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Technetium-99m Radiolabeling Using a Phage-Derived Single-Chain Fv with a C-Terminal Cysteine

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Single-chain Fv (scFv) antibody fragments have potential for clinical imaging studies because of their rapid tumor penetration and high tumor-to-tissue ratios at early time points. ScFvs clear rapidly from the circulation so radiolabels such as ^{99m}Tc which have short half-lives are desirable, but the free thiol groups necessary for labeling with ^{99m}Tc are not normally found on these molecules. **Methods:** We constructed a vector which enabled a free cysteine to be linked to the C-terminus of scFvs. MFE-23, a scFv directed against carcinoembryonic antigen (CEA), was cloned into this vector and cys-tagged MFE-23 was labeled with ^{99m}Tc using a D-glucarate transfer method. **Results:** The radiolabeled product was stable in vivo and in vitro and showed favorable tumor-to-blood ratios in vivo at early time points (4:1 at 24 hr and 8:1 at 48 hr), although high kidney levels were also detected. **Conclusion:** Our study demonstrates an effective method to enable scFvs radiolabeling with ^{99m}Tc and also shows the potential of using a ^{99m}Tc-labeled scFv for clinical imaging studies.

Key Words: single-chain Fv antibody fragments; technetium-99m

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Small antibody fragments have potential for good tumor targeting since they penetrate rapidly and give high tumor-to-background ratios at early time points (*1–3*). Single chain Fvs (scFvs) consist of a variable heavy (VH) and a variable light (VL) chain of an immunoglobulin tethered together with a flexible linker (*4*) and as such these are the smallest antibody fragments (mol wt 27 kD) which maintain full antibody binding capacity. In accordance with their low molecular weight, scFvs are cleared rapidly from the circulation and, when combined with their rapid tumor penetration, allows patient imaging within 24 hr (*5*). MFE-23 used for this study is a high affinity, high specificity scFv directed against carcinoembryonic antigen (CEA). It was produced by phage technology, which made it possible to select for high affinity, and it was expressed in *E. coli* with a high yield. MFE-23 has already shown favorable biodistribution in an animal model, when radiolabeled with ^{125}I (*6*).

As for radiolabel choice, a half-life appropriate to the rate of antibody uptake by the tumor is desirable (*7*). Moreover, with the rapid tumor uptake of scFvs, radiolabels with short half-lives such as ^{99m}Tc and ^{123}I are most suitable. Radiolabeling scFvs with ^{123}I has some disadvantages. First, if tyrosine residues are situated in the antigen-binding site, radiolabeling these residues may lower the antigen-binding capacity of the scFv. Further, ^{123}I is very expensive and thyroid blocking agents are necessary to prevent iodine uptake by the thyroid. These disadvantages may be overcome by using ^{99m}Tc, but for radiolabeling with ^{99m}Tc, free sulphydryl groups are shown to be essential (*8*). These groups are not available on scFvs unless specifically added, for example by chemically modifying the scFv (*9*). We achieved this by engineering a free cysteine to the C-terminal amino acid of MFE-23 and, using this as a labeling attachment for ^{99m}Tc, developed a simple technique for inserting free thiol groups in genetically engineered scFvs. The cys-tagged product was called MFE-23-cys.

MATERIALS AND METHODS

Expression Vector and Cloning of MFE-23-cys

To create a new expression vector with a cysteine in the C-terminal tail, inverse PCR site-directed mutagenesis (*10*) was used to replace a histidine in the previously described (*11*) pUC119-based expression vector containing a C-terminal hexahistidine tag. Modification was achieved using 25 cycles of PCR with the oligonucleotides Cys-His-For (5′-TGGTGATGACATGCGGCCGCC CGTTTGAT-3′) and His6-Back (5′-TCATCACTAATAAGAATTCACTGGCCG-3′) followed by self-ligation. Clones containing the required sequence (Fig. 1) were identified by DNA sequencing. MFE-23 (*6*) was subcloned into this vector as an NcoI/NotI fragment.

Expression of MFE-23-cys in *E. coli*

E. coli 'Sure' cells were transformed with the plasmid construct shown in Figure 1. Cells were shaken at 37°C in 2X TY medium with 100 μg/ml ampicillin and 0.1% glucose until an optical density of 0.9 at 600 nm was obtained. Protein expression was induced by adding 1 m*M* isopropyl beta-D thiogalactoside overnight at 30°C. The cells were then pelleted and the supernatant containing MFE-23-cys decanted and stored at 4°C.

