, Basel, Switzerland) [37].

Cell lines

Eight human cell lines were used: four were HER-2-positive (SKOV3 ovary carcinoma, CALU3 lung carcinoma, MDA-MB361 and SKBR3 breast carcinoma, provided by the American Type Culture Collection, ATCC) and four HER-2-negative (A431 epidermoid carcinoma and OVCAR3 from ATCC, IGROV1 ovarian carcinoma, a gift from Dr. J. Benard, and Mewo melanoma, kindly provided by Dr. J. Fogh). Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were routinely tested for mycoplasma contamination and were consistently found negative.

Cloning the Ig variable domain of MAb MGR6

The V-genes of MAb MGR6 were reverse-transcribed, amplified and assembled to encode scFv fragments using the polymerase chain reaction essentially as described [6], but using the Recombinant Phage Antibody System (RAPAS, Pharmacia Biotech) according to the manufacturer's instructions. The assembled scFv was cloned into the phagemid pHEN1 [16].

Preparation of phage displaying scFv

Escherichia coli TG1 cells containing the pHEN-MGR6 plasmid were grown at 37 °C with shaking in 50 ml 2 × TY broth [23], 100 μg/ml ampicillin, 1% glucose. At $O.D._{600}$ of 0.5, the culture was infected with 10^9 transforming units (t.u.)/ml of M13KO7 helper phage and incubated for 30 min at 37 °C without shaking and for 30 min at 37 °C with shaking. After centrifugation at 2500*g* for 10 min, cells were resuspended in 500 ml 2 × TY broth, 100 μg/ml ampicillin, 25 μg/ml kanamycin and grown at 30 °C for 16 h with shaking. Phages were precipitated from the culture supernatant with polyethylene glycol as described [22] and resuspended in 5 ml phosphate-buffered saline (PBS) (giving $\sim 10^{12}$ t.u. phage/ml).

Selection of functional scFv

Selection was performed on a monolayer of SKOV3 cells in 100-mm Petri dishes as described [13]. To determine the correct and functional combination of VH-VL, individual phage clones displaying scFv were assayed for binding by phage ELISA on a panel of cells, performed essentially as described [6] but using horseradish peroxidase (HRP)-conjugated anti-M13 Ab (Pharmacia Biotech). For phage ELISA, cells were seeded and grown in 96-well microtiter plates and grown to confluence. Cells were fixed with 0.1% glutaraldehyde and saturated for 2 h with PBS, 2% Marvel (MPBS) at 37 °C before commencing the ELISA.

FACS analysis

Phage binding was analyzed by FACS as described [13]. FACS analysis of soluble scFv was performed on a panel of HER-2-positive cells but detected with 10 μg/ml of mouse MAb 9E10 followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ab (Sigma). Epitope specificity was assessed by competition FACS analysis, in which the selected biotinylated scFv (final concentration 150 nM) was mixed with serial dilutions of purified MAb MGR6 in 100 μl of PBS, 1% bovine serum albumin and

incubated for 1 h at room temperature. Binding was detected by FITC-streptavidin (Amersham).

DNA sequencing

Nucleic acid sequences of selected V-regions were determined by the dideoxy chain termination method [33] using the Sequenase kit (USB). scFv variable regions were sequenced with the primers S1, S3, S4 and S6, supplied in the RAPAS kit (Pharmacia).

Subcloning, expression and purification

Soluble scFv fragments from pHEN-MGR6 were produced as described above by inoculating bacterial cells bearing the plasmid into 2 × TY, 100 μg/ml ampicillin, 0.1% glucose and induction with 1 mM isopropyl-b-D-thiogalactoside as described [11]. After induction, the culture was shaken overnight at 30 °C and scFv fragments harvested from the periplasm essentially as described [2]. scFv fragments containing the myc tag were purified by affinity chromatography on immobilized 9E10 MAb.

To facilitate purification, the MGR6 scFv gene was subcloned into the expression vector pUC119*Sfi*I-*Not*I-Hismyc [15], which resulted in the addition of a hexa-histidine tag at the C-terminal end of the scFv gene. The pHEN1 vector containing the MGR6 scFv was prepared using a Wizard miniprep kit (Promega), digested with *Sfi*I and *Not*I, and fragments purified on a 1.5% agarose gel. Fragments were ligated into pUC119*Sfi*I-*Not*I-Hismyc digested with *Sfi*I and *Not*I and the ligation mixture used to transform electrocompetent *E. coli* TG1 cells.

scFv fragments were purified by immobilized metal affinity chromatography as described [2]. Monomeric and dimeric forms of the scFv were separated by gel filtration chromatography on a Superdex 75 HR 10/30 column (Pharmacia) using high-pressure liquid chromatography (HPLC, Bio-Rad). Coomassie-stained SDS-PAGE assessed purity of protein preparations.

