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Folate Receptor-Specific Positron Emission Tomography Imaging with Folic Acid-Conjugated Tissue Inhibitor of Metalloproteinase-2

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The tissue inhibitor of metalloproteinase-2 (TIMP-2) inhibits matrix metalloproteinases activity and modulates cellular proliferation and apoptosis. The human serum albumin-TIMP-2 with folic acid conjugate (termed HT2-folate) was synthesized to promote uptake through folate receptors (FRs), and a corresponding radiolabeled compound was prepared for tumor diagnosis by positron emission tomography (PET). ^{68}Ga-NOTA-HT2-folate was synthesized from ^{68}Ga and the NOTA chelator with HT2-folate. The fusion protein was identified using MALDI-TOF mass spectrometry. The radioligand was prepared with a high radiochemical yield. Cell-surface association of ^{68}Ga-NOTA-HT2-folate significantly increased over time in FR-positive tumor cells. In animal PET and biodistribution studies, tumor uptake was very high as early as 1 h after radioligand injection. Folate conjugation enhanced the selective receptor-targeting efficacy of HT2 in FR-expressing tumors, and its radioligand will be useful as an *in vitro* tool and for *in vivo* tumor diagnosis by PET imaging.

Key Words : Folic acid, Human serum albumin-TIMP-2, ^{68}Ga-NOTA-HT2-folate, Tumor, Radioligand

Introduction

Cancer is generally characterized by uncontrolled cell division and metastasis, leading to death. Cancer cell metastasis is largely regulated by the active forms of matrix metalloproteinases (MMPs). MMPs play an important role in cell division, migration, and tissue reorganization.[1] MMP activity is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs), which form high-affinity complexes with the active form of MMPs.[2] One member of the TIMP family, TIMP-2, inhibits MMP activity and modulates proliferation and apoptosis.[2a,3] The antitumor activity of TIMP-2 is attributed to its antiangiogenic properties; it inhibits endothelial cell proliferation and is thought to be linked to the direct control of MMP proteolysis and down-regulation of vascular endothelial growth factor.[4] Many therapeutic proteins have been conjugated to human serum albumin (HSA) due to its long half-life (approximately 19 days). The HSA portion of the conjugate typically increases circulating half-life, improves biological activity, and enhances the stability of its fusion partner *in vivo*.[5] An HSA-TIMP-2 conjugate (HT2) was prepared by linking TIMP-2 to the C terminus of HSA; the fusion protein inhibited tube formation of human umbilical vein endothelial cells by approximately 81%.[6] HT2 has been proposed as a candidate for therapeutic treatment of angiogenesis-related diseases.

The folate receptor (FR) is a glycosyl phosphatidylinositol-anchored glycoprotein that is strongly expressed in tumor cells of epithelial origin.[7] The receptor consists of 3 isoforms: FRα, FRβ, and FRγ– the α and β isoforms are membrane-bound. FRα is highly expressed in various non-mucinous tumors, including over 90% of ovarian carcinomas, but its expression is otherwise restricted in normal epithelial cells.[8] FRβ is frequently expressed in acute and chronic myelogenous leukemias.[9] Folic acid is a small molecule (molecular weight = 441.4) that is involved in pathways linked to cell proliferation and plays an essential role in tumor growth. Because of its high specific binding affinity to FR-α on the cell surface, folic acid has been used for targeted drug delivery of conjugated bioactive probes to tumor cells.[10] Folic acid has been conjugated to many types of probes, such as anticancer agents, antisense oligonucleotides, proteins, liposomes, and radioligands, for the treatment and diagnosis of cancer cells overexpressing FR, which is common in many types of human cancers.[11]

Selective targeting of FR-overexpressing tumors can be achieved with a bioactive agent covalently linked to folic acid, which acts as an uptake moiety. We attempted this strategy using the bioactive TIMP-2 protein. ^{68}Ga-labeled HT2-folate was prepared and the radioligand was tested *in vitro* and *in vivo* by positron emission tomography (PET) to evaluate its tumor-targeting properties and potential utility as a tool for tumor diagnosis.