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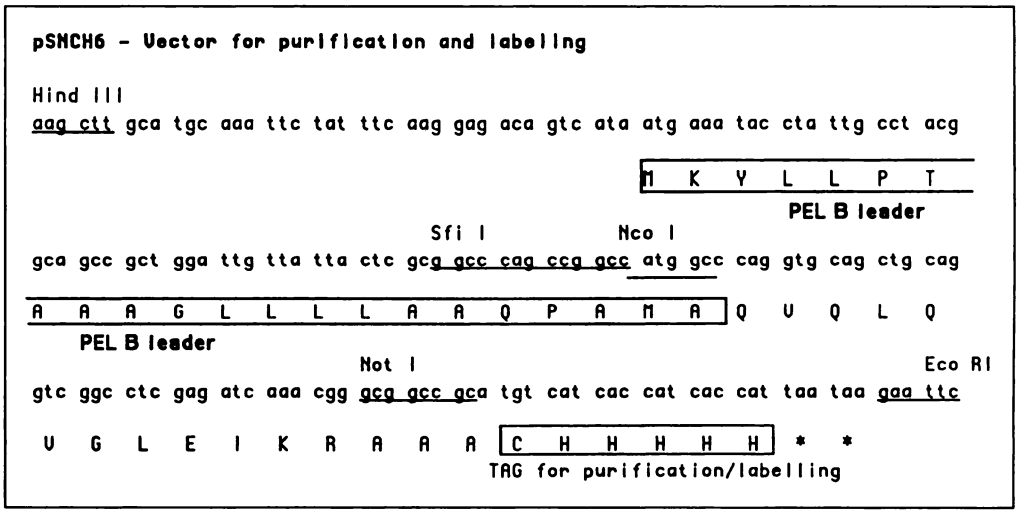

FIGURE 1. pUC 119 construct used to clone MFE-23-cys. The pelB signal sequence directs antibody fragments into the bacterial periplasm where they fold into antigen binding conformation (*18*).

Purification of MFE-23-cys

Supernatant was concentrated 10-fold using a Spiral cartridge (S1Y10 Amicon) and dialyzed against PBS/Az (50 m*M* phosphate, 150 m*M* NaCl pH 7, containing 0.02% sodium azide). The concentrate was applied to a CEA/Sepharose-4B column containing 7 mg of CEA. Unbound material was washed through with PBS/Az. Bound MFE-23-cys was eluted with 50 m*M* diethylamine (pH 11), after which the fractions containing the protein were immediately neutralised with 1 *M* phosphate buffer (pH 7.5). The collected fractions were pooled, dialysed against PBS/Az and stored at 4°C. Pooled eluates were then concentrated using Diaflo ultrafiltration membranes (YM10 Amicon), after which the concentrated material was purified by size-exclusion gel filtration on Sephacryl S-100 (Pharmacia). The yield of MFE-23-cys during the purification process was estimated using the optical density measured at 280 nm. Purified MFE-23-cys monomer was concentrated and stored at 4°C.

Labeling of MFE-23-cys

MFE-23-cys monomer was radiolabeled with ^{99m}Tc using a ^{99m}Tc-D-glucarate transfer method (*12*). One milliliter of [^{99m}Tc]-sodium pertechnetate (40.5 mCi) was added to 12.5 mg monopotassium D-glucarate, 16.8 mg of sodium bicarbonate and 100 μg stannous chloride (stannous chloride was made up directly before use at a concentration of 0.2 mg/ml). This solution was left for 1 min at room temperature. Then 0.5 ml was mixed with 200 μg MFE-23-cys in PBS/1 mM EDTA (600 μl). The mixture was incubated at room temperature for 30 min and then applied to a PD-10 column (Pharmacia; primed with 3% human serum albumin in PBS) to separate the radiolabeled protein from the free pertechnetate. The percentage of ^{99m}Tc bound to the protein was tested by thin-layer chromatography using acetonitrile/water (30:20) as running solvent.