Biotinylation was carried out using a kit (Pierce) according to the manufacturer's suggested procedure.

Western blot analysis

Cell lysates and blots were prepared as described [24]. MGR6 scFv was detected using the supernatant of MAb 9E10 at a 1:100 dilution or 10 μg/ml of purified MAb IdM6.4. An HRP-conjugated anti-mouse antibody (1:2000 dilution) served to visualize immunoreactive proteins using enhanced chemiluminescence (ECL, Amersham).

Kinetics analysis

The kinetic analysis was performed by plasmon resonance with BIAcore equipment (Pharmacia).

HER-2 ECD was covalently bound to a CM5 sensor chip using the amine coupling kit (Pharmacia) at an antigen concentration of 20 μg/ml in 10 mM sodium acetate, pH 5.2. The apparent kinetic dissociation rate constants (K_{off}) were calculated under saturating conditions of antibody from 200 nM to 1 μM, with a 5 μl/min or 10 μl/min flow buffer rates. Evaluation was performed for at least 30 min. Irrelevant antigens were tested in BIAcore to assess the specificity of the analysis system. Both the natural and recombinant antibodies were unable to bind any antigens except the ECD of HER-2. Subsequently, detachment of residual antibody bound to the sensor chip was performed with 2 M $MgCl_2$.

In vivo preclinical evaluation

Intact MAb MGR6 was labeled with ^{125}I by the lactoperoxidase-catalyzed reaction, and both myc- and myc-His tag containing scFv were ^{125}I-labeled by the lactoperoxidase method or Bolton-Hunter reagent [21]. The final specific activity ranged, respectively, from 296 to 480 MBq/mg and from 37 to 148 MBq/mg.

Experiments were performed on 6- to 8-week-old female Balb/c mice (Charles River, Calco, Como, Italy). The animals were maintained and treated according to the provisions of the European Economic Community Council adopted by the Italian government. Treatments were approved by the institutional ethics committee for animals. Mice received a Lugol solution (0.02% I_2 and 0.6 mg/ml $KClO_4$) in their drinking water from 3 days before radiolabeled scFv administration and throughout the experiments to block free iodine uptake by the thyroid gland and the stomach mucosa.

Groups of three mice received a single i.v. bolus injection of ^{125}I-scFv (0.5 μg/mouse). Blood samples were collected at fixed times after injection and animals were killed at 1, 2, 5 and 24 h post-injection. Major tissues were obtained at necropsy, weighed and counted in a gamma counter. Pharmacokinetic analysis was carried out on serum samples as described above.

Results

Cloning the MGR6 scFv

Standard cloning and sequencing procedures detected at least two different VH and five VL chains in the original MGR6 hybridoma (data not shown). Thus, the phage display methodology was used to select for a single functional variable domain. The V-genes of MAb MGR6 were cloned into the vector pHEN1 for expression as scFv in the VH-linker-VL configuration, fused to pIII on the surface of filamentous phage. To select for only functional rearrangements of antibody V-genes, independent clones were pooled and phagemid particles both rescued and selected on a panel of cell lines with known HER-2 levels. After a single round of selection, 20 of 96 tested clones bound to SKOV3 target cells in phage ELISA. Sequence analysis of five of these clones revealed the same VH and Vk sequences. The MGR6 VH and Vk gene segments are members of the Kabat MISC family, subgroups IA and V respectively. Figure 1 shows the deduced protein sequence of MGR6 scFv.