Experimental

General. HT2 was obtained from Biocure Pharm (Daejeon, Korea). Folic acid, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), and 6-(fluorescein-5(6)-carboxamido)hexanoic acid (FITC) were pur-

chased from Sigma-Aldrich (St. Louis, MO, USA). Centrifugal filter units (Amicon® Ultra-4) from Millipore (Billerica, MA, USA) were used to concentrate and purify solutions. Mass spectra were obtained by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry at the Korea Basic Science Institute (KBSI). A ^{68}Ge/^{68}Ga generator was purchased from Eckert & Ziegler (Obinisk, Russia). Radioactivity was counted with a Cobra II gamma scintillation counter (Perkin-Elmer, Inc., MA, USA). Animal PET images were scanned by an Inveon PET/CT/SPECT (Siemens, TN, USA) at KBSI.

Synthesis of HT2-folate. A mixture of folic acid (0.1 mg, 226.6 nmol in dimethylsulfoxide at a concentration of 1 mg/mL), HBTU (0.5 mg, 1.32 ìmol), and HOBt (0.18 mg, 1.33 ìmol) in phosphate-buffered saline (PBS) was allowed to react for 10 min after addition of 10 μL DIEA at room temperature. The mixture was added to HT2 (1 mg, 11.4 nmol) and incubated for 90 min at room temperature. The crude mixture was purified by size exclusion chromatography (PD-10 column) and product fractions were collected and concentrated with centrifugal filters (molecular size cut-off, 50 kDa). Molecular weights were calculated by MALDI-TOF mass spectrometry. The final product was stored at –20 °C.

Synthesis of 1,4,7-Triazacyclononane-*N,N′,N″*-triacetic acid (NOTA)-HT2 and NOTA-HT2-folate. NOTA-conjugated proteins were synthesized with 2-(*p*-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bz-NOTA) (1 mg, 2.2 μmol) and HT2 or HT2-folate (4 mg/mL) in 0.1 M sodium carbonate buffer. The solution was incubated overnight at room temperature in the dark. The mixture was purified through a PD-10 column with PBS elution buffer. The product fractions were collected and concentrated with centrifugal filters. The molecular weight and number of folic acid residues per HT2 molecule were calculated by MALDI-TOF mass spectrometry.

Labeling of NOTA with ^{68}Ga. The resulting ^{68}Ga was eluted from a ^{68}Ge/^{68}Ga generator with 0.1 N HCl (1 mL) and the second fraction of the eluted ^{68}Ga solution was used for radiolabeling. ^{68}Ga solution (37 MBq in 1 mL of 0.1 N HCl) was added to the NOTA-conjugated proteins described above. Phosphate buffer (0.2 mL of 0.5 M) was added and the pH of the solution was adjusted to 6.0 with 7% sodium bicarbonate.[12] The reaction mixture was incubated for 20 min at room temperature with constant shaking (400 rpm) in a thermomixer (Eppendorf, Hamburg, Germany). ^{68}Ga-NOTA-HT2 was purified through a PD-10 column with PBS buffer and filtered by Alumina Sep-Pak to remove free ^{68}Ga.

Cells and Animals. The Human oral cancer cell (KB) line was obtained from ATCC (America Type Culture Collection, USA). The KB cells were known to overexpress the folate receptor.[13] The cells were grown in RPMI1640 (GIBCO, BRL Life Technologies Inc.) supplemented with penicillin (100 U/mL), streptomycin (100 g/mL), and 10% fetal bovine serum (FBS). The cells were maintained at 37 °C in a 5% CO_2 atmosphere. Animal experiments were performed according to a protocol approved by the local Institutional Review Committee on Animal Care (KBSI-AEC1001). Five-week-old male BALB/c nu/nu mice were maintained under specific pathogen-free conditions. To induce tumors, 1×10^6 KB cells were subcutaneously injected into the dorsal region of the right thigh of each mouse.