An aliquot (100 μl) of diluted radiolabeled scFv (1:30 in PBS/Tween 0.05%) was applied to a 1-ml CEA-sepharose column, containing 1 mg of CEA, to test CEA-binding. Unbound material was washed through with PBS/az. Bound material was obtained by adding 3 *M* ammonium thiocyanate. Furthermore, the stability of the labeled scFv was tested by applying the product (diluted 1:30) to Sephacryl S-100. To test the stability of the radiolabeled product in vivo, serum taken from mice injected with the labeled scFv 24 hours after administration was also applied to Sephacryl S-100. Stability of the ^{99m}Tc-labeled product was also tested by gel autoradiography as follows: radiolabeled MFE-23-cys was subjected to SDS-PAGE using a 15% nonreducing polyacrylamide gel. The protein was then blotted onto Immobilon P (Millipore) and visualized by autoradiography. To assess the serum stability of purified technetium labeled MFE-23-cys in vitro, the protein peak eluted from a PD-10 column was applied to Sephacryl S-100, after which the eluted radiolabeled product was incubated for 24 hr at 37°C with normal human serum. The product was then re-applied to Sephacryl S-100 and the radioactive fractions were collected.

Xenograft Study

The human colon adenocarcinoma cell line LS174T was used to develop a xenograft tumor model (*13*). Technetium-99m-MFE-23-cys (11 μg/36.4 μCi per mouse) was administered through the tail vein. Mice were killed at 24 and 48 hr after tracer administration and blood, liver, kidney, lung, spleen, colon, muscle, femur and tumor were removed. Activity was assessed by counting on the gamma counter after digestion with 7 *M* KOH. Activity was expressed as the percent injected dose per gram of tissue. Four mice were used for each time point.

RESULTS

Yields during MFE-23-cys Purification

After affinity chromatography and concentration, a protein yield of 13.3 mg of MFE-23-cys was obtained from 4 liters of supernatant. The elution profile from the Sephacryl S-100 showed two peaks (Fig. 2); the second peak corresponded with a molecular weight of 27 kD, the correct molecular weight for monomer MFE-23-cys. The first peak was shown to be dimer

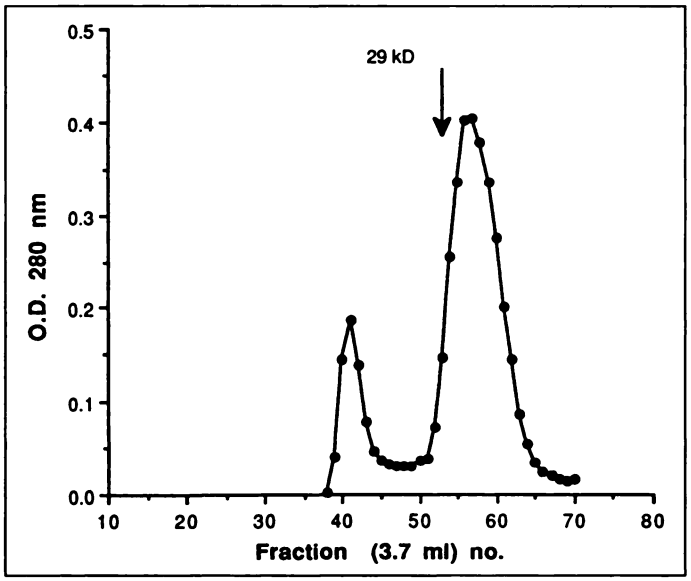

FIGURE 2. Isolation of MFE-23-cys by gel filtration using Sephacryl S-100.

