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Targeting human prostate cancer with $^{111}$In-labeled D2B IgG, F(ab$\prime$)$_2$ and Fab fragments in nude mice with PSMA-expressing xenografts

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D2B is a new monoclonal antibody directed against an extracellular domain of prostate-specific membrane antigen (PSMA), which is overexpressed in prostate cancer. The potential of D2B IgG, and F(ab$\prime$)$_2$ and Fab fragments of this antibody for targeting prostate cancer was determined in mice bearing subcutaneous prostate cancer xenografts. The optimal time point for imaging was determined in biodistribution and microSPECT imaging studies with $^{111}$In-D2B IgG, $^{111}$In-capromab pendetide, $^{111}$In-D2B F(ab$\prime$)$_2$ and $^{111}$In-D2B Fab fragments in mice with PSMA-expressing LNCaP and PSMA-negative PC3 tumors at several time points after injection. All $^{111}$In-labeled antibody formats specifically accumulated in the LNCaP tumors, with highest uptake of $^{111}$In-D2B IgG and $^{111}$In-capromab pendetide at 168 h p.i. (94.8 ± 19.2% injected dose per gram (ID/g) and 16.7 ± 2.2% ID/g, respectively), whereas uptake of $^{111}$In-D2B F(ab$\prime$)$_2$ and $^{111}$In-D2B Fab fragments peaked at 24 h p.i. (12.1 ± 3.0% ID/g and 15.1 ± 2.9% ID/g, respectively). Maximum LNCaP tumor-to-blood ratios were 13.0 ± 2.3 (168 h p.i.), 6.2 ± 0.7 (24 h p.i.), 23.0 ± 4.0 (24 h p.i.) and 4.5 ± 0.6 (168 h p.i.) for $^{111}$In-D2B IgG, $^{111}$In-F(ab$\prime$)$_2$, $^{111}$In-Fab and $^{111}$In-capromab pendetide, respectively. LNCaP tumors were clearly visualized with microSPECT with all antibody formats. This study demonstrates the feasibility of D2B IgG, F(ab$\prime$)$_2$ and Fab fragments for targeting PSMA-expressing prostate cancer xenografts. Copyright © 2014 John Wiley & Sons, Ltd.

**Keywords:** prostate cancer imaging; D2B IgG; Fab fragments; capromab pendetide

1. **INTRODUCTION**

Prostate cancer is the second leading cause of cancer-related deaths in men in the Western world, and since metastatic prostate cancer is incurable, early diagnosis represents the most effective strategy with a change towards curative treatment options. Additionally, differentiation between malignant and nonmalignant prostate tissue is essential for the initiation of adequate treatment and the evaluation of response to therapy and relapse.

At present, prostate cancer is usually diagnosed by a combination of digital rectal examination, evaluation of prostate-specific antigen serum concentrations and transrectal ultrasound (TRUS)-guided biopsy (1). However, the ability of TRUS to delineate small cancer foci and differentiate between benign prostatic hyperplasia, atrophy, inflammatory processes and malignancies is limited. Magnetic resonance imaging (MRI) can be used for primary diagnostic purposes of prostate cancer, for instance if clinical suspicion for prostate cancer persists despite negative prostate biopsies, or for local tumor staging purposes (1). Positron emission tomography (PET) imaging is a useful imaging modality to detect metastatic disease in several cancers (2). However, $^{18}$F-FDG-PET is not suited for staging patients with prostate cancer, owing to the relatively low avidity of prostate cancer for $^{18}$F-FDG (3). The most commonly used approach to identify bone metastases in prostate cancer patients are bone scans. In equivocal cases, $^{11}$C-Choline- or $^{18}$F-fluoride-PET/CT can be an option (1). Recent developments in molecular imaging include radiolabeled monoclonal antibodies (mAb) used as tracers for single-photon emission tomography (SPECT) and PET.

