Pretargeting of Carcinoembryonic Antigen–Expressing Cancers with a Trivalent Bispecific Fusion Protein Produced in Myeloma Cells

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Abstract

Purpose: To characterize a novel trivalent bispecific fusion protein and evaluate its potential utility for pretargeted delivery of radionuclides to tumors.

Experimental Design: hBS14, a recombinant fusion protein that binds bispecifically to carcinoembryonic antigen (CEA) and the hapten, histamine-succinyl-glycine (HSG), was produced by transgenic myeloma cells and purified to near homogeneity in a single step using a novel HSG-based affinity chromatography system. Biochemical characterization included size-exclusion high-performance liquid chromatography (SE-HPLC), SDS-PAGE, and isoelectric focusing. Functional characterization was provided by BIAcore and SE-HPLC. The efficacy of hBS14 for tumor pretargeting was evaluated in CEA-expressing GW-39 human colon tumor–bearing nude mice using a bivalent HSG hapten (IMP-241) labeled with ^{111}In.

Results: Biochemical analysis showed that single-step affinity chromatography provided highly purified material. SE-HPLC shows a single protein peak consistent with the predicted molecular size of hBS14. SDS-PAGE analysis shows only two polypeptide bands, which are consistent with the calculated molecular weights of the hBS14 polypeptides. BIAcore showed the bispecific binding properties and suggested that hBS14 possesses two functional CEA-binding sites. This was supported by SE-HPLC immunoreactivity experiments. All of the data suggest that the structure of hBS14 is an 80 kDa heterodimer with one HSG and two CEA binding sites. Pretargeting experiments in the mouse model showed high uptake of radiopeptide in the tumor, with favorable tumor-to-nontumor ratios as early as 3 hours postinjection.

Conclusions: The results indicate that hBS14 is an attractive candidate for use in a variety of pretargeting applications, particularly tumor therapy with radionuclides and drugs.

There are two major approaches for using antibodies to target radionuclides to tumors. Initial strategies involved direct coupling of the radionuclide to antibodies (1). The slow blood clearance of directly labeled antibodies likely results in a dose-limiting amount of radiation delivered to the red marrow before there is sufficient radioactivity accumulated in the more radioresistant solid tumors to cause significant antitumor effects. With more recently developed strategies, a nonradiolabeled antibody is first "pretargeted" to the tumor and the radioactivity, now attached to a small, fast-clearing compound, is given sometime later (2). Exposure of the red marrow is reduced when the radioactivity is associated with a small compound that clears rapidly from the blood and body. The decreased toxicity expands the therapeutic window, with the potential for improved therapy. The greatly improved tumor-to-nontumor ratios, combined with high uptake in the tumor, provide superior signal strength and contrast for improved imaging.

The majority of pretargeting methods involve the use of either a bispecific antibody with a radiolabeled hapten or some variations of a procedure based on the interaction of streptavidin and biotin (2). Hapten valency is a major determinant for tumor uptake and retention of an effector when employing the bispecific antibody pretargeting approach. Bivalent hapten effectors are markedly superior to those possessing a single hapten (3). This affinity enhancement system is believed to be due to the ability of the bivalent hapten to cross-link the pretargeted macromolecule at the tumor site, resulting in the formation of a more stable complex and, therefore, a longer tumor residence time (3).

Although pretargeting approaches were first used for enhanced imaging capabilities (2), interest in therapeutic applications for pretargeting advanced with the finding of Axworthy et al. (4) that by using their streptavidin-biotin pretargeting

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approach, concentrations of a radiolabeled biotin localized in a tumor could rival those of a directly radiolabeled antibody. Clinical trials with a radioiodinated peptide using a bispecific antibody pretargeting approach have also been reported with some responses noted (5, 6). However, due to human antimouse antibody to the bispecific antibody used in these early trials, a new bispecific antibody that was prepared by coupling a humanized anti–carcinoembryonic antigen (CEA) antibody (hMN-14) with a murine anti–diethylenetriaminepentaacetic acid antibody (m734) is being evaluated, and seems to reduce the incidence of anti-antibody responses (7, 8).

One chemically conjugated bispecific antibody (Fab′ × Fab′) prepared from hMN-14 and m734 (9) was used initially for evaluation of pretargeted delivery of ^{99m}Tc/^{188}Re-labeled peptides, but more recently, we have focused on the development of a series of bispecific antibody pretargeting systems that use an antibody (m679) directed against HSG (10). Such pretargeting methods offer considerable flexibility in the types of effectors that can be used because the antibody binds to the HSG hapten and not to the effector moiety (e.g., diethylenetriaminepentaacetic acid). Initial studies with two chemically linked bispecific antibodies (mMu-9 × m679 and hMN-14 × m679) in human colon cancer xenografts suggest that both could be applicable for imaging with ^{99m}Tc or ^{111}In, or for therapy with ^{90}Y or ^{177}Lu (11). Pilot studies with a ^{90}Y-labeled 7,10-tetra-azacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid-di-HSG peptide have also shown positive antitumor effects in experimental animals (12).

These promising pretargeting results have encouraged us to embark on a program to develop recombinant bispecific antibodies derived from variable domains of 679 and hMN-14. Initially, we developed a series of bispecific diabodies that simultaneously bind CEA via hMN14 variable domains and HSG via murine or humanized 679 variable domains (13). Pretargeting studies in tumor-bearing mice showed that the bispecific diabodies produced similar tumor uptake, but with considerably higher tumor-to-nontumor ratios than hMN-14 × m679 chemical conjugate (13). These bispecific diabodies were produced as soluble protein from an *E. coli* expression system. Although yields were not at a commercially viable level, enough material was produced for characterization and proof of principle in the animal model.

