Noninvasive In Vivo Imaging and Biologic Characterization of Thyroid Tumors by ImmunoPET Targeting of Galectin-3

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Abstract

The high prevalence of thyroid nodules in the adult population and the relatively low incidence of thyroid cancer make the preoperative identification of malignant lesions challenging. The β-galactoside-binding protein galectin-3 is widely expressed in well-differentiated thyroid carcinomas, but not in normal thyrocytes and benign thyroid nodules. This molecule offers a candidate biomarker to improve thyroid cancer diagnosis. Here we report the development of an immunoPET approach for noninvasive imaging of thyroid cancer. The method employs a 89Zr-labeled mAb to galectin-3, which shows high specificity and binding affinity in vitro. Reliable and specific immunoPET imaging was obtained of thyroid cancer in vivo in murine xenograft models of human thyroid cancer. Our findings provide a method to improve the clinical management of patients with thyroid nodules while reducing unnecessary surgery and social costs.

Introduction

Thyroid nodules are a common clinical problem. About 40% of the adult population has one or more clinically evident nodules, but the number is much higher (up to 67%) when subclinical nodules discovered during thyroid ECHO-scan are also counted (1). Fortunately, 80%–85% of these lesions are benign. Fine-needle aspiration biopsy (FNAB) has substantially improved the preoperative characterization of thyroid nodules, but the method fails to provide a definitive diagnosis in the large majority of follicular thyroid proliferations (2). Ultrasonography (thyroid ECHO-scan) can correctly drive FNAB toward suspicious nodules, allowing the evaluation of their structure, size, and vascularization; but the method per se does not provide any biologic information. On a different clinical ground, thyroid scintigraphy with radioiodine is widely used preoperatively. Although the method provides functional information on iodine uptake (cold or hot nodules), it invariably fails to distinguish among benign and malignant lesions. Indeed, a diagnostic method, which combines in vivo imaging and biologic characterization of thyroid nodules is needed.

The use of PET in combination with different PET tracers like [18F]-2-fluoro-2-deoxy-d-glucose ([18F-FDG]), [18F]-DOPA, and 68Ga-somatostatin analogues has been considered for this purpose (3). 18F-FDG PET has been used but its sensitivity and specificity are questioned, although a cancer diagnosis is generally ruled out in the presence of negative result (4–6). PET imaging of a thyroid cancer–associated marker would be preferable.

The β-galactoside-binding protein galectin-3 (Gal-3), the expression of which has been extensively investigated in normal, benign and malignant thyroid condition (7–13), seems to be the candidate molecule for exploring immunoPET targeting of thyroid cancer in vivo.

Several molecular and clinical studies provide a solid biologic rationale to support this work hypothesis: (i) Gal-3 is undetectable in normal and benign thyroid conditions, whereas well-differentiated thyroid cancers consistently express Gal-3 in the cytosol, on the cell plasma membrane, and in pericellular milieu (7–13); (ii) the forced expression of Gal-3 in thyroid cells (via eDNA transfection) generates a transformed phenotype (14, 15). Conversely, inhibition of Gal-3 expression (via mRNA interference) reverts the transformed phenotype in a papillary thyroid carcinoma cell line and in a breast carcinoma cell line as well (15, 16); (iii) the aberrant expression of Gal-3 in thyroid follicular cells blocks the apoptotic program, a feature that favors the development of cancer (17–19); (iv) Gal-3 is a physiologic target of p53 transcriptional activity and p53-mediated downregulation of Gal-3 is required for p53-induced apoptosis (20); (v) genetic studies provide the evidence that the hypomethylation state of 5 CpG sites in the Gal-3 gene is highly associated with thyroid malignancies (21); (vi) an immunohistochemical test method based on Gal-3 expression analysis has been developed for improving the diagnostic accuracy of conventional thyroid FNA cytology and histology (8, 9, 22–24). The method has been validated for clinical use in two large multicenter studies (8, 9). The

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larger one, performed retrospectively on well-characterized thyroid lesions (histologic samples), showed 94% sensitivity and 98% specificity of Gal-3 immunodetection in distinguishing benign from malignant thyroid lesions, with positive predictive value 98%, negative predictive value 94%, and diagnostic accuracy 96% (8). With this background, an immunooPET strategy for imaging thyroid cancer in vivo based on Gal-3 immunotargeting has been developed.