The prostate-specific membrane antigen (PSMA) is a type II membrane glycoprotein (4) that provides an excellent target for prostate cancer imaging owing to its selective overexpression on the surface of prostate cancer cells (5). Initial validation of PSMA as an in vivo target resulted from imaging with the $^{111}$In-labeled mAb capromab pendetide (7E11/CYT-356, Prostascint$^\text{®}$) (6), which is approved by the US Food and Drug Administration for diagnostic imaging in patients with biopsy-proven prostate cancer, who are at high risk for pelvic lymph node metastases (7). However,
Targeting PSMA-Expressing Prostate Cancer

capromab pendetide is directed against an epitope on an intracellular domain of PSMA (8). Therefore, binding of this antibody to tumor cells requires uptake of the antibody into the cells. It has been postulated that capromab pendetide may only bind to dying or necrotic cells with cellular membranes that are permeable for IgG. The mAb J591, which is directed against an extracellular epitope of PSMA, has shown great potential for diagnostic radio-nuclide imaging in prostate cancer patients (9–11), especially when labeled with \(^{111}\)In, showing excellent tumor targeting of metastases (12).

In the present study, we use a new mAb D2B IgG, a murine IgG1 mAb directed against an epitope on the extracellular domain of PSMA (13). We characterized the tumor targeting capacity of D2B IgG and compared it with those of F(ab\(^\prime\))\(_2\) and Fab fragments of D2B IgG to evaluate possible limitations such as low tumor-to-blood ratios that can occur owing to relatively slow clearance of intact antibodies compared to fragments (14). In these experiments, capromab pendetide was used as a reference.

2. MATERIALS AND METHODS

2.1. Production and purification of antibodies and fragments

The monoclonal antibody D2B (IgG1) was purified from hybridoma culture supernatant by Protein A affinity chromatography. The antibody secreting cells were obtained according to the hybridoma technology from mice that were immunized with a cell lysate of LNCaP cells. Mice were injected three times (days 0, 14 and 28) i.p. with 800 μg of lysate and Freund’s adjuvant (Sigma-Aldrich, St Louis, MO, USA). On the last three days before the spleen of the mice was harvested, mice were boosted with 10 μg of recombinant PSMA protein (Novagen, Milan, Italy) intravenously. F(ab\(^\prime\))\(_2\) fragments were produced by pepsin digestion essentially as described earlier (15). Briefly, after incubation of D2B IgG for 3 h at 37 °C with pepsin in 0.15 M sodium citrate (pH 3.8), digestion was stopped by adjusting the pH of the solution to pH 7.0 with 1.0 M Tris. F(ab\(^\prime\))\(_2\) fragments were purified using a cation exchange column (Mono-S H/R 5/5, Pharmacia, Uppsala, Sweden) that was eluted with 40 mM sodium acetate buffer (pH 6.0) with a 0–400 mM lithium chloride gradient. The F(ab\(^\prime\))\(_2\) fragment containing fractions were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and pooled. Fab fragments were generated by papain digestion (6 h at 37 °C) of D2B IgG using papain immobilized on agarose resin according to the protocol of the manufacturer (Thermo Scientific, Pierce Fab Preparation Kit) followed by purification using protein A affinity chromatography and dialysis.

2.2. Cell culture

The human prostate cancer cell lines PC3 (PSMA\(^{-}\)) and LNCaP (PSMA\(^{+}\)) derived from a bone metastasis and a supraclavicular lymph node, respectively, were used. Both cell lines were obtained from the ATCC and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum (Life Technologies, Bleiswijk, The Netherlands) and glutamine 2 mM.

2.3. Mouse models

Male BALB/c nude mice (Janvier, Le Genest Saint Isle, France), 8–9 weeks old, housed in filter-topped cages (five mice per cage) under nonsterile standard conditions with free access to standard animal chow and water, were adapted to laboratory conditions for 1 week before experimental use. Mice were subcutaneously inoculated with \(3 \times 10^6\) LNCaP cells (right flank) suspended in 200 μl of 33% complete RPMI 1640 medium with 67% Matrigel (BD Biosciences) and with \(3 \times 10^6\) PC3 cells (left flank) suspended in 200 μl of 67% complete RPMI 1640 medium with 33% Matrigel. LNCaP cells were inoculated 7 days prior to PC3 cells. LNCaP and PC3 tumors grew to approximately 0.1 g in 14 and 7 days after tumor cell inoculation, respectively.