We have shown that a chemically prepared bispecific antibody from hMN-14 and c734 with bivalency to the tumor antigen could improve the amount and longevity of effector binding in the tumor (14). However, it is desirable to further optimize the pharmacokinetic properties of such multivalent constructs (e.g., by reducing their molecular size) because we would especially prefer to develop the approach without the need for a clearing step that is used for streptavidin-biotin pretargeting systems (2). Such multivalent proteins were designed and produced with the *E. coli* system (13); however, yield was not acceptable for extensive testing. Herein, we describe the design of hBS14, a novel trivalent bispecific construct, and its production in myeloma cell culture. We show that hBS14 possesses the biochemical and functional properties as per its design, and investigate its potential for use as a tumor pretargeting agent.

Materials and Methods

Molecular cloning. Each of the vector constructs was engineered using standard molecular biology methods. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized with an Applied Biosystems 392 DNA/RNA synthesizer. PCR reactions were done using AmpliTaq polymerase (Applied Biosystems, Foster City, CA) and a Perkin-Elmer (Wellesley, MA) GeneAmp PCR system 9600. Some subcloning procedures used pGem3Z vector (Promega, Madison, WI). The genes encoding the hBS14 polypeptides were ultimately engineered into the mammalian expression vector pdHL2, which permits the amplification of antibody production (15, 16). The pdHL2 vector contains the genes for immunoglobulin G (IgG) constant regions (C_H and C_K) and was originally designed to accept variable domain cassettes and direct the synthesis of whole IgG. Because we were interested in expressing novel single chain–based constructs devoid of constant region sequences, it was necessary to create a new shuttle vector to facilitate the assembly and transfer of the hBS14 genes into the pdHL2 vector.

Functional features of the SV3 shuttle vector. Overlapping synthetic oligonucleotides (85-mer) were annealed to form duplex DNA possessing the features shown above. This duplex was ligated into the *Hin*dIII and *Eco*RI restriction endonuclease sites of the pGEM3z cloning vector (Promega) to generate the SV3 shuttle vector. The variable domain genes were amplified by PCR from the humanized bispecific diabody construct BS1.5H-pET-ER (13) and assembled into open reading frames (ORF) in the SV3 shuttle vector via the *Nco*I and *Sal*I restriction endonuclease sites. SV3 constructs were generated for both ORF1 and ORF2, which encode polypeptides 1 and 2, respectively (Fig. 1A).

Each ORF includes the IgG light chain leader peptide, which directs secretion of the nascent polypeptides, preceding the variable domain genes, which are in turn followed by the codons for six histidines and two stop codons. The variable domains are separated by linker peptides consisting of five or six amino acid residues. ORF1 and ORF2 were subcloned into a single pdHL2 expression vector. ORF1 was excised from its shuttle vector with *Xba*I and *Bgl*II restriction endonucleases and cloned into the *Xba*I and *Bam*HI sites of pdHL2 to generate the intermediate construct hBS14ORF1-pdHL2. ORF2 was then excised from its shuttle vector with *Xho*I and *Eag*I restriction enzymes and cloned into those same sites of the intermediate hBS14ORF1-pdHL2 construct to generate the final dicistronic expression vector, hBS14-pdHL2 (Fig. 1C).

Stable transfection and amplification of hBS14 genes in Sp2/0 myeloma cells. Sp2/0-Ag14 mouse myeloma cells have been used previously in conjunction with the pdHL2 expression vector for high-level expression of recombinant IgG (16). The hBS14-pdHL2 DNA vector was linearized by digestion with *Eco*RI restriction endonuclease and successfully transfected into Sp2/0-Ag14 (4×10^6 cells) by electroporation (450 V, 25 μF). The pdHL2 vector contains the gene for dihydrofolate reductase, allowing clonal selection as well as gene amplification with methotrexate (Calbiochem, La Jolla, CA).

Transfectants were cloned by plating in 96-well plates in the presence of 0.05 μmol/L methotrexate, and the primary screening for hBS14-expressing clones was accomplished by ELISA. The ELISA screening format consisted of an HSG peptide, IMP-239 (synthetic peptide composed of Ac-Lys(HSG)-Cys-NH_2; Immunomedics, Inc., Morris Plains, NJ), coupled to bovine serum albumin that was first adsorbed to microplate wells. Conditioned media from the putative clones were transferred to the microplate wells to allow hBS14 binding to the HSG conjugate. Bound hBS14 was detected with WI2 (17), a rat anti-idiotype IgG to MN-14, and horseradish peroxidase–conjugated goat anti-rat IgG. Expression of hBS14 was confirmed by BIAcore using an HSG-coupled (IMP-239) sensorchip. An increase in response units following injection of culture media signified expression of hBS14. A further increase in response units by the subsequent injection of WI2 showed that the expressed protein was bispecific and fully functional. With this method, standard concentration curves were generated using purified 679 proteins, allowing for accurate real-time measurements of productivity. Gene amplification and the resulting increase in productivity were accomplished by stepwise increase in methotrexate concentration in the culture media over several months.