***In vitro* Binding Assay and Measurement of Cellular Uptake.** Uptake of radiolabeled HT2-folate into KB cells was determined as follows. Cells were seeded in 24-well plates at a density of 1×10^6 cells/well. After attachment, the cells were treated with radiolabeled HT2 or HT2-folate (37-74 kBq) by incubation for 30, 60, and 120 min at room temperature, and then collected by filtration through a vacuum manifold and washed twice with PBS. ^{68}Ga-NOTA-HT2-folate was treated in a specific binding assay with folic acid (100 μg/mL) for 2 h. The cells were dissolved in 0.1 N NaOH, collected, and radioactivity was measured in a NaI (Tl) γ-counter. Experiments were performed twice with triplicate samples.

PET Analysis and Data Reconstruction. KB tumor-bearing mice were prepared by subcutaneous injection of 1×10^6 tumor cells/mouse (n = 3). ^{68}Ga-NOTA-HT2 or ^{68}Ga-NOTA-HT2-folate (7.4 MBq) was injected intravenously. PET scans were performed for 20 min at 1 and 3 h post-injection. All micro PET images were reconstructed by a three-dimensional ordered-subsets expectation maximum algorithm with a 128 × 128 matrix and 4 iterations, then displayed using the INVEON Research workplace software (Siemens, USA). A region of interest (ROI) was identified in the tumors in the coronal, sagittal, and transaxial micro PET images.

Biodistribution Studies. To induce tumors, 1×10^6 KB cells were subcutaneously injected into nude mice (n = 3). Mice bearing tumor xenografts were injected with radioligands (3.7 MBq) in 0.2 mL saline *via* the tail vein. Mice were sacrificed at 30 min, 1 h, and 2 h after injection. For blocking studies, the animals were sacrificed 2 h after co-injection of the ^{68}Ga-NOTA-HT2-folate and blocking folic acid (100 μg). Organ samples (blood, heart, lung, liver, spleen, kidney, muscle, femur, and tumor tissue) were collected and weighed, and radioactivity was determined in a ã-counter. Data are expressed as the percent injected dose per gram tissue (%ID/g).

Results

Synthesis of Unlabeled Compounds. The fusion protein HT2-folate was synthesized to improve tumor targeting by receptor-mediated uptake (Fig. 1(a)). Folic acid was covalently coupled to amine residues of the HT2 protein through peptide bonds using HBTU and HOBt under basic conditions. The ratio for folic acid conjugation of the MMP2-targeted TIMP-2 albumin conjugate (HT2) was 1:20 HT2: folic acid. Unreacted folic acid was removed by size exclusion chromatography. The NOTA-conjugated proteins were prepared for micro PET imaging with ^{68}Ga. HT2 (*m/z* = 88,420.5),[14] HT2-folate (*m/z* = 89,194.9), and NOTA-HT2-folate (*m/z* = 95,985.8) were confirmed by mass

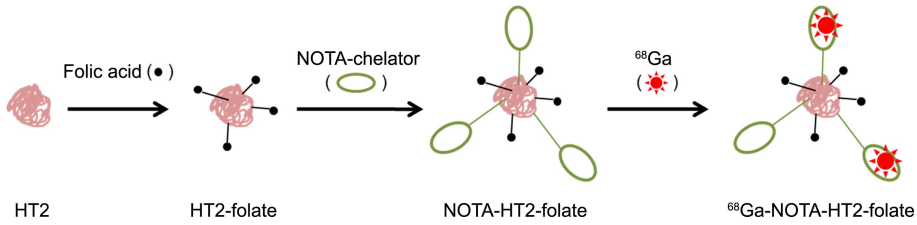

Figure 1. (a) Scheme of HT2-folate and ^{68}Ga-labeled HT2-folate, (b) MALDI-TOF mass spectra of HT and HT2-folate.

spectrometry (Fig. 1(b)). The number of folic acid and NOTA moieties per molecule of protein was around 1.8 and 15, respectively, as calculated by MALDI-TOF mass spectrometry.