All experiments have been approved by the institutional Animal Welfare Committee of the Radboud University Medical Center and were conducted in accordance with the principles set forth by the Revised Dutch Act on Animal Experimentation.

2.4. Radiolabeling of D2B

The murine mAb D2B IgG and all fragments were conjugated with p-isothiocyanatobenzyl-diethylenetriaminepentaacetic acid (ITC-DTPA; Macrocyclics) in 0.1 M NaHCO\(_3\), pH 9.5, with a 10-fold molar excess of ITC-DTPA (2.5 mg D2B IgG with 110 μg ITC-DTPA, 4.5 mg F(ab\(^\prime\))\(_2\) with 250 μg ITC-DTPA and 0.75 mg Fab with 100 μg ITC-DTPA). After incubation for 1 h at room temperature, the reaction mixture was dialyzed for 3 days in a Slide-A-Lyzer (20 kDa cutoff, Pierce) against 0.25 M NH\(_4\)Ac, pH 5.4.

For biodistribution studies, D2B IgG-DTPA (28 μg), F(ab\(^\prime\))\(_2\)-DTPA (22 μg), Fab-DTPA (20 μg) and capromab pendetide (30 μg) were radiolabeled with 3.7 MBq \(^{111}\)In chloride (Covidien, Petten, Netherlands) in 0.1 M MES buffer pH 5.4 (three times the volume of \(^{111}\)In chloride) and incubated during 30 min at room temperature under metal-free conditions. For SPECT/CT studies, D2B IgG-DTPA (20 μg), F(ab\(^\prime\))\(_2\)-DTPA (25 μg), Fab-DTPA (25 μg) and capromab pendetide-DTPA (30 μg) were labeled with \(^{111}\)In in 0.1 M MES buffer pH 5.4 at specific activities of 2.57 GBq/nmol (D2B IgG), 982.3 MBq/nmol (F(ab\(^\prime\))\(_2\)), 232.4 MBq/nmol (Fab), and 56 MBq/nmol (capromab pendetide). Volumes of the radiolabeling reaction were 1.3 ml (D2B IgG), 1.5 ml (F(ab\(^\prime\))\(_2\)), 2.0 ml (Fab) and 1.5 ml (capromab pendetide). Following incubation, 50 mM EDTA was added to the final concentration of 5 mM to chelate unincorporated \(^{111}\)In.

The labeling efficiency was determined by instant thin-layer chromatography on silicagel strips (ITLC-SG; Gelman Sciences, Ann Arbor, MI, USA) using 15 μm citrate buffer, pH 6.0, as the mobile phase. The radiochemical purity of the preparations that were used in the biodistribution experiments was 95, 58, 90 and 95%, for \(^{111}\)In-labeled D2B IgG-DTPA, F(ab\(^\prime\))\(_2\)-DTPA, Fab-DTPA and capromab-DTPA, respectively. For SPECT-imaging, it was 70, 79, 97 and 95%, respectively. \(^{111}\)In-labeled D2B IgG-DTPA, F(ab\(^\prime\))\(_2\)-DTPA, Fab-DTPA, and capromab-DTPA were purified by gel filtration on a PD-10 column. The radiochemical purity was determined by ITLC and exceeded 97% for all preparations used in the studies.

2.5. Immunoreactivity

The immunoreactive fractions of the radiolabeled antibody/fragment preparations were determined using freshly trypsinized LNCaP cells, essentially as described by Lindmo et al. (16,17). The activity in the vials and in the cell pellet after washing with 500 μl binding buffer was determined in the gamma counter (Wizard 3\(^{\mathrm{TM}}\) 1480, LKB-Wallac, Oy, Finland) after incubation of 1 h at 37 °C. Immunoreactive fractions of all radiolabeled preparations used in the experiments exceeded 80%. For \(^{111}\)In-capromab pendetide,
the assay was carried out on live and permeabilized LNCaP cells using the Fix & Perm Cell Fixation and Permeabilization Kit (Invitrogen) to permeabilize the cells.

