Specific Localization, Gamma Camera Imaging, and Intracellular Trafficking of Radiolabelled Chimeric Anti-GD3 Ganglioside Monoclonal Antibody KM871 in SK-MEL-28 Melanoma Xenografts


ABSTRACT

The chimeric monoclonal antibody KM871, directed against the GD3 antigen, is under evaluation for its potential to target melanoma. To facilitate the in vivo evaluation of biodistribution properties and measurement of pharmacokinetics, KM871 was radiolabeled with 125I via tyrosine residues and with 111In via the bifunctional metal ion chelator C-functionalized trans-cyclohexyl diethylenetriaminepentaacetic acid (CHX-A’-DTPA) to lysine residues. Using antigen-positive SK-MEL-28 melanoma cells, immunoreactivities of 42 and 40% cell binding were obtained, respectively, for the two radioconjugates. Binding was enhanced in the presence of added unlabeled antibody. A humanized A33 antibody was similarly labeled with the two isotopes and used as a control. To determine and compare in vivo biodistribution characteristics of KM871 radiolabeled with 111In or 125I, mixtures of the radioconjugates were injected i.v. into BALB/c nude mice bearing GD3-positive SK-MEL-28 melanoma xenografts. Gamma camera images were acquired; groups of five mice were sacrificed at various time intervals, and tumors, blood, and tissues were analyzed. 111In-labeled CHX-A’-DTPA-KM871 showed a maximum tumor uptake of 41.9 ± 7.0% injected dose/g at 72 h with prolonged retention over a 15-day period. The tumor/blood ratio was 3:1 by 72 h, and higher ratios were observed at later time points. No abnormal accumulation of 111In-labeled conjugate was found in normal tissues. In contrast, there was little accumulation of 125I-labeled KM871 in the same tumors. The specificity of antibody localization was confirmed by the low tumor uptake values for radiolabeled control antibody. Gamma camera imaging demonstrated excellent uptake of 111In-labeled CHX-A’-DTPA-KM871 in the xenografts. Chromatographic analyses of xenograft cytosolic extracts demonstrated tumor internalization and internalization and internalization and internalization of radiolabeled KM871 with the formation of small molecular weight metabolites. Laser scanning confocal microscopy demonstrated that the majority of intracellular KM871 is localized to lysosomes. Despite the catabolism of the radioconjugate, a dose-dependent increase in KM871 tumor localization was shown through immunohistochemical examination of xenograft biopsies. This study demonstrates for the first time the in vivo localization of a radiolabeled anti-GD3 monoclonal antibody to GD3-expressing xenografts using gamma camera scanning techniques and tumor cell internalization of KM871 tagged with a green fluorescent dye, Alexa Fluor 488, through confocal microscopy. KM871 has potential for targeting tumors in patients with melanoma.

INTRODUCTION

There has been an intensive search for cell surface antigens of human cancer that could serve as targets for antibody-based therapies. Although serological analysis of human cancer cells has identified a wide range of surface antigenic systems (1), only a relatively small number of antigens have shown the degree of cancer specificity required for safe and effective antibody therapy. GD3 ganglioside has been the focus of considerable interest as a therapeutic target for melanoma since the generation of the mouse mAb2 R24. This IgG3 antibody was isolated from a mouse injected with human melanoma cell line SK-MEL-28 and showed strong cell surface reactivity with a range of human melanoma cell lines and other tumor cell lines of neuroectodermal origin but not cultured cells from epithelial cancers. Biochemical characterization revealed that the antigen detected by mAb R24 was GD3 ganglioside (3).

Gangliosides are a series of neuraminidase-containing glycosphingolipids originally found as plasma membrane components in normal brain but which are now known to be expressed by many other different cell types, although in smaller quantities. Malignant transformation of cells, especially those of neuroectodermal origin, often results in elevated expression of gangliosides such as GM2, GD2, GD3, and 9-O-acetylganglioside GD3 (2–5). Further analysis of mAb R24 showed that it mediated strong complement-dependent cytotoxicity of GD3-positive target cells in vitro (6) and that it suppressed growth of xenotransplanted tumors in nude mice (6). The initial Phase I clinical trial with mAb R24 in patients with advanced melanoma showed marked inflammatory reactions at tumor sites in some patients with cutaneous disease and tumor regressions in 3 of 12 treated patients (7). Although additional trials of mAb R24 therapy alone (8, 9), R24 in combination with cytokines (10, 11), or chemotherapeutic agents (12) have been conducted, assessment of the potential therapeutic value of R24 has been limited because of the strong immunogenicity of R24 in humans, leading to the development of high titers of antismouse antibodies in treated patients. Another obstacle to the evaluation of R24 in the clinic was our inability to trace label R24 with an isotope appropriate for exploring the pharmacokinetics and localization of this antibody.

