Title of Thesis

Development of novel nuclear medical imaging methods for multiple diagnoses of disease-related molecules

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(Doctor Course)
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Abstract

Inflammatory bowel disease (IBD) and cancer are intractable diseases causing great mental and financial stresses on patients. These diseases involve multiple and complex molecular interactions, and the methods for curing these diseases remain largely undeveloped. Thus, for improving the therapeutic benefits, it is necessary to develop noninvasive methods providing the necessary information on multiple disease-related molecular species, and to establish the systems combining molecular diagnostics and therapy. In this thesis, I have considered nuclear medical imaging as a candidate method for addressing the needs described above.

Nuclear medical imaging enables noninvasive visualization of molecules in all body parts, including areas deep within the body. Therefore, nuclear medical imaging is useful for providing information on disease-related molecules in affected areas. In fact, several different nuclear medical imaging probes targeting pathogenic molecules have been developed for clinical diagnosis (e.g. $^{111}$In-Zevalin). However, the number of probes applied for clinical diagnosis is extremely low; consequently, developing the probes targeting various types of pathogenic molecules is highly desired. Furthermore, it is extremely difficult to simultaneously visualize multiple disease-related molecular
species using the existing nuclear medical imaging modalities. Thus, the ultimate objective of the present study was to develop a nuclear medical imaging method allowing to acquire the information on multiple molecular species relevant to IBD and cancer. Developing the methods for noninvasive probing of the presence of multiple disease-related molecular species is expected to assist in developing personalized therapies.

Four stages were envisioned for achieving the ultimate objective of the present study: (1) Exploration of imaging targets or selection of targets from previously reported disease-related molecules, (2) Development of novel nuclear imaging probes for visualizing the target molecules, (3) Development of a novel nuclear imaging technique allowing to visualize multiple molecular species simultaneously. In this thesis, I addressed objective stages (1) to (3) by studying the mice model of IBD and cancer. The main findings are summarized below.

Chapter 1 presents the research on target molecules for IBD imaging. I examined alterations in the expression of pathognomonic molecules in the inflamed region of the mouse intestines and found that interleukin (IL)-6, IL-1β, and S100 calcium binding protein A8 (S100A8) were particularly promising target molecules for IBD imaging because the expression levels of these molecules were altered during the early stages of
inflammation and depended on the extent of inflammation. Hence, utilization of nuclear medical imaging probes targeting these inflammation-related molecules would be helpful for grasping in greater detail the inflammatory conditions of IBD during the early stages of its development, which would eventually assist in developing evidence-based therapy.

Chapter 2 describes the development of a novel antibody probe for cancer diagnostics. Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) was selected as the imaging target because CTLA-4 has been used as a target molecule for cancer immunotherapy, and fully human anti-CTLA-4 monoclonal antibodies (mAbs), ipilimumab and tremelimumab, were developed for cancer treatment. In this study, $^{64}$Cu-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-anti-CTLA-4 monoclonal antibody (mAb) was developed, and the probe usefulness was evaluated by using positron emission tomography (PET) and ex-vivo biodistribution analyses of tumor-bearing mice. These results suggested that although $^{64}$Cu-DOTA-Control IgG (isotype control) accumulated slightly in the CT26 tumor tissues likely owing to enhanced permeability and retention (EPR) effects, the accumulation of $^{64}$Cu-DOTA-anti-CTLA-4 mAb was more pronounced. In addition, $^{64}$Cu-DOTA-anti-CTLA-4 mAb exhibited significantly higher tumor-to-blood and
tumor-to-muscle ratios compared with $^{64}$Cu-DOTA-Control IgG. These results suggest that $^{64}$Cu-DOTA-anti-CTLA-4 mAb can be useful for evaluating CTLA-4 expression in tumors.

Chapter 3 describes the imaging study of multiple molecular species performed by using GREI. In this study, $^{89}$Zr-deferoxamine (DFO)-anti-epidermal growth factor receptor (EGFR) mAb and $^{111}$In-DOTA-anti-human epidermal growth factor receptor 2 (HER2) mAb were intravenously administered to tumor-bearing mice, and distributions of these probes in the tumor-bearing mice were visualized by using GREI. Although the quality of $^{111}$In-labeled probe data remains to be further improved, these results suggest that GREI can be used for noninvasively visualizing multiple disease-related molecular species in tumors.

As mentioned above, these studies provide the fundamental information for developing the novel methods for evaluating the expression of multiple pathogenic molecular species relevant to IBD and cancer. Thus, the conclusions of the present study can assist in developing evidence-based medical care methods.
Abbreviations

BSA: bovine serum albumin

CD: cluster of differentiation

CTLA-4: cytotoxic T lymphocyte-associated antigen-4

DFO: deferoxamine

DMSO: dimethyl sulfoxide

DOTA: 1,4,7,10-tetraazacyclododecane-\(N,N',N'',N'''\)-tetraacetic acid

DSS: dextran sulfate sodium

EDTA: ethylenediaminetetraacetic acid

EGFR: epidermal growth factor receptor

EPR: enhanced permeability and retention

flCTLA-4: full-length CTLA-4

Foxp3: forkhead box P3

FR: folate receptor

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GREI: Gamma-Ray Emission Imaging

HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HER2: human epidermal growth factor receptor 2
HPLC: high-performance liquid chromatography
IBD: inflammatory bowel disease
IL: interleukin
IL-1R type 1: IL-1 receptor type 1
IL-6Rα: IL-6 receptor-α
IND: indomethacin
LiCTLA-4: ligand independent CTLA-4
mAb: monoclonal antibody
MALDI: matrix-assisted laser desorption-ionization
MS: mass spectrometry
MIP: maximum intensity projection
MRI: magnetic resonance imaging
NOTA: 1,4,7-triazacyclononane-1,4,7-triacetic acid
PBS (-): phosphate-buffered saline without calcium and magnesium
PET: positron emission tomography
RT: room temperature
RT-PCR: reverse transcription polymerase chain reaction
sCTLA-4: soluble CTLA-4

SD: standard deviation

SEM: standard error of mean

SPECT: single-photon emission computed tomography

SUV: standardized uptake value

TLC: thin layer chromatography

TLR: toll-like receptor

TNF: tumor necrosis factor

TOF: time-of-flight

Treg: regulatory T cell

VEGF: vascular endothelial growth factor

%ID/g: the percentage of injected dose per gram

^{18}F-FDG: fluorine-18-fluorodeoxyglucose

1/4CTLA-4: CTLA-4 variant consisting of exons 1 and 4 only
Introduction

An extraordinarily large number of molecules exist in our body, and interact with one another to sustain the essential biological functions of the body. Current research in life sciences focuses on elucidating the molecular functions as one of the more important research themes for the development of novel methods for treatment or diagnosis. With such a background, the demand for molecular imaging techniques is ever increasing. These technologies can be used for in vivo visualization of biomolecules; thus, they find applications in clinical diagnosis as well as in research using animal models. Various types of molecular imaging modalities, such as positron emission tomography (PET), single-photon emission computed tomography (SPECT), optical imaging, and magnetic resonance imaging (MRI) have already been put to practical use in life science research and/or clinical diagnosis. Furthermore, our laboratory has developed a novel nuclear medical imaging modality, called the Gamma-Ray Emission Imaging (GREI). A well-suited imaging modality must be chosen from these modalities in accordance with the study purposes because each modality has its own strengths and weaknesses. In what follows, we introduce the characteristics of these modalities and provide the rationale for using the PET and GREI in the present study.
1. **Positron emission tomography (PET)**

PET is a nuclear medical imaging modality utilizing an imaging probe labeled with a positron-emitter such as $^{11}$C, $^{18}$F, $^{64}$Cu, or $^{89}$Zr. The positron emitted from the positron-emitter annihilates a nearby electron, resulting in the generation of a pair of annihilation radiations, each with an energy of 511 keV, traveling at opposite directions to one another, 180° apart (Fig. 1). Coincidence measurement of the annihilation radiations by PET detectors provides the information regarding the position of PET probe.

The advantages of PET are that it provides high resolution and high sensitivity. PET can visualize tiny amounts of a probe (of concentration $\sim 10^{-11}$–$10^{-12}$ M) and is practically independent of the probe-depth owing to the accurate methods employed to correct for radiation attenuation [1]. The spatial resolution of PET is $\sim 4$–$8$ mm$^3$ and $\sim 1$–$2$ mm$^3$ in clinical imaging and small animal imaging systems, respectively. However, PET imaging cannot separately localize two simultaneously injected probes, because the energies of annihilation radiations are the same for both probes and it is impossible to distinguish the each radiation from multiple positron emitters.
2. **Single-photon emission computed tomography (SPECT)**

SPECT is a nuclear medical imaging modality utilizing an imaging probe labeled with a single-photon (gamma-ray) emitter such as $^{99m}$Tc, $^{111}$In, or $^{123}$I. Importantly, SPECT needs a collimator in front of gamma-ray detectors to obtain the direction in which the gamma-rays travel from the probe (Fig. 2). A collimator is a Pb or W septa to permit the passage of only gamma-rays oriented in certain directions and stop the others. The
position of the SPECT probe can be determined by multiple information regarding the traveling direction of the gamma-rays.

Although the sensitivity and quantitative capability of SPECT is lower than PET, SPECT is cheaper than PET. Small animal SPECT has a spatial resolution of ~ 1 mm$^3$ and clinical systems have resolutions of ~ 12–15 mm$^3$ [1]. Moreover, although SPECT imaging with multiple radioisotopes has been attempted, the choice of radionuclides is strictly limited in SPECT, because high-energy (more than 300 keV) gamma-rays pass through the collimator irrespective of direction.

