A Radiolabeled Self-assembled Nanoparticle Probe for Diagnosis of Lung-Metastatic Melanoma

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Melanoma is a highly malignant skin cancer that frequently metastasizes to the lung, bone, and brain at an early phase. Therefore, noninvasive detection of metastasized melanoma could be beneficial to determine suitable therapeutic strategies. We previously reported a biocompatible ternary anionic complex composed of plasmid DNA (pDNA), polyethyleneimine (PEI), and γ-polyglutamic acid (γ-PGA) based on an electrostatic interaction, which was highly taken up by melanoma cells (B16-F10), even if it was negatively charged. Here, we developed a radiolabeled γ-PGA complex by using indium-111 (111In)-labeled polyamidoamine dendrimer (4th generation; G4) instead of pDNA and iodine-125 (125I)-labeled PEI instead of native PEI, and evaluated its effectiveness as a melanoma-targeted imaging probe. This ternary complex was synthesized at a theoretical charge ratio; carboxyl groups of 111In-diethylenetriaminepentaacetic acid (DTPA)-G4 : amino groups of PEI : carboxyl groups of γ-PGA was 1 : 8 : 16, and the size and zeta potential were approximately 29 nm and ~33 mV, respectively. This complex was taken up by B16-F10 cells with time. Furthermore, a biodistribution study, using normal mice, demonstrated its accumulation in the liver, spleen, and lung, where macrophage cells are abundant. Almost the same level of radioactivity derived from both 111In and 125I was observed in these organs at an early phase after probe injection. Compared with the normal mice, significantly higher lung-to-blood ratios of radioactivity were observed in the B16-F10-lung metastatic cancer model. In conclusion, the radiolabeled γ-PGA complex could hold potentialities for nuclear medical imaging of lung metastatic melanoma.

Key words melanoma; self-assembled nanoparticle; nuclear medical imaging

INTRODUCTION

Melanoma, which typically develops due to malignant proliferation of melanocytes in the skin, is one of the most aggressive types of skin cancer, and the number of cases of malignant melanoma is increasing annually.1 Melanoma has metastatic potential in other organs, including the lung, brain, liver, and bone2; therefore, accurate in vivo diagnosis of metastasized melanoma is considered essential for determining suitable therapeutic strategies.

At present, fluorine-18-fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) plays an important role in detecting and staging recurrent lung melanoma.5,6 However, it is difficult for 18F-FDG-PET to distinguish tumor regions from inflammatory regions, and it is poor at detecting brain and lung metastases. Thus, there might be limitations for patients with advanced melanoma.5,6 Furthermore, indium-111 (111In)-labeled octreotide derivative and lanreotide derivative targeting somatostatin receptor highly expressed in melanoma have been developed,7 however, the detection rates are not high.

We previously reported a self-organized ternary anionic complex composed of plasmid DNA (pDNA), polyions such as protamine, and γ-polyglutamic acid (γ-PGA), which can be simply prepared via electrostatic interaction, without requiring purification.8 Generally, the cellular uptake of anionic nanoparticles is not notable because they are repelled to the anionic cellular membrane. However, this γ-PGA complex showed marked cellular uptake by mouse melanoma cells via the clathrin-mediated pathway and macropinocytosis (although this complex was negatively charged).8 γ-PGA is a water-soluble, biocompatible, and non-immunogenic polymer, which is used in a variety of applications, including medicine and cosmetics.9,10 Nanoparticles coated with γ-PGA showed good biocompatibility characteristics, such as no agglutination with erythrocytes and hemolysis, and no effects on the tight junctions of the cells and the transepithelial electrical resistance.11,12 Furthermore, we have recently reported a radiolabeled γ-PGA complex composed of In-111 (111In)-labeled polyamidoamine (PAMAM) dendrimer (111In-diethylenetriaminepentaacetic acid (DTPA)-G4) instead of pDNA, cationic polyethyleneimine (PEI), and γ-PGA as a biocompatible nuclear medical imaging probe for sentinel lymph node detection.13 As nuclear medical imaging with single photon emission computed tomography (SPECT) is widely used clinically because of its capability to perform a noninvasive whole body scan,14 in this study, we applied 111In-labeled γ-PGA complexes to in vivo imaging of metastasized melanoma. For this purpose, the 111In-labeled γ-PGA ternary complexes should be delivered into the melanoma in intact form. In order to evaluate the stability of γ-PGA complexes in vitro and in vivo, we prepared complexes composed of 111In-DTPA-G4, iodine-125-labeled PEI (125I-PEI), and γ-PGA for tracking each component. We examined their cellular uptake by melanoma cells and their biodistribution in normal mice and mice with metastasized melanoma in the lung, and evaluated the usefulness of radiolabeled γ-PGA complexes as nuclear medi-
cal imaging probes targeting metastasized melanoma.