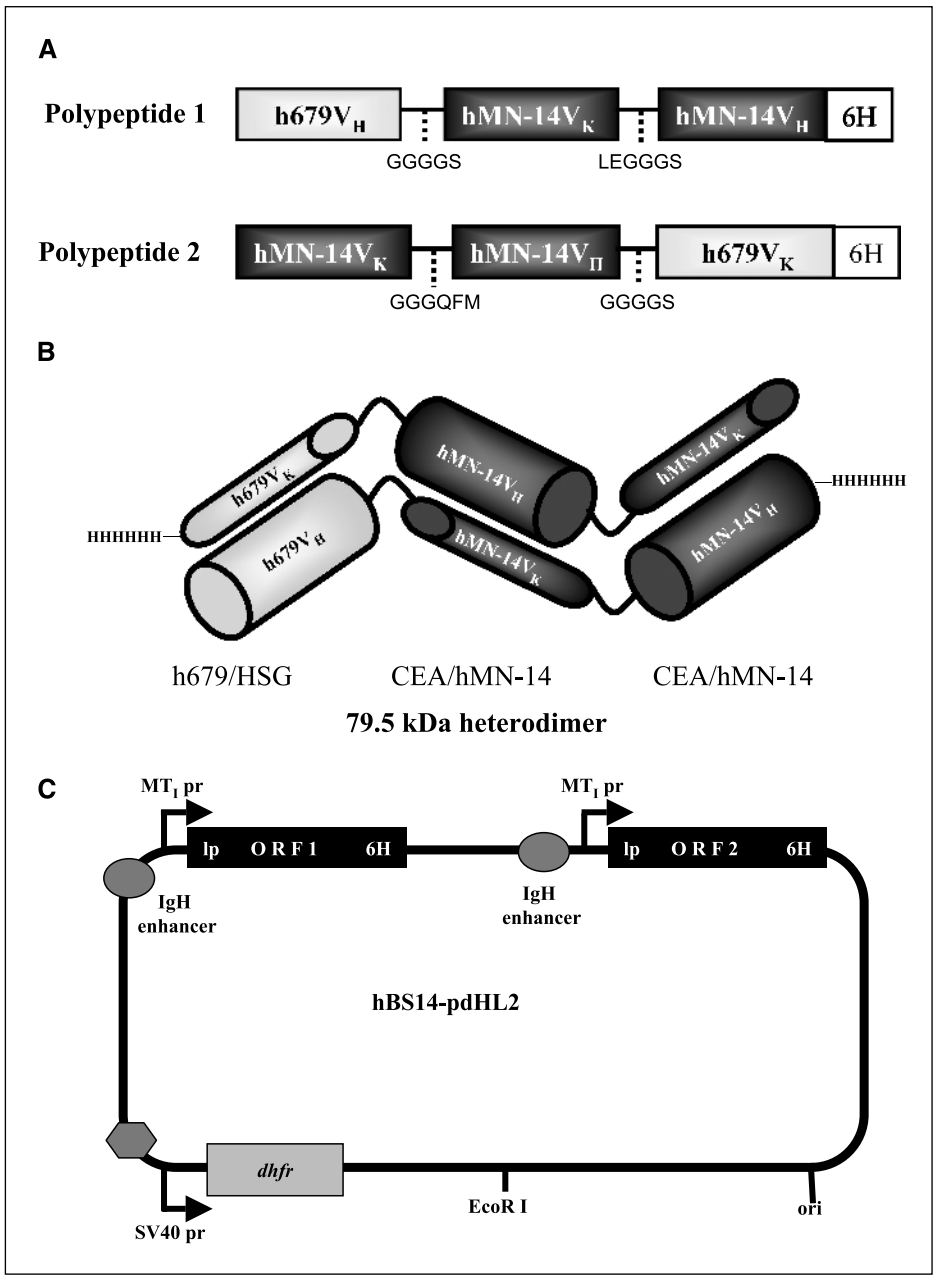

Fig. 1. Schematic representation of the two polypeptides that are synthesized from the hBS14-pdHL2 vector (*A*) that form the trivalent bispecific hBS14 protein (*B*). *C,* schematic representation of the hBS14-pdHL2 vector. For the coding sequences, the κ chain leader peptides (*lp*), ORF, and hexahistidine (*6H*) are indicated. Other features include immunoglobulin H enhancers, metallothioneine promoter (*MT*$_I$*pr*), and the gene for dihydrofolate reductase (*dhfr*).

Production, purification, and biochemical characterization of hBS14. For production of hBS14, terminal roller bottle cultures were grown in Hybridoma-SFM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1 μmol/L methotrexate.

IMP-291, which was designed to have a lower affinity for 679, was synthesized and conjugated to Affigel (Bio-Rad, Richmond, CA) by standard methods. The high binding capacity (>20 mg/mL) Affigel-IMP-291 proved to be exquisite for affinity purification of hBS14.

Culture media was clarified by centrifugation and filtered. Larger culture volumes were concentrated by ultrafiltration. The clarified media was supplemented with EDTA (2 mmol/L) and Triton X-100 (0.02%) and adjusted to pH 6.5 to 7.5, if necessary, and applied to the affinity column. The hBS14 was purified by affinity chromatography using 1 mL of Affigel-IMP-291 for every 10 mg of hBS14 contained in the media. The column was washed to baseline with 0.1% Tween 20-PBS and eluted with 10 column volumes of elution buffer [1 mol/L imidazole, 150 mmol/L sucrose, 0.02% Tween 20, 50 mmol/L citrate (pH 4.5)]. The eluate was concentrated by ultrafiltration and dialyzed into formulation buffer [150 mmol/L sucrose, 0.02% Tween 20, 10 mmol/L NaAc (pH 4.5)]. Nearly 100% of the hBS14 was bound and eluted from the affinity column.

Size-exclusion high-performance liquid chromatography (SE-HPLC) was done on a Beckman System Gold Model 116 with a Bio-Sil SEC 250 column (Bio-Rad, Hercules, CA). A variety of standards were used to calibrate the column including hMN-14 IgG (~150 kDa), hMN-14 Fab′ (~50 kDa) modified with *N*-ethyl maleimide, hMN-14 F(ab′)$_2$ (~100 kDa), hMN-14 diabody (53 kDa), and hMN-14 triabody (80 kDa). The bispecific binding properties were analyzed using a BIAcoreX system (Biacore, Inc., Piscataway, NJ) with a high-density HSG-coupled biosensor chip prepared according to the instructions of the manufacturer. Culture media or purified samples were injected over the HSG sensorchip before injection of WI2 (20 μg/mL). Soluble CEA (Scripps Laboratories, La Jolla, CA) was also used in place of WI2 and gave similar results.