Ohta et al. (13) have recently developed a murine IgG3 anti-GD3 mAb KM641 that also shows potent immune effector functions and antitumor activity in animal models. This antibody has been genetically engineered into a mouse-human chimeric IgG1 (KM871) to reduce immunogenicity in patients while retaining immune effector functions and antitumor properties (14). Our previous studies have demonstrated that KM871 has a relatively low apparent affinity for the GD3 antigen (Kd = 1.05 × 10−7 M−1) with fast association and dissociation rates as determined by biosensor analysis (15). The chimeric KM871 IgG1 is a stable protein amenable to conjugation with the bifunctional metal ion chelator CHX-A’-DTPA (16), enabling radiolabeling with the radiometal 111In. This isotope has lower...
gamma emission energy and $T_{1/2}$ than radiodiodes, rendering it suitable for gamma camera imaging during in vivo biodistribution.

The objectives of this study were to prepare a stable, immunoreactive, radioiodinated anti-GD3 conjugate and to perform the preclinical evaluation of the radioiodinated antibody in an animal model of melanoma. We report here the successful use of $^{111}$In to radiolabel a CHX-A-DTPA-conjugated KM871 and to demonstrate specific tumor uptake in BALB/c nude mice bearing GD3-expressing melanoma xenografts. A comparison of $^{125}$I- and $^{111}$In-labeled KM871 tumor localization, internalization, and catabolism as a function of time after injection was performed to provide a rationale for radiolabeled KM871 clinical trials. These studies were extended with investigations into the intracellular fate of KM871 through scanning laser confocal microscopy of an intracellular acidic organelle probe and KM871 tagged with a green fluorescent dye, Alexa Fluor 488.

**MATERIALS AND METHODS**

**Antibodies and Cells.** The chimeric anti-ganglioside GD3, mAb KM871 (IgG1 derived from the murine IgG3 KM641; Ref. 14) and class-matched control anti-GM2 chimeric antibody KM966 (IgG1, derived from a murine IgG3; Ref. 14) were provided by the Institute for Cancer Research (New York, NY), together with the A33-expressing colorectal cell line SW1222. The ganglioside GD3-expressing melanoma cell line SK-MEL-28 was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 (TRACE Biosciences Pty. Ltd., Sydney, New South Wales, Australia) with 5% v/v FCS (CSL, Melbourne, Victoria, Australia).

**Radio labeling.** All analytical grade reagents, except when stated, were obtained from Merck Pty. Ltd. (Darmstadt, Germany). The mAb KM871 was minimally labeled with $^{125}$I (AMRAD-NEN, Melbourne, Victoria, Australia) using the standard chloramine-T method and purified on a centrifugal desalting column in 0.2-ml solution containing 18.7 mg/ml tribromoethanol dissolved in 12.5 ml isopentyl alcohol/ml water for injection. Radioiodination was measured with an Atomlab-100 dose calibrator (Biodex, Brookhaven, NY). Radiochemical purity of labeled antibody was analyzed by ITLC-SG (Gelman Sciences, Inc., Ann Arbor, MI), developed using 10% v/v trichloroacetic acid as solvent. Radioactivity was measured with a Cobra II automated gamma counter (Canberra-Packard, Melbourne, Victoria, Australia).

The antibody KM871 was also labeled with $^{111}$In (AMRAD) via the bifunctional metal ion chelate CHX-A-DTPA according to methods described previously (16, 17). For radio labeling, $^{111}$In was added to 100 μl of chelated KM871 (7.2 mg/ml), and the pH was maintained at pH 5.5 by addition of 1.0 N HCl. After 20 min, the pH was increased by the addition of 2.0 M sodium acetate, and the mixture was quenched with EDTA. The radioiodinated mixture was purified by Sephadex G50 chromatography (Sigma Chemical Co., St. Louis, MO) using PBS as solvent. For controls, the mAb huA33 was similarly labeled with $^{125}$I and $^{111}$In. Radiochemical purity of labeled antibodies was analyzed by ITLC-SG developed using 10 mm EDTA and 0.9% w/v saline/10 mm NaOH mixture (16).

The mAb KM871 was also dual-labeled with $^{125}$I and $^{111}$In. This was achieved by labeling an aliquot of $^{111}$In-CHX-A-DTPA-KM871 with $^{125}$I. This colabeled antibody, $^{125}$I$^{111}$In-labeled CHX-A-DTPA-KM871, was injected into mice bearing SK-MEL-28 melanoma xenografts to compare the biodistribution properties with mixtures of single-labeled antibody, $^{125}$I-labeled KM871 and $^{111}$In-labeled CHX-A-DTPA-KM871.

**In Vitro Properties of Radioiodinated Antibodies.** The immunoreactivities of radioiodinated antibody KM871 were determined using the Lindmo assay (19). Twenty ng of radioiodinated KM871 were added to 0–1.0 × 10^5 SK-MEL-28 cells in 1.0 ml of medium. The cells were incubated for 45 min at room temperature with continuous mixing throughout to keep the cells in suspension. Cells were harvested by centrifugation and washed once to remove unbound antibody, and pellets were measured in a gamma counter (Cobra II, Model 5002, Auto-gamma; Canberra-Packard). Three samples of radioiodinated KM871, at the same concentration as that initially added to the cells, were measured at the same time as cell pellets (“standards”), and percentage binding of KM871 to SK-MEL-28 cells was calculated by the formula: (cpm cell pellet/mean cpm radioactive antibody standards) × 100. Percentage binding was graphed against SK-MEL-28 cell concentration and immunoreactivities calculated as the y intercept of the inverse plot of both values. A further immunoreactivity experiment was performed. Prior to addition to the melanoma cells, 20 μg of unlabeled KM871 were mixed with the 20-ng radioiodinate, and antigen binding was determined as described above.