**Figure 2**

**Schematic of single-photon emission computed tomography (SPECT).**
3. Optical imaging

Optical imaging can be broadly classified into fluorescence- and bioluminescence-imaging. Fluorescence imaging is an imaging modality utilizing a fluorochrome such as a near-infrared fluorescent probe. Fluorescence imaging is based on the absorption of energy from an external excitation light followed by re-emission by the fluorochrome at a longer wavelength (i.e. of lower energy) compared to the excitation light. The position of the probe (fluorochrome) is determined by detecting the fluorescence with a thermoelectrically cooled charge-coupled device camera. In bioluminescence imaging, light, which is generated by a chemiluminescent reaction between an enzyme and its substrate, is externally detected and is an indicator of a biological/molecular process. The advantages of optical imaging are high sensitivity (up to $\sim 10^{-15}$ M), low cost, and relatively high throughput capability [1]. Furthermore, fluorescence imaging can visualize multiple molecular species simultaneously by probes labeled with different fluorophores [2]. These advantages make them particularly suited to the development and validation of drugs. However, optical imaging has limited clinical applications, since the light transmission from tissues is limited, which limits spatial resolution and the depth of imaging, resulting in very limited signal quantification [3].
4. Magnetic resonance imaging (MRI)

MRI is an imaging modality utilizing the magnetic properties of atoms: their interactions with strong external magnetic fields and radiowaves to produce images. MRI is characterized by high spatial resolution (< 50 µm for preclinical devices and 300 µm in ultra-high-field experimental clinical devices) [3]. In addition, MRI is applicable to both anatomical-/morphological- and functional-imaging without exposure to ionizing radiation. For these reasons, MRI is widely used for clinical diagnosis and for assessment of the effect of treatment. However, unlike other modalities of nuclear medical imaging and optical imaging, MRI has relatively low sensitivity ($10^{-3}$–$10^{-4}$ M) [1]. Therefore, at least millimolar levels of probe concentration are generally required to visualize a molecule. In general, due to the sensitivity restrictions, MRI instruments receive signals from protons of water molecules owing to the abundance of water in our body. In other words, application of MRI is mainly restricted to anatomical/morphological imaging.

5. Gamma-Ray Emission Imaging (GREI)

GREI is a nuclear medical imaging modality, which employs two double-sided orthogonal-strip germanium (Ge) detectors arranged in parallel. GREI has a feature to
detect gamma-rays with a wide energy range (200–2000 keV) simultaneously and noninvasively [4-6]. Thus, it has the potential to visualize multiple molecular species simultaneously by several probes labeled with radioisotopes emitting gamma-rays of different energies. Furthermore, since this process does not need mechanical collimators, there is no loss of gamma-rays caused by collimation. Thus, GREI has the potential to conduct nuclear medical imaging with high sensitivity. The spatial resolution of GREI is about 3 mm [7].

The principles of GREI are explained as follows. If Compton scattering of an incident gamma-ray occurs at the front Ge detector and then the scattered gamma-ray is fully absorbed at the rear Ge detector (photoelectric absorption), the gamma-ray energy of the original source \( E_\gamma \) can be calculated by summing the energies deposited at the front detector \( E_1 \) and the rear detector \( E_2 \) (Fig. 3). Scattering angles \( \theta \) are calculated from \( E_\gamma, E_1, E_2, \) and the straight lines connecting the interaction points at the front detector with those at rear detector, using the equation of motion of Compton scattering \( \cos \theta = 1 + m_e c^2 \{1/E_\gamma - 1/ (E_\gamma - E_1)\} \). The circular cone, which has \( \theta \) as its half-angle, indicates the direction of the radiation source. The locations of gamma-ray sources can be derived from the cones generated by a number of these events.
Figure 3

**Schematic of Gamma-Ray Emission Imaging (GREI),**

where $E_\gamma$, $E_1$, $E_2$, $\theta$, and $m_c^2$ are source gamma-ray energy, scattered gamma-ray energy, absorbed gamma-ray energy, Compton scattering angle, and the rest-mass energy of an electron, respectively.
The characteristics of these different modalities are summarized in Table 1. Nuclear medical imaging modalities are the best molecular imaging modalities for clinical translation, because these modalities provide high sensitivity and allow for noninvasive visualization of molecules in the body, including areas deep within the body. Several different nuclear medical imaging probes have therefore been developed and applied in the diagnosis of various diseases including inflammatory bowel diseases (IBD) and cancer. Among them, fluorine-18-fluorodeoxyglucose ($^{18}$F-FDG) is a representative nuclear medical imaging probe for the detection of inflammation and cancer. However, since $^{18}$F-FDG PET in principle evaluates glucose metabolism in cells, it is difficult to elucidate the relationship between the diseases and the responsible molecules. Furthermore, although many pathological conditions are characterized by multiple and complex molecular interactions, it is difficult to analyze multiple molecular species using PET and SPECT. Therefore, the ultimate objective of the present study was to develop a method for acquiring the information on multiple molecular species for IBD and cancer diagnostics. Four stages were envisioned for realizing this objective: (1) Exploration of imaging targets or selection of the targets from previously reported pathogenic molecules, (2) Development of novel nuclear imaging probes for visualizing the target molecules, (3) Development of a novel nuclear imaging technique enabling
simultaneously visualizing multiple molecular species. Therefore, the studies in this thesis were aimed at exploring target molecules for nuclear medical imaging of IBD (Chapter 1), the development of novel imaging probes targeting cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), which is a target molecule for cancer immunotherapy (Chapter 2), and the development of a method for simultaneous imaging of multiple pathogenic molecular species (Chapter 3). Developing the methods for noninvasive evaluation of the expression of multiple pathogenic molecular species of IBD and cancer is expected to assist in developing more accurate evidence-based medical care strategies.

In this thesis, GREI was used for developing a novel nuclear imaging technique allowing to simultaneously visualize multiple molecular species (Chapter 3), because GREI is the only molecular imaging modality that can be potentially used for simultaneous and noninvasive visualization of multiple molecular species in different body parts, including areas deep within the body. On the other hand, from the viewpoint of sensitivity, quantitative capability, and spatial resolution, PET is the most suitable nuclear medical imaging modality. Therefore, GREI is not likely to be suitable for developing novel nuclear medical imaging probes, when compared with PET. In the study addressing the development of a novel nuclear medical imaging probe (Chapter 2),
PET (but not GREI) was used for evaluating the usefulness of a novel nuclear medical imaging probe.

**Table 1**

**Summary of the characteristics of molecular imaging modalities**

<table>
<thead>
<tr>
<th>Imaging modality</th>
<th>Major advantages</th>
<th>Major disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PET</td>
<td>High sensitivity</td>
<td>High cost</td>
</tr>
<tr>
<td></td>
<td>Accurate quantification</td>
<td>Multiple radioisotopes cannot be detected simultaneously</td>
</tr>
<tr>
<td></td>
<td>Unlimited depth penetration</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clinical translation</td>
<td></td>
</tr>
<tr>
<td>SPECT</td>
<td>Unlimited depth penetration</td>
<td>Relatively low spatial resolution of clinical devices</td>
</tr>
<tr>
<td></td>
<td>Clinical translation</td>
<td>Limited detectable gamma-ray energy (Less than 300 keV)</td>
</tr>
<tr>
<td></td>
<td>Multiple radioisotopes can be detected simultaneously</td>
<td></td>
</tr>
<tr>
<td>Optical imaging</td>
<td>High sensitivity</td>
<td>Low depth penetration</td>
</tr>
<tr>
<td></td>
<td>Low cost</td>
<td>Limited clinical translation</td>
</tr>
<tr>
<td></td>
<td>High throughput</td>
<td>Relatively low spatial resolution</td>
</tr>
<tr>
<td>MRI</td>
<td>High spatial resolution</td>
<td>Relatively low sensitivity</td>
</tr>
<tr>
<td></td>
<td>Clinical translation</td>
<td>High cost</td>
</tr>
<tr>
<td>GREI</td>
<td>Multiple radioisotopes can be detected simultaneously</td>
<td>Relatively low spatial resolution</td>
</tr>
<tr>
<td></td>
<td>Wide detectable gamma-ray energy (200-2000 keV)</td>
<td>High cost</td>
</tr>
<tr>
<td></td>
<td>High energy resolution</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unlimited depth penetration</td>
<td></td>
</tr>
</tbody>
</table>
Reference


7. RIKEN NEWS No. 390 December 2013: 2-5
Chapter 1

Exploration of target molecules for nuclear medical imaging of inflammatory bowel disease

1.1 Abstract

Nuclear medical imaging technology is a powerful tool for the diagnosis of inflammatory bowel disease (IBD) and the efficacy evaluation of various drug therapies for it. However, it is difficult to elucidate directly the relationships between the responsible molecules and IBD using existing probes. Therefore, the development of an alternative probe that is able to elucidate the pathogenic mechanism and provide information on the appropriate guidelines for treatment is earnestly awaited. Thus, the present study aimed at identifying the inflammation-related molecules, the expression levels of which are altered early during inflammation and depend on the extent of inflammation.

First, the accumulation of fluorine-18 fluorodeoxyglucose ($^{18}$F-FDG) in the intestines of dextran sulfate sodium (DSS)- or indomethacin (IND)-induced IBD model mice was measured by positron emission tomography (PET) and autoradiography to presume the
inflamed area in the intestine. Second, the gene expression levels of cytokines and cytokine receptors, including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, S100 calcium binding protein A8 (S100A8), IL-6 receptor-α (IL-6Rα), IL-1R type 1, toll-like receptor (TLR)-4, in the inflamed area of the intestines of both model mice were evaluated by reverse transcription polymerase chain reaction (RT-PCR).

The results of PET and autoradiography suggested the possibility that the inflammation was induced particularly in the colons of mice by the administration of DSS, whereas it was induced mainly in the ilea and the proximal colons of mice by the administration of IND. RT-PCR analysis revealed that the expression levels of IL-6, IL-1β, and S100A8 were significantly elevated during the progression of IBD, whereas the expression levels decreased as the mucosa began to heal. In addition, the expression levels of these molecules were altered before the IBD symptoms appeared. These results suggest that these molecules participate in the pathogenesis of IBD and are promising target molecules for IBD imaging. Finally, the development of an imaging probe for these target molecules is expected to improve our understanding of the inflammatory conditions of IBD.
1.2 Introduction

Inflammatory bowel disease (IBD) is an inflammatory disorder of the intestinal tract that is most commonly found in developed countries, affecting the quality of life. Nuclear medical imaging technology is a valuable tool for the noninvasive evaluation and monitoring of pathological conditions including IBD. In particular, radiolabeled autologous leukocytes and fluorine-18 fluorodeoxyglucose ($^{18}$F-FDG) are widely used as radiopharmaceutical agents for IBD diagnosis [1,2]. Radiolabeled autologous leukocytes are considered to be the gold standard for imaging IBD. This imaging technology has also shown high sensitivity and specificity for the precise detection of the involved bowel segment [3-6]. However, imaging with radiolabeled autologous leukocytes is time-consuming and requires a dedicated laboratory and personnel for handling blood products. On the other hand, $^{18}$F-FDG positron emission tomography (PET) has become an increasingly important methodology in clinical oncology because of its ability to image increased glucose uptake by tumor cells [7,8]. Nevertheless, several studies have shown that increased glucose metabolism is not restricted to malignant cells. $^{18}$F-FDG is also able to visualize inflammatory lesions on the basis of increased glucose uptake by inflammation cells and is thus useful for the diagnosis of
various inflammatory diseases [9-11].