2.6. Competitive binding assay

The IC50 of D2B IgG, F(ab′)2, and Fab fragments was determined in a competitive binding assay on LNCaP cells using 111In-labeled D2B as a tracer. LNCaP cells were grown to confluence in six-well plates, and incubated on ice for 2 h in 1 ml of binding buffer with 1.85 kBq of 111In-labeled D2B IgG and increasing concentrations (0.005–300 nM) of unlabeled D2B IgG, F(ab′)2, and Fab fragments. Cells were washed with binding buffer after incubation and the cell-associated activity was measured in a gamma counter. IC50 values were calculated using GraphPad Prism software, version 5.03 (GraphPad, La Jolla, CA, USA).

2.7. Flow cytometric analysis

For cell surface staining, LNCaP cells were incubated with either the mAbs D2B IgG, capromab pendetide or an isotype control murine IgG (Jackson ImmunoResearch) in phosphate-buffered saline (PBS) with 2% of bovine serum albumin (BSA) and 2% human serum (HS) for 30 min on ice. Part of the cells were fixed in 2% paraformaldehyde and incubated in PBS, 2% HS and 0.1% saponin to permeabilize the cells. After the incubation, samples were washed three times in PBS or PBS and 0.1% saponin and incubated for 20 min on ice with the secondary anti-mouse-alexa 488 antibody for staining. Cells were analyzed in a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences).

2.8. Biodistribution studies

The effect of mAb protein dose on the biodistribution of 111In-D2B in nude mice with subcutaneous LNCaP tumors was determined in groups of five mice that received different protein doses of 111In-D2B IgG (0.1, 1, 3, 10, 30 or 100 μg of D2B IgG per mouse, 0.4 MBq). Mice were euthanized with CO2/O2 asphyxiation 3 days after injection of 111In-D2B IgG. Blood samples were obtained by heart puncture and tissues (LNCaP tumor, muscle, lung, spleen, kidney, liver, pancreas, stomach, duodenum and prostate) were dissected, weighed, and the radioactivity was measured in a gamma counter. For calculation of the uptake of radioactivity in each tissue as a fraction of the injected dose, an aliquot of the injection dose was counted simultaneously.

In a subsequent study, 111In-D2B IgG (0.4 MBq, 3.0 μg/mouse), 111In-F(ab′)2 (0.4 MBq, 2.2 μg/mouse), 111In-Fab fragments (0.4 MBq, 1 μg/mouse) or 111In-capromab pendetide (0.4 MBq, 5 μg/mouse) was injected i.v. into groups of five mice with subcutaneous PC3 tumors (left flank) and LNCaP tumors (right flank). In addition, for Fab and F(ab′)2, five mice per antibody format received an excess of unlabeled D2B IgG (300 μg/mouse) two days before injection of the 111In-labeled fragments. For D2B IgG and capromab pendetide, the excess of unlabeled D2B IgG or capromab was injected on the same day as the 111In-labeled antibodies. Mice were euthanized by CO2/O2 asphyxiation and the biodistribution of 111In-D2B IgG was determined at 1, 2, 4, 24, 72 and 168 h after injection. Because of their more rapid clearance, biodistribution of the 111In-F(ab′)2 and 111In-Fab fragments was determined at 1, 2, 4 and 24 h after injection. Activity concentrations in the tissues (% ID/g) were determined as described above.

2.9. MicroSPECT/CT imaging

Four groups of five mice with subcutaneous LNCaP (right flank) and PC3 (left flank) tumors were injected with 37 MBq of 111In-D2B IgG (3.0 μg/mouse), 111In-F(ab′)2 (1.3 μg/mouse), 111In-Fab (1 μg/mouse) and 111In-capromab pendetide (5 μg/mouse),
respectively. The mice that received $^{111}$In-labeled D2B IgG and capromab pendetide, SPECT/CT scans were imaged 0, 1, 3 and 7 days after injection using a small animal SPECT/CT scanner (U-SPECT II, MILabs, Utrecht, Netherlands; acquisition time 30–90 min, depending on the time point) with a 1.0 mm diameter pinhole collimator tube. Mice injected with $^{111}$In-F(a$b'$)$_2$ and $^{111}$In-Fab fragments underwent SPECT/CT at 0, 1 and 2 days after injection. For Fab and F(ab')$_2$, two additional mice were injected with an excess of unlabeled D2B IgG 2 days before injection of the $^{111}$In-labeled antibody fragments. For D2B IgG, the excess of unlabeled antibody formats was coadministered on the same day as $^{111}$In-D2B.