Synthesis of Radioligand. The ^{68}Ga-labeled protein was prepared using a ^{68}Ga/^{68}Ge generator system. ^{68}Ga was eluted with 0.1 N HCl, diluted with 0.5 M phosphate buffer, and titrated to pH 5-6 with 7% sodium bicarbonate. The entire labeling procedure, including the purification step, was completed within 30 min. The radiochemical yield of ^{68}Ga-NOTA-HT2-folate was 35-50%.

***In vitro* Cell Binding Assay and Rate Measurement.** Cell binding studies were performed with ^{68}Ga-NOTA-HT2-folate in KB tumor cells (Fig. 2(a)). Uptake was greatest in the KB cells and increased in a time-dependent manner. The KB tumor cell uptake of ^{68}Ga-NOTA-HT2-folate *vs.* ^{68}Ga-

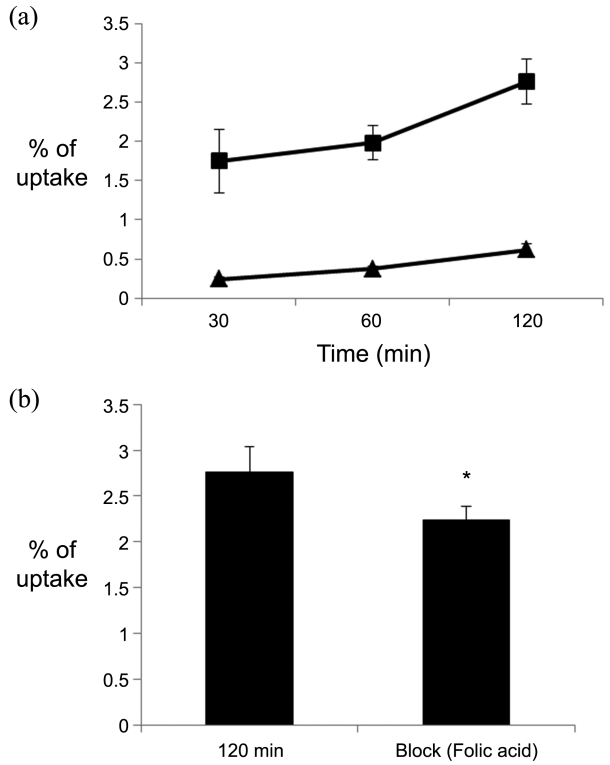

Figure 2. KB tumor cell uptake of ^{68}Ga-NOTA-HT2-folate and ^{68}Ga-NOTA-HT2 *in vitro*. (a) Tumor cell uptake (% of uptake) of the radioligand is shown as a function of time. ■: KB tumor cell uptake of ^{68}Ga-NOTA-HT2-folate; ▲: KB tumor cell uptake of ^{68}Ga-NOTA-HT2. (b) KB tumor cell uptake of ^{68}Ga-NOTA-HT2-folate with and without 100 μg folic acid at 2 h incubation. *P < 0.05.