On the other hand, inflammation imaging using these probes cannot elucidate the causative molecules. The identification of molecules contributing to the pathogenesis may be useful for grasping more detailed inflammation conditions based on disease-related molecules, and/or potentiate the medical care of expensive biological therapy including molecular-targeted drugs. However, no nuclear medical imaging probes targeting inflammation-related molecules have been developed to an extent satisfactory for clinical diagnosis of IBD. Therefore, in this study I explored potential target molecules for the nuclear medical imaging of IBD. The candidate molecules for IBD imaging must be inflammation-related, and the expression of these molecules must be altered during the progression and healing of IBD. Moreover, IBD is characterized by exacerbations and remission, and the risk of inflammation-related cancer increases with increasing IBD duration (particularly in ulcerative colitis) [12]. Therefore, IBD should be treated as soon as possible for preventing the development of inflammation-related cancer. The suitable candidate molecules for IBD imaging are the disease-related molecules, the expression of which is altered early during the inflammation. Thus, the present study aimed at determining the inflammation-related molecules, the expression levels of which are altered early during the inflammation and
depend on the extent of inflammation.

Cytokines are key instigators and regulators of immune responses. They are thought to be major contributors to various inflammatory diseases [13-20]. To date, more than 90 cytokines have been identified, nine of which are the basis for current therapeutics on the market [21]. In this study, the cytokines and cytokine receptors, including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, S100 calcium-binding protein A8 (S100A8), IL-6 receptor-α (IL-6Rα), IL-1R type 1, and Toll-like receptor (TLR)-4, which are target molecules of inflammatory diseases, were focused as candidate molecules for IBD imaging.

In this study, DSS- or IND-treated mice were used as animal models of IBD. DSS is a polyanionic derivative of dextran esterified with chlorosulfonic acid [22]. It has been reported that DSS is directly toxic to gut epithelial cells of basal crypts, resulting in loss of the integrity of the mucosal barrier [23]. The DSS model is one of the most widely used rodent models of IBD (particularly in ulcerative colitis). The DSS model is similar to human IBD in terms of etiology, pathology, pathogenesis, and therapeutic response.

In this experiment, the concentration of DSS solution was 5.0% because the symptoms (body weight change, rectal bleeding, and diarrhea) were induced in all BALB/c mice and all mice survived by administering 5.0% DSS in drinking water for 7 days. On the
other hand, non-steroidal anti-inflammatory drugs, including IND, are known to induce bowel inflammation resembling Crohn's disease. The gastrointestinal injury was caused by suppression of mucosal prostaglandin production via inhibition of cyclooxygenase activity [24,25]. PET imaging and autoradiography were conducted using $^{18}$F-FDG to presume the inflamed areas in the intestines. Furthermore, time-dependent changes in cytokine and cytokine receptor expression levels in the intestines were analyzed and compared with the observed symptoms and histological findings.
1.3 Materials and Methods

Preparation of IBD model mice

Female BALB/c mice (6 weeks old) were purchased from CLEA Japan Inc. The mice were maintained at 25°C and 55% humidity with free access to standard chow and water. Dextran sulfate sodium (DSS)-treated mice were prepared by the administration of 5.0% DSS (mol wt 36,000–50,000, MP Biomedicals Co., Ltd.) in drinking water for one to seven days. Indomethacin (IND)-treated mice were prepared by the administration of IND (Sigma-Aldrich Co., Ltd.) suspended in Ringer's solution (Otsuka Pharmaceutical Co., Ltd.) with a drop of polysorbate 80 (Alfa Aesar Co., Ltd.) at the dose of 10 mg/kg. Investigations were initiated after receiving approval from the committee on animal experiments of Okayama University.

Assessment of symptoms of colitis

Daily changes in body weight and symptoms, such as rectal bleeding and diarrhea, of DSS-treated IBD model mice were classified by laboratory examination and scored
from 1 to 4, as mentioned below.

Bleeding Score: 1, normal; 2, commingling of blood in stool; 3, adhesion of blood to mouse anus; 4, constant effluent blood at mouse anus. Diarrhea Score: 1, normal; 2, loose corporeal stool; 3, loose incorporeal stool; score 4, watery diarrhea.

\textit{\textsuperscript{18}F-FDG PET and autoradiography}

\textsuperscript{18}F-FDG was provided by the Institute of Biomedical Research and Innovation Hospital, Kobe, Japan. After administering IND for one day or feeding 5.0\% DSS in drinking water for seven days, \textsuperscript{18}F-FDG uptake in both model mice was measured by using a small-animal PET scanner (microPET Focus220; Siemens Medical Solutions Inc.). \textsuperscript{18}F-FDG at an activity of 5 MBq was injected to each mouse via the tail vein while the animal was conscious. Twenty min after the injection, the mouse was anesthetized with 1.5\% isoflurane gas and placed in the prone position. Forty min after the injection, emission data were acquired for 10 min. The acquired data were summed into sinograms and three-dimensional images were reconstructed by maximum a posteriori and the filtered back-projection method using a ramp filter with cutoff at the Nyquist frequency with attenuation correction.
Fifty-five min after the injection, the mouse was deeply anesthetized with diethyl ether and then euthanized. The whole intestine was rapidly removed and opened along the longitudinal axis. After washing with saline, the intestine was stretched and contacted with an imaging plate. The plate was scanned with a Typhoon™ FLA 7000 biomolecular imager (GE Healthcare Life Science, Co., Ltd.).

**Histological assessment of IBD**

For the histological assessment of IBD, samples of distal colonic (DSS-treated mice) or ileal tissue (IND-treated mice) were resected. Then, the tissue samples were embedded in Optimal Cutting Temperature compound (Sakura Finetek Japan Co., Ltd.) and frozen with dry ice. Ten-µm-thick tissue sections were prepared and mounted on APS-coated slide glasses (Matsunami Glass Ind., Co., Ltd.). The tissue sections were fixed with 4% paraformaldehyde/PBS and then stained with hematoxylin and eosin (H&E). Changes in histological morphology were observed with an inverted microscope (Nicon TE-2000-U Microscope; Nikon Co., Ltd.). For each group, the assessments were conducted at least three times.
Reverse transcription polymerase chain reaction (RT-PCR) analysis of cytokines and cytokine receptors

Total RNA was isolated from distal colonic or ileal tissues using a PureLink™ RNA Mini Kit (Invitrogen Co., Ltd.). One microgram of total RNA was used as the template for single-strand cDNA synthesis with a Transcriptor First Strand cDNA Synthesis Kit (Roche Co., Ltd.). Analysis of IL-6, IL-1β, TNF-α, S100A8, IL-6Ra, IL-1R type 1, TLR-4, and GAPDH mRNA expression levels was carried out with RT-PCR using TaKaRa Ex Taq (TaKaRa Co., Ltd.). The amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as internal control. Primers are listed in Table 2. PCR products were analyzed on agarose gels. Fluorescence intensities of the PCR products were quantitated with NIH ImageJ software. mRNA expression data were normalized to GAPDH mRNA expression and each bar represents fold increase (means ± standard deviation (SD)).
Table 2

List of primer sequences for reverse transcription polymerase chain reaction

(RT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>atggatgcctaccaaaactgga</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ggaattggggttaggaagga</td>
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<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>ggtgtgtgacgttcccattag</td>
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<td></td>
<td>Reverse</td>
<td>tcgtgcttggttctctctg</td>
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<tr>
<td>S100A8</td>
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Statistical analysis

Parametric data are presented as means ± SD. Statistical significance was determined using the Student’s t-test, Bonferroni’s test, or Dunn’s test.
1.4 Results

Preparation of IBD model mice and assessment of symptoms

DSS-treated mice were examined for daily changes compared with normal mice. As shown in Fig. 4A, significant body weight loss was observed from days 5 to 7. Rectal bleeding significantly increased from days 6 to 7 (Fig. 4B), while diarrhea scores increased from days 5 to 7 (Fig. 4C).
Figure 4

Assessment of symptoms of dextran sulfate sodium (DSS)-treated inflammatory bowel disease (IBD) model mice.

A. Body weight changes following DSS induction of colitis. B. Rectal bleeding scores. C. Diarrhea scores. Data are expressed as means ± standard deviation (SD). For statistical evaluation of A, the Student’s t-test was applied. For statistical evaluation of B and C, Dunn’s test was applied. Symbols ** and *** denote $P < 0.01$ and $P < 0.001$ vs Day 0, respectively (n = 7-9).
Small-animal $^{18}$F-FDG PET and autoradiography studies of intestinal inflammation

Small animal $^{18}$F-FDG PET was performed on normal and DSS- and IND-treated mice (Fig. 5) to presume the inflamed areas of the intestines in DSS- and IND-treated mice. Maximum intensity projection (MIP) images are shown in Fig. 5A. Accumulation of $^{18}$F-FDG in the abdominal regions of DSS- and IND-treated mice was successfully visualized. In addition, high uptake of $^{18}$F-FDG was likely observed in the brain, brown adipose tissue, heart, muscles, bones, and bladder. For more detailed evaluation, transverse, sagittal, and coronal $^{18}$F-FDG PET images of normal, DSS-treated, and IND-treated mice are also shown in Fig. 5. High $^{18}$F-FDG accumulation was observed in the intestines of DSS- and IND-treated mice compared with those of normal mice (Figs. 5B and 5C). However, the regions of $^{18}$F-FDG accumulation in the intestines of DSS-treated mice were different from those of IND-treated mice.
Figure 5

Positron emission tomography (PET) studies of DSS- and indomethacin (IND)-treated model mice using fluorine-18 fluorodeoxyglucose (\(^{18}\text{F}-\text{FDG}\)).