Immunoreactivity and* in vivo *studies. Immunoreactivity was assayed by SE-HPLC. Soluble CEA was mixed with ^{125}I-labeled hBS14 (molar ratios ranging from 1:1 to 10:1) before the analysis and radioactive peaks were detected with an in-line γ-radiation detector. The bispecific binding properties of the hBS14 were also tested by SE-HPLC by first mixing a molar excess of unlabeled hBS14 with ^{111}In-labeled IMP-241 [synthetic peptide composed of 7,10-tetra-azacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid-Phe-Lys(HSG)-D-Tyr-Lys(HSG)-NH_2] before adding CEA (11).

In vivo studies were done with ~20 g female NCr nude mice (Taconic, Germantown, NY) bearing s.c. CEA-producing GW-39 human colon cancer xenografts (18). To assess direct targeting, mice were injected i.v. with ^{131}I-labeled hBS14 (32 μg, 6 μCi). A separate group of tumor-bearing mice were injected with a similar amount of the hMN-14 × m679 Fab′ × Fab′ bispecific antibody (11) that was affinity purified and was immunoreactivity tested in a similar manner as the hBS14. For pretargeting studies, 32 μg (0.4 nmol) of hBS14 trace labeled with ^{125}I were injected i.v., and 27 hours later, IMP-241 (0.04 nmol) labeled with 24 μCi of $^{111}InCl_3$ was given i.v. In each study, groups of five animals were necropsied at the times indicated, tissue weighed, and radioactivity determined. Uptake is defined as percent injected dose per gram (% ID/g) using a standard prepared from the injected materials, and tumor-to-nontumor ratios determined based on the percent injected dose per gram in the tumor and each tissue. Radioiodination was done as described by Weadock et al. (19).

Results

The molecular design of hBS14, shown in Fig. 1, was devised to only allow proper heterodimer formation with pairing of complementary domains. The two polypeptides pair to form an ~80 kDa heterodimer with one HSG-binding domain and two CEA-binding domains.

The expression cassettes for the hBS14 polypeptides were engineered into a single construct in the pdHL2 mammalian expression vector (Fig. 1C). The construct was stably transfected into Sp2/0-Ag14 myeloma cells. Selection and amplification of clones were accomplished with methotrexate.

hBS14 was purified from culture media in a single-step using HSG-based affinity chromatography. Greater than 95% recovery of fully active protein was achieved using 1 mL of Affigel-IMP-291 for every 10 mg of hBS14. Even when purified from media supplemented with 10% fetal bovine serum, affinity purification resulted in a nearly homogeneous product. SE-HPLC shows a single sharp protein peak with a retention time of 9.33 minutes, consistent with an 80 kDa protein (Fig. 2A). As standards, BS1.5H diabody (54 kDa), hMN-14 triabody (78 kDa), and hMN-14 F(ab′)$_2$ (100 kDa) had retention times of 9.80, 9.35, and 8.77 minutes, respectively. Figure 2B shows the results of SDS-PAGE analysis on three separate batches of hBS14 that were purified from serum-supplemented media. The molecular weights for polypeptides 1 and 2 were calculated from the deduced amino acid sequences. Only two polypeptide bands are evident and, as expected, these bands are of equal intensity and are consistent with the calculated molecular weights of the hBS14 polypeptides (39.94 and 39.52 kDa). There is no evidence of protein degradation. Isoelectric focusing gel electrophoresis of the three batches of purified hBS14 (Fig. 2C) shows a major band near the isoelectric point (pI) of hBS14 (pI = 7.73) as calculated from the deduced amino acid sequence. There are trace bands at lower p*I* that are likely product-related and may be the result of negligible deamidation of some basic amino acid residues. Taken together, this combination of standard biochemical analyses suggests that the transgenic myeloma cells correctly synthesize and secrete hBS14 as designed and that we have developed a robust process capable of generating highly purified material.

Functional characterization was provided by BIAcore experiments to show bispecific binding properties (Fig. 3). hBS14 bound tightly to HSG that was immobilized on a sensorchip. The HSG-bound proteins were able to capture subsequently added CEA or WI2, demonstrating that they can simultaneously bind both antigens. If the WI2 binding is allowed to approach saturation, the stoichiometry of the binding can be determined. The additional increase in response units resulting from WI2 binding was compared with the initial response unit increase of the hBS14 on binding to the HSG sensorchip. As each increase in response unit (RU) level is directly proportional to the mass bound, the WI2/bispecific antibody molar binding ratio can be calculated using the formula $(RU_{WI2} / RU_{hBS14}) \times (MW_{hBS14} / MW_{WI2})$. hBS14 was designed to be bivalent for CEA (and monovalent for HSG) and, as such, should bind WI2 (also bivalent) with a 1:1 molar ratio. Indeed, the experimentally determined molar binding ratio of WI2 to hBS14 was found to be between 0.7 and 0.8, approaching the theoretical maximum of 1.0.

Immunoreactivity experiments also supported that hBS14 possesses two functional CEA binding sites (Fig. 4). ^{125}I-labeled hBS14 was mixed with increasing amounts of CEA before analysis by SE-HPLC. Two distinct peak shifts were detected. The peak at 11.16 minutes is unbound ^{125}I-labeled hBS14. The peak at 7.95 minutes is hBS14 bound to one CEA whereas the peak at 7.21 minutes is hBS14 bound to two CEAs. The bispecific binding properties of the hBS14 were also tested by SE-HPLC by first mixing a molar excess of unlabeled hBS14 with ^{111}In-labeled IMP-241 with >98% of the ^{111}In-labeled di-HSG peptide shifting to a molecular size indicative predominantly of a complex containing two hBS14 molecules. This was then followed by the addition of a mole excess of CEA, with a further shift of >95% of the ^{111}In-labeled IMP-241 to a higher molecular weight, indicating the hBS14 binding to both the HSG peptide and CEA.