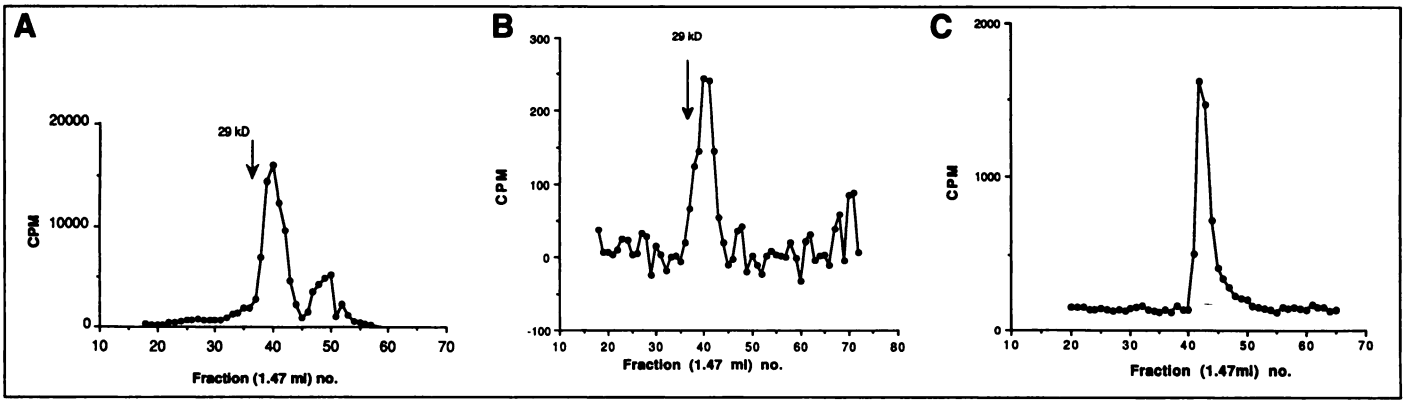

FIGURE 3. (A) Sephacryl S-100 gel filtration showing stability of ^{99m}Tc-labeled MFE-23-cys in vitro. (B) Sephacryl S-100 gel filtration showing stability of ^{99m}Tc-labeled MFE-23-cys in vivo. (C) Sephacryl S-100 gel filtration showing stability of ^{99m}Tc-labeled MFE-23-cys after incubation in normal human serum for 24 hr.

MFE-23-cys on nonreducing SDS-PAGE. After concentrating MFE-23-cys monomer, a final yield of 8 mg was achieved compared to only 1 mg from the dimer peak (ratio 8:1). Purity of monomer MFE-23-cys was confirmed on SDS-PAGE, which revealed only one band at the correct molecular weight (result not shown).

Analysis of Technetium-99m-MFE-23-cys

MFE-23-cys monomer was radiolabeled using a ^{99m}Tc-D-glucarate transfer method. Technetium-99m uptake appeared to be low, 5%–10%, at the protein concentration used in this study so the radiolabeled MFE-23-cys was separated from free pertechnetate by PD-10 chromatography. Thin-layer chromatography of the protein peak obtained from the PD-10 column showed that more than 80% of the ^{99m}Tc was associated with the protein. CEA-binding activity of this radiolabeled product appeared to be very good; after applying the radiolabeled product to the CEA/Sepharose column, 55% of counts was recovered in the bound fraction.

Stability of MFE-23-cys in vitro was confirmed by applying the radiolabeled product to Sephacryl S-100 (Fig. 3A); little degradation was detected. No sign of dimer formation was seen and no aggregation was detected. In accordance with this result, gel autoradiography showed only one band at the correct molecular weight for this scFv (Fig. 4). Serum from mice injected with ^{99m}Tc-labeled scFv was taken 24 hr after administration and applied to Sephacryl S-100. Results showed a single peak at the correct molecular weight (Fig. 3B), which also confirmed its stability in vivo. Since counts were low, the background counts accounted for 30% of the activity. We therefore performed another experiment with the radiolabeled MFE-23-cys to test its stability in normal human serum. After 24 hr incubation at 37°C the ^{99m}Tc-labeled product gave only one peak at the appropriate molecular weight for the scFv, confirming its stability in serum (Fig. 3C).

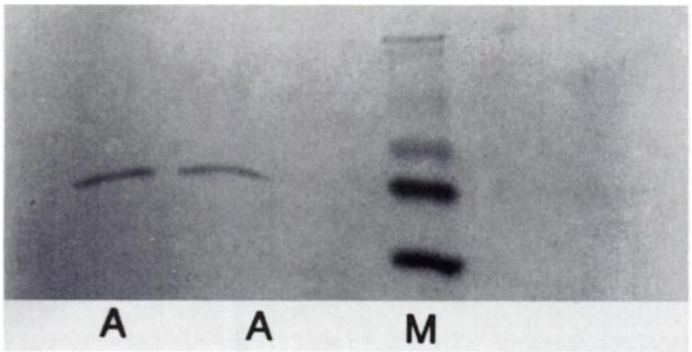

FIGURE 4. Gel autoradiography with ^{99m}Tc-labeled MFE-23-cys. A=^{99m}Tc-labeled MFE-23-cys and M=marker (22-29.7-35-50-97.2.-142kD). A single band was detected at the correct molecular weight (27 kD) for a single-chain Fv.