A. Maximum intensity projection (MIP) rotation images. Arrows indicate the characteristic accumulation of \(^{18}\text{F}-\text{FDG}\) (n = normal mouse, d = DSS-treated mouse, and i = IND-treated mouse) (n = 2). B. Abdominal PET images (transverse, sagittal, and coronal images) of normal mouse (left) and DSS-treated mouse (right) (n = 2). C. Abdominal PET images of normal mouse (left) and IND-treated mouse (right) (n = 2). Arrows indicate the characteristic accumulation of \(^{18}\text{F}-\text{FDG}\) in the abdominal regions. Bln, BAT, M, Bo, Bld and H are brain, brown adipose tissue, muscle, bone, bladder, and heart, respectively.
Then, autoradiography was carried out to presume the region of $^{18}$F-FDG accumulation in the intestine (Fig. 6). A low distribution of $^{18}$F-FDG was observed throughout the intestines of normal mice (Fig. 6A). However, in DSS-treated mice, $^{18}$F-FDG uptake was focally increased in the colons (Fig. 6B). On the other hand, $^{18}$F-FDG accumulation was increased mainly in the ilea and proximal colons of IND-treated mice (Fig. 6C). These high uptakes of $^{18}$F-FDG were not due to the accumulations in stool and blood, because the intestine of each mouse was sufficiently washed with saline before the autoradiography measurement.
Figure 6

 Autoradiography studies of DSS- and IND-treated model mice using $^{18}$F-FDG.

A. Autoradiography of intestine of normal mouse (n = 2). B. Autoradiography of intestine of DSS-treated mouse (n = 2). C. Autoradiography of intestine of IND-treated mouse (n = 2). Yellow arrows indicate the characteristic accumulation of $^{18}$F-FDG.
Assessment of histological findings of intestines in DSS- and IND-treated mice

To evaluate histological changes in the intestines of DSS- and IND-treated mice, H&E staining of intestinal tissues was performed (Fig. 7). Colon tissues of normal and DSS-treated mice are shown in Fig. 4A. Normal colon showed good mucosal architecture (Fig. 7A, panel a). Compared with normal colon, atrophy of mucosa was observed in colon tissues one day after DSS administration (Fig. 7A, panel b). Loss of mucosal architecture was observed from day 4 (Fig. 7A, panel c). These damages became more extensive on day 7 (Fig. 7A, panel d).

H&E-stained ileal sections of IND-treated mice are shown in Fig. 7B. Normal ileum is shown in Fig. 7B panel a. Denuded epithelium and edema in the submucosa were observed on day 1 (Fig. 7B, panel b). Recovery of the damaged area was observed on day 4 (Fig. 7B, panel c).
Figure 7

Assessment of histological findings of intestines in DSS- and IND-treated mice.

A. Microscopic observations of DSS-treated colitis during inflammation progression in mice. (a) day 0 (normal colon), (b) day 1, (c) day 4, and (d) day 7. Representative hematoxylin and eosin (H&E)-stained colon sections are shown. B. Microscopic observations of IND-treated small-intestine ulceration during healing in mice. (a) day 0 (normal ileum), (b) day 1, and (c) day 4. Representative H&E-stained ileal sections are shown. Scale bar = 200 µm.
**Analysis of cytokine and cytokine receptor gene expression by RT-PCR**

Whether the mRNA expression levels of cytokines and cytokine receptors changed at the inflamed region was examined by RT-PCR (Fig. 8). The mRNA expression levels of cytokines, such as IL-6, IL-1β, S100A8, and TNF-α, showed a time-dependent increase in the colonic mucosa of mice that received DSS (Fig. 8A). Notably, TNF-α expression was increased from day 1. Compared with normal colon, the expression levels of cytokine receptors, such as IL-6Rα and IL-1R type 1, were also increased. However, these levels reached a plateau from day 1. In addition, TLR-4 expression levels were increased in a time-dependent manner.

The expression levels of cytokine and cytokine receptor genes in the ilea of IND-treated mice were also assessed by RT-PCR (Fig. 8B). The expression levels of IL-6, IL-1β, S100A8, IL-1R type 1, IL-6Rα, and TLR-4 in ileal tissue of IND-treated mouse were increased on day 1, in comparison with normal ileal tissue. In particular, the expression levels of IL-6, IL-1β, S100A8, and IL-1R type 1 were significantly increased compared to those in normal ileum, whereas the mRNA expression levels of IL-6, IL-1β, S100A8, and IL-1R type 1 were decreased on day 4. Although the expression levels of IL-6Rα and TLR-4 also tended to decrease on day 4, the decrease
was not statistically significant. No significant changes were found in the expression of TNF-α.
Figure 8

Analysis of cytokine and cytokine receptor gene expression by RT-PCR.

A. Time course of mRNA expression levels of cytokines and cytokine receptors in the colon treated with DSS. B. Time course of mRNA expression levels of cytokines and cytokine receptors in the ileum treated with IND. RT-PCR was performed for cytokines (IL-6, IL-1β, TNF-α, and S100A8) and cytokine receptors (IL-6Rα, IL-1R type 1, and TLR-4). Amplification of GAPDH is shown as internal control. Expression was normalized to that of GAPDH, and each bar represents means ± SD. For statistical evaluation, Bonferroni’s test was applied. Symbols *, **, and *** denote P < 0.05, P < 0.01, and P < 0.001 vs Day 0, respectively (n = 3). Symbols ## and ### denote P < 0.01 and P < 0.001 vs Day 4, respectively (n = 3).
1.5 Discussion

Body weight loss and inflammation-associated rectal bleeding are frequently examined to monitor IBD in DSS-treated mice [23, 26]. Thus, DSS-treated mice were examined daily for differences relative to normal mice. IBD symptoms occurred 5 days after DSS treatment. Symptoms of IND-treated mice were not scored, because the symptoms pertaining to the IND-treated IBD model were different from those pertaining to the DSS-treated IBD model and the evaluation method of the symptoms pertaining to the IND-treated model has not been established.

Several studies using $^{18}$F-FDG-PET have shown very promising results for imaging of different inflammation types, including IBD [10]. PET imaging with $^{18}$F-FDG was therefore performed to ascertain that intestinal inflammation was indeed induced in DSS- and IND-treated mice. These results indicate a possibility that inflammation was induced in the intestines of both DSS-treated and IND-treated mice but the inflamed regions of the DSS-treated mice were different from those of the IND-treated mice. On the other hand, strong accumulation of $^{18}$F-FDG was observed in the organs other than intestine. Although ex-vivo biodistribution analysis or autoradiography should be conducted for determining the regions of $^{18}$F-FDG accumulation, the
$^{18}$F-FDG-accumulating organs other than the intestine seemed to be the brain, the brown adipose tissue, the heart, the muscles, the bones, and the bladder. Because $^{18}$F-FDG acts like a glucose, the $^{18}$F-FDG properties enable physiological accumulation in the brain, brown adipose tissue, and muscles [27,28]. The accumulation of $^{18}$F-FDG in bladder is frequently observed because the bladder serves as the excretion pathway for $^{18}$F-FDG [28]. The accumulation in bones might be explained by elimination of osteotropic $^{18}$F from the body $^{18}$F-FDG. The autoradiography images obtained from these measurements also suggested that inflammation was induced particularly in the large intestines of mice after administering the DSS, whereas it was induced mainly in the ilea and proximal colons after administering the IND. However, these $^{18}$F-FDG-based evaluations were qualitative. For quantitative evaluations, the sample size (number of animals) has to be increased and quantification of radioactivity accumulation of $^{18}$F-FDG in the intestines should be performed by using a gamma counter or a Ge detector.

Atrophy of mucosa and loss of mucosal architecture have been observed in colon tissues after DSS administration [22]. In this study, similar histological changes were also observed in DSS-treated mice and the colitis became advanced in a time-dependent manner. In addition, Yamato et al. observed a denuded epithelium and severe edema in
the submucosa on day 1 in IND-treated rats, and the ulcer healed as time advanced [29]. In this study, severe intestinal ulcers in IND-treated mice were observed on day 1 and healing was observed on day 4.

Time-dependent changes in cytokine and cytokine receptor expression levels in the inflamed areas of the intestines were analyzed by RT-PCR. The goal of this experiment was to determine cytokines and cytokine receptors, the expression levels of which are altered early during inflammation and depend on the extent of inflammation. RT-PCR analysis indicated that the expression levels of cytokines and cytokine receptors, such as IL-6, IL-1β, S100A8, TNF-α, and TLR-4, increased significantly during the progression of bowel inflammation in the DSS-treated mice. The upregulation of these molecules (particularly TNF-α and TLR-4) appeared earlier than the symptoms of IBD, including body weight changes and rectal bleeding. These results suggested that these cytokines or cytokine receptors, such as IL-6, IL-1β, S100A8, TNF-α, and TLR-4, participate in the pathogenesis of IBD at an early stage, and that evaluating the expression of these molecules may detect weak inflammation before the onset of IBD symptoms. Furthermore, the expression levels of cytokine and cytokine receptors (particularly IL-6, IL-1β, S100A8, and IL-1R type 1) in inflamed ileal tissue of IND-treated mice were significantly higher than those in normal ileal tissue, whereas the expression levels of
these molecules decreased as the mucosa began to heal. Considering the histological findings (Fig. 4B), in the IND-treated mice, these molecular expression levels depended on the degree of inflammation. From the results of RT-PCR analysis of DSS and IND-treated mice, IL-6, IL-1β, and S100A8 were identified as particularly promising target molecules for IBD imaging because the expression levels of these molecules are altered early during inflammation and depend on the extent of inflammation. However, both DSS- and IND-treated mice used in these experiments were acute bowel inflammation models. On the other hand, human IBD is actually a chronic inflammatory disease. In the future experiments, it will be necessary to examine whether IL-6, IL-1β, and S100A8 are promising target molecules for IBD imaging by using chronic-stage IBD models. In addition, the altered expression of these molecules has been observed only on a genetic level. Hereafter, it will be necessary to develop nuclear medical imaging probes targeting the molecules described above, and to investigate whether the probes can be used for visualizing the target molecules in IBD models and patients.

On the other hand, TNF-α expression was increased in the DSS-induced IBD model mice at an early date (from day 1) while no significant changes were found in the IND-treated mice. These results suggested that not all cytokines and cytokine receptors are fundamental to the pathogenesis of the inflammatory disease, and each molecule
exhibits a different expression pattern during IBD progression depending on the pathogenic mechanism or the region of inflammation. Hence, these molecules may show potential for use as biomarkers for the detection of the different conditions underlying inflammation. However, this reasoning contains a weak point: IBD imaging targeting cytokines and/or cytokine receptors can under-detect the inflammation if the probe is targeting the molecules that are not fundamental to the pathogenesis. To avoid such under-detection of IBD, multiple molecular species should be imaged, rather than single molecules. Therefore, diagnosis of multiple molecular species using nuclear medical imaging modalities (such as Gamma-Ray Emission Imaging (GREI)) would be useful for determining the disease-related molecules that are fundamental to the pathogenesis.