The stability of hBS14 has been followed for over 12 months. Samples stored at 4°C in formulation buffer remained unchanged when tested at the 12-month time point. SE-HPLC showed no protein loss or degradation and no loss of bifunctional activity was measured by BIAcore (data not shown).

Initial *in vivo* studies included an assessment of the biodistribution of ^{125}I-labeled hBS14 in nude mice bearing GW-39 tumors (Fig. 5). Four hours postinjection, tumor uptake was 6.1 ± 0.9% ID/g with a blood concentration of 14.8 ± 1.8% ID/g. By 24 hours, the blood concentration decreased to 0.92 ± 0.16, but tumor uptake remained unchanged (5.7 ± 0.7% ID/g), which was more than 2-fold higher than that seen with the ^{125}I-labeled hMN14 × m679 Fab′ × Fab′ chemically conjugated bispecific antibody (2.2 ± 0.4% ID/g) that we reported earlier (11). Activity in the blood and tissues continued to decrease over time at a more rapid rate than the tumor (Fig. 5, *top*). Tumor uptake at 96 hours was 1.7 ± 0.5% ID/g compared with 0.08 ± 0.03% ID/g in the blood. Fig. 5 (*bottom*) shows the distribution of radioactivity in all sampled tissues at 24 hours, when blood concentrations first decreased to <1.0% ID/g. We had shown previously that this was a critical concentration of bispecific antibody in the blood

for pretargeting, marking the earliest time that the peptide should be given to obtain good tumor uptake with minimal normal tissue accretion (20).

The utility of hBS14 for tumor pretargeting was evaluated in GW-39 tumor–bearing mice using ^{111}In-labeled IMP-241. Based on our prior work (9, 11, 12), a mole injection ratio of 10:1 bispecific antibody/peptide was used, and an interval of 27 hours was selected to ensure that the blood concentration of the bispecific antibody had decreased to <1% ID/g. The data shown in Fig. 6 illustrate the uptake and clearance kinetics of the ^{125}I-labeled hBS14 and the ^{111}In-labeled IMP-241 peptide starting at 3 hours after the ^{111}In-labeled IMP-241 injection (i.e., 30 hours after the hBS14). At the first sampling time (3 hours after the peptide was given), there was 4.6 ± 1.5% ID/g of the hBS14 in the tumor with 19.1 ± 8.7% ID/g of the ^{111}In-labeled IMP-241, but with only 0.25 ± 0.08% ID/g in the blood, corresponding to tumor/blood, tumor/liver, and tumor/kidney ratios of 83 ± 44, 59 ± 20, and 5.2 ± 2.3, respectively. Whereas tumor uptake of the peptide decreased slowly over time at a rate that was similar to that of the ^{125}I-labeled hBS14, the peptide cleared from the blood more rapidly than hBS14, indicating that the residual levels of hBS14 in the blood were sufficiently low as not to bind much peptide, thus allowing the peptide to clear at a more rapid rate.

Discussion

Bispecific antibody pretargeting of radionuclides has a number of advantages over a directly radiolabeled antibody for imaging and therapeutic applications. Tumor-to-nontumor ratios are superior, even when compared with a directly radiolabeled antibody fragment, and tumor uptake of the radiolabeled peptide can rival that of a directly radiolabeled IgG when the pretargeting system is optimized (11, 20). Although most pretargeting systems have used anti-hapten antibodies that are specific for a particular chelate (2, 5, 6), we are