A putative thyroid cancer–specific probe was created ad hoc by radiolabeling a rat mAb to a Gal-3 amino-terminal epitope, with a long half-life positron emitter \( ^{89}\text{Zr} \). The radiotracer showed high binding specificity to Gal-3 in vitro and was able to provide a reliable imaging of thyroid cancer xenografts in vivo. This innovation, if optimized for clinical use, promises a selective imaging of Gal-3–positive (malignant) thyroid nodules in vivo, allowing a better preoperative selection of the lesions candidate to surgery.

Materials and Methods

Chemical, reagents, and instruments

All reagents were obtained from Sigma-Aldrich unless otherwise stated. A panel of mAbs to different Gal-3 epitopes was produced by using as immunogen a full-length human Gal-3 synthetic peptide (R&D Systems). Comparative experiments on Gal-3 binding in vitro and ex vivo did not show any substantial difference in immunoreactivity among the Gal-3 mAbs available so far (unpublished data; refs. 7–12 and 20). For in vitro imaging experiments, a well-characterized rat mAb (M3/38) to an amino-terminal common epitope of human and mouse Gal-3, was instrumental (25). Sterile and protein G affinity–purified M3/38 mAb was derived from TIB-166 hybridoma (ATCC) and used for preparing the PET radiotracer. Zirconium-89 \( (^{89}\text{Zr}; t_{1/2} = 74.8\) hours, \( \beta^+ = 22.6\%\); 1 GBq/mL in 1 mol/L oxalic acid) was produced from BV Cyclotron. Activity measurement were made using a Capintec CRC-15R Dose Calibrator (Capintec) with a calibration factor of 495 for \( ^{89}\text{Zr} \). Accurate quantification of activity was performed by counting experimental samples for 1 minute in a Perkin-Elmer Automatic Wizard Gamma Counter, with energy window of 800 to 1,000 keV for \( ^{89}\text{Zr} \) (909 keV emission). Desferrioxamine–thiouryl–phenyl–isothiocyanate (DFO) was purchased from Macrocyclics, Inc.

Cell cultures

Anaplastic thyroid carcinoma cells FRO82-1 (ATCC), follicular thyroid carcinoma cells WRO82-1 (kindly provided by Dr. Silvia Soddu, National Cancer Institute Regina Elena, Rome, Italy), and papillary thyroid carcinoma cells BCPAP (DSMZ-German Collection of Microorganisms and Cell Cultures), were described previously (26–28). Cells were cultured in complete RPMI1640 culture medium in standard condition (29) and tested for integrity, Gal-3 expression, and tumorigenicity before each experimental procedure as reported elsewhere. All in vitro experiments using the aforementioned cell lines were repeated at least four times.

Preparation of Gal-3 immunoPET radiotracer

Gal-3–specific rat mAb M3/38 was modified with DFO as reported previously (30, 31). In short, while gently shaking, a three molar excess of DFO (5 mmol/L; 15 μL) was added dropwise to the purified anti-Gal-3 mAb solution (25 nmol/L in 0.5 mL of 0.1 mol/L NaHCO\(_3\), pH 9.0) incubating at 37°C for 30 minutes. Nonconjugated chelate was removed by size exclusion chromatography using a Sephadex G-25 column (GE Healthcare Life Sciences) and 0.9% sodium chloride as eluent. \( ^{89}\text{Zr-DFO-anti-Gal-3} \) probe was labeled by reaction of \( ^{89}\text{Zr-oxalate} \) and DFO-mAb to Gal-3 for 30 minutes at 37°C at pH 7.0 (detailed description is shown in Supplementary Data). \( ^{89}\text{Zr-DFO-mAb} \) to Gal-3 was purified by size exclusion chromatography (Sephadex G-25, PD-10 column, >30 kDa, GE Healthcare Life Sciences) using 0.25 mol/L sodium acetate buffer/0.5% gentisic acid (pH 5.5) as eluent. Radioimmunoconjugate stability was assessed incubating 100 μL \( ^{89}\text{Zr-DFO-mAb} \) to Gal-3 in human serum, sodium acetate/0.5% gentisic acid pH 5.5 at 37°C, and in 50 mmol/L DTPA at 4°C, over 120 hours. Every 24 hours, samples of radiolabeled antibody (1.5 μL) were spotted on ITLC silica gel strip and developed in 0.02 mol/L citrate buffer/DTPA 50 mmol/L (pH 5.0) used as eluent. The number of DFO molecules to mAb molecules was determined according to the tracer method described by Meares and colleagues adapted for the bifunctional chelator and radioisotope used (32).