In conclusion, I examined alterations in the expression of pathognomonic molecules in the inflamed regions of the mouse intestines and found that IL-6, IL-1β, and S100A8 were particularly promising molecules for IBD imaging because the expression levels of these molecules are altered early during the inflammation and depend on the extent of inflammation. Hence, utilization of nuclear medical imaging probes targeting these inflammation-related molecules would be helpful for grasping in greater detail the inflammatory conditions of IBD early during its development, which would eventually
lead to evidence-based therapy.
1.6 References


Chapter 2
Development of a novel nuclear imaging probe for visualization of cytotoxic T lymphocyte-associated antigen-4 in the tumor

2.1 Abstract

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) targeted therapy by anti-CTLA-4 monoclonal antibody (mAb) is highly effective in cancer patients. However, it is extremely expensive and potentially produces autoimmune-related adverse effects. Therefore, the development of a method to evaluate CTLA-4 expression prior to CTLA-4-targeted therapy is expected to open doors to evidence-based and cost-efficient medical care and to avoid adverse effects brought about by ineffective therapy. Thus, this study aimed to develop a nuclear medical imaging probe for CTLA-4 visualization in tumor. First, the expression levels of CTLA-4 in CT26 cells, in normal colon tissues, and in CT26 tumor xenografts obtained from BALB/c mice and BALB/c nude mice were examined by reverse transcription polymerase chain reaction (RT-PCR) analysis. Second,
$^{64}$Cu-1,4,7,10-tetraazacyclododecane-$N,N',N'',N'''$-tetraacetic acid-anti-mouse CTLA-4 mAb ($^{64}$Cu-DOTA-anti-CTLA-4 mAb) was newly synthesized and the usefulness of it was evaluated by positron emission tomography (PET) and ex-vivo biodistribution analysis in CT26-bearing BALB/c mice.

High CTLA-4 expression was confirmed in the CT26 tumor tissues of tumor-bearing BALB/c mice. However, CTLA-4 expression was extremely low in the cultured CT26 cells and the CT26 tumor tissues of tumor-bearing BALB/c nude mice. The results suggested that T cells were responsible for the high CTLA-4 expression. Furthermore, PET experiments suggested that although $^{64}$Cu-DOTA-Control IgG (isotype control) accumulated slightly in the CT26 tumor tissues likely owing to EPR effects, the accumulation of $^{64}$Cu-DOTA-anti-CTLA-4 mAb was more pronounced. In addition, $^{64}$Cu-DOTA-anti-CTLA-4 mAb exhibited significantly higher radioactivity accumulation in the CT26 tumor as well as significantly higher tumor-to-blood and tumor-to-muscle ratios, compared with $^{64}$Cu-DOTA-Control IgG. These results suggested that $^{64}$Cu-DOTA-anti-CTLA-4 mAb is useful for evaluating CTLA-4 expression in the tumor.
2.2 Introduction

Cancer is a complex mixture of host and tumor cells. Whereas the human body has the ability to produce an anti-tumor immune response, cancers develop multiple strategies to evade the host immune system [1]. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), also known as cluster of differentiation 152 (CD152), is one of the most important molecules that are involved in the downregulation of the immune system and the anti-tumor response. CTLA-4 is expressed predominantly on the surface of two major subsets of CD4\(^+\) T cells: regulatory T cells (Tregs) and activated CD4\(^+\) effector cells, and activated CD8\(^+\) effector T cells [2, 3]. In addition, recent research showed that various tumor cells also express CTLA-4 [4].

CTLA-4 targeted therapy augments endogenous response to tumor cells, thereby leading to tumor cell death when utilized on its own or with other therapeutic interventions [3]. It is for this reason that CTLA-4 has attracted attention as a target molecule for cancer immunotherapy [5]. Fully human anti-CTLA-4 monoclonal antibodies (mAbs), ipilimumab and tremelimumab, were developed for the treatment of cancer patients. Ipilimumab is the first drug to demonstrate survival benefits in metastatic melanoma patients, and was approved by the US Food and Drug
Administration for the treatment of advanced melanoma in 2011. Pre-clinical and clinical trials of anti-CTLA-4 mAbs have been conducted for the treatment of other cancers, including colon, breast, lung, ovarian, and prostate cancers [3, 6].

Although CTLA-4-targeted therapy is an attractive method for the treatment of various cancers, the therapy is beset by several problems. First, the enhanced T cell response by the CTLA-4 blockade frequently produces autoimmune-related adverse effects, such as rash, diarrhea, colitis, hepatitis, and hypophysitis [7, 8]. Furthermore, a super-agonist antibody for CD28 (TGN1412), which directly stimulates T cells, caused life-threatening inflammatory reactions in a London clinical trial [9]. Extreme precaution must be taken when CTLA-4-targeted antibodies are used for the treatment because CTLA-4 is an antagonist of CD28–ligand interactions [10]. Second, antibody drugs are extremely expensive. One treatment course of ipilimumab in the United States consists of four doses at US$30,000 per dose [2, 11]. Clearly, there is an urgent need to develop a method to screen patients for sensitivity to the CTLA-4-targeted therapy, to eliminate adverse effects brought about by ineffective therapy and reduce unnecessary financial burden in non-sensitive patients. The identification of CTLA-4 expression in tumor prior to molecular-targeted therapy would lead to evidence-based and cost-efficient medical care.
Biopsy is principally conducted to evaluate the expression of molecules of interest. However, it is an invasive and stressful procedure. Moreover, biopsy evaluates the expression of target molecules only in a localized region of the tumor. Thus, it is difficult to acquire information of a patient’s sensitivity to a molecular-targeted drug for tumors existing in whole body.

Molecular imaging can provide molecular information of the whole body in a noninvasive manner and be used for the determination of sensitivity to antibody drugs. Tumor imaging probes for human epidermal growth factor receptor 2 (HER2) [12-14], epidermal growth factor receptor (EGFR) [15-18], and vascular endothelial growth factor (VEGF) [19, 20], which are the target molecules of trastuzumab, cetuximab/panitumumab, and bevacizumab, respectively, have been developed. The expression of those molecules in tumor was detected with their respective probes by positron emission tomography (PET) or single-photon emission computed tomography (SPECT). However, a molecular imaging probe that targets CTLA-4 has yet to be developed. Therefore, this study aimed to develop a molecular imaging probe for CTLA-4 visualization in tumor.

There are four functionally different variants of CTLA-4 (Fig. 9) [21-26]. In this study, first, the expression and variant of CTLA-4 was examined in CT26 tumor tissues and
cultured CT26 cells by reverse transcription polymerase chain reaction (RT-PCR) analysis.

![Diagram of CTLA-4 variants](image.png)

**Figure 9**

**Structures of CTLA-4 variants.**

The CTLA-4 gene consists of four exons. The leader peptide corresponds to exon 1 in the CTLA-4 gene, the ligand-binding domain to exon 2, the transmembrane region to exon 3, and the cytoplasmic tail to exon 4. There are four different variants of the CTLA-4 protein: full-length CTLA-4 (flCTLA-4) (exons 1, 2, 3, and 4), soluble CTLA-4 (sCTLA-4) (exons 1, 2, and 4), ligand independent CTLA-4 (liCTLA-4) (exons 1, 3, and 4), and a variant consisting of exons 1 and 4 only (1/4CTLA-4).
Second, $^{64}$Cu-1,4,7,10-tetraazacyclododecane-$N,N',N'',N'''$-tetraacetic acid (DOTA)-anti-mouse CTLA-4 mAb was newly developed by introducing DOTA groups to anti-mouse CTLA-4 mAb and subsequent radiolabeling with $^{64}$Cu. The utility of $^{64}$Cu-DOTA-anti-CTLA-4 mAb as an imaging probe was assessed by PET imaging and ex-vivo biodistribution analysis. In this study, tumor-bearing mice was prepared by syngeneic implantation of CT26 cells (mouse colon tumor cell line) to BALB/c mice. Immune-deprived mice bearing human tumor cell lines were not used because T cells might be responsible for the CTLA-4 expression in the tumor tissues.
2.3 Materials and Methods

Cell culture

CT26 was purchased from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 10 U/mL penicillin, and 10 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Preparation of subcutaneous tumor model mice

Female BALB/c and BALB/c (nu/nu) nude mice (4-6 weeks old) were purchased from CLEA Japan Inc. Tumor-bearing BALB/c and BALB/c nude mice were prepared by subcutaneously implanting CT26 cells (1-4 × 10⁶ cells). Investigations were initiated after receiving approval from the committee on animal experiments of Okayama University.
**RT-PCR analysis**

RNA extraction and cDNA synthesis were conducted by using the same methods as my previous report [27]. Total RNA was isolated from cultured cells and tissues with TRIZOL® reagent (Life Technologies Co., Ltd.) and a PureLink™ RNA Mini Kit (Life Technologies Co., Ltd.). One microgram of total RNA was used as the template for single-strand cDNA synthesis with a Transcriptor First Strand cDNA Synthesis Kit (Roche Co., Ltd.). Analysis of mRNA expression levels was carried out with RT-PCR using TaKaRa Ex Taq (TaKaRa Co., Ltd.). The amplification of β-actin is shown as internal control. Primer sequences are listed in Table 3. The amplicons were separated on agarose gel (AGAROSE™, Amresco, Inc.), stained with ethidium bromide, and visualized with a Benchtop 2UV Transilluminator (UVP, Inc.).
Table 3

List of primer sequences for reverse transcription polymerase chain reaction (RT-PCR)

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**64 Cu-DOTA-anti-CTLA-4 mAb production**

Anti-mouse CTLA-4 mAb (200–500 µg) (R&D Systems, Inc.) was conjugated to DOTA-mono-N-hydroxysuccinimide ester (DOTA-mono-NHS ester; Macrocyclics, Inc.) in phosphate-buffered saline without calcium and magnesium (pH 7.5) (PBS (-)), by using a 100-fold molar excess of DOTA-mono-NHS ester. The mixture was stirred at room temperature (RT) for three hours to give the DOTA-anti-CTLA-4 antibody. The DOTA-anti-CTLA-4 antibody was purified with a PD-10 column (GE Healthcare Co., Ltd.) and an Amicon-Ultra 50K device (Millipore Co., Ltd.). The DOTA-anti-CTLA-4 antibody was analyzed by size-exclusion high-performance liquid chromatography (HPLC) using TSK-GEL Super SW3000 (Tosoh Co., Ltd.). The mobile phase of 10 mM PBS (-) containing 0.3 M NaCl was used and the flow rate was 0.35 mL/min.