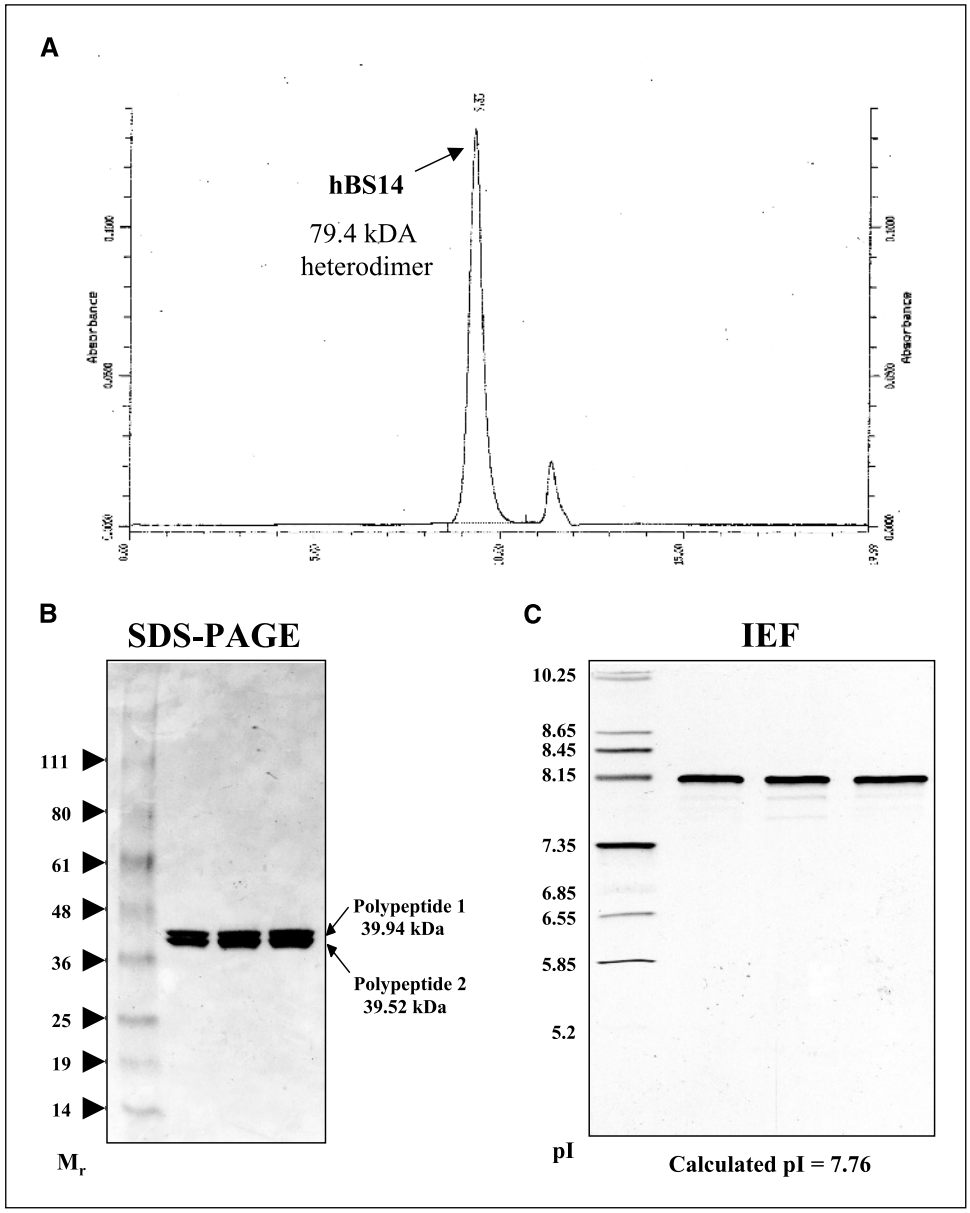

Fig. 2. *A,* SE-HPLC of hBS14 following single-step purification with Affigel-IMP-291 affinity chromatography. The position of the hBS14 peak is indicated. The smaller peak with a retention time at ~11.5 minutes is nonprotein and derived from buffer. *B,* SDS-PAGE analysis of three batches of hBS14 following single-step purification with Affigel-IMP-291 affinity chromatography. The calculated molecular weights, obtained from the deduced amino acid sequences of hBS14 polypeptides 1 and 2, are given; arrows, gel positions. *C,* isoelectric focusing analysis of three independent batches of hBS14 following single-step purification with Affigel-IMP-291 affinity chromatography. The location of isoelectric focusing standards and the calculated pI of hBS14 are indicated.

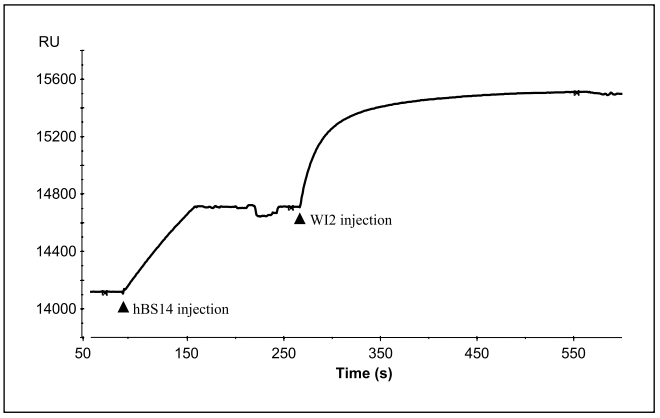

Fig. 3. BIAcore sensorgram showing simultaneous HSG and CEA binding for hBS14. hBS14 (50 ng) was loaded on a high-density HSG-coupled (IMP-239) sensor chip and then WI2 (2 μg) was allowed to bind to the immobilized hBS14. Arrowheads, injection times.

developing a pretargeting system that uses an anti-hapten antibody that is independent of the compound used for radiolabeling, and therefore is highly flexible in its ability to be used with a number of radionuclides (11). Bispecific antibodies have traditionally been prepared by chemically coupling Fab′ fragments of the antitumor and anti-hapten antibodies, and others have prepared a bispecific antibody IgG using a cell fusion quadroma technology (21). Advances in recombinant engineering have further advanced the ability to prepare bispecific antibody (22, 23). Our group previously described initial work in developing bispecific diabodies and multivalent multispecific agents produced in *E. coli*. Although our efforts were limited due to low yield, particularly with the more complex multivalent constructs, the results were encouraging (13). We then investigated whether similar constructs could be expressed and produced with a reasonable yield in mammalian cells. *In vitro* and *in vivo* targeting data presented herein clearly show that a bispecific antibody with divalent binding to CEA and monovalent binding to the HSG hapten can be prepared and used highly effectively in a pretargeting setting.