Immunocomplex characterization and integrity assays

The radioimmunoconjugate was analyzed by high-performance liquid chromatography (HPLC) and SDS–PAGE followed by phosphor imager analysis for integrity. Radiochemical purity was measured by HPLC analysis of \( ^{89}\text{Zr-DFO-mAb} \) to Gal-3 with a SEC-s3000 size exclusion column (Phenomenex, PBS buffer [0.15 mol/L NaCl, 0.05 mol/L sodium phosphate (pH 6.8)] as eluent (flow rate 1.0 mL/minute, UV detector set at 280 nm combined with a radioactivity detector). Protein concentration was determined with bichinolinic acid reagent (Pierce Chemical). SDS–PAGE analysis was performed under nonreducing conditions on a 10% Tris-HCl Precast gel (Bio-Rad Laboratories) by loading an equal amount of protein (25 μg). Molecular species corresponding to the native anti-Gal-3 mAb and immunocomplexes were visualized by Blue Coomassie staining. Phosphor imaging analysis was performed to confirm the biomolecular integrity of the \( ^{89}\text{Zr-labeled mAb} \) to Gal-3. Briefly, after drying, the gel was exposed against Fuji imaging plates (FUJI) for 5 seconds followed by image acquisition with a high-speed Phosphor Imager scanner (C35 Bio, Raytest) and analyzed by Aida Image Software.

Western blot and FACS analyses

FRO82-1, WRO82-1, and BCPAP cell lysates were prepared using RIPA buffer (NaCl 150 mmol/L, Tri-HCl 50 mmol/L, Triton-100 2%, SDS 0.1%, EDTA 1 mmol/L) supplemented with 1% protease inhibitors (Sigma) as reported (29). Protein concentration was determined (BCA assay) and aliquots of cell lysate (38 μg) were loaded and resolved on 10% SDS-Precast gel (Bio-Rad Laboratories) under nonreducing conditions and then blotted onto polyvinylidene difluoride membrane (PVDF; Millipore). Gal-3 molecular species were visualized as reported previously (20). Gal-3 expression on the target thyroid cancer cell lines was also assessed by FACS analysis by using a Gal-3–specific mAb followed by incubation with a specie-specific secondary antisera conjugated with AlexaFluor488 (Abcam). The specificity of signal was demonstrated by omitting the primary mAb. Measurements were performed using FACSCalibur System (Becton Dickinson) and data were analyzed using FlowJo 8.8.6 software.
Binding studies

**Saturation binding assay.** Saturation binding studies were performed with **89**Zr-DFO-mAb to Gal-3 and FRO82-1 thyroid carcinoma as target cells. For each experiment, seven test solutions were prepared in triplicate. They contained increasing amounts of **89**Zr-labeled mAb to Gal-3 (1 × 10^{-12} mol/L to 1 × 10^{-6} mol/L) and 6 × 10^5 FRO82-1 cells in a total volume of 200 μL of PBS/1% BSA (pH 7.4). The solutions were incubated for 2 hours at 4°C, then centrifuged and washed twice with ice-cold PBS/1% BSA. For each concentration of the radiotracer, nonspecific binding was determined in the presence of 100-fold molar excess of cold unmodified mAb. Data were analyzed with nonlinear regression method using GraphPad Prism software 4.0 to determine the KD and Bmax values, and a Scatchard transformation was performed.

**Radiotracer immunoreactivity test.** Immunoreactivity of **89**Zr-labeled anti-Gal-3 probe was assessed using a modified protocol derived from the Lindmo method (33), which extrapolates the binding of the radiolabeled antibody at an infinite excess of antigen. Six serial dilutions of FRO82-1 tumor cells were prepared in triplicate (3.2 × 10^6 to 8 × 10^6 cells/well) and incubated with the radiolabeled mAb to Gal-3 (5 ng, 34,000 cpm) for 1 hour at 4°C, in a total volume of 500 μL of PBS/1% BSA (pH 7.4). Nonspecific binding was determined in the presence of 1,000-fold molar excess of the unmodified antibody (5 μg/well). Cells were then centrifuged, washed twice with cold PBS 1% with BSA, and radioactivity associated with the pellets was finally counted in a γ-counter. The count data were background corrected and compared with the total number of counts in standard samples. Immunoreactive fractions were determined by linear regression analysis of a plot of (total/bound) activity versus (1/[normalized number of cells]), and calculated as 1/γ intercept. The data were analyzed using GraphPad Prism software 4.0.