The stability of radioiodinated KM871 was determined by incubation in serum at 37°C and analyzed by ITLC-SG as described previously (17). Retention of immunoreactivity was determined as detailed above. These evaluations were repeated on radioiodinated antibody in mouse blood samples collected at time points p.i. For immunoreactivity analyses, aliquots of blood containing ~20 ng of antibody were mixed with SK-MEL-28 melanoma cells. The concentrations of labeled KM871 were estimated from the blood clearance curve.

**Animal Model.** A group of 10–20 5 × 10^5 SK-MEL-28 cells in 0.1 ml of PBS were injected intradermally into the underside flank of female BALB/c nude mice (CULAS, Sydney, New South Wales, Australia) 3–4 weeks of age. Tumors started to develop by 3–4 weeks, and mice were used when tumors weighed 0.2–0.7 g. A dose containing a sterile filtered mixture of 5 μg of $^{125}$I-labeled KM871 (5 μCi) and 5 μg of $^{111}$In-labeled CHX-A-DTPA-KM871 (10 μCi) in 0.1 ml of PBS was administered via retro-orbital injection while mice were under inhaled ethane anesthesia. From 10 min p.i., groups of five mice were sacrificed by cervical dislocation, and blood, tumors, and normal tissues (liver, spleen, kidney, heart, lung, muscle, skin, brain, bone, stomach, and small bowel) were removed for analysis. Blood clearance kinetics were determined using a curve fitting program, SAAM II (The University of Washington, Seattle, WA), assuming a two-compartment model. Cumulative retention of radioiodinated KM871 was determined by integrating the area under the curve for biodistribution in melanoma xenografts. These values are expressed as %ID/g. To demonstrate specificity of tumor uptake of radioiodinated antibodies, the experiments were repeated with labeled control mAb huA33 in five mice bearing melanoma, with sacrifice and tissue collection 3 days p.i. In separate experiments, the biodistribution properties of $^{125}$I-labeled and $^{111}$In-labeled CHX-A-DTPA-KM871 conjugate mixture, or KM871 mAb colabeled with both isotopes, were studied in groups of five mice with SK-MEL-28 xenografts. Animals were sacrificed at 48 h p.i., and residual activity (cpm) of labeled antibodies in blood and tumors was determined.

**Gamma Camera Imaging.** Mice were anesthetized by i.p. injection of 0.2-ml solution containing 18.7 mg/ml tribromoethanol dissolved in 12.5 μl of isopentyl alcohol/ml water for injection and placed supine on a dual-headed Biad Trionix gamma camera (Twinsbury, OH) equipped with a medium energy collimator. A standard containing 10% of injected dose was placed within the field of view. $^{111}$In images were acquired over 10 min, and data were recorded on a Sun Sparc Station 10 computer.

**Procedure for Evaluating Catabolism of Radioiodinated Antibodies by Tumors.** Tumors from three mice were pooled for each time point (4 h, 24 h, day 3, and day 7), minced, and homogenized in 4 volumes of ice-cold 10 mM Tris/HC1 (pH 7.4) buffer containing 0.25 M sucrose and Complete protease inhibitor mixture (1:50 dilution; Boehringer Mannheim, Melbourne, Victoria, Australia). Homogenization was performed with a Kinematica Polytron PT-1200 with a 5-mm probe (Lucerne, Switzerland) using a setting of 3.0 with three 30-s bursts and cooling on ice. All fraction volumes were recorded. Ultraceutriguration [100,000 × in TLS 55 rotor; Optima TLX Ultraceutrigue (Beckman Instruments, Melbourne, Victoria, Australia)] of the homogenate generated the tumor cell cytosol containing the extractable radiocatabolites (20). Aliquots of the homogenates and cytosolic were counted (cpm) for quantification of radioactivities.

To access the formation of the catabolites $^{111}$In-labeled CHX-A-DTPA-peptide(s) and $^{125}$I-labeled tyrosine or peptides within the xenografts, soluble cytosolic fractions (1.0 ml) were subjected to gel filtration chromatography on a Biogel P6DG column (1.5 × 7.20 cm; exclusion, Mt, 6000) equilibrated in PBS. Fractions (1.0 ml) were collected and counted together with an aliquot of cytosol for determining radioactive recovery. The column was calibrated using sodium iodide ($^{125}$I; Mt, 150), $^{111}$In-labeled DTPA complex (Mt, 510), and mAb KM871 (Mt, 150,000). The elution peak fractions of the markers were determined through either radioactive counting or by protein UV absorption at 280 nm.