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                                                    CDR1
AAQPAMAQVKLQQSGAELVKPGASVKLSCTASGFNIKDTYMHWVKQRPEE
          CDR2
GLEWIGRIDPANGNTKYDPKFQGKATITADTSSNTAYLQLSSLTSEDTAV
      CDR3                LINKER
YYCARGEYDYPFPYWGQGTSVTVSSGGGGSGGGGSGGGGSYIELTQSPAS
                  CDR1                                CDR2
LAVSLGQRATISCRASQSVSTSRYSYMHWYQQKPGQPPKLLIKYASNLES
                                        CDR3
GVPARFSGSGSGTDFTLNIHPVEEEDTATYYCQHSWEIPRTFGGGTKLEL

KRAA
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Fig. 1 The protein sequence of the MGR6 scFv gene, deduced from the sequence obtained by the dideoxy chain termination method. Five clones positive for binding to HER-2-overexpressing SKOV3 cells in phage ELISA were sequenced and found all to contain identical VH and Vk sequences belonging to the Kabat MISC family. A flexible synthetic linker [*underlined*, $(G_4S)_3$] inserted between the VH and the Vk sequences allowed for functionality. The final protein had an estimated molecular mass of 30 kDa and a pI of 7.63

ELISA analysis of phage-bound scFv revealed specific binding to HER-2-positive cells at levels that directly paralleled known HER-2 expression. No binding was evident in unrelated cell lines that do not express HER-2 (Fig. 2).

Purification and characterization of soluble MGR6 scFv

Soluble scFv was expressed as a recombinant fusion protein with either a C-terminal myc tag or His-myc tag, harvested from the bacterial periplasm and purified. MGR6 scFv containing a C-terminal myc tag was purified by affinity chromatography on immobilized anti-myc tag MAb 9E10 in yields of 2.7 mg/l of *E. coli* grown overnight in shaker flasks. MGR6 myc-His tag was affinity-purified on a Ni column with a purification yield of 6 mg/l of bacterial culture.

Western blot analysis under non-reducing conditions with both anti-myc tag MAb 9E10 (Fig. 3A) and anti-MGR6 idiotype IdM6.4 MAb (Fig. 3B) indicated the presence of two bands with apparent molecular weights compatible with the monomeric and dimeric forms of the MGR6 scFv. The specific reactivity of our idiotype MAb with both bands and the absence of reactivity to a non-relevant control scFv (D1.3 anti-lysozyme scFv, data not shown), suggests that also upon dimerization, the MGR6 scFv retains its binding capacity.

Soluble forms of both myc and myc-His tag scFv bound to HER-2-positive cells (Fig. 4). Since soluble scFv purified by gel filtration was monovalent, binding capacity appeared to be lower than that of phage surface-bound scFv.

Immunohistochemistry further demonstrated the specific binding of soluble MGR6 scFv to HER-2-positive human tissues (data not shown).

Epitope specificity and kinetic analysis of scFv binding

The epitope recognized by scFv was compared to that recognized by the entire murine IgG molecule. FACS analysis of competition between biotinylated scFv and non-biotinylated intact MAb MGR6 for binding to HER-2-overexpressing SkBr3 cells (Fig. 5) showed that approximately 83% of scFv binding was inhibited when competed against the intact MAb used at a concentration of 650 nM. Binding inhibition of the scFv decreased with decreasing MAb concentrations. At the lowest MAb concentration used (2.5 nM), binding was still inhibited by 18%. At equimolar concentrations of MAb and scFv (150 nM), binding of the biotinylated scFv was inhibited by 75%, a possible indication of the role of valency in inhibition of scFv binding.

We further investigated the antigen-scFv complex formation by kinetic analyses using plasmon resonance. HER-2 antigen was covalently coupled to a BIAcore sensor chip obtaining an antigen density correlating to 4000 resonance units (RU). MGR6 scFv myc tag purified by affinity anti-myc column and gel filtration or entire MAb MGR6 were injected onto the sensor chip. The K_{off} values were not affected by either the antibody

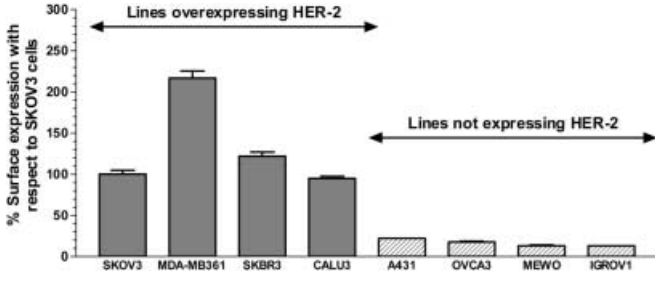

Fig. 2 Binding of phage-bound MGR6 scFv to a panel of human tumor cell lines. Phage-bound MGR6 scFv was incubated with monolayer cells fixed with 0.1% glutaraldehyde. Bound MGR6 scFv was detected by HRP-conjugated anti-M13 antibody. Results were standardized against SKOV3 cells, which were used as a positive control for MGR6 binding. HER-2 expression levels below 20% of SKOV3 HER-2 expression were considered negative