After the last scan, mice were euthanized and biodistribution of the $^{111}$In-D2B, $^{111}$In-F(a$b'$)$_2$, $^{111}$In-Fab or $^{111}$In-capromab pendetide was determined as described above. Scans were reconstructed with MILabs reconstruction software (MILabs, Utrecht, Netherlands), which uses an ordered-subset expectation maximization algorithm.

2.10. Statistical analysis

Statistical analyses were performed with Graphpad Prism, version 5.03. Results are presented as mean ± standard deviation (SD). Differences in tumor uptake of the antibody formats were tested for significance using the two-tailed t-test. A $p$-value below 0.05 was considered significant.

3. RESULTS

3.1. In vitro characterization

Flow cytometric analysis (FACS) with live and permeabilized LNCaP cells revealed high binding of D2B IgG to live (95.9 ± 2.2%) as well as to permeabilized cells (96.9 ± 1.7%). In contrast, for capromab pendetide, binding to live cells was low (7.6 ± 0.7%), while binding to permeabilized cells was high (96.1 ± 1.1%), showing that D2B recognizes an epitope on the cell surface, while capromab is directed against an intracellular epitope (Fig. 1).

For $^{111}$In-D2B IgG the immunoreactive fraction on live LNCaP cells was 84.1%, and the immunoreactive fractions of $^{111}$In-capromab pendetide on permeabilized LNCaP cells were 93.6%, indicating that the immunoreactivity of both antibodies was preserved during the labeling procedure. The IC$_{50}$ of D2B IgG and F(a$b'$)$_2$ fragments was 3.7 nM (95% confidence interval, CI: 2.8–4.8 nM) and 1.9 nM (CI 1.6–2.3 nM), respectively, whereas it was slightly lower for Fab fragments (8.9 nM; CI 7.1–11.0 nM Fig. 2), which could be due to the monovalency of Fab fragments.

Figure 2. Competitive binding curves of IC$_{50}$ determination of D2B IgG, F(a$b'$)$_2$, and Fab fragments on prostate-specific membrane antigen (PSMA)-positive LNCaP cells.

Figure 3. Biodistribution results of the dose escalation of $^{111}$In-D2B in mice ($n=5$) with subcutaneous LNCaP xenografts. Data are presented as mean ± SD.
3.2. Biodistribution studies

In the dose escalation study an inverse correlation between the D2B antibody protein dose and the uptake in the LNCaP tumor was observed: LNCaP tumor uptake of $^{111}$In-D2B IgG decreased from $52 \pm 5\%$ ID/g at 0.1 $\mu$g/mouse to $26 \pm 1.5\%$ ID/g at 100 $\mu$g/mouse. Highest tumor uptake ($54 \pm 4\%$ ID/g)

Figure 4. Ex vivo biodistribution of nude mice bearing subcutaneous PSMA-negative PC3 and PSMA-positive LNCaP xenografts at several time points after intravenous injection of (a) $^{111}$In-D2B IgG, (b) $^{111}$In-F(ab')$_2$, (c) $^{111}$In-Fab fragments and (d) $^{111}$In-capromab pendetide. Data are presented as mean ± SD.
was observed at D2B doses ≤ 3 μg/mouse at 3 days p.i. (Fig. 3). At that time point, tumor-to-blood ratios ranged from 4.3 (at 0.1 μg/mouse) to 1.8 (at 100 μg/mouse). Tumor weight was 0.04 g ± 0.02 g (D2B IgG group), 0.08 g ± 0.03 g (Fab′ group), 0.07 g ± 0.02 g (Fab group) and 0.10 g ± 0.02 g (capromab group).