In Vivo Studies

The biodistribution of ^{99m}Tc-labeled MFE-23-cys was examined over 48 hr. The percent injected activity per gram of tissue and the tissue-to-blood ratios were assessed (Figs. 5 and 6). At 24 hr, approximately 4% of injected activity was localized in the tumor, which dropped to 2.4% at 48 hr. Favorable tumor-to-blood ratios at both time points were seen with ^{99m}Tc-labeled MFE-23-cys because of the fast clearance of the scFv: 4:1 at 24 hr and 8:1 at 48 hr.

Significant activity in normal tissues was only observed in the kidney. Technetium-99m-MFE-23-cys showed 9% injected activity/gram in the kidney at 24 hr and 4.2% at 48 hr, which resulted in high kidney-to-blood ratios at both time points: 10:1 at 24 hr and 17:1 at 48 hr, respectively.

DISCUSSION

We have constructed a vector containing a cysteine in a C-terminal tag for scFvs. The vector is compatible with that used for phage antibody libraries (*6*) and thus allows simple

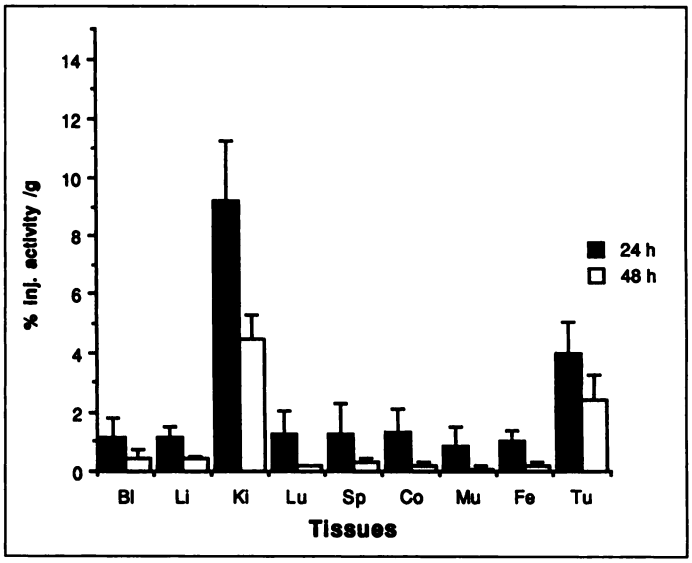

FIGURE 5. Localization in the LS174T colon xenograft of ^{99m}Tc-MFE-23-cys. Results are expressed as percent injected activity per gram of tissue at 24 and 48 hr after injection. Blood (Bl), liver (Li), kidney (Ki), lung (Lu), spleen (Sp), colon (Co), muscle (Mu), femur (Fe) and tumor (Tu). Four mice per group were used. Bars = s.d.

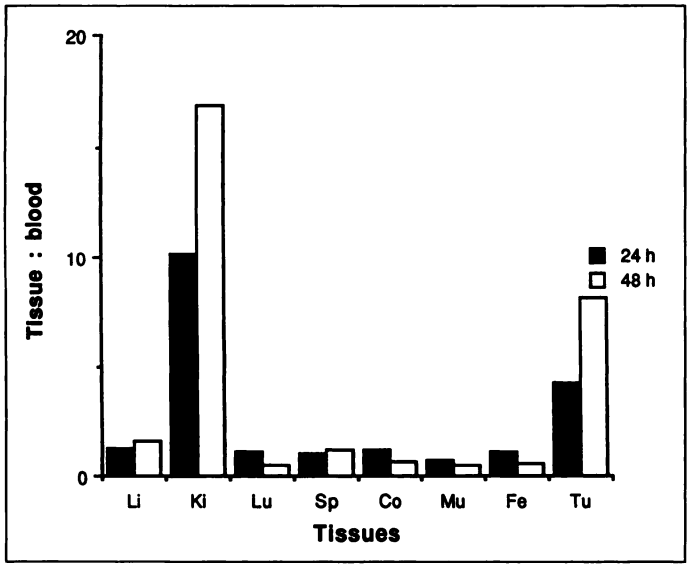

FIGURE 6. Tissue-to-blood ratios of ^{99m}Tc-MFE-23-cys in LS174T colon xenograft at 24 and 48 hr after injection. Liver (Li), lung (Lu), spleen (Sp), colon (Co), muscle (Mu), femur (Fe) and tumor (Tu). Four mice per group were used.