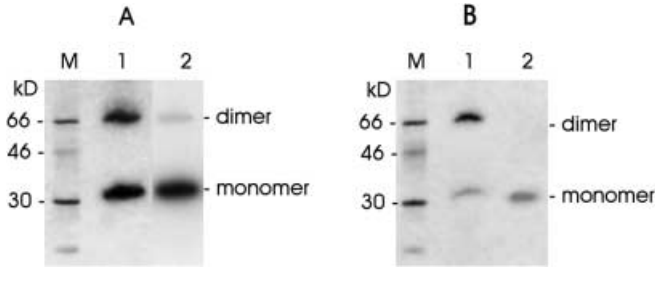

Fig. 3A, B Western analysis of purified myc or myc-His tag MGR6 scFv. Soluble scFv was purified by affinity chromatography on either an anti-myc tag column or by IMAC. Samples were electrophoresed on a 12% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose and immunoblotted with either **A** anti-myc tag MAb 9E10 or **B** anti-Id M6.4 MAb, followed by ^{125}I-labeled anti-mouse antibody

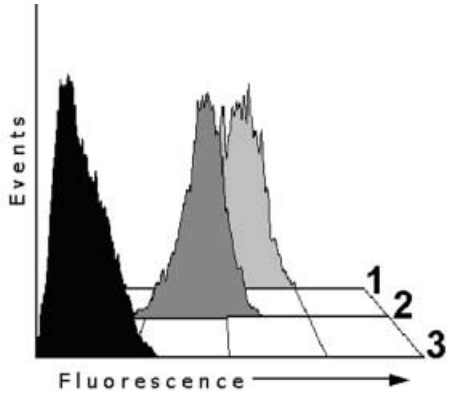

Fig. 4 FACS analysis of MGR6 scFv binding to SkBr3 cells. HER-2-overexpressing SkBr3 cells were incubated with intact MAb MGR6 (*1*) or soluble MRG6 scFv (*2*), followed by anti-mouse-FITC or MAb 9E10 plus anti-sheep-FITC, respectively. Both MGR6 MAb and scFv bound to SkBr3 cells at elevated levels with respect to the negative control (*3*)

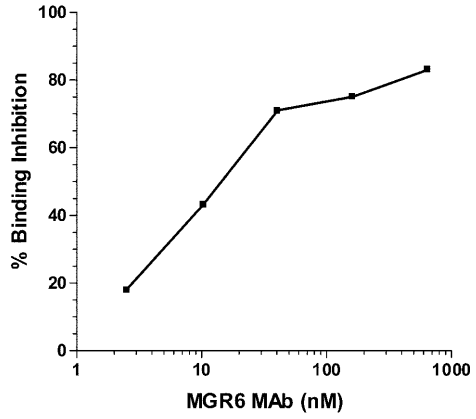

Fig. 5 Competition FACS of biotinylated MGR6 scFv against MAb MGR6. Biotinylated MGR6 scFv (150 nM) competed for binding to SKOV3 cells against the entire non-biotinylated MAb. At 650 nM, scFv binding was inhibited by 83%, whilst at equimolar concentration of MGR6 MAb and scFv (150 nM), inhibition was approximately 75%

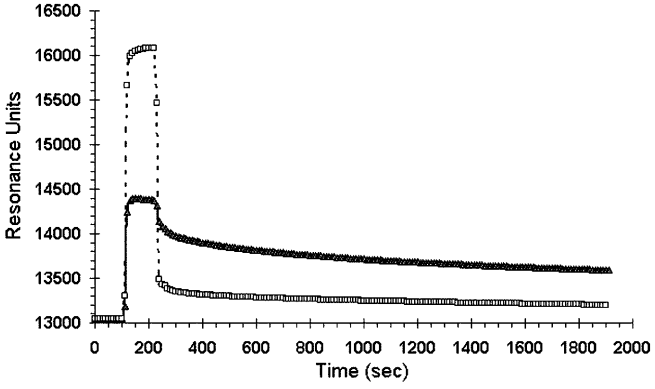

Fig. 6 Comparison of the BIAcore sensorgrams obtained for the binding of MAb MGR6 (▲) and scFv MGR6 (□) on immobilized antigen. The respective K_{off} rates were calculated from these sensorgrams

concentration or the buffer flow rate (data not shown). This suggests the absence of the antibody molecules rebinding to the immobilized antigen during the dissociation phase. The calculated K_{off} (9.3×10^{-4} s^{-1}) of the scFv was similar to that of the intact MGR6 antibody molecule (6.0×10^{-4} s^{-1}) (Fig. 6).