**Confocal Laser Scanning Microscopy.** Internalization was examined by confocal microscopy using two different fluorescent tags attached to KM871. In the first set of experiments (n = 6), KM871 mAb in 0.1 ml NaHCO3 (pH 8.2)
was conjugated with FITC (Sigma Chemical Co.) at a 10-fold molar excess for 15 min at room temperature. The mixture containing FITC-KM871 was purified on a Sephadex G50 column equilibrated in PBS. For controls, FITC-huA33 was similarly prepared. SK-MEL-28 cells were grown overnight on coverslips in medium, and the cells were maintained throughout the experiment at 37°C (using a Nikon Eclipse incubator, Model ICT-32, for an Eclipse TE300 microscope) and superfused with 5% CO2/95% O2 at 100% humidity. Images were collected using a Bio-Rad MRC-1024ES confocal microscope attached to a Nikon Eclipse TE300 equipped with a Nikon CFI Plan Apo 60X, 1.2NA water immersion lens with a coverslip correction collar. The fluorescein-labeled antibodies were excited with the 488-nm line of a 100-mW argon ion laser (Ion Laser Technology), and the resulting fluorescence was filtered with an orange glass barrier filter (OG 515). Images were collected just prior to and at 2-min intervals after addition of the FITC-KM871 antibody or control antibody at final concentrations of 40 μg/ml.

In the second set of confocal experiments (n = 6), KM871 and control huA33 were conjugated to the green fluorescent dye Alexa Fluor 488, according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). SK-MEL-28 cells were grown on coverslips as described previously and then incubated in medium with LysoTracker Red DND-99 (1:400; Molecular Probes). LysoTracker is a fluorescent acidotrophic probe for labeling and tracking acidic organelles in live cells. After 15 min incubation at 37°C, cellular uptake of LysoTracker was confirmed by confocal microscopy. The excess LysoTracker Red was then removed by washing with medium and replaced with 20 μg/ml KM871-Alexa Fluor 488 in medium. The cells were incubated in the excess antibody over the time course as described for the KM871-FITC experiments. Images were collected sequentially at two excitation wavelengths to minimize interference between channels (Alexa Fluor 488 excited at 488 nm; LysoTracker at 514 nm). The resulting green (Alexa Fluor 488) and red (LysoTracker Red) fluorescent signals were separated using a dichroic mirror (565LP) and further filtered using either a 522BP32 (Alexa Fluor 488) or 585LP (LysoTracker Red) barrier filter in front of separate detectors. In addition to collecting red and green fluorescent images of the cultures at different time points, images were acquired at different depths through the labeled cells (z series or serial optical sections).

**Immunohistochemical Analysis.** Localization of injected unlabeled antibody KM871 in xenograft models was investigated using a modified immunohistochemical technique (21). Two antibody protein doses (100 and 300 μg) were injected into groups of four mice with tumor masses 200–500 mg. Animals were sacrificed at 10 min, 4, 24 h, and 4 days p.i. Saline (0.9% w/v) and huA33 mAb were used as controls. Tumors were snap frozen, and 5-μm sections were cut using a Zeiss Microm cryomicrotome (Melbourne, Victoria, Australia). All immunohistochemical reagents used were from Sigma Chemical Co. The sections were fixed in ice-cold acetone for 10 min, and nonspecific binding sites were saturated with a protein blocking agent for 10 min. Specific binding sites were saturated with a protein blocking agent for 10 min. Minimal radioiodination of KM871 tyrosine residues was performed using chloramine-T, resulting in a specific activity of 1.0 mCi/mg and 60% labeling efficiency. After chromatographic purification, >99% of 125I was bound to protein, as determined by TLC. Labeling with radiometal 111In was effected via the chelate CHX-A'-DTPA. A molar ratio of 2:1 chelate:mAb was achieved, as determined by differential TLC separation of total radiolabeled antibody mixtures (17). Specific activities of 2–3 mCi/mg with a labeling efficiency of 40–50% were obtained. Greater than 95% of radioactivity was associated with the radioconjugate, as determined by TLC. The retention of immunoreactivity after radiolabeling and stability of isotopes bound to antibody were evaluated. Immunoreactive fractions of the two radioconjugates were determined by binding to GD3-expressing SK-MEL-28 melanoma cells in the presence of excess antigen (Fig. 1A). The binding of low levels (20 ng) of labeled antibody alone was compared with binding in the presence of excess unlabeled antibody. The latter condition is normally used to demonstrate specificity of binding because unlabeled antibody should act as a competitive inhibitor in the binding assay. However, apparent enhanced binding of labeled antibody was observed instead under such conditions, an observation reported previously with the anti-GD3 mAb R24 (22).

Double reciprocal plots of values derived from Fig. 1A and linear extrapolation to the ordinate (Fig. 1B) provided a measure of the percentage of immunoreactivity (19). At low levels (20 ng) of antibody, values of 42.2 and 39.4% were obtained for the 125I-labeled and 111In-labeled conjugates, respectively. In the presence of 20 μg of unlabeled KM871 mAb, immunoreactive fractions were calculated to be 80% (125I-labeled KM871) and 64.5% (111In-labeled KM871). Control IgG1 KM966 antibody did not affect binding of radiolabeled conjugates.

**Fig. 1.** A, binding of 125I-labeled KM871 (□) and 111In-labeled CHX-A'-DTPA-KM871 (○) monoclonal antibody to GD3 ganglioside-expressing SK-MEL-28 melanoma cells. Increased binding was observed in the presence of excess unlabeled antibody (■) and (●) for the two radioconjugates, respectively. B, double inverse plots of the data in A allow the determination of immunoreactive fraction by linear extrapolation to the ordinate.

