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Novel Human Single Chain Antibody Fragments that Are Rapidly Internalizing Effectively Target Epithelioid and Sarcomatoid Mesotheliomas

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Abstract

Human antibodies targeting all subtypes of mesothelioma could be useful to image and treat this deadly disease. Here we report tumor targeting of a novel internalizing human single chain antibody fragment (scFv) labeled with ⁹⁹mTc (⁹⁹mTc-M40) in murine models of mesothelioma of both epithelioid (M28) and sarcomatoid (VAMT-1) origins. ⁹⁹mTc-M40 was taken up rapidly and specifically by both subtype tumor cells in vitro, with 68% to 92% internalized within 1 hour. The specificity of binding was evidenced by blocking (up to 95%) with 10-fold excess of unlabeled M40. In animal studies, tumors of both subtypes were clearly visualized by SPECT/CT as early as 1 hour postinjection of ⁹⁹mTc-M40. Tumor uptake measured as percent of injected dose per gram tissue (%ID/g) at 3 hours was 4.38 and 5.84 for M28 and VAMT-1 tumors, respectively, significantly greater than all organs or tissues studied (liver, 2.62%ID/g; other organs or tissues <1.7%ID/g), except the kidneys (130.7%ID/g), giving tumor-to-blood ratios of 5:1 and 7:1 and tumor-to-muscle ratios of 45:1 and 60:1, for M28 and VAMT-1, respectively. The target-mediated uptake was confirmed by a nearly 70% reduction in tumor activity following administration of 10-fold excess of unlabeled scFv. Taken together, these results indicate that M40 can rapidly and specifically target epithelioid and sarcomatoid tumor cells, demonstrating the potential of this agent as a versatile targeting ligand for imaging and therapy of all subtypes of mesothelioma.

Introduction

Malignant mesothelioma (MM), caused primarily by exposure to asbestos, is a highly aggressive tumor arising from serosal surfaces of the pleura, peritoneum, and pericardium (1, 2). MM has 3 major subtypes: epithelioid (EM) that is more likely to respond to treatment and accounts for 50% to 70% of all cases, sarcomatoid (SM) that rarely responds to any treatment and represents 7% to 20%, and mixed/biphasic for the remaining 20% to 30%. Patients with MM have a median survival time of 8 to 14 months (3). There is an urgent unmet need to develop new diagnostics and therapeutics for MM (4) as the disease has a long latency period with past and ongoing exposure to asbestos contributing to the development of new cases worldwide.

One approach to detect and treat cancer is to conjugate imaging and/or therapeutics to molecules which can recognize internalizing antigens, receptors or cell surface markers that are overexpressed on tumor cells, leading to efficient localization and tumor cell killing (5, 6). However, presently there are very few MM-associated cell surface antigens that are overexpressed by all subtypes of MM, especially the SM (7). One well established marker—mesothelin, a 40 kDa cell surface glycoprotein, has been reported to be expressed by EM cells (8), but not SM (9). Recently we have identified a panel of internalizing human scFv antibodies by phage display selection that target cell surface antigens associated with both EM and SM (6). The selected scFvs bind to human mesothelioma cells in vitro, thereby recognizing clinically represented tumor antigens (6) and thus offer the potential to deliver high levels of imaging probes to tumor cells but not to nontarget normal tissues based on intracellular delivery. By screening the yeast surface human cDNA display library with mesothelioma targeting phage antibody, we have further identified MCAM/CD146/MUC18 as one of the target antigens for MM cells that was overexpressed in more than 80% of EM and SM tissues, but not other tissues (10). In the present study, we investigated both the in vitro and in vivo tumor targeting and imaging potential of an additional scFv (M40) for both EM (M28) and SM (VAMT-1) subtypes.

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Expression and purification of M40

The M40 was produced as previously reported (6, 11–13).

99mTc radiolabeling of M40

The scFv was radiolabeled as previously reported (14, 16). The carbonyl-kit (IsoLink Tyco/Mallinckrodt) was used to prepare the [99mTc(CO)3(OH2)3] moiety. An aliquot (40 μg) of M40 was mixed with 100 to 500 μL of [99mTc(CO)3(OH2)3] solution and the mixture was heated at 37°C for 60 minutes. The product was purified using a PD-10 column. Labeling efficiency and purity were determined by size exclusion HPLC and thin-layer chromatography (TLC).