Preclinical in vivo evaluation

SDS-PAGE and live cell binding assays initially evaluated the quality of the labeled antibodies. Under non-reducing conditions, more than 98% of intact MGR6 migrated at approximately 150 kDa, while the scFv migrated at approximately 32 kDa.

In live cell binding assays the immunoreactive fraction of intact ^{125}I-MGR6 MAb ranged from 60% to 80%, while that of ^{125}I-MGR6 scFv labeled by the lactoperoxidase method was less than 10%. In the case of the scFv, immunoreactivity was increased to 50% when the Bolton-Hunter reagent was used for labeling, but yields of labeled product were very low. The latter radiolabeled reagent was used for preclinical studies in vivo.

Table 1 Pharmacokinetic parameters of ^{125}I-labeled MGR6 scFv in Balb/c mice

	MAb	MGR6 scFv
Dose (moles)	1.59×10^{-11}	1.35×10^{-11}
Alpha half-life (min)	225.6	13.4
Beta half-life (h)	144.2	6.2
AUC[a] ($M \times 10^{-4}$ min)	0.29	0.03
Clearance (μl/min)	0.55	44

[a] Area under plasma concentration curve calculated by trapezoidal rule

Plasma concentrations of ^{125}I-MGR6 scFv at each time point were used to construct a pharmacokinetic curve. Table 1 lists the results of data using an open two-compartment pharmacokinetic model. The pharmacokinetic behavior of the ^{125}I-MGR6 entire MAb [12] is presented for comparison.

The elimination curves of the radiolabeled reagents were biphasic, with an initial rapid α-phase (approximately 13.4 min for the scFv) followed by a slower β-phase. Intact MAb MGR6 showed a long serum elimination phase (144 h), in direct contrast to a t1/2β of 6.2 h for the scFv, which was cleared rapidly due to its small size; only 0.8%ID/g was present in the blood after 24 h.

Discussion

Experience gained from the in vivo phase I clinical trial carried out with the murine MGR6 MAb suggested the need for improvements in localization and clearance rates to increase both the specificity and efficacy of this MAb in RIT. The production of the MGR6 scFv by phage display enabled selection of a smaller molecule (approximately 32 kDa) specific for the same epitope on the ECD of HER-2. Preclinical in vitro and in vivo data indicated that MGR6 scFv exhibited similar affinity but an increased clearance rate with respect to the intact murine MAb.

Although the therapeutic capacity of MAbs remains to be fully confirmed, targeting MAb alone or in combination with cisplatin to HER-2-overexpressing tumor cells recently gave promising results in patients with very poor prognosis [1]. The humanized antibody Herceptin has since entered an international double-blind and placebo-controlled randomized phase III trial in metastatic breast cancer patients. This antibody significantly enhanced the effect of chemotherapy, both in time to progression and in response [35].

Downregulation of the HER-2 molecule kills by single-cell mechanisms, so that the probability of destroying cells in nearby tumor sites not reached by the MAb is minimal. Radioimmunoconjugates can exert their toxic

effect over several cell diameters from the source of radiation and do not require internalization for their activity. Striking results were obtained in different clinical trials using radiolabeled conjugates of the anti-CD20 MAb, with rates of complete response greater than 80% in highly pretreated patients with recurrent low-grade lymphoma and 62% progression-free survival at 2 years [39]. Promising results were also obtained with radiolabeled MAb in ovary carcinoma patients in an adjuvant setting [26]. However, the large size of intact MAbs makes blood and total body clearance rates extremely slow [32], seriously limiting the efficacy of therapy and diagnosis based on entire MAbs. In fact, the phase I clinical trial conducted using MAb MGR6 showed slow clearance rates, thus increasing the background in tumor imaging to the point that only peripheral lesions in positive patients were clearly identifiable (manuscript in preparation).