### RESULTS

**Properties of 125I-labeled and 111In-labeled CHX-A’-DTPA-KM871 Antibodies.** Minimal radioiodination of KM871 tyrosine residues was performed using chloramine-T, resulting in a specific activity of 1.0 mCi/mg and 60% labeling efficiency. After chromatographic purification, >99% of 125I was bound to protein, as determined by TLC. Labeling with radiometal 111In was effected via the chelate CHX-A’-DTPA-KM871. A molar ratio of 2:1 chelate:mAb was achieved, as determined by differential TLC separation of total radio-labeled antibody mixtures (17). Specific activities of 2–3 mCi/mg with a labeling efficiency of 40–50% were obtained. Greater than 95% of radioactivity was associated with the radioconjugate, as determined by TLC. The retention of immunoreactivity after radiolabeling and stability of isotopes bound to antibody were evaluated. Immunoreactive fractions of the two radioconjugates were determined by binding to GD3-expressing SK-MEL-28 melanoma cells in the presence of excess antigen (Fig. 1A). The binding of low levels (20 ng) of labeled antibody alone was compared with binding in the presence of excess unlabeled antibody. The latter condition is normally used to demonstrate specificity of binding because unlabeled antibody should act as a competitive inhibitor in the binding assay. However, apparent enhanced binding of labeled antibody was observed instead under such conditions, an observation reported previously with the anti-GD3 mAb R24 (22).

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antibody (data not shown), suggesting that enhancement of binding was not attributable to Fc interactions.

To determine the stability of the radioisotope conjugation to mAb protein, radiolabeled antibodies were incubated in human serum at 37°C for 7 days. TLC analysis of aliquots sampled from 0 to 7 days demonstrated that radiolabels were firmly bound to protein (>95%), whereas there was a small reduction in immunoreactivities to 35.6 and 32.7% for 125I-labeled and 111In-labeled conjugates (20 ng of antibody), respectively. The stability evaluation of radioconjugate in mouse serum from 4 h to 7 days p.i. showed a small reduction in immunoreactivity from 50 to 42% for 125I-labeled and 40 to 36% for 111In-labeled KM871 conjugates. These results demonstrated that the two radioconjugates in the blood circulation were stable for up to 7 days with retention of antigen binding activity.

**Biodistribution and Pharmacokinetics.** The relative levels of radiolabeled antibody KM871 in melanoma xenografts and blood at times p.i. are shown in Fig. 2. The blood clearance rates of 125I-labeled and 111In-labeled KM871 were similar. Assuming a two compartmental model with a four-parameter fit of mean blood levels, the T1/2 (α) for 125I-labeled and 111In-labeled conjugates were 3.3 and 2.5 h, respectively, and the T1/2 (β) for both the radioconjugates was identical at 69.3 h.

The relative concentration of 125I-labeled and 111In-labeled conjugates in melanoma xenografts differed markedly over time (Fig. 2). By 4 h p.i., the tumor values for 125I-labeled and 111In-labeled conjugates were comparable at 7.2 ± 0.8 and 10.4 ± 2.2%ID/g (Table 1). From 24 h, the mean 111In-labeled KM871%ID/g levels in the xenografts were significantly higher than 125I-labeled KM871 (t test; P < 0.001). The accumulation of 111In-labeled conjugate in tumors continued to increase until 72 h, whereas the 125I-labeled conjugate did not. The mean uptake of 111In-labeled conjugate peaked at 41.9 ± 7.0%ID/g at 72 h p.i. with prolonged retention at the xenograft over the next 5 days, gradually decreasing to 7.5 ± 1.4%ID/g at day 15 p.i. The tumor:blood ratio increased from 3:1 to 6:1 from 3 to 7 days p.i. The weight of tumors at 168 and 288 h p.i. were 0.36 ± 0.10 g and 0.53 ± 0.03 g, respectively. The apparent decrease in %ID/g may reflect a combination of increase in tumor masses and turnover of 111In in the tumor. The cumulative retention of radioconjugates by the tumors was determined by integrating the area under the respective curves in Fig. 2. Results were 1,472 and 11,900%ID-h/g tumor for the 125I-labeled and 111In-labeled conjugates, respectively. The cumulative tumor:blood ratios were 0:6:1 and 4:3:1, respectively, indicating greater accumulation of the radiometal conjugate in tumors compared with the iodinated antibody.

The biodistribution properties at 72 h p.i. of both radiolabeled KM871 antibodies in various tissues are shown in Fig. 3. The overall distribution of both radioconjugates in normal tissues were comparable except for spleen and liver. The 111In-labeled conjugate showed significantly greater localization to the spleen (P = 0.038) and liver (P = 0.005) compared with the 125I-labeled KM871 conjugate at 72 h (Fig. 3). Similar normal tissue distributions have been reported previously for 111In-labeled conjugates. The level of radiolabeled KM871 in normal tissues decreased with time without marked retention, consistent with reduction in blood pool activity.