**In vivo studies**

**Thyroid cancer xenografts.** Female athymic Nude-Foxn1nu/nu mice (CD1-Foxn1nu, 5 weeks old (Charles River Laboratories), were used for establishing thyroid cancer xenografts in vivo by subcutaneous injection of FRO82-1, WRO82-1, and BCPAP cells. Mice were kept in cages of 3 animals each with water and food ad libitum. Cells were harvested at 80% confluence, washed twice in sterile PBS, and injected subcutaneously into nude mice in 100 μL sterile PBS. Tumor growth was checked by measuring the long and short diameter with a caliper twice a week. For tissue biodistribution analysis, five groups of animal (5 mice each) were transplanted with 5 × 10^6 cells; one group (5 mice) was used for blocking experiments. For imaging studies, three groups of mice (n = 8 mice/group) were xenografted in the right thigh with 1.0–1.5 × 10^7 FRO82-1, WRO82-1, and BCPAP cells and used for Gal-3 immunoPET experiments as described elsewhere. Mice were handled according to guidelines for the welfare and use of animals in cancer research (34). In vivo experiments were approved by the local authorities (animal license numbers: 55.2-1-54-2531-52-07; 55.2-1-54-2531-146-08; 55.2-1-54-2532-119-13).

**Time-dependent tissue accumulation of **89**Zr-DFO-mAb to Gal-3.** Five groups of female athymic Nude-Foxn1nu/nu mice (CD1-Foxn1nu, 5 mice/group) were injected intravenously with 1.5 MBq (40 μCi) of **89**Zr-DFO-mAb to Gal-3 via lateral tail vein, under isoflurane anesthesia. ImmunoPET imaging was performed on an Inveon Small-Animal PET/CT scanner (Siemens) at 24, 48, 72, 96, and 120 hours after injection of the radiotracer. Mice were placed in prone position on the scanner bed, and 60-minute static images were acquired using a 400–650 keVwindow. Images were reconstructed by a three-dimensional ordered subsets expectation maximum algorithm (OSEM3D/MAP).

The resulting matrix was 128 × 128 pixels with 159 transverse slices (voxel size 0.78 × 0.78 × 0.80 mm^3). Data were normalized and corrected for random, dead time, and decay with no correction for attenuation or scatter. Anesthetized mice were then, bled, killed, and dissected after each imaging session at different time points (24, 48, 72, 96, and 120 hours). Tumor and organs were weighted and counted in a γ-counter for ex vivo radioactivity accumulation analysis. Radioactivity uptake was calculated as percentage of injected dose per gram of tissue (%ID/g; mean ± SD).
pathologists. Gal-3 immunoreactivity of tumor xenografts and tissues was scored as follows: ++, homogeneous staining >70% of the lesion; +, variable staining >10 <70% of the lesion; +/−, heterogeneous staining <10% of the lesion; and negative staining. A coherent constitutive Gal-3 expression in some cell types (i.e., macrophages) was expected in several organs (i.e., liver) and reported in detail when observed.

Results
Production and characterization of $^{89}$Zr-DFO-anti-Gal-3 probe
Purified rat anti-Gal-3 mAb was functionalized with DFO and labeled with the long-lived positron emitter zirconium-89 ($^{89}$Zr) under mild condition as described elsewhere. The 3:1 chelate: mAb conjugation ratio, chosen to be kept below 2 the substitution ratio, did not affect mAb immunoreactivity and provided a chelate:mAb coupling ratio of 0.9. The labeling process did not impair mAb structural integrity as demonstrated in FACS analysis on the same target cells by using a radiolabeled anti-Gal-3 mAb (Fig. 2B). The binding specificity was confirmed by the absence of signal in tumor cells incubated for 30 minutes at 4°C with the secondary AlexaFluor488 conjugated anti-rat IgG-specific antisera (Fig. 2C).

Gal-3 expression in thyroid carcinoma cell lines
Cell lysates obtained from $5 \times 10^6$ FRO82-1, WRO82-1, and BCPAP thyroid carcinoma cell lines were analyzed for constitutive Gal-3 expression in Western blot analysis. As expected, a sharp molecular species of 31 kDa MW, compatible with Gal-3, was detected in all the instances (Fig. 2A). Gal-3 expression was also demonstrated in FACS analysis on the same target cells by using a rat mAb to Gal-3 (Mabtech; Fig. 2B). The binding specificity was confirmed by the absence of signal in tumor cells incubated for 30 minutes at 4°C with the secondary AlexaFluor488 conjugated anti-rat IgG-specific antisera (Fig. 2C).