64Cu was produced by irradiating a 99.6% 64Ni-enriched nickel target with 12 MeV protons using a cyclotron (CYPRIS-HM12, Sumitomo Heavy Industries, Ltd.). Then, 64Cu was purified with a Muromac column (Muromachi Technos Co., Ltd.). The buffer solution of DOTA-anti-CTLA-4 mAb was replaced with 0.1 M acetate buffer (pH 6.5) three times by using an Amicon-Ultra 50K device (Millipore Co., Ltd.). DOTA-anti-CTLA-4 mAb was radiolabeled with 64Cu by incubating at 40 °C for one
hour. To remove excess $^{64}$Cu, the buffer was replaced with 0.2 M glycine buffer by using the Amicon-Ultra 50K device. Buffer of the purified antibody solution was replaced with PBS (-) by using the Amicon-Ultra 50K device. The resultant solution was used for injection.

The radiochemical purity of $^{64}$Cu-DOTA-antibodies in PBS (-) was confirmed by reversed phase radio-thin layer chromatography (TLC). This analysis was performed with a TLC aluminum sheet, RP-18 F254 S (Merck Chemicals Co., Ltd.) and methanol: water: acetic acid (4:1:1) was used as the mobile phase. TLC chromatograms were obtained by autoradiography (FLA-7000IR; GE Healthcare Co., Ltd.). $^{64}$Cu-DOTA-isotype IgG$_{2A}$ ($^{64}$Cu-DOTA-Control IgG) was produced in the same way as that for negative control by using rat IgG$_{2A}$ isotype control (R&D Systems, Inc.).

**Assay for CTLA-4 binding activity**

The CTLA-4 binding activity of DOTA-anti-CTLA-4 mAb and DOTA-Control IgG was examined by enzyme-linked immunosorbent assay (ELISA) and compared with that of original anti-CTLA-4 mAb and DOTA-Control IgG. Twenty ng of recombinant mouse CTLA-4 (R&D Systems, Inc.) in 50 mM carbonate buffer (pH 9.6) per well was
added into a 96-well ELISA plate (R&D Systems, Inc.). After blocking with 3% bovine serum albumin (BSA) and 1% Tween 20 in PBS (-) containing 0.05% Tween 20, 5 ng of the antibodies in PBS (-) containing 1% BSA and 0.05% Tween 20 was added to each well and incubated for one hour. After incubation, each well was treated with 50 uL of HRP-conjugated anti-rat IgG (R&D Systems, Inc.) diluted 1:6000 with PBS (-) containing 1% BSA and 0.05% Tween 20. Peroxidase activity was visualized with a TMB Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories, Inc.) and the absorbance at 450 nm was measured. The absorbance was corrected by performing a blank trial. The corrected absorbance values of DOTA-anti-CTLA-4 mAb and DOTA-Control IgG were respectively divided by the absorbance of anti-CTLA-4 mAb, and relative immunoreactivities were calculated. The experiment was repeated, and each bar represents the mean value.

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS was conducted to determine the extent of DOTA conjugation to antibodies using a method similar to that reported by Lu et al. [28]. MALDI-TOF-MS
was performed by using an Ultraflex III MALDI TOF/TOF (Bruker Daltonics Co., Ltd.). Non- and DOTA-conjugated antibodies were desalted with PD Spin Trap G-25 (GE Healthcare Co., Ltd.). Sinapinic acid (Nacalai Tesque, Inc.) at 20 mg/mL in 2:1 acetonitrile/H$_2$O with 0.1% trifluoroacetic acid (Wako Pure Chemical Industries, Co., Ltd.) was used as the MALDI matrix.

**PET imaging study**

$^{64}$Cu-DOTA-anti-CTLA-4 mAb (4 µg, approximately 16 MBq) or $^{64}$Cu-DOTA-Control IgG (4 µg, approximately 14 MBq) was intravenously administered to CT26-bearing BALB/c mice via the tail vein. Forty-eight hours after administration of the radiolabeled antibodies, probe uptake in the CT26-bearing mice was measured with a small-animal PET scanner (microPET Focus220; Siemens Medical Solutions Inc.). During PET imaging, the mice were anesthetized with 1.5% isoflurane and 1.5% N$_2$O gas, and placed in the prone position. Emission data were acquired for 60 min. The image intensity was expressed by standardized uptake value (SUV). SUV$_{\text{max}}$ was calculated by ASIPRO software package (Concorde Microsystems, Inc.).
**Biodistribution study**

Forty-eight hours after administration of $^{64}$Cu-DOTA-anti-CTLA-4 mAb (4 µg, 1 MBq) or $^{64}$Cu-DOTA-Control IgG (4 µg, 1 MBq), the animals were immediately sacrificed and the organs and blood were removed. The organs and blood were weighed and radioactivity was measured using a gamma counter (ARC-7001B, ALOKA Co., Ltd.). Decay-corrected uptake was expressed as the percentage of injected dose per gram (%ID/g) and calculated as the ratio to blood or muscle for comparison of the accumulation abilities in the CT26 tumor between $^{64}$Cu-DOTA-anti-CTLA-4 antibody and $^{64}$Cu-DOTA-Control IgG.

**Immunohistological staining**

Tumor-bearing BALB/c mice were sacrificed and CT26 tumor tissues including the normal tissues around them were resected and embedded in Optimal Cutting Temperature compound (Sakura Finetek Japan Co., Ltd.). Ten-µm-thick frozen tissue sections were prepared and mounted on MAS-coated glass slides (Matsunami Glass Ind.,
Co., Ltd.). The tissue sections were fixed with 4% paraformaldehyde in PBS (-), blocked with 5% goat serum in PBS (-), and incubated with anti-CTLA-4 antibody (R&D Systems, Inc.). Then, the tissue sections were subjected to endogenous peroxidase inactivation with 0.19% H₂O₂/methanol (Wako Pure Chemical Industries, Ltd.), followed by incubation with horseradish peroxidase conjugated anti-rat IgG antibody (R&D Systems, Inc.). Immunocomplexes were visualized with a DAB substrate kit (Dako Co., Ltd.).

**Statistical analysis**

SUV<sub>max</sub> data are expressed as means ± standard deviation (SD) and other data are expressed as means ± standard error of mean (SEM). Statistical significance was determined using the Student’s t-test. The tests were performed using GraphPad Prism software (GraphPad Software, Inc.).
2.4 Results

*CTLA-4 expression in CT26 tumor tissues and cultured CT26 cells.*

First, RT-PCR was carried out to examine CTLA-4 expression in CT26 tumor tissues and cultured CT26 cells, and the results were compared to those obtained with normal colon tissues (Fig. 10). CTLA-4 (amplicon length: 920 base pairs [bp]) was strongly expressed in CT26 tumor tissues compared with normal colon tissues. On the other hand, CTLA-4 expression was extremely low in cultured CT26 cells. Moreover, Treg markers, such as forkhead box P3 (Foxp3) and folate receptor 4 (FR4) [29, 30], were more strongly expressed in the tumor tissues than the normal colon tissues and the cultured CT26 cells. The expression of CD25 and CD69, which are molecules expressed on regulatory and activated T cells [29-31], was also increased in the tumor tissues compared to the normal colon tissues and the cultured CT26 cells. CD154, which is induced on T cells by T cell activation [32], was also more strongly expressed in the tumor tissues than the normal colon tissues and the cultured CT26 cells.
<table>
<thead>
<tr>
<th></th>
<th>Tissues</th>
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<tr>
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**Figure 10**

RT-PCR analysis in normal colon tissues, CT26 tumor tissues, and cultured CT26 cells.

Expression of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), regulatory T cell (Treg) markers, and T cell activation markers in normal colon tissues, CT26 tumor tissues, and cultured CT26 cells.
**CTLA-4 and T cell marker expression in CT26 tumor tissues from tumor-bearing BALB/c nude mice.**

From the results of Fig. 10, I assumed that T cells were involved in CTLA-4 expression in the CT26 tumor tissues from the tumor-bearing BALB/c mice, and CT26 tumor tissues from the tumor-bearing BALB/c nude mice did not express CTLA-4 due to a marked decrease of T cells in those mice. Thus, two subcutaneous tumor models were prepared by syngeneic subcutaneous transplantation of CT26 into normal BALB/c mice or BALB/c nude mice, and compared CTLA-4 and T cell marker expression in the CT26 tumor tissues from the tumor-bearing BALB/c mice with those from the tumor-bearing BALB/c nude mice by RT-PCR analysis (Fig. 11).

RT-PCR showed that CTLA-4 expression was dramatically decreased in the CT26 tumor tissues from the tumor-bearing BALB/c nude mice, compared with those from the tumor-bearing BALB/c mice. Furthermore, the expression of CD4 and CD8 as well as Foxp3, FR4, CD69, CD154, and CD25 was markedly decreased in the tumor tissues from the tumor-bearing BALB/c nude mice.
<table>
<thead>
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<th>Mice</th>
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<th>Nude</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Tissues</td>
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<td>CT26 xenograft</td>
<td>CT26 xenograft</td>
<td></td>
</tr>
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<td>flCTLA-4</td>
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<tr>
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<td><img src="image" alt="β-actin" /></td>
<td><img src="image" alt="β-actin" /></td>
<td></td>
</tr>
</tbody>
</table>

Figure 11

Gene expression analyses in tissues from tumor-bearing BALB/c and BALB/c nude mice.

CTLA-4 and T cell marker expression in normal colon tissues from normal BALB/c mice, CT26 tumor tissues from tumor-bearing BALB/c mice, and CT26 tumor tissues from tumor-bearing BALB/c nude mice.
**Synthesis of DOTA-conjugated antibody probe**

DOTA chelators were conjugated to each mAb, as shown in Figure 12A. The chromatograms of all the mAbs showed a single peak. In addition, the retention times of anti-CTLA-4 mAb, DOTA-anti-CTLA-4 mAb, Control IgG, and DOTA-Control IgG were 9.42, 9.36, 10.57, and 10.33, respectively (Fig. 12B). The retention times of DOTA-conjugated antibodies were slightly shorter than those of the original antibodies, suggesting that anti-CTLA-4 mAb or Control IgG conjugated to DOTA and was well purified.
Figure 12

Preparation of 1,4,7,10-tetraazacyclododecane-\(N,N',N''',N''''\)-tetraacetic acid (DOTA)-conjugated mAb.