For biodistribution studies, 111In-D2B IgG (0.4 MBq, 3.0 μg/mouse), 111In-F(ab′)2 (0.4 MBq, 2.2 μg/mouse), 111In-Fab fragments (0.4 MBq, 1 μg/mouse) or 111In-capromab pendetide (0.4 MBq, 5 μg/mouse) was injected ivanecine. All three 111In-labeled D2B antibody formats specifically accumulated in the s.c. PSMA+ LNCaP tumors (right flank), while uptake in PSMA-PC3 xenografts (left flank) remained low. LNCaP tumor uptake increased for 111In-D2B IgG from 2.2 ± 2.0% ID/g at 1 h p.i. to 94.8 ± 19.2% ID/g at 168 h after injection, the latter representing the highest tumor uptake observed in these studies (Fig. 4). For 111In-F(ab′)2 and 111In-Fab, LNCaP tumor uptake ranged from 1.6 ± 0.6 and 4.1 ± 0.9% ID/g at 1 h p.i. to 12.1 ± 3.0 and 15.1 ± 2.9% ID/g at 24 h after injection, respectively. For 111In-labeled capromab pendetide, LNCaP tumor uptake ranged from 0.7 ± 0.4% ID/g at 1 h p.i. to 16.7 ± 2.2% ID/g at 168 h after injection, reaching a maximum of 17.4 ± 2.8% ID/g at 3 days after injection.

Tumor uptake of the three D2B formats could be blocked by coinjection of an excess of unlabeled mAb. The uptake of 111In-D2B IgG decreased from 60.9 ± 11.0 to 12.3 ± 7.1% ID/g at 72 h p.i. The LNCaP uptake of 111In-F(ab′)2, decreased from 4.8 ± 2.1% ID/g to 3.5 ± 1.8% ID/g (4 h p.i.), while the LNCaP uptake of 111In-Fab decreased from 8.5 ± 2.4% ID/g to 3.9 ± 0.4% ID/g, respectively at 4 h p.i. Maximum uptake in the PSMA-negative PC3 tumor was 9.1 ± 1.3% (24 h p.i.), 6.8 ± 1.3% (4 h p.i.) and 6.3 ± 1.1% ID/g (2 h p.i.) for 111In-labeled IgG, F(ab′)2 and Fab, further confirming the specific uptake of the D2B antibody formats in the PSMA+ tumor.

Maximum LNCaP tumor-to-blood ratio for 111In-D2B IgG was 13.0 ± 2.3 (168 h p.i.). For D2B Fab and (Fab′)2 fragments, high tumor-to-blood ratios were reached faster compared with tumor-to-blood ratios of D2B IgG, with a maximum of 23.0 ± 4.0 and 1.8 (at 100 μg/mouse), 0.08 g ± 0.03 g (F(ab′)2 group), 0.10 g ± 0.02 g (capromab group).