Saturation binding and immunoreactivity studies
Binding affinity and immunoreactivity of the radiolabeled anti-Gal-3 mAb were analyzed in vitro on FRO82-1 target cells. The saturation binding curves generated were characteristic for high-affinity binding to a single class of antigen with $K_d$ of 8.5 ± 0.5 nmol/L (Fig. 3A). Immunoreactivity of $^{89}$Zr-labeled anti-Gal-3 mAb was tested on serial dilutions of FRO82-1 tumor cells ($2 \times 10^6$ to $8 \times 10^5$ cells/well) incubated in the presence or absence of unmodified mAb using a modified Lindmo assay (see Materials and Methods for details). To calculate the immunoreactive fraction, a double inverse plot of total activity applied over specific binding ([TA]/[SB]) as function of the inverse cell concentration (1/[cells]) was employed. By fitting a straight line through these data, we determined the intercept value at the ordinate, which equals 1/r where r represents the immunoreactive fraction of the total amount of antibody. Under these conditions, the $^{89}$Zr-labeled anti-Gal-3 mAb showed to retain 75% of the activity of 148 kBq/nmol was routinely achieved. Radiochemical purity measured by size exclusion chromatography (SEC-HPLC) after gel chromatography purification was higher than 99% (Fig. 1A). According to SDS-PAGE and autoradiographic analysis, DFO functionalization and radiolabeling procedures did not impair mAb structural integrity (Fig. 1B). $^{89}$Zr-DFO-anti-Gal-3 probe showed high in vitro stability with less than 5% of $^{89}$Zr lost over 120 hours (data not shown), confirming what was previously reported for other $^{89}$Zr-labeled mAbs (33).

Figure 1. DFO-mAb conjugate characterization. A, SEC-HPLC analysis of $^{89}$Zr-DFO-mAb to Gal-3. UV profile (dotted line) shows one peak with retention time (Rt) of 9 minutes, indicating high integrity of the immunonjugate and high radiochemical purity (solid line) of the radioimmunoconjugate. A small radioactive peak due to formation of dimers is visible. B, SDS-PAGE performed under nonreducing conditions and autoradiography analysis of mAb to Gal-3. No molecular modifications occurred to the rat mAb to Gal-3 during conjugation and radiolabeling reactions. More than 99% of the radioactivity was associated with the expected molecular species resolved in the gel. The band at 170 kDa corresponds to the mAb with posttranslational modifications, whereas the weak band at 73 kDa corresponds to the heavy chains of antibody due to the thaw freezing process.
immunoreactivity (Fig. 3B). The binding specificity was further confirmed in Western blotting on cell lysates from FRO82-1, WRO82-1, and BCPAP thyroid carcinoma cells under nonreducing conditions. A sharp molecular species of 31 kDa corresponding to Gal-3 is visible. β-Actin (49 kDa) was used as control. B, plasma membrane expression of Gal-3 assessed by FACS analysis in FRO82-1, WRO82-1, and BCPAP cells. Staining was performed with a rat mAb to Gal-3 (black line); unstained cells served as negative control (blue line). C, no signal was detected when the respective cell lines were incubated with the AlexaFluor488-conjugated secondary antibody alone (black line).

Figure 3.
Radiotracer cell binding assays. A, saturation binding test of 89Zr-labeled mAb to Gal-3 on FRO82-1 cells incubated with increasing concentrations of radiotracer. Nonspecific binding was determined in presence of 100-fold molar excess of unlabeled mAb to Gal-3. Inset shows the Scatchard plot analysis of the same data. B, Lindmo immunoreactivity analysis of radio labeled anti-Gal-3 mAb in presence of increasing number of FRO82-1 cells. (TA/[SB] data are plotted as a function of the reciprocal of cell concentration (1/[cells]). The y intercept gives the reciprocal of the immunoreactivity (75%, r = 1/1.34). C, the figure shows the specific binding of 89Zr-DFO-mAb to Gal-3 to cell lysates obtained from FRO82-1, WRO82-1, and BCPAP cells (top) and the blockage by 100-fold excess of unlabeled rat mAb to Gal-3 (bottom).