MATERIALS AND METHODS

Materials PAMAM dendrimer (generation 4th, G4) and branched PEI (average molecular weight of 25000) were purchased from Sigma-Aldrich Co. (Milwaukee, WI, U.S.A.). γ-PGA was supplied by Yakult Pharmaceutical Industry Co., Ltd. (Tokyo, Japan). 111InCl₃ in HCl aq. (0.02 M) was kindly supplied by Nihon Medi-Physics (Tokyo, Japan). Na⁺/125I in 10 µM NaOH aq. was purchased from PerkinElmer, Inc. (Waltham, MA, U.S.A.). p-SCN-Bn-DTPA was obtained from Macrosciences, Inc. (Dallas, TX, U.S.A.).

Preparation of ¹¹¹In-Labeled G4 (¹¹¹In-DTPA-G4) DTPA-G4 was synthesized by conjugation of G4 with p-SCN-Bn-DTPA according to the procedure described in our previous report.13) Thereafter, ¹¹¹In-labeling was conducted by incubating ¹¹¹InCl₃ with DTPA-G4 (100 µg) in acetate buffer (0.1 M, pH 6.0) for 1 h at room temperature, followed by purification twice with a diafiltration membrane (Amicon Ultra-4 L 5% glucose), containing 1 µg DTPA-G4, was intravenously injected into ddY mice (6-week-old). Mice were sacrificed 10 and 30 min, and 1, 3, 6, and 24 h post-injection of probes. The blood, spleen, pancreas, stomach, intestines, kidneys, liver, heart, lung, muscle, thyroid, urine, and feces were excised, and the weight and radioactivity were measured. The data are expressed as a percentage of the injected dose (% ID) for the thyroid, urine, and feces, or as a % ID per gram of tissue (% ID/g). A similar biodistribution study using Balb/c mice (8-week-old) was performed 3 h post-injection of the probes.

Biodistribution Studies Using Mice with Metastasized Melanoma in the Lung For preparation of mice with metastasized melanoma in the lung, B16-F10 cells (1.0×10⁶ cells/100 µL PBS) were intravenously injected into Balb/c mice (male, 5-week-old). At 3 weeks after injection of melanoma cells, these mice were injected with ¹¹¹In-DTPA-G4/¹²⁵I-PEI/γ-PGA (18.5 kBq ¹¹¹In and 18.5 kBq ¹²⁵I/100 µL 5% glucose), containing 1 µg DTPA-G4, was intravenously injected into ddY mice (6-week-old). Mice were sacrificed 10 and 30 min, and 1, 3, 6, and 24 h post-injection of probes. The blood, spleen, pancreas, stomach, intestine, kidneys, liver, heart, lung, muscle, thyroid, urine, and feces were excised, and the weight and radioactivity were measured. The data are expressed as a percentage of the injected dose (% ID) for the thyroid, urine, and feces, or as a % ID per gram of tissue (% ID/g). A similar biodistribution study using Balb/c mice (8-week-old) was performed 3 h post-injection of the probes.