subcloning of scFvs manufactured by filamentous phage technology. Our work demonstrates that a cloned antibody fragment can be easily subcloned into this vector and expressed as a soluble scFv linked to the amino acid cysteine, which contains a thiol group necessary for radiolabeling with ^{99m}Tc. Furthermore, we have shown that the ^{99m}Tc labeling procedure was effective with a cloned scFv, resulting in a stable, biologically active, radiolabeled product.

We used MFE-23 in our study, because this high affinity anti-CEA scFv had already proved to be a good tumor targeting agent when radiolabeled with iodine (*6*). Free thiol groups are shown to be necessary for radiolabeling with ^{99m}Tc (*8*), but reducing this scFv to produce free SH-groups would be likely to denature the protein. Since MFE-23 was produced by phage technology, its genes were already cloned and MFE-23-cys, containing a free thiol group, could be readily derived.

Purification of MFE-23-cys was easy and rapid. The yield was relatively high; 8 mg MFE-23-cys was obtained from 4 liters of supernatant. This is in accordance with the high yield of MFE-23, from which the cys-tagged product was derived. We chose to purify on a CEA-affinity column and the yield was good, but since a histidine tag was also attached to the protein the product could be purified on a metal chelating column. This method appears to be less expensive, more rapid and quite useful when producing a clinical batch (*14*). The high yield of MFE-23-cys allows purification of sufficient quantities for diagnostic imaging studies. Although size-exclusion chromatography did show some dimer formation, the percentage was small (12.5%). Dimer formation is likely to be due to the cysteine group, since the original MFE-23 was all in monomeric form (*15*).

Technetium-99m labeling of MFE-23-cys monomer was performed using a D-glucarate transfer method (*12*). Such a direct labeling method is advantageous since this procedure can be reduced to a one-step labeling process beneficial for making a marketable pharmaceutical product. Furthermore, Pak et al. (*16*) demonstrated that this technique showed selective labeling of SH-groups and the absence of nonspecific binding. The ^{99m}Tc uptake of MFE-23-cys was low, which was probably due to the low protein concentration. This is consistent with the results of Pak et al. (*16*), who showed that a lower labeling efficiency was observed using a low protein concentration. The specific activity (μCi/μg) of the ^{99m}Tc-labeled MFE-23-cys was similar to that with the iodinated MFE-23, which was appropriate for comparable purposes. Although we did not attempt to optimize the radiolabeling efficiency, this could be achieved by increasing the protein concentration, as described by Pak et al.

After radiolabeling with ^{99m}Tc, MFE-23-cys appeared to be stable both in vivo and in vitro. Importantly, MFE-23-cys did not show any sign of dimer formation after ^{99m}Tc radiolabeling.

The ^{99m}Tc-labeled product showed better CEA-binding activity than previously observed with iodinated MFE-23 when tested on a CEA/Sepharose column (*15*). This can be explained by the fact that direct ^{99m}Tc labeling methods are site-specific, using the groups distal to the antigen binding site, which allows the antibody to retain its immunoreactivity (*12,17*).

In vivo biodistribution showed a higher percentage of injected activity in the tumor for ^{99m}Tc-MFE-23-cys than we have reported for iodinated MFE-23 (*15*). This high tumour uptake is advantageous for imaging studies, especially when using short-lived radiolabels such as ^{99m}Tc. The superior tumor uptake can be partly explained by the greater retention of immunoreactivity after radiolabeling with technetium. Higher counts, however, were also detected in the other organs compared to iodinated MFE-23 (*14*), which suggests that ^{99m}Tc remained attached to the protein longer than iodine, which is cleaved off by deiodinases (*7*). This mechanism might have also contributed to the superior tumor localization when using the ^{99m}Tc-labeled scFv.