To demonstrate the specificity of localization of radiolabeled KM871, a separate group of five mice bearing melanoma xenografts were injected with similar amounts of control 125I-labeled and 111In-labeled CHX-A-DTPA huA33 antibodies. The maximal tumor uptake (at 72 h p.i.) was only 5.8 ± 1.8 and 10.1 ± 1.1%ID/g, respectively.
At 4 h p.i., the proportion of 125I and 111In catabolites were comparable, at selected time points p.i. tumors were excised, homogenized, and subjected to ultracentrifugation. The supernatant fraction was defined as containing cytosolic or soluble cell content. The intact KM871 used as a high molecular weight marker (M₉, 150,000) eluted as a peak in fraction 5, indicating the void volume (Vo) of the column (Fig. 5A). The 111In-labeled DTPA complex (M₉, 510) and 125I-labeled sodium iodide (M₉, 150) eluted at fractions 11 and 16, respectively (Fig. 5A). At 4 h p.i., ~80% of the 111In activity eluted as a low molecular weight (M₉ > 510) peak in fraction 9 (Fig. 5C). This major 111In-labeled catabolite persisted in the tumor to day 7 (Fig. 5D). The 125I-labeled catabolites eluted as three main radioactive peaks (Fig. 5). The peak at fraction 5 was of high molecular weight and was trichloroacetic acid precipitable (data not shown). The peak observed in fractions 10–11, M₉ ~510, in size, and the final small peak in fraction 16 corresponded with the elution of 125I-labeled sodium iodide (M₉, 150). The high molecular weight 125I peak became the main catabolite after 24 h (Fig. 5, B–D). The overall recovery of radioactivity during chromatography was determined by summing the fractions cpm and comparing this recovery to the cpm loaded onto the column. Recovery was >90%, indicating no significant loss of material during chromatography.

KM871 Internalization into SK-MEL-28 Cells. Internalization of FITC-KM871 into SK-MEL-28 cells was evaluated by laser scanning confocal microscopy after incubating conjugated antibody with live cells grown on a coverslip (n = 6 cultures). Initially, after 2 min incubation, FITC-KM871 was bound mainly to cell surfaces, which
showed a variation of fluorescence intensities. Formation of intracellular fluorescent vesicles adjacent to the cell membrane was just discernible at this early time point (data not shown). These fluorescently labeled vesicles became more prevalent and intense by 8 min of incubation, reflecting the continuous internalization and accumulation of FITC-KM871 in peripheral cytoplasmic vesicles or early endosomes (data not shown). Fluorescein is, however, quenched in acidic environments, resulting in reduced fluorescent signal as the vesicles progressed to become the more acidic late endosomes, or lysosomes, thus preventing visualization of FITC-labeled KM871 mAb in vesicles other than early endosomes. Accordingly, further investigations into the internalization of KM871 were performed with the Alexa Fluor dyes (Molecular Probes), which are brighter, more resistant to photobleaching, and are resistant to changes in pH.

SK-MEL-28 cells were labeled with LysoTracker Red and imaged during incubation with Alexa Fluor 488-KM871. At 6 min of the time course, fluorescent vesicles formed close to the cell membrane, as observed with the FITC-labeled antibody (data not shown). At 35 min, vesicles were imaged deeper within the cytoplasm of the cell, and results for optical sections through the base of a cell (i.e., close to the coverslip) at 75 min are presented in Fig. 6, A–C. The red fluorescence of LysoTracker Red in Fig. 6A indicates the location of acidic vesicles such as lysosomes and late endosomes close to the nucleus. The arrows indicate the same typical labeled vesicle that is close to the nucleus. The green fluorescence (Fig. 6B) indicates the distribution of Alexa-Fluor 488-KM871 mAb, and Fig. 6C presents the result of merging Fig. 6A and Fig. 6B. The yellow color results from the combination of the red and green images and is illustrative of areas of possible colocalization of Alexa-Fluor 488-KM871 and LysoTracker Red. Alexa Fluor 488-KM871 mAb was observed to internalize and localize to acidic vesicles close to the nucleus. Fig. 6, D and E, shows a series of red/green merged optical sections (z-series) through the same cell where z indicates the depth of the optical section into the cell. It is apparent, through examination of the z-series that Alexa Fluor 488-KM871 mAb (displayed as green or yellow) is found throughout the cell and appears to accumulate in the same region as the lysosomes close to the nucleus. This result is very strong evidence that the labeled antibody is internalized. Fig. 6F is an optical section close to the “base” of the cell (coverslip) and shows Alexa Fluor 488-KM871 mAb containing vesicles (green) close to the cell membrane. These are presumably newly formed early endosomes and, therefore, are not stained with LysoTracker Red.