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Statistical Analysis Data are represented as mean ± standard deviation (S.D.). Statistical analyses were performed using the statistics program GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, U.S.A.). Mean values were compared using two-way ANOVA, followed by Tukey’s test for the cellular uptake study. To compare the distribution of ¹¹¹In and ¹²⁵I in ddY mice, a two-way ANOVA followed by Bonferroni test was performed. Furthermore, Tukey’s test was performed to compare the distribution of radioactivity in the organs and tissues of normal Balb/c mice, mice with metastasized melanoma in the lung, and mice with metastasized melanoma in the lung injected with excess γ-PGA. Unpaired t-test was carried out to analyze lung-to-blood radioactivity ratios. Differences at the 95% confidence level (p < 0.05) were considered significant.
RESULTS

Physicochemical Characteristics of $^{111}\text{In}$ and $^{125}\text{I}$-Labeled Ternary Complexes

$^{111}\text{In}$-DTPA-G4 was synthesized with a radiochemical yield of 95%. $^{125}\text{I}$-PEI was synthesized with a radiochemical yield of 53%. $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA was prepared at a charge ratio of 1:8:16 of the carboxyl groups of $^{111}\text{In}$-DTPA-G4: amino groups of $^{125}\text{I}$-PEI: carboxyl groups of γ-PGA. The particle size and zeta potential of $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA were 29.3 ± 8.7 nm and −32.6 ± 3.8 mV, respectively, which were almost equal to non-radiolabeled DTPA-G4/PEI/γ-PGA (particle size: 36.7 ± 4.0 nm, zeta potential: −28.6 ± 1.8 mV) (Table 1).

The radiochemical purities of $^{111}\text{In}$-DTPA-G4, $^{125}\text{I}$-PEI, and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA were determined with CAE and TLC, and were greater than 90% for both $^{111}\text{In}$ and $^{125}\text{I}$ (Fig. 1).

| Complex                  | Size (d.nm) | Zeta-potential (mV) |
|--------------------------|-------------|---------------------|
| G4-DTPA/PEI/γ-PGA       | 36.7 ± 4.0  | −28.6 ± 1.8         |
| G4-DTPA/$^{125}\text{I}$-PEI/γ-PGA | 25.5 ± 3.5  | −42.0 ± 5.7         |
| $^{111}\text{In}$-G4-DTPA/$^{125}\text{I}$-PEI/γ-PGA | 29.3 ± 8.7  | −32.6 ± 3.8         |

In Vitro Cellular Uptake Studies

The cellular uptakes of $^{111}\text{In}$-DTPA-G4, $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI, and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA in B16-F10 cells are summarized in Fig. 2. $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA with anionic charge was taken up by mouse melanoma B16-F10 cells over time. The cellular uptake of $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA was comparable to that of cationic $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI, while it was significantly higher than that of anionic $^{111}\text{In}$-DTPA-G4 ($4$, $58$, and $47\%$ dose $^{111}\text{In}$/mg protein for $^{111}\text{In}$-DTPA-G4, $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI, and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA, respectively, 6 h after incubation). The uptake of $^{125}\text{I}$ radioactivity was significantly higher than that of $^{111}\text{In}$ radioactivity at each time point when B16-F10 cells were incubated with $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI or $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA.

Biodistribution Studies Using ddY Normal Mice

The biodistribution of $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA was evaluated at 10 and 30 min, and 1, 3, and 6 h after probe injection into normal mice via the tail vein. $^{111}\text{In}$ ($^{111}\text{In}$-DTPA-G4) and $^{125}\text{I}$ ($^{125}\text{I}$-PEI) exhibited extremely similar distribution patterns in the liver, spleen, and lung for both $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA (Figs. 3A, B, Supplementary Table 1) and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI (Figs. 3C, D, Supplementary Table 2), except for in the kidney and blood. $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA showed relatively slower clearance from the blood, and higher accumulation in the spleen, liver, lung, and kidney compared to $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI.