Fluorescence labeling of M40 (Cy5.5-M40)

The M40 was labeled with Cy5.5 by incubation with 3:1 molar excess of Cy5.5-NHS ester in a carbonate/bicarbonate buffer (pH 7.2–8.5) for 1 hour followed by purification using PD-10 column.

In vitro cell culture assay

Internalization experiments were performed as described previously (12, 15, 16). Briefly, 1 million VAMT-1, M28, or BPH-1 cells (control) were incubated with 0.05 to 2 μCi 99mTc-M40 in various concentrations for 1 or 3 hours. All cell lines had been tested for mycoplasma contamination and characterized by cell proliferation and morphology evaluation (6). The cells were washed to determine cell surface–bound (acid releasable) and internalized (acid resistant) radioligand/radioactivity expressed as the percentage of applied activity normalized to 1 million cells. For nonspecific uptake, the aforementioned procedure was repeated after addition of 10-fold excess unlabeled M40. For microscopy study, 1 × 106 cells were incubated with 20 nmol/L (10 MBq) of Cy5.5-M40 and tumor cells for 1 and 3 hours. The cells were washed to determine cell surface binding and internalization (−) curves of 99mTc-M40 at varying concentrations ranging from 1 to 80 nmol/L in M28 cells (A) and VAMT-1 cells (B) at 37°C at 1 hour. C, the 99mTc-M40 at a concentration of 20 nmol/L was incubated with 1 × 106 cells of M28, VAMT-1, or control (BPH-1) cells for 1 hour at 37°C and 4°C. Blocking study was performed using 10-fold excess unlabeled M40 1 hour prior to incubation with the 99mTc-M40 at 37°C. Inset, internalization of total cell accumulation for 99mTc-M40 after a 1-hour incubation with M28 and VAMT-1 at 37°C. D, fluorescence microscopy of Cy5.5-M40 (red) on mesothelioma tumor cells (VAMT-1), and control cell line (BPH-1) at 1 hour incubation. The cells were stained with lysotracker (green) to demarcate the lysosomal compartments.

Biodistribution studies

Animal procedures were performed according to a protocol approved by the UCSF Animal Care and Use Committee. Six-week-old male nu/nu mice were purchased from Charles River Laboratories. For tumor inoculation, 106 M28 and VAMT-1 cells in 200 μL of PBS were administered subcutaneously into the right and left shoulders of the animal, respectively. The mice were studied when tumor size reached approximately 2 to 4 mm in diameter (30–60 mm3 in volume). Tumor mice in groups of 4 animals were injected each with 18.5 to 37 MBq (0.5–1.0 μCi) of 99mTc-M40 containing approximately 2 to 4 μg of scFv. A control group (blocking study) were injected with 10-fold excess unlabeled M40 1 hour before 99mTc-M40. The biodistribution at 1, 3, or 6 hours of the study group was determined and compared with that of the control group.
SPECT/CT imaging

Mice were imaged with a small animal SPECT/CT system (GE healthcare). For anatomical correlation, CT was first performed after injection. SPECT imaging was initiated 1 and 3 hours after injection.

PET/CT imaging

Mice were fasted overnight prior to 18F-FDG injection of 3.7 to 7.4 MBq [100–200 mCi] and imaged with a microPET/CT system (Siemens Medical Solutions USA, Inc.) 1 hour after injection for a 20-minute acquisition time.

Results

In vitro characterization of 99mTc-M40

The 99mTc labeling yield of M40 (from 99mTc(CO)3(OH)3) was 70% to 85%. The radiochemical purity of the 99mTc-M40 was greater than 95%. The final 99mTc-M40 had a high specific activity of approximately 9.3 MBq/μg (6.3 x10^7 Ci/mol). The 99mTc radiolabeling was stable in phosphate buffer for at least the duration investigated (24 hours), as reported previously (14, 16).