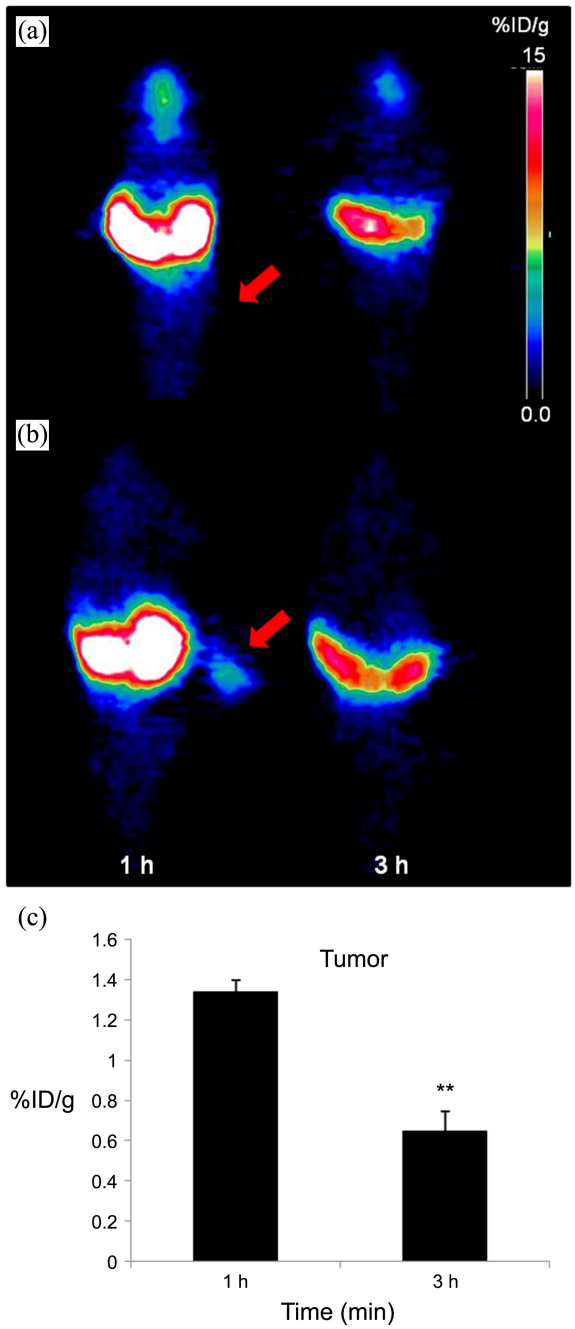

Figure 3. MicroPET images. Coronal microPET images of ^{68}Ga-NOTA-HT2 (a) and ^{68}Ga-NOTA-HT2-folate (7.4 MBq) (b) injected into mice bearing KB tumor cells at 1 and 3 h after injection. Arrows indicate the position of the KB tumor. The images are displayed at different values of %ID/g. (c) ROIs of the tumor with ^{68}Ga-NOTA-HT2-folate 1 and 3 h after injection. **P < 0.01.

NOTA-HT2 was approximately 5 times higher at all sampled time points. Cellular uptake of ^{68}Ga-NOTA-HT2-folate in KB cells at 30, 60, and 120 min was 1.75 ± 0.41, 1.98 ± 0.22, and 2.76 ± 0.28% of uptake, respectively, and was 0.24 ± 0.02, 0.37 ± 0.03, and 0.62 ± 0.07% of uptake, respectively, for ^{68}Ga-NOTA-HT2. In the blocking studies, FR specificity was demonstrated by co-incubation with folic acid; KB cell uptake of ^{68}Ga-NOTA-HT2-folate was inhibited by 20% at 2 h incubation ($P < 0.05$) (Fig. 2(b)).

PET Image Analysis in Tumor Bearing Mouse. The micro PET study was performed 2 weeks after the implantation of KB cells in nude mice (n = 3). ^{68}Ga-NOTA-HT2-folate and ^{68}Ga-NOTA-HT2 (7.4 MBq) were injected into the tail vein of KB tumor-bearing mice. The mice were scanned for 20 min and static images were obtained 1 and 3 h after radioligand injection (Fig. 3). There were high levels of uptake by the liver, indicating major hepatobiliary clearance, and the high initial uptake of radioligand declined rapidly. When ^{68}Ga-NOTA-HT2 was injected into mice, microPET images showed no tumor uptake (position of tumor indicated by red arrow) (Fig. 3(a)). In contrast, ^{68}Ga-NOTA-HT2-folate showed high tumor uptake (Fig. 3(b)). The ROI of the tumor was around 2-fold higher at 1 h *vs*. 3 h after injection of radioligand (1.34 ± 0.06 *vs.* 0.65 ± 0.1 %ID/g, respectively) (Fig. 3(c)). The results in tumor-bearing mice demonstrate its potential utility as a new tool for diagnosis of FR-positive tumors *in situ*.