Time-dependent tumor accumulation of 89Zr-DFO-mAb to Gal-3 in vivo
For this experiment, athymic Nude-Foxn1 nu mice-bearing FRO82-1 tumor xenografts (5/group) were injected with 1.5 MBq
(40 μCi, 4 μg) of 89Zr-labeled anti-Gal-3 mAb. A time-dependent increase of radiotracer tumor accumulation was visualized by performing static PET/CT acquisitions at 24, 48, 72, 96, and 120 hours after injection. Tumor uptake was already visible at 24 hours after injection and a high contrast image (tumor/background) was achieved at 48 hours after injection, with no detectable tracer accumulation in normal tissues, except for the liver (Fig. 4A), in which 89Zr residualized after catabolism of the conjugate and joins where 89Zr accumulates. Using image-derived uptake calculations, the major increase of the specific tumor signal was registered between 48 and 72 hours, with 16.30 ± 1.51 and 18.92 ± 3.01 %ID/g (mean ± SD), respectively. The specificity of 89Zr-DFO-mAb to Gal-3 binding was confirmed by the reduction of tracer uptake in mice (n = 5) preinjected intravenously (1 day before injection of radiotracer) with a 100-fold excess of unlabeled anti-Gal-3 mAb (Fig. 4B). Ex vivo analysis of radiotracer biodistribution performed at different time points (24, 48, 72, 96, and 120 hours after injection) showed a time-dependent tumor accumulation ranging from 14.65 ± 1.74 %ID/g to 21.41 ± 1.76 %ID/g (Fig. 4C). Differences in radiotracer tumor accumulation were found statistically significant: P < 0.05 for mice measured between 24 and 72 hours, and P < 0.01 for mice measured between 24 and 96 hours, and between 24 and 120 hours (Fig. 4D). A strong reduction of activity in the blood (50.92%) measured between 24 and 48 hours after injection (24 hours: 14.41 ± 6.74 %ID/g, 48 hours: 8.24 ± 2.53 %ID/g), and reduction of measured activity in heart and kidneys during the 120 hours selected for the study, yielded a positive tumor/organ ratio and high contrast images, accounting for a relatively fast blood clearance through renal excretion (Fig. 4E–G; Supplementary Table S1). As expected, no uptake of the radiotracer was registered in the mouse thyroid gland, which was used as internal negative control for Gal-3 mAb binding.

In vivo Gal-3 immunoPET targeting in thyroid carcinoma models

In vivo Gal-3 immunoPET targeting was demonstrated in three different thyroid cancer models that constitutively express Gal-3. FRO82-1, WRO82-1, and BCPAP thyroid cancer xenografts were established in athymic Nude-Foxn1nu/nu mice (n = 8 mice/group) by cell injection in the right thigh. Two weeks after cell transplantation (tumor size ranging from 2.5 to 5 mm), mice were injected in the tail vein with 1.5 MBq (40 μCi) of 89Zr-labeled mAb to Gal-3 and subjected to imaging sessions at 48 hours after injection. Static PET/CT images showed high tumor to background contrast in all the xenografted animals with a clear accumulation of the radiotracer in the tumor, rapid blood clearance, and accumulation of activity in the liver (Fig. 5A–C). After imaging session, mice were sacrificed, the organs extracted, dried, weighted, and analyzed in a γ-counter for %ID/g measurement. Ex vivo analysis of organ biodistribution of 89Zr-labeled probe revealed a tumor accumulation ranging between 6.0 and 9.0 %ID/g, a specific reduction of radiotracer binding in mice preinjected with an excess (100-fold) of cold mAb to Gal-3, and a positive tumor/organ ratio in all the animals (Fig. 6A–C; Supplementary Table S2). Radiotracer tumor accumulation (%ID/g of tissue) was measured in vivo by using image-derived uptake calculations and ex vivo by using a γ-counter. For these specific experiments, 6 mice from each group with visible tumor growth were finally considered. In 4 mice, the tumor growth was not measurable, and in two instances the animals died for other reasons.

A good correlation between in vivo and ex vivo measurements was found: R² = 0.729, R² = 0.879, and R² = 0.798 for FRO82-1, WRO82-1, and BCPAP, respectively (Fig. 6D). Analyzing all tumors, independently by the cell line of origin, the correlation between in vivo and ex vivo measurements was confirmed with a R² = 0.756 (Fig. 6E).

Gal-3 expression analysis in tumor xenografts and normal mouse tissues ex vivo

After in vivo imaging experiments, mice were sacrificed. Formalin-fixed and paraffin-embedded tissue sections were obtained at autopsy from the excised tumors and normal organs for further immunohistochemical studies. As expected, Gal-3 was detected on the cell plasma membrane, in the cytoplasm and pericellular

Figure 4.