**Immunohistochemical Detection of Injected Antibody.** After observing the tumor catabolism of radiolabeled KM871, we investigated in vivo KM871 intratumoral distribution. Mice bearing SK-MEL-28 tumors were injected i.v. with KM871 (100 and 300 μg) or controls (100 and 300 μg of huA33 mAb or isotonic saline). Immunohistochemical analyses performed on tissue biopsies indicated that melanoma cells were viable (H&E staining; Fig. 7A), and uniform expression of G D3 ganglioside was observed (Fig. 7B). By day 1 p.i., detection of 100 μg of KM871 dose was patchy (Fig. 7C), whereas controls gave negative staining (Fig. 7D). A time course of detection of the 300 μg of KM871 dose is shown (Fig. 7E–H). By 10 min p.i., minimal staining for the high antibody dose was observed (Fig. 7E). By 4 h p.i., penetration of the 300 μg of KM871 dose into the tumor was weakly detectable in the perivascular region and connective tissue (Fig. 7F). By 24 h p.i., antibody was more readily detectable (Fig. 7G) and was uniformly distributed throughout the tumor section by 4 days p.i. (Fig. 7H). These results indicate that the injected antibody pene-

![Confocal microscopy images of a G D3 expressing SK-MEL-28 cell labeled with LysoTracker Red DND-99 (red fluorescence; A) and 20 μg/ml Alexa Fluor 488-KM871 (green fluorescence; B). C, the merge of images (A and B) with yellow, indicating colocalization of the two fluorescent probes. The arrows indicate the same typical labeled vesicle in A–C that is close to the nucleus (N). D and E, a series of red/green merged optical sections (z-series) through the same cell, where z indicates the depth of the optical section into the cell. F, an optical section close to the “base” of the cell (i.e., close to the coverslip) shows Alexa Fluor 488-KM871 antibody-containing vesicles (green) close to the cell membrane (CM).](cancerres.aacrjournals.org)
trated into melanoma xenografts in a time-dependent manner, and despite catabolism, KM871 can still be detected 4 days p.i.

**DISCUSSION**

Our study is the first to demonstrate the specific targeting and imaging of an antibody to the ganglioside GD3 in an animal xenograft system. The successful radiolabeling of KM871 allowed quantitative evaluation of tumor targeting by the antibody in vivo and also facilitates the translation of the antibody to initial proof-in-principle Phase I clinical trials. Many factors influence the localization of injected mAbs from blood circulation into tumor (24). One critical issue is the need to prepare radiolabeled antibodies that retain immunoreactivity with stable attachment of isotope.

Previously, attempts to radioiodinate anti-GD3 antibodies, particularly the murine IgG3 antibody R24, were unsuccessful, and clinical trial data on targeting and biodistribution in melanoma patients could not be obtained. Optimal selection of antibody dose, evaluation of tumor uptake of antibody with time, and the distribution of antibody in normal tissues in vivo can only be determined with radiolabeled antibody trials. The difficulty in radioiodinating R24 is related to the physicochemical properties of the antibody. The CHX-A-DTPA chelate technology has only recently been described and has not been applied to R24. The chimeric anti-GD3 antibody KM871 (14), which is an IgG1, has several important properties for development for clinical trials. The IgG1 isotype is more stable and amenable to conjugation with the chelate CHX-A-DTPA, permitting labeling with the radiometal 111In. The mAb KM871, similar to R24, has potent immune effector functions, and the chimeric nature of the KM871 antibody should minimize immunogenicity in humans. Taken together, KM871 has advantages over murine R24, which showed promise in early clinical trials in melanoma patients (7).

125I- and 111In-conjugated KM871 retained binding affinity to GD3-expressing melanoma cells and was also stable in vitro as assessed by TLC. The binding affinity of KM871 was retained, despite prolonged incubation at 37°C in serum and in vivo animal experiments. Interestingly, unlabeled mAb KM871 enhanced the binding of radiolabeled KM871 to melanoma cells (Fig. 1). The mechanism of this enhancement in these studies is not clear, but it did not appear to be a result of Fc interaction. This phenomenon has been described previously for the anti-GD3 antibody R24 (22), where it was suggested that the antibody was able to bind onto another immobilized antibody molecule, via the Fv region (“homophilic binding”).

The uptake of 111In-labeled CHX-A-DTPA-KM871 in GD3-expressing xenografts reached a peak at 2–3 days p.i., with a maximal uptake of 41.9 ± 7.0%ID/g. The retention of the 111In-labeled conjugate was prolonged, with ~10%ID/g still present in xenografts at 15
days p.i. (Fig. 2). Importantly, there was an almost 8-fold higher uptake from 24 h p.i. of 111In-labeled CHX-A-DTPA-KM871 in xenografts compared with 125I-labeled KM871 (Table 1), indicating a marked difference in processing/catabolism of the radioiodine conjugate compared with the radiometal conjugate in xenografts. The specificity of uptake was demonstrated using an IgG1 control antibody (huA33) labeled with 111In, which did not demonstrate localization in xenografts. The localization of 111In-labeled CHX-A-DTPA-KM871 in xenografts was also demonstrated with gamma camera imaging, with excellent uptake identified in tumor (Fig. 4), and apart from blood pool activity in the heart, there were no other normal organs detected, indicating the stability of the antibody in vivo and the lack of accessible antigen in normal tissues.

The blood clearance kinetics (Fig. 2) and normal tissue distribution (Fig. 3) of 125I-labeled KM871 and 111In-labeled CHX-A-DTPA-KM871 were similar. Trans-chelation of 111In from antibody to serum proteins such as transferrin did not appear to have occurred to a significant degree, because there was no major loss of immunoreactivities. Previous studies using DTPA anhydride as a source of chelating agent for binding 111In resulted in substantial liver, spleen, and kidney uptake (25). The relatively low liver uptake of 111In-labeled CHX-A-DTPA-KM871 in this study attests to the current improvement in chelation chemistry (16), permitting the preparation of stable radioconjugates. The lack of significant liver and other normal tissue uptake of 111In indicate the suitability of this chelated form of KM871 for imaging biodistribution studies using whole body gamma camera scans in future clinical trials.