A. Scheme of the synthesis of DOTA-conjugated mAb. B. High-performance liquid chromatography (HPLC) analysis of original and DOTA-conjugated mAbs.
Furthermore, MALDI-TOF-MS analysis was carried out to measure the average number of DOTA chelators that were conjugated to anti-CTLA-4 mAb or Control IgG (Table 4). The mass differences between anti-CTLA-4 mAb and DOTA-anti-CTLA-4 mAb, and between Control IgG and DOTA-Control IgG were 1634 and 1686, respectively. The mass differences were divided by the mass value of single DOTA conjugation (386 mass units), and the resulting values represented the average number of DOTA chelators that were conjugated to anti-CTLA-4 mAb or Control IgG. From the calculations, 4.2 or 4.4 DOTA chelators on average were conjugated into a single molecule of anti-CTLA-4 mAb or Control IgG.

Table 4

Average molecular weights of original and DOTA-conjugated antibodies, and estimated numbers of DOTA chelators per unit antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Average molecular weight</th>
<th>Mass difference</th>
<th>The number of DOTA per antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CTLA-4 mAb</td>
<td>150957</td>
<td>1634</td>
<td>4.2</td>
</tr>
<tr>
<td>DOTA-anti-CTLA-4 mAb</td>
<td>151731</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control IgG</td>
<td>147870</td>
<td>1686</td>
<td>4.4</td>
</tr>
<tr>
<td>DOTA-Control IgG</td>
<td>148557</td>
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</tbody>
</table>
Then, the binding activity of DOTA-anti-CTLA-4 mAb to CTLA-4 was measured by ELISA (Figure 4). The binding activity of DOTA-anti-CTLA-4 mAb to CTLA-4 was 86.3% of that of the original anti-CTLA-4 mAb. The binding activity of DOTA-Control IgG was 0.3%.

![Evaluation of CTLA-4 binding activity of DOTA-anti-CTLA-4 mAb.](image)

**Figure 13**

Evaluation of CTLA-4 binding activity of DOTA-anti-CTLA-4 mAb.
Visualization of CTLA-4 in the CT26 tumor using PET.

$^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG were obtained in radiochemical purities of 94% and 97%, respectively. To evaluate the $^{64}$Cu-DOTA-anti-CTLA-4 mAb uptake by CTLA-4 positive tumor (CT26), PET and ex-vivo biodistribution analysis were performed. Representative coronal and sagittal images are shown in Figure 5. At 48 hours after administration of the probes, $\text{SUV}_{max}$ values of $^{64}$Cu-DOTA-anti-CTLA-4 mAb were 2.72 and 2.58, while those of $^{64}$Cu-DOTA-Control IgG were 1.83 and 2.28. Thus, $^{64}$Cu-DOTA-anti-CTLA-4 mAb exhibited higher accumulation in the tumors, compared with $^{64}$Cu-DOTA-Control IgG.
Figure 14

Positron emission tomography (PET) images of $^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG.

A. Representative coronal (a) and sagittal (b) PET images of $^{64}$Cu-DOTA-anti-CTLA-4 mAb in CT26-bearing mice (n = 2). B. Representative coronal (a) and sagittal (b) PET images of $^{64}$Cu-DOTA-Control IgG in CT26-bearing mice (n = 2).
The results were consistent with those of the ex-vivo biodistribution study (Fig. 15). 

$^{64}$Cu-DOTA-anti-CTLA-4 mAb showed significantly higher accumulation in the CT26 tumors than $^{64}$Cu-DOTA-Control IgG ($7.49 \pm 0.32 \%$ID/g vs. $5.84 \pm 0.38 \%$ID/g, $p < 0.01$). Moreover, $^{64}$Cu-DOTA-anti-CTLA-4 mAb showed higher tumor-to-blood and tumor-to-muscle ratios than $^{64}$Cu-DOTA-Control IgG (tumor-to-blood ratio: $0.58 \pm 0.03$ vs. $0.40 \pm 0.02$, $p < 0.001$; tumor-to-muscle ratio: $8.48 \pm 0.63$ and $5.31 \pm 0.35$, $p < 0.01$).
Figure 15

Biodistribution analysis of $^{64}$Cu-labeled mAb probes.

A. Accumulation (the percentage of injected dose per gram (%ID/g)) of $^{64}$Cu-DOTA-anti-CTLA-4 mAb (n = 7) and $^{64}$Cu-DOTA-Control IgG (n = 6) in each organ. B. Tumor-to-blood and tumor-to-muscle ratios of $^{64}$Cu-DOTA-anti-CTLA-4 mAb (n = 7) and $^{64}$Cu-DOTA-Control IgG (n = 6). Data are expressed as means ± SEM. Symbols* and ** denote p < 0.01 and p < 0.001 vs. $^{64}$Cu-DOTA-Control IgG, respectively.
In addition, CTLA-4 protein expression in the CT26 tumor was confirmed by immunohistochemical staining (Fig. 16A). CTLA-4 was weakly expressed in the normal tissues surrounding the tumor (Fig. 16B).

Figure 16

Confirmation of CTLA-4 expression in the CT26 tumor and normal tissues.

A. CT26 tumor tissue section. B. Normal tissue section surrounding CT26 tumor tissue.

Scale bar = 50 µm.
2.5 Discussion

CT26 is a N-nitroso-N-methylurethane-induced, undifferentiated colon carcinoma cell line and recent cancer immunotherapy studies have shown that CTLA-4 blockade reduced CT26 colon tumor size and was effective in CT26 tumor models [33, 34]. Therefore, in this study, the CT26 cell line was used to prepare subcutaneous tumor models for PET imaging.

First, CTLA-4 expression in CT26 tumor tissues, normal colon tissues, and/or cultured CT26 cells was compared by RT-PCR analyses and confirmed that CTLA-4 was strongly expressed in the CT26 tumor tissues compared to the normal colon tissues. In this experiment, the full-length form of CTLA-4 (flCTLA-4) (amplicon length: 920 bp), which is a representative immunosuppressive variant of CTLA-4, was expressed in the CT26 tumor tissues.

On the other hand, CTLA-4 was not expressed in the cultured CT26 cells, although it was strongly expressed in the CT26 tumor tissues. Contardi et al. reported some human tumor cell lines that expressed CTLA-4 [4]. On the other hand, CTLA-4 is expressed also on CD25^+ (and/or) Foxp3^+ (and/or) FR4^+ CD4^+ Tregs, activated CD4^+ effector T cells, and activated CD8^+ effector T cells [2, 3, 24]. In addition, flow cytometry analysis
by Valzasina et al. revealed that almost all CD4$^+$ T cells in the CT26 tumor tissues expressed CD25 [35]. From those reports, I hypothesized that CTLA-4 expression in the CT26 tumor tissues regulated by T cells. To prove my hypothesis, the expression of several T cell markers in normal colon tissues, CT26 tumor tissues, and cultured CT26 cells was examined. RT-PCR analysis showed that Treg markers and T cell activation markers were strongly expressed in the CT26 tumor tissues but not the normal colon tissues or cultured CT26 cells. Therefore, I assumed that the T cells were responsible for CTLA-4 expression in the CT26 tumor tissues. Then, the expression of CTLA-4 and T cell markers in the CT26 tumor tissues of tumor-bearing BALB/c mice with those of tumor-bearing BALB/c nude mice was compared in hopes of elucidating the relationship between CTLA-4 expression in the CT26 tumor tissues and T cells. BALB/c nude mice are thymus-deficient and thus, these mice are T cell-deficient. Interestingly, the expression of CTLA-4 as well as T cell markers was quite low in the CT26 tumor tissues from the BALB/c nude mice. The results indicated that T cells were responsible for the CTLA-4 expression.

Second, a nuclear medical imaging probe that targets CTLA-4 was developed and its utility was examined in mice bearing CTLA-4-expressing CT26 tumor. The anti-CTLA-4 mAb for the imaging probe synthesis was made by using recombinant
mouse CTLA-4 representing an extracellular domain of mouse CTLA-4 (Ala36-Phe161) as the immunogen. This mAb was selected for CTLA-4 imaging probe synthesis because clinically used anti-CTLA-4 mAb (ipilimumab) also recognizes the extracellular domain of CTLA-4 and a mAb probe that recognizes the extracellular domain of CTLA-4 is suited for the prediction of the efficacy and drug disposition of ipilimumab.

For CTLA-4 imaging, anti-CTLA-4 mAb was conjugated to DOTA. DOTA-conjugated mAb was prepared by reacting the nucleophilic amino group in the amino acid residue (particularly in lysine) of mAb with the electrophilic DOTA-mono-NHS ester. The binding activity of antibodies to CTLA-4 may be reduced, particularly when DOTA conjugates to lysine residues critical for mAb binding to CTLA-4 [36]. Therefore, it is necessary to check in advance the binding activities of the antibodies. The binding activity of DOTA-conjugated anti-CTLA-4 mAb was examined and compared with that of control IgG. ELISA confirmed that the binding activity of DOTA-anti-CTLA-4 mAb was preserved for use in CTLA-4 imaging although a slight reduction (86.3%) was observed relative to the binding activity of the original anti-CTLA-4 mAb. The binding activity of DOTA-Control IgG was extremely low (0.3%) compared with that of DOTA-anti-CTLA-4 mAb. The results suggest successful
preparation of metal-chelator-conjugated anti-CTLA-4 mAb with CTLA-4 binding activity.

The choice of the positron emitter is an important factor for successful PET imaging. Intact antibodies diffuse into tumors more slowly and are retained for longer durations than small molecules (i.e., enhanced permeability and retention (EPR) effects). Thus, imaging with intact antibody probes typically requires about 2 days (ideally at a later time-point) post-injection before high-contrast images can be obtained [36-37]. Therefore, relatively long-lived radioisotope is needed for imaging using antibody probe. In this study, $^{64}$Cu was used for labeling DOTA-mAbs. $^{64}$Cu decay generates positron emissions applicable to PET and the half-life of $^{64}$Cu ($T_{1/2} = 12.7$ h) is sufficiently long for imaging up to 48 h after administration to accommodate the mAb localization time. Therefore, $^{64}$Cu has been used for the development of mAb-based radiopharmaceuticals [13, 15, 19].