### Table 1. Ex vivo biodistribution (tissue uptake % ID/g) and LNCaP tumor-to-organ ratios of 111In-capromab pendetide, 111In-D2B IgG, 111In-F(ab′)2 and 111In-Fab fragments in nude mice with subcutaneous prostate-specific membrane antigen (PSMA)-negative PC3 xenografts and PSMA-positive LNCaP xenografts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>111In-Capromab (168 h p.i.) (% ID/g tissue uptake)</th>
<th>111In-D2B IgG (168 h p.i.) (% ID/g tissue uptake)</th>
<th>111In-F(ab′)2 (24 h p.i.) (% ID/g tissue uptake)</th>
<th>111In-Fab (24 h p.i.) (% ID/g tissue uptake)</th>
<th>111In-Fab (24 h p.i.) (% ID/g tissue uptake)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>3.7 ± 0.3</td>
<td>4.5 ± 0.6</td>
<td>7.3 ± 0.7</td>
<td>13.0 ± 2.3</td>
<td>2.0 ± 0.3</td>
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<td>Muscle</td>
<td>0.5 ± 0.1</td>
<td>31.9 ± 6.5</td>
<td>0.9 ± 0.2</td>
<td>110.4 ± 41.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>3.0 ± 0.4</td>
<td>5.5 ± 0.7</td>
<td>4.3 ± 0.8</td>
<td>22.8 ± 6.3</td>
<td>2.1 ± 0.1</td>
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<tr>
<td>Spleen</td>
<td>5.3 ± 0.4</td>
<td>3.5 ± 0.6</td>
<td>3.1 ± 0.13</td>
<td>30.4 ± 6.4</td>
<td>5.5 ± 0.7</td>
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<td>Pancreas</td>
<td>1.1 ± 0.1</td>
<td>14.9 ± 1.0</td>
<td>1.0 ± 0.2</td>
<td>98.9 ± 18.6</td>
<td>0.9 ± 0.0</td>
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<tr>
<td>Kidney</td>
<td>9.0 ± 0.6</td>
<td>1.9 ± 0.3</td>
<td>2.7 ± 0.4</td>
<td>35.5 ± 7.5</td>
<td>57.8 ± 6.4</td>
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<tr>
<td>Stomach</td>
<td>1.00 ± 0.1</td>
<td>17.6 ± 2.5</td>
<td>0.9 ± 0.2</td>
<td>104.0 ± 31.0</td>
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<tr>
<td>Duodenum</td>
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<td>11.9 ± 2.4</td>
<td>1.5 ± 0.1</td>
<td>63.4 ± 14.3</td>
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<tr>
<td>Liver</td>
<td>5.5 ± 0.7</td>
<td>3.1 ± 0.7</td>
<td>3.4 ± 0.3</td>
<td>28.2 ± 4.6</td>
<td>7.3 ± 0.4</td>
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<tr>
<td>Prostate</td>
<td>1.8 ± 0.2</td>
<td>9.5 ± 1.8</td>
<td>1.7 ± 0.5</td>
<td>59.2 ± 14.4</td>
<td>1.6 ± 0.3</td>
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<tr>
<td>PC3 tumor</td>
<td>7.1 ± 1.2</td>
<td>2.4 ± 0.4</td>
<td>6.7 ± 0.9</td>
<td>14.0 ± 1.2</td>
<td>6.5 ± 1.0</td>
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<tr>
<td>LNCaP tumor</td>
<td>16.7 ± 2.2</td>
<td>94.8 ± 19.2</td>
<td>12.1 ± 3.0</td>
<td>1.9 ± 0.2</td>
<td>2.2 ± 0.4</td>
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3.3. MicroSPECT/CT Imaging

Coronal sections of SPECT/CT images of all 111In-labeled antibody formats through the center of subcutaneous PSMA+ LNCaP and PSMA-PC3 tumors are displayed in Fig. 5. At 24 h p.i., LNCaP tumors were clearly visualized with all formats. The optimal time point for imaging with 111In-D2B IgG and 111In-capromab pendetide was 168 h p.i. and for 111In-F(ab′)2 and 111In-Fab this was 48 h p.i. Tumor uptake was specific for 111In-D2B IgG, 111In-F(ab′)2 and 111In-Fab since tumor uptake was markedly lower in mice coinjected with an excess of unlabeled D2B IgG (Fig. 5). Uptake in the PSMA+ PC3 tumors remained low for all antibody formats at all time points.

For D2B Fab′ and Fab fragments, high accumulation of 111In in the kidneys, especially the renal cortex, was observed, indicating that these antibody preparations were cleared via the kidneys apparently with tubular reabsorption of the 111In-labeled metabolites.

4. DISCUSSION

In the present study, the in vivo tumor targeting characteristics of 111In-D2B IgG, 111In-F(ab′)2, and 111In-Fab fragments for targeting of prostate cancer were examined in mice with human prostate cancer xenografts using 111In-labeled capromab pendetide as a reference.