Evidence for the mechanism of internalization and catabolism of 111In-labeled CHX-A-DTPA-KM871 and 125I-labeled KM871 after binding to cell surface G D3 was obtained from a number of experimental approaches. Subcellular fractionation of tumor homogenates clearly indicated that rapid internalization occurred, as shown by the presence of both 111In and 125I labels in the cytosol fraction, which consisted of both high molecular weight and low molecular weight labeled species (Fig. 5). The cytosolic labeled species most likely consist of both intact antibody and breakdown products, and the lower amount of 125I-labeled species would suggest that the catabolic process present within the melanoma cells has resulted in preferential retention of 111In-labeled compared with 125I-labeled species. The selective retention of 111In-labeled compared with 125I-labeled antibody in tumor cells after internalization is well established in numerous tumor antigen systems (26–30). Although radiometal catabolites are retained within the lysosomes after catabolism, the 125I-labeled catabolites diffuse from tumor cells. Mono- and di-iodo-tyrosines are formed and leave the lysosomes rapidly via carrier-mediated transport (31). The catabolites are dehalogenated extracellularly and unless incorporated into thyroxine, are mainly excreted into the urine. In the case of radiometal-conjugated antibodies, the major catabolites are small isotope-DTPA-lysyl peptide complexes, which are trapped within the intact lysosomes. Such complexes, ionic in nature, are unlikely substrates for carrier-mediated transport and cannot exit the lysosomes by diffusion.

Rapid internalization of KM871 into cultured G D3-expressing SK-MEL-28 cells was also demonstrated using confocal microscopy. Using FITC-labeled KM871, movement of KM871 into vesicles adjacent to the cell membrane of SK-MEL-28 cells was observed by 8 min incubation. However, the true extent of vesicular trafficking was likely to be underestimated with this approach because of the potential problems of quenching of FITC in acidic (i.e., lysosome) environments. To more accurately define the internalization of KM871, labeling of KM871 with green fluorescent dye was performed (Fig. 6). Similar results of rapid internalization to small vesicles were observed, and trafficking to acidic vesicles (late endosomes and lysosomes) was demonstrated. These internalization results are consistent with subcellular fractionation studies and provide confirmation of the proposed mechanism for the differential retention of 111In-labeled conjugates compared with 125I-labeled conjugates in the G D3-expressing xenograft model.

Rapid internalization of an antibody may be potentially detrimental to its anticanancer activity, particularly immune effector mechanisms such as activating complement (complement-dependent cytotoxicity) or peripheral blood lymphocytes (antibody-dependent cell cytotoxicity). However, in the present study, our estimation from cell binding assays is that there are approximately 5.4 × 10^9 antibody binding sites/SK-MEL-28 cell. Using a mean molecular weight of 1000–2000 for gangliosides (5), it was estimated from the sensitive liposome immune lysis assay that there were 3.3 to 6.6 × 10^6 G D3 gangliosides per SK-MEL-28 cell (32), in agreement with our determination. Cytotoxicity assays for complement-dependent cytotoxicity and antibody-dependent cell cytotoxicity have shown KM871 to be very effective in killing G D3-positive target cells, achieving a 50% cell lysis at a concentration of 3.0 μg/ml of antibody (13). In such assays, a relatively small amount of antibody is used, continuously bathing the cells, resulting in cell death in the presence of complement and blood cells. In the in vivo situation, there will be a concentration gradient of circulating KM871 antibody between blood vessels, tumor stroma, and tumor cells. Contact must be made between antibody and G D3 antigen before internalization can occur. Given the relatively dense antigen present on the tumor cell surface, this should provide sufficient time for the surface-bound antibody to interact with the immune system for killing cancerous cells. We have also shown by immunohistochemical techniques that injected unlabeled antibody can be detected in xenograft sections, despite the internalization process and the barriers to antibody penetration into tumor (including tumor vasculature and interstitial pressure), and the high "off-rate" of KM871 for G D3 antigen demonstrated by biosensor (BIAcore) analysis (15).

In conclusion, we have demonstrated that 111In-labeled CHX-A-DTPA-KM871 but not 125I-labeled KM871 is suitable for in vivo targeting of G D3 ganglioside-expressing melanoma. Because gangliosides are expressed in a wide variety of normal human tissues, gamma camera scanning techniques using 111In-labeled CHX-A-DTPA-KM871 should be able to identify normal organs (if any) with substantial expression of accessible G D3 antigen. The retention of 111In in tumor cells should also provide the ability to visualize KM871 uptake in tumors with time and provide quantitative data on the localization ability of KM871 in vivo. In conjunction with the potent immune effector functions mediated by this antibody and the potentially reduced immunogenicity of its chimeric form, KM871 is a promising therapeutic agent for the treatment of metastatic melanoma.


Specific Localization, Gamma Camera Imaging, and Intracellular Trafficking of Radiolabelled Chimeric Anti-G D3 Ganglioside Monoclonal Antibody KM871 in SK-MEL-28 Melanoma Xenografts

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