The binding affinity of 99mTc-M40 to the mesothelioma cells exhibited an apparent Kd of 20 to 21 nmol/L and the antigen density for M40 derived from the saturation cell binding assay was comparable on both tumor cells (Fig. 1A, 1B). M40 was rapidly internalized over the concentrations tested with about 68% to 92% of total cell-associated uptake, following 1 hour incubation at 37°C (Fig. 1C), whereas the binding and uptake in the control BPH-1 cells was much less (Fig. 1C). Specificity was further demonstrated by blocking of uptake/ internalization into both M28 and VAMT-1 (>95%) cells with a 10-fold excess of unlabeled M40 (Fig. 1C).

Fluorescence microscopy

As shown in Figure 1D, Cy5.5-M40 was rapidly internalized into mesothelioma cells within 1 hour after incubation at 37°C, whereas there was negligible uptake in the control (BPH-1) cells under identical conditions.

In vivo SPECT/CT imaging and biodistribution of 99mTc-M40

Mice were imaged with small animal SPECT/CT at 1 and 3 hours after injection with concomitant assessment of ex vivo biodistribution at 1, 3, and 6 hours (Fig. 2A). Imaging with SPECT/CT showed high tumor uptake (Fig. 2B) as early as 3 hours postinjection. At 3 hours, tumor uptake was 4.38%ID/g and 5.84%ID/g for M28 and VAMT-1 tumors, respectively (Table 1), greater than for all organs/tissues studied (liver 2.62%ID/g, other organs/tissues <2%ID/g) except the kidney (130.7%ID/g), giving M28 and VAMT-1 tumor/muscle ratios of 45:1, 60:1, and tumors/blood ratios of 5:1 and 7:1, respectively (Fig. 2A). In contrast, the control blocking study with 10-fold excess of unlabeled M40 at 3 hours reduced by more than 70% of tumor uptake of the 99mTc-M40 to 0.9%ID/g and 1.6%ID/g for M28 and VAMT-1, respectively (Table 1 and Fig. 2C).

Micro-PET/CT imaging of 18F-FDG

18F-FDG PET imaging was performed to confirm the distinct identity of the 2 subtypes of tumors in a single animal. As
shown in Figure 2D, the 18F-FDG PET/CT image at 1 hour detected SM (VAMT-1) preferentially.

**Discussion**

We report here the *in vitro* and *in vivo* tumor targeting and imaging potential of a rapidly internalizing human scFv (M40) selected from a panel of scFvs targeting internalizing epitopes present on both EM and SM cells (6).

Several important findings have been uncovered in this study. First, the 99mTc-M40 showed strikingly rapid and selective binding and internalizing ability *in vitro* into the mesothelioma tumor cells but not into the control nontumorigenic cells, consistent with our previous findings (6). Although we did not perform the Lindmo assay (17) to determine what percentage of our labeled antibodies retained reactivity (the immunoreactive fraction), our results nonetheless demonstrate that the M40 binds with high affinity to both subtypes of mesothelioma cells and that either the site-specific labeling of M40 with 99mTc through the hexahistidine tag or conjugation to Cy5.5 does not significantly affect its targeting ability. Second, although the M40 accumulated in the tumors, its clearance from blood and other normal organs was rapid (except from the kidney which is the site for all scFv clearance) making it feasible to label the M40 with positron-emitting radionuclides with short half-lives such as gallium-68 (68Ga) for quantitative PET imaging. Finally, unlike other available antibodies against MM, this antibody has the ability to target both EM and SM. If such dual targeting can be maintained in the clinical setting, this antibody would have a major imaging and therapeutic potential.

The 99mTc-M40 had a high uptake in the kidneys as observed with other 99mTc-scFvs (10, 16), consistent with the route of scFv clearance *in vivo*. Nonetheless, additional engineering of M40 may further increase the kidney clearance rate and improve the contrast. In addition, developing other forms of the antibody such as diabody, minibody, or Affibody (Affibody AB) could further improve the tumor binding and homing efficiency as well as overall pharmacokinetic profile (10, 18).