Table 1. Particle Size and Zeta-Potential of Complexes Prepared in This Study

Fig. 1. Chromatographic Analyses of Each Complex by Using Cellulose Acetate Membrane Electrophoresis

(A) $^{111}\text{In}$-DTPA-G4, (B) $^{125}\text{I}$-PEI, (C) $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA. The symbols ■ and ▲ present $^{111}\text{In}$ and $^{125}\text{I}$, respectively.

Fig. 2. In Vitro Cellular Uptake of Complexes by B16-F10 Melanoma Cells

(A) Time-dependent cellular uptake of $^{111}\text{In}$-DTPA-G4, $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI, and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA. † $p < 0.01$ and * $p < 0.001$ vs. $^{111}\text{In}$-DTPA-G4. (B, C) Cellular uptake study of $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI (B) and $^{111}\text{In}$-DTPA-G4/$^{125}\text{I}$-PEI/γ-PGA (C). ■ and ▲ represent $^{111}\text{In}$ and $^{125}\text{I}$, respectively. Results are expressed as means ± S.D. (n = 4). † $p < 0.05$ and * $p < 0.001$ vs. $^{111}\text{In}$.
G4/125I-PEI. While the accumulation of 111In-DTPA-G4/125I-PEI in the spleen and liver was highly retained up to 6h post-injection, a gradual decrease in the lung and heart was observed. In the lung, the clearance of 125I was slower than that of 111In. There was low uptake of radioactivity in the pancreas, stomach, intestine, muscle, and thyroid. At 6h after probe injection, approximately 5% excretion of radioactivity into urine was observed for both the probes.

Biodistribution Studies Using Mice with Metastasized Melanoma in the Lung

The biodistribution of 111In-DTPA-G4/125I-PEI in mice with metastasized melanoma in the lung at 3h post-injection is summarized in Fig. 4 and Supplementary Table S3. Compared with the lung of normal Balb/c mice (25.1% ID/g for 111In in Supplementary Table 3), a relatively higher accumulation of radioactivity was observed in the lung with metastasized melanoma in the B16-F10 lung metastatic cancer model (33.7% ID/g for 111In). The accumulation of 125I radioactivity in the lung of normal mice (40.8%
ID/g) was approximately same level as that in the lung of mice with metastasized melanoma (42.0% ID/g). The radioactivity ratios between lung with metastasized melanoma and blood (22.4 ± 11.2 for 111In and 121.1 ± 17.7 for 125I) were significantly higher than those between normal lung and blood (6.6 ± 2.0 for 111In and 44.4 ± 7.2 for 125I) (p < 0.05).

In the blocking study (Fig. 4 and Supplementary Table S3), the accumulation of 111In-DTPA-G4/125I-PEI/γ-PGA in the lung with metastasized melanoma was markedly suppressed by co-injection of excess γ-PGA (approximately 3.8% ID/g for 111In and 5.5% ID/g for 125I), while the uptake of radioactivity (111In and 125I) in the liver was slightly increased. The renal uptake of 125I was significantly reduced by co-injection of excess γ-PGA.

DISCUSSION

In this study, we evaluated the feasibility of using radiolabeled γ-PGA ternary complexes as nuclear medical imaging probes targeting metastasized melanoma in the lung. By using radiolabeled DTPA-G4 and PEI with different radionuclides, we traced the distribution of each component in vitro and in vivo, and investigated the accumulation of probes in the lung with metastasized melanoma.

As shown in Table 1, 111In-DTPA-G4/125I-PEI/γ-PGA showed similar particle size and zeta potential to non-radiolabeled DTPA-G4/PEI/γ-PGA, indicating that radiolabeling of each component did not greatly affect the self-assembly of each component by electrostatic interaction and physicochemical properties of self-assembled nanoparticles.