Numerous constructs have been designed based on scFv technology (23). In some cases, novel molecules having the predicted structural and functional features are produced. In other cases, the molecule is misfolded or nonfunctional or the host cell is incapable of synthesizing the construct all together. The likelihood of failure increases with the complexity of the construct. A further complication is the tendency of scFv-based constructs to form multimers via domain swapping, resulting in a heterogeneous product consisting of monomers, dimers, trimers, etc. (24, 25). The molecular design of hBS14 favors the formation of an 80 kDa heterodimer having one h679 and two hMN-14 Fvs. The expression cassettes of hBS14 encode two heterologous polypeptides. Polypeptide 1 (39.94 kDa) consists of a central hMN-14 V_K flanked by an NH_2-terminal h679 V_H and a COOH-terminal hMN-14 V_H domain. Polypeptide 2 (39.52 kDa) consists of a central hMN-14 V_H flanked with an NH_2-terminal hMN-14 V_K and a COOH-terminal V_K domain. Homodimers in which each V domain is paired with a complementary domain cannot form. If incomplete homodimers do form, they would likely be unstable and degraded due to exposed hydrophobic surfaces. Heterodimers, in which each V_H domain can pair with a V_K, can potentially form in either of the two linear orientations, parallel or antiparallel. In the parallel orientation, V domains are mispaired (e.g., h679 V_H/hMN14 V_K), resulting in an inactive molecule that is unable to bind HSG. If such parallel heterodimers or any homodimers were to form, they would be eliminated during the HSG-based affinity chromatography. To determine the extent of inactive dimer formation, material was isolated from culture media by immobilized metal affinity chromatography, which captured all product-related proteins, because both polypeptides possess COOH-terminal hexahistidine tags. The immobilized metal affinity chromatography–purified sample was indistinguishable from IMP-291 affinity–purified hBS14 by SE-HPLC and SDS-PAGE (data not shown). Further, all of the immobilized metal affinity chromatography–purified material was found to bind to and elute from Affigel-IMP-291 as well as show bispecific (HSG and CEA) binding capabilities by BIAcore (data not shown). Thus, no homodimers or inactive parallel heterodimers were detected. Apparently, only the bispecifically active antiparallel heterodimers are formed.

Two sets of experiments indicate that hBS14 has three functional binding domains. We have previously used the BIAcore bispecific binding ratio to determine valency for CEA

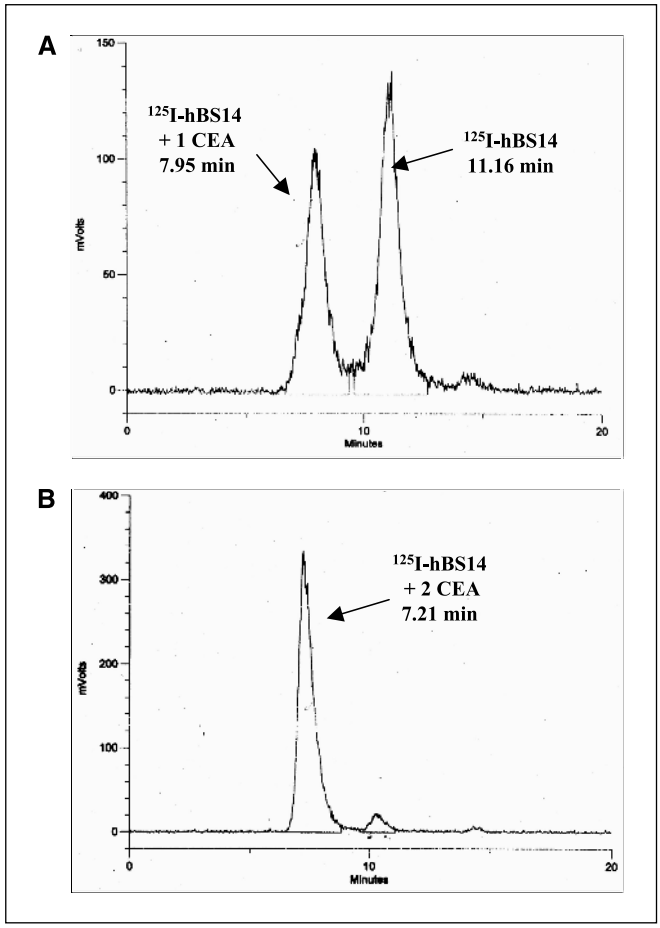

Fig. 4. Analysis of hBS14 immunoreactivity with CEA. ^{125}I-labeled hBS14 was mixed with soluble CEA at 1:1 (*A*) and 10:1 (*B*) molar ratios before size exclusion HPLC analysis. Radioactive peaks representing unbound ^{125}I-labeled hBS14 at 11.16 minutes, ^{125}I-labeled hBS14 bound to one CEA at 7.95 minutes, and ^{125}I-labeled hBS14 bound to two CEA molecules at 7.21 minutes are indicated by arrows.

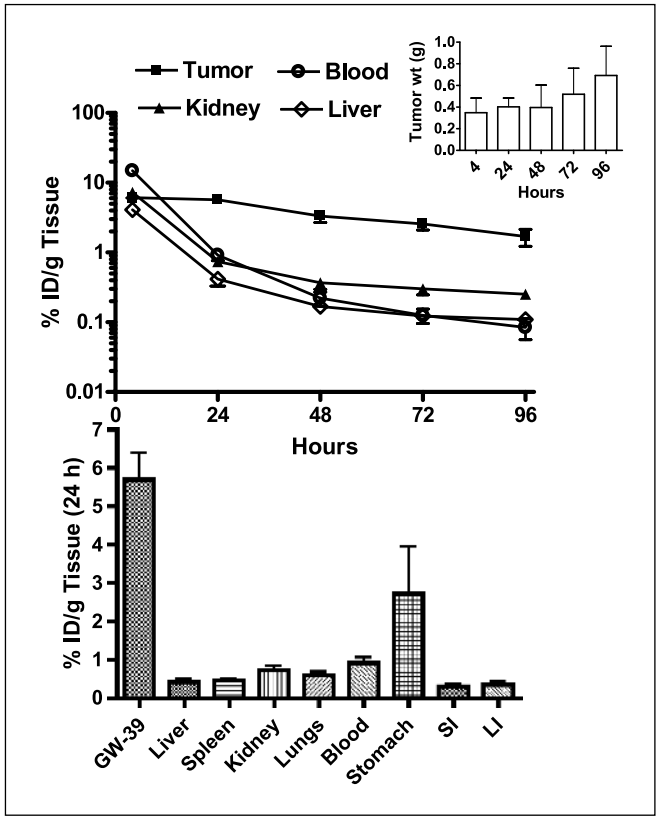

Fig. 5. Biodistribution of ^{125}I-labeled hBS14 (6 μCi, 40 μg) in nude mice bearing GW-39 human colonic tumor xenografts (*inset,* tumor sizes). Points and columns, mean (n = 5 mice); bars, SD. Top, clearance in the tumor, blood, liver, and kidneys over the course of the entire study; bottom, a more comprehensive set of data for the 24-hour interval necropsy. Enhanced activity in the stomach is due to radioiodinated catabolites.