The 99mTc-M40 rapidly accumulated into both M28 and VAMT-1 cells as early as 3 hours with comparable tumor uptake at 4.38 ±0.39%ID/g and 5.84±0.72%ID/g, respectively. Because we observed the difference of 18F-FDG uptake in our system between the 2 cell types, M28 and VAMT-1, with approximately 8%ID/g and 16%ID/g, respectively, similar to other reports (19, 20), 18F-FDG was used to confirm the identity of the 2 subtypes implanted in a single mouse and thus to highlight the dual targeting by M40. The characteristics of 99mTc-M40 are representative of a promising new class of radiotracers that recognize cell surface markers highly expressed at comparable level on both types of mesothelioma (Fig. 1A & 1B). As such, they may find utility for tumor characterization and staging as well as treatment planning and monitoring complementary to 18F-FDG.

In conclusion, we demonstrated that 99mTc-M40 selectively binds and internalizes into both M28 and VAMT-1 cells.

### Table 1. Biodistribution of 99mTc-M40

<table>
<thead>
<tr>
<th>Organ</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>2.43 ± 0.08</td>
<td>2.62 ± 0.34</td>
<td>2.48 ± 0.22</td>
<td>2.40 ± 0.21</td>
</tr>
<tr>
<td>Heart</td>
<td>0.26 ± 0.10</td>
<td>0.28 ± 0.03</td>
<td>0.23 ± 0.01</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>100.5 ± 4.45</td>
<td>130.7 ± 32.7</td>
<td>95.3 ± 19.31</td>
<td>146.6 ± 12.1</td>
</tr>
<tr>
<td>Lung</td>
<td>0.42 ± 0.13</td>
<td>1.61 ± 0.24</td>
<td>0.51 ± 0.22</td>
<td>0.65 ± 0.34 a</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.55 ± 0.21</td>
<td>1.02 ± 0.36</td>
<td>0.48 ± 0.12</td>
<td>0.66 ± 0.42</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.11 ± 0.01</td>
<td>0.25 ± 0.04</td>
<td>0.18 ± 0.15</td>
<td>0.13 ± 0.03 a</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.36 ± 0.21</td>
<td>0.45 ± 0.13</td>
<td>0.33 ± 0.12</td>
<td>0.29 ± 0.21</td>
</tr>
<tr>
<td>Sm. Int.</td>
<td>0.32 ± 0.16</td>
<td>0.31 ± 0.04</td>
<td>0.40 ± 0.03</td>
<td>0.53 ± 0.44</td>
</tr>
<tr>
<td>Ig. Int.</td>
<td>0.25 ± 0.06</td>
<td>0.25 ± 0.07</td>
<td>0.31 ± 0.08</td>
<td>0.25 ± 0.26</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.06 ± 0.02</td>
<td>0.10 ± 0.05</td>
<td>0.10 ± 0.03</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>Fat</td>
<td>0.11 ± 0.01</td>
<td>0.13 ± 0.03</td>
<td>0.06 ± 0.01</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>Blood</td>
<td>1.20 ± 0.21</td>
<td>0.82 ± 0.13</td>
<td>0.81 ± 0.15</td>
<td>2.40 ± 0.21 a</td>
</tr>
<tr>
<td>Tumor (VAMT-1)</td>
<td>3.74 ± 0.07</td>
<td>5.84 ± 0.72</td>
<td>2.60 ± 0.19</td>
<td>1.62 ± 0.16 a</td>
</tr>
<tr>
<td>Tumor (M-28)</td>
<td>2.68 ± 0.24</td>
<td>4.38 ± 0.39</td>
<td>2.17 ± 0.21</td>
<td>0.91 ± 0.22 a</td>
</tr>
</tbody>
</table>

**NOTE:** The biodistribution of 99mTc-M40 was assessed at 1, 3, and 6 hours after injection in tumor-bearing nude mice. A control (blocking) study was performed with 10-fold excess unlabeled M40 injected 1 hour prior to injection of 99mTc-M40 in tumor-bearing nude mice (*n* = 4). Data are %ID/g ± SD.

*a*Organs with significant difference in uptake at 3 hours after injection between study group and blocking control group (*P* < 0.05).
in vitro, and furthermore rapidly and specifically targets both subtypes of mesothelioma in vivo, demonstrating its potential as a novel agent for imaging and therapy of all subtypes of mesothelioma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**


**Acknowledgments**

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