Since intact antibodies clear slowly from the circulation and maximum tumor uptake is reached at 3–7 days after injection, we included Fab′ and Fab fragments of D2B in our studies to investigate whether further improvements in prostate cancer targeting would be possible by using D2B antibody fragments. The slow accumulation of intact antibodies has been attributed to tubular reabsorption of the 111In-labeled metabolites.
Figure 5. Representative SPECT/CT images of mice bearing subcutaneous PSMA-negative PC3 (left flank, green arrows) and PSMA-positive LNCaP (right flank, blue arrows) prostate cancer xenografts at several time points after intravenous injection of $^{111}$In-D2B IgG, $^{111}$In-F(ab′)$_2$, $^{111}$In-Fab fragments and $^{111}$In-capromab pendetide.
to the slow blood clearance of intact IgG in combination with the presence of various physiological barriers between the circulation and the tumor cell surface (18). It has been pointed out that the vascular endothelium, the relatively large transport distances in the tissue and the enhanced interstitial fluid pressure in the tumor tissue hamper the penetration of antibodies into the tumor tissue to bind to their target antigen. Antibody fragments such as F(ab)′2 and Fab fragments are cleared more rapidly from the blood, mainly because they lack the CH2 domain, which causes IgG molecules to recycle into the bloodstream via the FcRn receptor (19). On the other hand, the sustained blood levels of intact IgG molecules are the driving force for their accumulation in tumors. Therefore, the uptake of antibody fragments in tumors is lower than that of intact IgGs, as was also observed in the present study. Because the diffusion rate of a molecule is linearly correlated to its molecular size (20), antibody fragments diffuse more rapidly in the interstitial fluid. Owing to the more rapid blood clearance, higher tumor-to-blood ratios are obtained. With the antibody fragments, tumors can be visualized more rapidly: optimal images can be acquired within 24 h p.i., whereas intact IgG can be used when high concentrations of the antibody in the tumor are required (e.g. in therapeutic applications when high payloads of toxic agents should be targeted to the tumor).

Here, we observed that the tumor uptake of 111In-D2B IgG was higher than that of 111In-F(ab)′2 and 111In-Fab at all time points, even as early as 24 h after injection. However, at early time points after injection, tumor–nontumor ratios of 111In-F(ab)′2 and 111In-Fab were higher than those of 111In-D2B IgG, indicating that the antibody fragments are preferred for imaging purposes. For the evaluation of response to therapy, when imaging early after tracer injection is required, and to determine tracer uptake before and shortly after intervention, antibody fragments should be used.

In contrast to 111In-D2B IgG, the renal clearance of 111In-F(ab)′2 and 111In-Fab resulted in high accumulation in the kidneys, most likely owing to tubular reabsorption after glomerular filtration of these antibody preparations. Usually, the threshold for glomerular filtration is approximately 60 kDa, suggesting that the antibody fragments are catabolized into smaller molecules in the kidneys (21). Renal clearance and accumulation of F(ab)′2 and Fab fragments might be a disadvantage, since it might interfere with visualization of malignant tissue located in the vicinity of the kidneys and the urinary bladder.

For the characterization of the tumor targeting capacities of 111In-D2B IgG, 111In-F(ab)′2 and 111In-Fab fragments, 111In-labeled capromab pendetide was used as a reference in this study. Since capromab pendetide targets an intracellular epitope of PSMA, internalization or uptake into the cell by another process is required to bind to PSMA. It has been postulated that capromab pendetide may only bind dying or necrotic cells with the presence of various physiological barriers between the circulation and the tumor cell surface. Antibody fragments such as F(ab)′2 and Fab fragments are cleared more rapidly from the blood, mainly because they lack the CH2 domain, which causes IgG molecules to recycle into the bloodstream via the FcRn receptor. On the other hand, the sustained blood levels of intact IgG molecules are the driving force for their accumulation in tumors. Therefore, the uptake of antibody fragments in tumors is lower than that of intact IgGs, as was also observed in the present study. Because the diffusion rate of a molecule is linearly correlated to its molecular size, antibody fragments diffuse more rapidly in the interstitial fluid. Owing to the more rapid blood clearance, higher tumor-to-blood ratios are obtained. With the antibody fragments, tumors can be visualized more rapidly: optimal images can be acquired within 24 h p.i., whereas intact IgG can be used when high concentrations of the antibody in the tumor are required (e.g. in therapeutic applications when high payloads of toxic agents should be targeted to the tumor).

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Still, specific tumor targeting of the PSMA+ xenografts was clearly visualized in SPECT/CT with specific differentiation between PSMA− and PSMA+ tumors. Compared with 111In-capromab pendetide, higher tumor-to-blood ratios were obtained. Owing to renal accumulation of F(ab)′2 and Fab fragments, visualization of malignant tissue located in the vicinity of the kidneys might be hampered. However, especially in settings where multiple images within a short time interval are required, 111In-D2B F(ab)′2 and Fab fragments can be used.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

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