binding (13). We have tested a number of bispecific proteins that bind CEA monovalently, including chemically conjugated hMN-14 × m679 (Fab × Fab) and a number of bispecific diabodies, and all display a WI2/bispecific antibody molar ratio of less than the theoretical maximum of 0.5. The molar ratios for constructs having two CEA-binding groups, such as hBS14, are always measured at >0.5 and approaching the theoretical maximum of 1.0. Immunoreactivity experiments with soluble CEA confirmed that hBS14 does, in fact, possess two functional CEA-binding domains. This does not necessarily mean that hBS14 can bind divalently to membrane-associated CEA on tumor cell surfaces. It is likely that some hBS14 molecules bind divalently to the cell surface whereas others only bind monovalently. *In vivo*, hBS14 is retained at a high level in tumors for a significantly longer period of time compared with bispecific diabodies, which bind monovalently (13).

We have developed a robust and easily scalable affinity chromatography system for the purification of any protein that has an HSG-binding group. Initial attempts involved the use of authentic HSG peptides (such as IMP-239) coupled to an Affigel matrix; however, the binding affinity of 679 monoclonal antibody for HSG is too strong and elution was not possible without protein denaturation. Morel et al. (10) measured the affinity of a number of HSG derivatives for binding to anti-HSG antibodies and reported that the dissociation constant for a histamine-succinyl-glycyl-glycine (HSGG)-acid peptide (6.8×10^{-9} mol/L) was about 60-fold lower than that of an HSG-amide peptide (1.1×10^{-10} mol/L). We envisioned that a further decrease of the binding affinity could be achieved by replacing HSG in IMP-239 with suitable HSGG analogues. IMP-291 was thus synthesized and the resulting Affigel-IMP-291 has a high protein-binding capacity of more than 20 mg/mL of resin. Using 1 mL resin/10 mg hBS14, nearly 100% of product was recovered from culture supernatant fluid. Even in the presence of 10% fetal bovine serum and only using mild wash conditions, hBS14 was purified to near homogeneity based on SE-HPLC, SDS-PAGE, and isoelectric focusing. This affinity purification will greatly simplify downstream steps when used as an initial capture step in a purification process.

In animals, hBS14 cleared rapidly from blood, similar to the clearance rate observed with the chemically conjugated Fab′ × Fab′ hMN14 × m679 bispecific antibody we reported earlier (11); however, tumor uptake for the hBS14 at 24 hours was found to be 2- to 3-fold higher than that of the chemical conjugate, which could be an advantage in pretargeting applications. Using standard pretargeting conditions, excellent tumor localization of the radiolabeled peptide was achieved within 3 hours of the peptide injection.

In conclusion, we have designed a trivalent bispecific construct capable of binding HSG and binding divalently to CEA, and produced this novel protein in mammalian cell culture. Affinity chromatography yields a homogeneous and fully active product. Preliminary studies with an animal model indicate that hBS14 is an attractive candidate for use in a variety of pretargeting applications, including imaging and therapy of CEA-producing tumors.

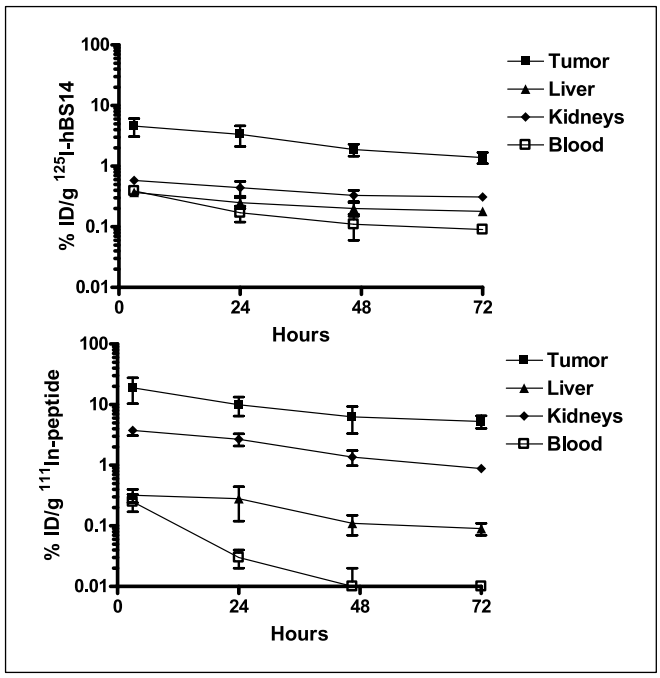

Fig. 6. Biodistribution of ^{125}I-labeled hBS14 (*top*) and ^{111}In-labeled IMP-241 (*bottom*) in nude mice bearing GW-39 tumors (weights ranging from 0.35 to 1.2 g). Animals were first injected with 32 μg of hBS14 and, after 27 hours, the ^{111}In-labeled IMP-241 was given i.v. The y-axis is given as a log scale to illustrate large differences between the tumor and tissue uptake. Points, mean (n = 5 mice); bars, SD.

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