Promising results have been reported when smaller targeting molecules have been used to redirect therapeutic agents, including isotopes. In the present study, we obtained a functional anti-HER-2 scFv expressed on the surface of the filamentous phage M13 or in the periplasm of *E. coli*. Consistent with previous reports [17, 19], the use of the phage display system was necessary to retrieve HER-2 binding clones, since the original MGR6 hybridoma contained a series of variable genes which were abortive, non-functional or not expressed. This system allowed for the rapid isolation of a functional rearrangement of VH and VL chains. The MGR6 scFv actively competed with the original MAb for HER-2 binding as shown by FACS analysis, indicating that the same ECD epitope was recognized. However, competition-binding experiments indicated a lower apparent affinity of scFv than that of the intact MGR6 molecule. The lower scFv affinity has been previously related to altered folding of the recombinant protein molecule or to the additive impact on affinity measurement of two binding sites (intact antibody) versus a single site (scFv).

BIAcore evaluation indicated an apparent off-rate of the scFv of 9.3×10^{-4} s^{-1}. This is within the K_{off} range calculated for other scFv [31] and approaches the estimated value (10^{-5} s^{-1}) needed for efficient tumor retention [34]. The calculated scFv K_{off} was similar to that of the intact MGR6 molecule (6.0×10^{-4} s^{-1}), suggesting the presence of scFv multimers bound to the antigen with avidity comparable to that of the whole antibody molecule. It has been previously demonstrated that scFv dimers (bivalent) bind the antigen more strongly than monomers (monovalent), influencing the dissociation rate of the antigen-antibody complex through an avidity effect. The presence and the role of these scFv multimers in tumor targeting and retention in vivo has still to be further investigated.

Since MGR6 V-genes were cloned using the phage display procedure, which couples genotype with phenotype, the introduction of point mutations and chain shuffling [41] would allow for the active selection of improved binding kinetics and affinities if necessary.

Immunoblots of the myc tag-containing scFv with both 9E10 and IdM6.4 revealed mixtures of monomeric and dimeric forms of the scFv in a molar ratio of approximately 2:1. Moreover, isolated monomers of MGR6 scFv myc tag purified by gel filtration retained the capacity to multimerize with a molar ratio similar to that of the unfractionated product (data not shown). This is consistent with previous studies demonstrating that soluble antibody fragments can aggregate into multimers. In contrast, after Ni affinity purification of the myc-His tag MGR6 scFv, only trace levels of the dimer were present. This decrease in the scFv dimeric form might reflect an increase in stability, consistent with the increased purification yields of myc-His tag scFv.

The MGR6 scFv was easily and reproducibly generated at high yields in the monomeric form when a His tag was added. Furthermore, in both myc and myc-His tag forms, immunoreactivity remained high even after biotin labeling (data not shown). In contrast, the recombinant MGR6 scFv molecule appeared to be very sensitive to the conventional labeling procedure for iodine isotopes, precluding more extensive evaluation of its in vivo targeting ability. Since the affinity and tumor specificity of our MGR6 scFv is comparable to published data on other scFv, it was thus expected that clearance rates were to be dramatically improved with respect to the intact MGR6 MAb. In the in vivo preclinical evaluation, the α- and β-half lives of the MGR6 scFv were indeed much shorter (13 min and 6.2 h, respectively) than those of the intact MAb. These values were consistent with those reported in different models. Our preliminary experiments using radiolabeled MGR6 scFv in athymic mice bearing xenotransplanted human tumors indicated accumulation in HER-2-expressing tumor cells at levels similar to those previously reported in other xenotransplanted models, whereas the amount in the HER-2-non-expressing tumor was similar to that in the blood pool at 24 h after scFv injection (manuscript in preparation). There was, however, some nonspecific uptake in the liver and kidneys, yet reports suggest that this uptake may be the result of the pI of the scFv, which is in our case 7.63.

The pharmacokinetics of the MGR6 scFv appear, based on these preliminary results, to be an improvement with respect to the MGR6 MAb. These results await confirmation using radiolabeled preparations with improved immunoreactivity or pre-targeting approaches [5, 28]. The latter, which has led to an improved therapeutic index by avoiding bystander toxicity in normal tissue, makes use of non-radiolabeled antibody fragments. Alternative genetic or chemical modifications of the scFv might serve to effect stable binding with radiolabeled α- or β-emitters.

Furthermore, there exists the possibility of directly modifying the isoelectric point of the scFv by introducing mutations in the framework regions [27], or chemical modifications with dextran, lactose or biotin [36]. Evidence suggests that a lower isoelectric point

(i.e., lower than 7) may greatly reduce non-specific uptake into tissues such as the liver and kidneys – a definite and well-known limitation to immunotherapies utilizing antibody fragment. We are currently in the process of determining the effects of such modifications on pharmacokinetics and biodistribution of the MGR scFv.

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