In the cellular uptake study, the radiolabeled γ-PGA complexes were taken up by melanoma cells in a time-dependent manner; however, significantly higher uptake of 125I than that of 111In for both 111In-DTPA-G4/125I-PEI and 111In-DTPA-G4/125I-PEI/γ-PGA was observed. This is probably because these complexes were partially dissociated during the cellular uptake process and positively charged PEI was intensely retained in cells via electrostatic interaction with anionic cellular membrane, while the uptake of negatively charged 111In-DTPA-G4 was limited. On the other hand, it has been reported that γ-PGA nanoparticles could be metabolized by the lysosome66; thus, most probes would be localized in the intracellular fraction.

The in vivo study using 111In-DTPA-G4/125I-PEI/γ-PGA demonstrated that almost the same level of radioactivity derived from both 111In and 125I was observed in the liver, spleen and lung at an early phase after probe administration, suggesting the relatively high in vivo stability. On the other hand, the radioactivity of 125I was cleared from the blood more rapidly than that of 111In, and approximately 2-fold the uptake of 125I was confirmed compared than that of 111In in the kidney. Therefore, each composition might be gradually dissociated in part, and PEI would preferentially accumulate in the kidney as previously reported.17 In the lung, although almost equal accumulation was observed for both radioisotopes, the retention of 125I was prolonged compared to that of 111In. Similar to the in vitro cellular uptake study, positively charged PEI would be retained in cells more rigidly following the partial dissociation of each component. 111In-DTPA-G4/125I-PEI/γ-PGA accumulated especially in the macrophage-rich spleen, liver, and lung, which was consistent with our previous report that pDNA/poly-l-arginine/γ-PGA (γ-PGA-coated complex) showed high transfection efficiency in the spleen.18 Furthermore, the accumulation of 111In-DTPA-G4/125I-PEI/γ-PGA in these normal tissues was significantly higher than that of 111In-DTPA-G4/125I-PEI, indicating the involvement of γ-PGA in targeting these tissues.

In a biodistribution study using mice with lung-metastasized melanoma, the relatively higher accumulation and three-fold higher lung-to-blood ratios of 111In-DTPA-G4/125I-PEI/γ-PGA were observed in the lung with metastasized melanoma than in the lung of normal mice, indicating the uptake of 111In-DTPA-G4/125I-PEI/γ-PGA by B16-F10 melanoma cells. Because of the large blood volume in the lung, the high background signals in the blood could compromise the visualization of metastasized melanoma in the lung. 111In-DTPA-G4/125I-PEI/γ-PGA exhibited high lung-to-blood ratios in the melanoma model mice, indicating the availability for in vivo imaging. Interestingly, the uptake of radioactivity by the lung with melanoma was mostly suppressed by co-injection of excess γ-PGA with 111In-DTPA-G4/125I-PEI/γ-PGA, suggesting the involvement of the γ-PGA-specific uptake pathway in the melanoma and alveolar macrophages in the lung. On the other hand, the high hepatic uptake of the radiolabeled γ-PGA complex might cause an artifact for diagnosis of metastasized melanoma in the lung (neighboring tissue). In the future, the drug design enabling decrease of hepatic activity would be required to detect lung metastatic melanoma with high contrast.

In conclusion, the radiolabeled γ-PGA complex showed a relatively high in vivo stability in normal mice and high lung-to-blood radioactivity ratios in mice with metastasized melanoma in the lung. Although further investigation is needed to improve melanoma-specific bindings of γ-PGA-coated nanoparticles, the γ-PGA complex would hold the potentiality for nuclear medical diagnosis of lung-metastatic melanoma. 111In and 125I could be replaced with yttrium-90 and iodine-131 (β-ray emitters), respectively; thus, this γ-PGA complex might be utilized for theranostics (therapy + diagnosis). Furthermore, G4 dendrimer and PEI could be labeled with paramagnetic metal (gadolinium) and fluorescence dyes, indicating the possibility of the use of γ-PGA complexes as multimodality imaging probes.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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