Biodistribution Studies. Biodistribution of radioligands was determined in KB xenograft-bearing nude mice sacrificed at 30 min, 1 h, and 2 h after injection of ^{68}Ga-NOTA-HT2-folate (3.4 MBq) and inhibition studies with folic acid co-injection (Fig. 4(a)). Radioactivity in blood slightly decreased over 2 h post-injection (1.93 ± 0.89, 1.74 ± 0.32, and 1.56 ± 0.48 %ID/g at 30 min, 1 h, and 2 h after injection, respectively). The highest tumor uptake of the folate-conjugated radioligand was at 1 h post-injection (0.41 ± 0.10, 1.54 ± 0.48, and 0.60 ± 0.04 %ID/g at 30 min, 1 h, and 2 h after injection, respectively) (Fig. 4(b)). When the folic acid was co-applied with radioligand, tumor uptake was significantly inhibited by 40% at 2 h after injection and there were no inhibition effects in other tissues (Fig. 4(c), and 4(d)). This result demonstrated that the radioligand was specific to FR. The greatest uptake was observed in the liver (14.0 ± 1.40 for the folate conjugate and 25.56 ± 0.23 %ID/g for unconjugated ligand, both 1 h after injection) (Fig. 5). These results are consistent with those from the micro PET image analysis. Tumor uptake of ^{68}Ga-NOTA-HT2-folate (1.54 ± 0.48 %ID/g) was slightly higher than that of the unconjugated ligand (0.49 ± 0.13 %ID/g) 1 h after injection (Fig. 5).

Discussion

TIMP2 exhibits strong MMP inhibitory activity with an IC_{50} ~7.5 nM;[15] however, the utility of TIMP2 as a diagnostic or therapeutic agent is limited due to its biological unavailability and relative instability. HT was synthesized for use as a tumor treatment,[6] as it consists of HSA and TIMP2 with a long circulating half-life and stability, and more specific MMP2 binding. Therefore, we aimed to synthesize and evaluate a more selective and specific protein for