In vivo characterization of 89Zr-labeled mAb to Gal-3. A, maximum intensity projection (MIP) of μ-PET images acquired at 48 hours postinjection (p.i.) of 40 μCi radiotracer shows a clear accumulation of 89Zr-labeled mAb to Gal-3 in the tumor in the right thigh. The axial projection shows a fusion image of CT and PET (bottom). B, a strong reduction of tracer uptake in mice preinjected with 100-fold excess of unlabeled mAb and imaged 48 hours postinjection of radiotracer confirmed the binding specificity. C, in biodistribution study, a time-dependent increase of radiotracer tumor uptake, fast renal clearance, and consequent reduction of blood pool activity was measured over 120 hours. D, the increase in tumor uptake was found statistically significant between 24 and 72 hours (P = 0.05), 24 and 96 hours (P = 0.01), and 24 and 120 hours (P = 0.01); E-G, positive tumor to blood (T/B), tumor to heart (T/H), and tumor to kidneys (T/K) ratios were responsible for the high contrast image obtained already at 48 hours postinjection.

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Figure 5.
Gal-3 targeting in different thyroid cancer xenograft models. Maximum intensity projection (MIP) and axial projection of static PET/CT images acquired at 48 hours postinjection of \(^{89}\)Zr-labeled mAb to Gal-3. High tumor to background contrast is visible in all three animal models due to a rapid blood clearance. Interestingly, tumors with a size of 5 mm for FRO82-1 (A), 3 mm for WRO82-1 (B), and 2.5 mm for BCPAP (C) were clearly visualized.

Activity accumulation in the joints was due to cartilage uptake of free \(^{89}\)Zr released by tracer metabolites. No radioactivity was found to be associated with the normal thyroid radioactivity was found to be released by tracer metabolites. No

6 models (pictures are representative of and comparison of all three animal max value were used for the analysis due to cartilage uptake of free89Zr

Activity accumulation in the joints was due to cartilage uptake of free. No

interstitium of FRO82-1, WRO82-1, and BCPAP thyroid cancer xenografts with a variable pattern of staining (>10% to <70% of the lesion).

A representative panel of pictures is shown in Supplementary Fig. S1D. Very importantly, normal thyroid gland was invariably Gal-3 negative. Only scattered gal-3–positive macrophages were visible (Supplementary Fig. S1D). The lack of Gal-3 immunoreactivity in the autologous normal thyroid epithelium (widely demonstrated in human studies also; see refs. 8 and 9), further confirms the restricted expression of gal-3 in thyroid malignancies, and the binding specificity of \(^{89}\)Zr-DFO- Gal-3 mAb to thyroid cancer in vivo.

As a general finding, in normal tissues, the expression of Gal-3 was restricted to scattered macrophages, mostly present in the alveolar spaces of the lung and hepatic sinusoids (Kupffer cells). Intercalated ductal cells of renal tubuli were also Gal-3 positive (Supplementary Fig. S2A–S2D).

Discussion

Gal-3 expression in thyroid gland is restricted to malignant-transformed thyrocytes, although scattered Gal-3–positive follicles associated to lymphoid aggregates may be observed in Hashimoto thyroiditis. Interestingly, benign thyroid proliferations (nodular hyperplasia and adenoma) do not express this marker (8–13). This fact, together with a strong biologic rationale, which explains the restricted expression of gal-3 in thyroid cancer (7–21), prompted us to explore the possibility to image thyroid cancer in vivo by using Gal-3 immunoPET. The imaging method we propose combines the high sensitivity and resolution of a PET camera with the biomolecular specificity of Gal-3 mAb for thyroid cancer.

A \(^{89}\)Zr-DFO-mAb to Gal-3 has been created, characterized by >90% isotope complexation, high radiochemical purity (>99%), and high specific activity. No impairment of structural integrity, immunoreactivity, retention of antibody affinity \((K_d \text{ in nmol/L, range})\), and stability in human serum over the time, was observed. Biodistribution studies performed in FRO82-1–xenografted mice showed a clear accumulation of the radiotracer in the tumors already after 24 hours postinjection. On the other side, very low accumulation of the radiotracer was observed in normal tissues, except for the liver, in which exogenous antibodies are cleared (35–37). This result speaks for a fast in vivo Gal-3 tumor targeting, which has been proven to be specific in xenografted mice pretreated with unlabeled Gal-3 mAb. The reliability of the proposed imaging approach has been verified in three different animal models of human thyroid carcinomas constitutively expressing Gal-3. Differences in tumor accumulation of the radiotracer among the thyroid cancer models considered in this study may be explained, at least in part, by the different specific activity of the radiotracer injected, (which means presence of unlabeled molecules in the formulation) or most likely by differences in Gal-3 expression among the target cells (38, 39). Furthermore, immunoPET targeting of Gal-3 on FRO82-1, WRO82-1, and BCPAP tumor xenografts, imaged at 2 weeks after cell transplantation, provided high tumor/background contrast at 48 hours after tracer injection. The different rate of growth of the tumor cell lines in vivo allowed us to evaluate the limit of detection of Gal-3 immunoPET. Tumors of 0.5 cm (FRO82-1), 0.3 cm (WRO82-1), and 0.25 cm (BCPAP) mean diameter were clearly visualized, demonstrating high imaging sensitivity.