Despite the activity detected in the blood with ^{99m}Tc-labeled MFE-23-cys, favorable tumor-to-blood ratios were still seen at both time points, 4:1 at 24 hr and 8:1 at 48 hr, respectively. In an earlier animal experiment in our laboratory with ^{99m}Tc-labeled MFE-23-cys, a tumor-to-blood ratio of 2:1 was already seen at 3 hr. Although higher tumor-to-blood ratios were seen with ^{125}I-labeled MFE-23 (13:1 at 24 hr and 24:1 at 48 hr) (*15*), our results show ^{99m}Tc-labeled MFE-23-cys to be a suitable targeting agent for early diagnostic imaging because of its greater total amount in the tumor.

The high kidney uptake appears to be a common problem with ^{99m}Tc-radiolabeled antibody fragments (*5,7*). Lower kidney uptake is seen with ^{125}I-MFE-23 (*15*), probably due to deiodinases. Kidney uptake might also be reduced by using a cleavable chelator (*9*). Since SPECT imaging enables separation of areas of uptake close together in the body, the high kidney uptake should not interfere with imaging studies. There was little uptake observed in other normal tissues.

CONCLUSION

Cloning of a scFv with an extra thiol group is a useful technique, which enables radiolabeling of scFvs with ^{99m}Tc. Technetium-99m-MFE-23-cys showed favorable biodistribution characteristics in vivo for early diagnostic imaging. Although in our experiment, ^{99m}Tc-MFE-23-cys had higher kidney uptake in vivo and a lower tumor-to-blood ratio than iodine-labeled MF3-23, these limitations should be balanced against the overall advantages of ^{99m}Tc radiolabeling—ready availability, low cost, ideal properties for gamma camera imaging, low patient radiation exposure per millicurie of radionuclide and no need for thyroid blocking agents—which make ^{99m}Tc much more practical for immunoscintigraphy.

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FIRST IMPRESSIONS:
Source of Soft-Tissue Activity?

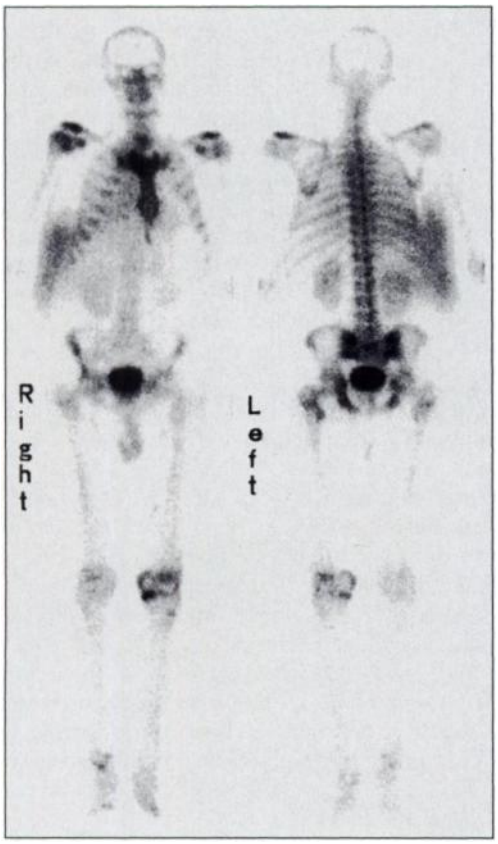

Figure 1.

PURPOSE
Anterior and posterior whole-body bone scan (Fig. 1) demonstrates activity in the right hemithorax as well as in the adjacent soft tissue of a 57-yr-old man with a right hilar mass and pleural effusion. The evening before the bone scan, the patient underwent diagnostic thoracentesis and pleural biopsy. Fluid collection was palpable in the area of the ^{99m}Tc-MDP activity, which was indicative of leakage of the pleural effusion and communication with the pleural cavity.

TRACER
Technetium-99m-MDP, 22 mCi

ROUTE OF ADMINISTRATION
Intravenous

TIME AFTER INJECTION
3 hours

INSTRUMENTATION
Picker Prism 2000

CONTRIBUTORS
Albert T. Lambert and Mike McBiles, Fitzsimons Army Medical Center, Aurora, Colorado

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