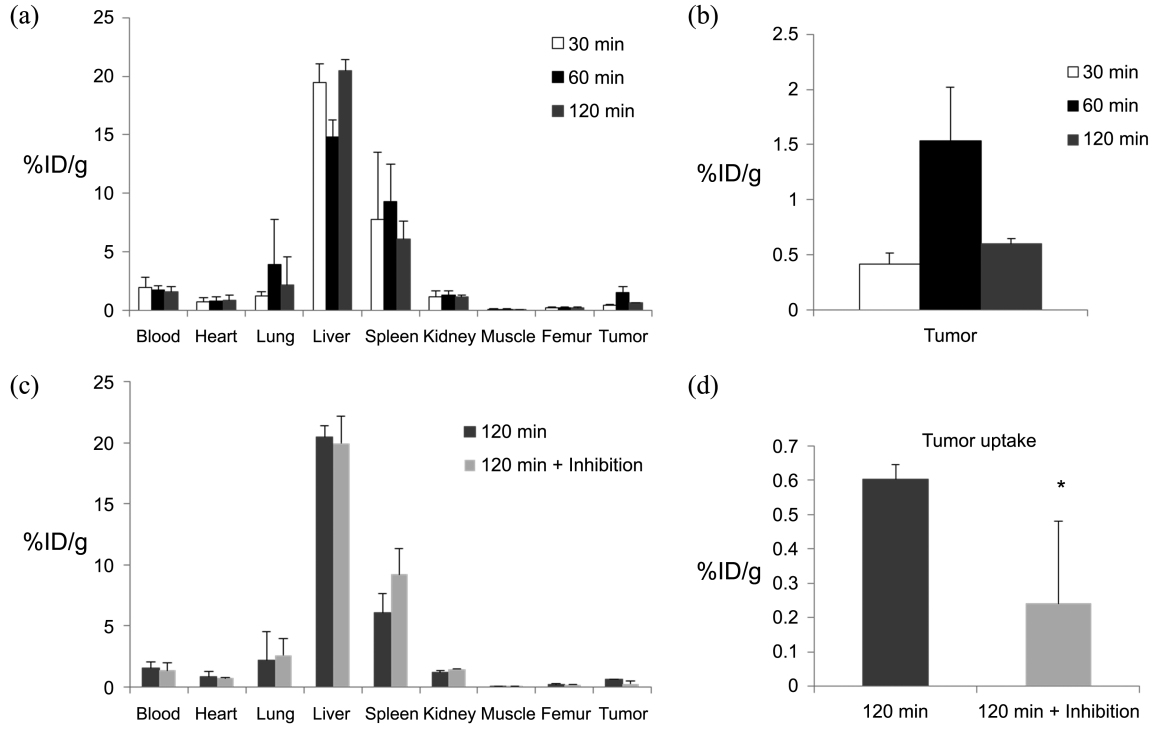

Figure 4. (a) Biodistribution of ^{68}Ga-NOTA-HT2-folate (3.7 MBq) in nude mice bearing KB cells at 30, 60, and 120 min after injection. (b) Tumor uptake at 30, 60, and 120 min after injection. (c) and (d) Tumor uptake and folic acid inhibition studies with the radioligand at 120 min after injection. Values represent mean %ID/g, and the error bars indicate SD (n = 3). *$P < 0.05$

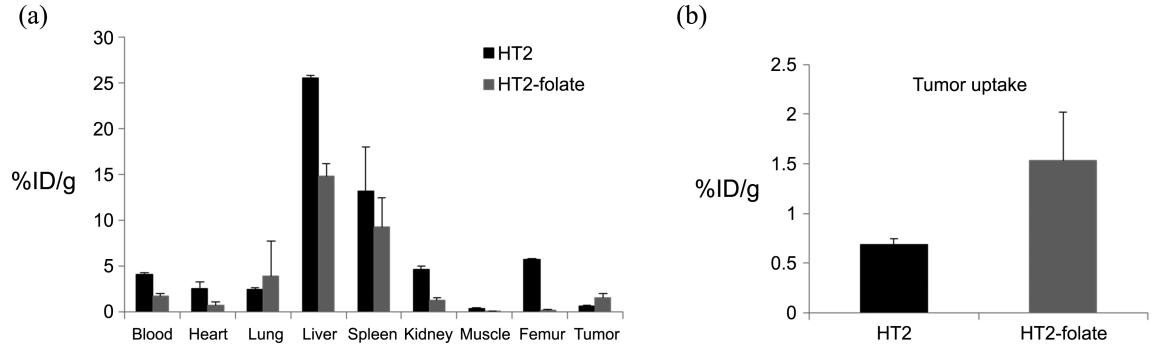

Figure 5. (a) Biodistribution of ^{68}Ga-NOTA-HT2-folate (gray) and ^{68}Ga-NOTA-HT2 (black) (3.7 MBq) in nude mice bearing KB cells at 1 h after radioligand injection *via* the tail vein. Values represent mean %ID/g and the error bars indicate SD (n = 3). (b) Tumor uptake (%ID/g) at 1 h after radioligand injection. $*P < 0.05$.

tumor diagnosis *via* conjugation with folic acid. In this study, labeled HT2-folate was prepared to enhance the diagnostic utility of TIMP-2 in an FR-positive solid tumor model. HT reportedly possesses antitumor and antiangiogenesis activity with passive targeting of a tumor site *in vitro* and *in vivo*. It is highly stable *in vivo* and may thus be applied to therapeutic proteins. However, large amounts of HT are required for cancer diagnosis or treatment *in vivo*, due to their non-specificity. Therefore, we sought to improve the selective receptor targeting efficacy for *in vivo* application. In the conjugated biomolecule, folic acid confers receptor-mediated tumor targeting, HSA extends the circulating half-life and stability of the protein, and TIMP-2 is associated with antitumor activity.