Residual activity visible in the heart speaks for residual blood pool activity that might be ascribed to a different animal-specific physiology, biodistribution, and renal excretion of the probe. The absence of radiotracer uptake in the autologous thyroid gland observed in all mice probed with \(^{89}\)Zr-DFO-mAb to Gal-3 reinforces the evidence that normal thyroid cells do not express Gal-3. This critical finding is confirmed by a plethora of immunohistochemical studies on human thyroid tissues published so far (7–13, 40–43).
A further support to the diagnostic value of Gal-3 immunoPET is the good correlation we found between %ID/g measured in tumor specimens ex vivo (via γ-counter) and the in vivo image-derived uptake calculations ($R^2 = 0.756$). The discrepancy between in vivo and ex vivo tumor accumulation, responsible for this correlation value, might be explained by partial volume effect in the image-derived uptake calculations, which can underestimate the uptake in small or heterogeneous target regions. Nevertheless, these results provide the first experimental evidence on the reliability and diagnostic potential of immunoPET targeting of Gal-3 for imaging thyroid cancer in vivo and represent a decisive step forward with respect to the previously published report (44). Because of the superior technical characteristics of a PET scanner in comparison with a SPECT camera (higher sensitivity, higher spatial resolution, faster acquisition of data, acquisition of dynamic images in tomographic mode), we believe that immunoPET targeting of Gal-3 for tumor imaging will perform better in a clinical setting (45, 46).

The proposed imaging approach is not intended for replacing conventional FNA-cytology in the preoperative characterization of follicular thyroid nodules, but should be used to integrate it in those thyroid conditions (about 30% of the case) in which conventional cytologic procedures are not conclusive (i.e., inadequate Thy-1/indeterminate Thy-3 follicular nodules). Furthermore, Gal-3 immunoPET has the potential to improve thyroid cancer diagnosis in the following conditions: (i) multiple thyroid nodules; (ii) small suspicious subcentimetric lesions (3–4 mm); (iii) small thyroid nodules in mediastinal position; (iv) suspicious high-risk thyroid lesions intimately associated to vascular structures, for which FNAB may be harmful. Gal-3 immunoPET targeting could also contribute to distinguish among normal thyroid tissue residues and minimal residual thyroid cancer after thyroidectomy. Although these conditions can be easily visualized with radioiodine, they will likely show a differential Gal-3 expression, according to their specific biologic behavior. Moreover, a fraction of poorly differentiated thyroid carcinomas (rare lesions) generally keep gal-3 expression but lose the ability to uptake radioiodine (8). In these instances, immunoPET targeting of Gal-3 might be of great utility in making a decision about the most appropriate therapeutic intervention (47, 48). Although discrimination among benign and malignant thyroid nodules represents per se the elective application of the proposed imaging method, this approach may be useful in other tumors. Gal-3, in fact, is expressed in different primary and...
metastatic tumors (i.e., melanoma, breast carcinoma, prostatic carcinoma; ref. 18). The possibility to image primary tumors in vivo or to detect melanoma or breast carcinoma metastasis in sentinel lymph nodes preoperatively represents interesting fields of research to be pursued.

Concluding, GaL-3 immunoPET targeting represents an innovative diagnostic method for in vivo detection and biologic characterization of thyroid nodules. This specific application is supported by a strong clinical and biologic rationale. The method could be potentially applied for imaging different tumor targets in vivo, but this possibility needs further investigations. Humanization of GaL-3 mAb and structural modifications of the probe (i.e., radiolabeled Fab-fragments) are in progress and may facilitate translation in the clinical setting. The proposed approach integrates the diagnostic procedure available so far and promises to improve the clinical management of patients bearing thyroid nodules, reducing unnecessary surgery, and social costs (49, 50).

Disclosure of Potential Conflicts of Interest
S. Braesch-Andersen is an owner/researcher and has ownership interest (including patents) in Mabtech. M. Schwaiger reports receiving a commercial research grant from Siemens Medical Solutions and has ownership interest (including patents) in Surgic Eye. A. Bartolazzi has ownership of a patent regarding the use of radiolabeled antibodies for galectin-3 imaging in vivo. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C. D’Alessandria, M. Schwaiger, A. Bartolazzi
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Thyroid Cancer Imaging In Vivo

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