Targeted drug delivery systems have been developed for specificity, selectivity, and efficacy. Folic acid vitamins are commonly used to increase the affinity for the folate receptor. Diverse folate conjugates have been applied as therapeutic agents, inhibitors, and in imaging procedures such as PET, magnetic resonance imaging, optical imaging modalities, *etc*. We developed a fusion protein with high binding affinity for the folate receptor, which was overexpressed in cancer cells. The FR-mediated uptake of HT2-folate was confirmed by cell binding assays with the radioligand. The difference in uptake of the folate conjugate *vs.* unconjugated molecule was approximately 5-fold higher in KB cells. Tumor cell uptake of folic acid with ^{68}Ga-NOTA-HT2-folate was significantly decreased by 20%, indicating that the radioligand was specific and receptor-binding. These results demonstrate the selective folate receptor targeting of the conjugate and radiolabeled fusion protein binding to KB cancer cells *in vitro*. We compared radioligands with or without folic acid in HT. Our radiolabeled HT2-folate exhibited 3-fold greater tumor uptake than unconjugated HT2, therefore providing enhanced targeting for cancer diagnosis.

Recently, blockade experiments demonstrated that 2-^{18}F-fluorofolic acid is efficiently and specifically taken up by tumor cells.[16] However, the radioisotope ^{18}F, which is commonly obtained from a cyclotron, is expensive and difficult to manage. In contrast, ^{68}Ga might be obtained from a ^{68}Ge/^{68}Ga generator system, which is easy to use, portable, and does not require an expensive cyclotron on site. Thus, the radioisotope ^{68}Ga can be produced easily. ^{68}Ga-NOTA-HT2-folate was prepared within 30 min of total labeling time. The uptake effect of folic acid-conjugated HT2 on KB tumor-bearing mice was confirmed by PET analysis using ^{68}Ga labeled HT2-folate. No tumor uptake was apparent in ^{68}Ga-NOTA-HT2-injected tumor-bearing mice. However, our novel radioligand ^{68}Ga-NOTA-HT2-folate showed significant uptake by FR-positive tumor cells at 1 h after injection through MicroPET imaging; this result was correlated with the biodistribution study results. Thus, the radioligand supports efficient imaging of FR-positive tumors in mouse models. In addition, FRs are significantly overexpressed in most human tumors.

In our study, radiolabeled folate-conjugated protein (^{68}Ga-NOTA-HT2-folate) provided more specific and selective cancer detection than HT2 protein alone, as folate receptor binding improved HT2 targeting. Kang *et al.* demonstrated the MMP inhibitory activity of HT2. Our results indicated time-dependent, improved tumor uptake of radiolabeled HT2 and HT2-folate, likely because our protein conjugates could bind folate and MMP2 receptors on the cell surface. The folate-conjugated protein provided better diagnostic results than HT2 alone; therefore, the ^{68}Ga-NOTA-HT2-folate radioligand might be a potent imaging agent for use in PET-based tumor diagnosis in preclinical studies. We also suggest the potential utility of the fusion protein as an anticancer agent, and will examine the mechanism of cancer inhibition with the fusion protein in xenograft mice models in the future.

Conclusion

We have found that folic acid conjugation to the HT2 fusion protein enhances targeting and selectivity of the biomolecule in FR-positive KB tumor cells. The radiolabeled molecule was taken up by FR-positive tumors. Thus, it may be a useful imaging agent for PET-based tumor diagnosis in animal models and preclinical studies. Further studies in animal models are warranted to investigate the antitumor effect of the fusion protein.

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