The utility of $^{64}$Cu-DOTA-anti-CTLA-4 mAb was examined by using PET and by performing ex-vivo biodistribution analysis. PET has high sensitivity and therefore can be used for examining pharmacokinetic profiles with sub-therapeutic micro-doses of mAb probes [37]. In this study, the dose of $^{64}$Cu-DOTA-anti-CTLA-4 mAb (4 µg, approximately 192 µg/kg) was much lower than the clinical therapeutic dose of
anti-CTLA-4 mAb (3000 µg/kg per dose for a total of 4 doses). Therefore, the diagnosis using $^{64}$Cu-DOTA-anti-CTLA-4 mAb is unlikely to induce side effects such as autoimmune diseases. The results of PET imaging suggested that although $^{64}$Cu-DOTA-Control IgG accumulated slightly in the CT26 tumor tissues likely owing to EPR effects, the accumulation of $^{64}$Cu-DOTA-anti-CTLA-4 mAb was more significant. It appears that the difference in accumulation between $^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG was owing to CTLA-4 expression in the CT26 tumor tissues. To demonstrate this, it was desirable to conduct a binding competition assay or autoradiography analysis. However, since $^{64}$Cu-DOTA-anti-CTLA-4 mAb exhibited significantly higher tumor-to-blood and tumor-to-muscle ratios compared with $^{64}$Cu-DOTA-Control IgG, it is likely that the accumulation of $^{64}$Cu-DOTA-anti-CTLA-4 mAb in the tumor resulted from the specific binding of $^{64}$Cu-DOTA-anti-CTLA-4 mAb to CTLA-4 in the tumor. Furthermore, PET images were quantified and $^{64}$Cu-DOTA-anti-CTLA-4 mAb/$^{64}$Cu-DOTA-Control IgG ratio in the CT26 was calculated. The mean ratio of SUV_{max} values in CT26 was 1.29 in the PET image and that in CT26 was 1.28 in the ex-vivo biodistribution analysis; further, the quantitative value of PET was similar to the ex-vivo biodistribution data. Thus, it is likely that quantitative characterization was ensured in the PET experiment. These
results suggested that $^{64}$Cu-DOTA-anti-CTLA-4 mAb is useful for evaluating CTLA-4 expression in the tumor. In addition, although further investigation is needed, $^{64}$Cu-DOTA-anti-CTLA-4 mAb could be used in the diagnosis of other types of tumor invaded by T cells, regardless of CTLA-4 expression in the tumor cells.

On the other hand, ex-vivo biodistribution analysis revealed that $^{64}$Cu-DOTA-anti-CTLA-4 mAb accumulated more significantly in the liver compared with $^{64}$Cu-DOTA-Control IgG. However, in this experiment, the expression of CTLA-4 in the liver was not examined. CTLA-4 expression could be confirmed by using methods such as immuno-histochemical staining and RT-PCR for confirming the difference between liver accumulations of $^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG corresponding to the CTLA-4 expression in the liver.

In addition, both $^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG were highly accumulated in the liver. This property makes it difficult to visualize CTLA-4 near the liver and increases the non-targeted organ radiation dosage. Therefore, the reduced antibody probe uptake in the liver leads to the development of a method for evaluating CTLA-4 expression in the tumor near the liver and reduced radiation exposure of the liver. The increased radioactivity of the liver for $^{64}$Cu-labeled mAb may reflect transchelation of the released $^{64}$Cu to copper-binding proteins in the liver (e.g.,
superoxide dismutase) [38] and/or the binding of mAbs to Fc receptor-bearing cells in the liver. Therefore, there are two possible approaches to decrease the accumulation in the liver. First, the transchelation of the released $^{64}\text{Cu}$ to copper-binding proteins in the liver could be prevented by improving the in vivo stability of the $^{64}\text{Cu}$-chelator complex. A recent study has shown that 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) has better in vivo stability with $^{64}\text{Cu}$ compared to DOTA [39]. In addition, NOTA can be radiolabeled with $^{64}\text{Cu}$ at room temperature in 30–60 min. This property is suitable for the radiolabeling of mAb probes because mAb is heat sensitive [40]. Thus, the use of NOTA for radiolabeling, instead of DOTA, may decrease the radioactivity in the liver. Second, the binding of mAbs to Fc receptor-bearing cells in the liver can be prevented by the development of antibody fragment probes such as Fab, F(ab')$_2$, and Fab'. Fab and F(ab')$_2$ can be prepared by the action of papain and pepsin, respectively [41] (Fig. 17). F(ab')$_2$ fragments produced by the pepsin digestion of IgG can be selectively reduced in the hinge region by mild reduction using a reducing agent such as dithiothreitol, tris(2-carboxyethyl)phosphine, or 2-mercaptoethamine [41]. Probes made of these antibody fragments do not bind to Fc receptors because they lack the Fc portion of IgG. However, the CTLA-4 binding of the antibody fragments (particularly the monovalent fragments such as Fab and Fab') may be lower than bivalent intact antibody. Therefore,
it is necessary to investigate to what extent the antibodies should be fragmented for preparing the antibody fragments exhibiting both high radioactivity accumulation and high ratio of the CTLA-4 imaging probe to the control probe.
Figure 17

Antibody fragmentation.

A. Digestion of IgG with papain. The heavy–light chain pairs that are formed are called Fab. B. Pepsin digestion of IgG. The bivalent fragments are called F(ab')$_2$. C. F(ab')$_2$ can be reduced at their heavy chain disulfides using a reducing agent. This reduced fragment is called Fab'.
In the PET experiments, $^{64}$Cu-DOTA-Control IgG was also slightly accumulated in CT26 tumor tissues, probably owing to EPR effects. This nonspecific uptake of control probe may result in false-positive diagnosis. For a more precise evaluation of CTLA-4 expression in the tumor, the ratio of the imaging probe of CTLA-4 ($^{64}$Cu-DOTA-anti-CTLA-4 mAb) to the control probe ($^{64}$Cu-DOTA-Control IgG) in the tumor should be increased. There are two probable methods to improve this ratio. The first is to change the time point of PET imaging. As described above, tumor penetration and clearance of the intact antibody probe were very slow; thus, imaging with intact antibody probes ideally requires later time-point than 2 days post-injection to obtain high-contrast images. In fact, for imaging probes targeting EGFR, a higher tumor-to-background ratio could be obtained at a later time point than 48 h [42]. Therefore, the use of radioisotopes with a longer half-life than $^{64}$Cu (e.g., $^{89}$Zr, $T_{1/2} = 78.4$ h) and later time point may improve the CTLA-4 imaging probe to control probe ratio in the tumor. Another potential method is antibody fragmentation. In fact, recent studies have reported that clearance and tumor penetration of the antibody fragments including Fab and F(ab)′$_2$ can be higher than those of intact antibodies [36]. Therefore, the development of antibody fragment probes may lead to the reduction of nonspecific uptake of control probe in the tumor and the establishment of high-specificity diagnosis.
In my thesis experiments, $^{64}$Cu-DOTA-anti-CTLA-4 mAb was made with anti-mouse 
CTLA-4 mAb. Hereafter, biodistribution analysis in humans using 
$^{64}$Cu-DOTA-anti-human CTLA-4 mAb and determination of the cutoff value for 
radioactivity accumulation of the mAb probe, such as SUV, are necessary for the 
development of a method for the clinical evaluation of CTLA-4 expression in tumors. 

In conclusion, $^{64}$Cu-DOTA-anti-CTLA-4 mAb was developed, and its potential as a 
new radiotracer was evaluated for the noninvasive evaluation of CTLA-4 expression in 
tumor. These results suggest that $^{64}$Cu-DOTA-anti-CTLA-4 mAb noninvasively 
visualized the CTLA-4 expression in the CT26 tumor. Therefore, 
$^{64}$Cu-DOTA-anti-CTLA-4 mAb can be useful for evaluating the CTLA-4 expression in 
the tumor. The evaluation of CTLA-4 expression in tumors using 
$^{64}$Cu-DOTA-anti-CTLA-4 mAb or mAb probe, in which the above problems are 
overcome, is likely to enable the selection of patients sensitive to CTLA-4-targeted 
therapy, thereby eliminating the adverse effects caused by ineffective therapy and 
reducing unnecessary financial burden on non-sensitive patients.
2.6 References


Chapter 3

Demonstration of multiple molecular species imaging using Gamma-Ray Emission Imaging

3.1 Abstract

Nuclear medical imaging modalities are molecular imaging technologies to visualize specific biological processes in living subjects at the molecular level. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have been widely used for clinical diagnosis because they can conduct noninvasive molecular imaging quantitatively with high sensitivity. However, these nuclear medical imaging modalities are intractable for visualizing multiple molecular species simultaneously. Since many diseases including cancer and inflammatory diseases are caused by complex interactions among multiple disease-related molecular species, a method that allows for noninvasive evaluation of the expression of multiple disease-related molecular species is expected to facilitate evidence-based medical care. Therefore, this study aimed to develop a method for multiple and simultaneous nuclear medical imaging of disease-related molecular species using Gamma-Ray Emission Imaging (GREI).
To demonstrate this method, mice bearing epidermal growth factor receptor (EGFR)-positive and human epidermal growth factor receptor 2 (HER2)-negative tumor xenografts (MDA-MB-468), and EGFR-negative and HER2-positive tumor xenografts (MDA-MB-361 or BT474) were prepared. Then, $^{89}\text{Zr}$-defereroxamine (DFO)-anti-EGFR monoclonal antibody (mAb) and $^{111}\text{In}$-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-anti-HER2 mAb were intravenously administered to tumor-bearing mice, and the biodistribution of these probes in the tumor-bearing mice was visualized by GREI after 72 h.

The GREI experiment results suggested that $^{89}\text{Zr}$-DFO-anti-EGFR mAb accumulated more in EGFR-positive tumors (MDA-MB-468) than in EGFR-negative tumors (BT474 or MDA-MB-361) at 72 h after mAb probe administration. Although further improvement is required to obtain well-contrasted images of the $^{111}\text{In}$-labeled probe, $^{111}\text{In}$-DOTA-anti-HER2 mAb tended to exhibit higher accumulation in the HER2-positive tumors (BT474 or MDA-MB-361) than in the HER2-negative tumors (MDA-MB-468). The results suggest that, although the quality of $^{111}\text{In}$-labeled probe data remains to be improved, GREI can be used for noninvasive visualization of multiple disease-related molecular species in tumors.
Introduction

Nuclear medical imaging technologies have features that enable molecules of interest to be traced in living subjects. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are representative nuclear medical imaging modalities. They have been used for fundamental research and clinical diagnosis owing to their ability to visualize biomolecules noninvasively with high sensitivity [1].

However, recent life science research has revealed that many pathological conditions, including cancer and inflammatory diseases, are not characterized by single molecules, but by multiple and complex molecular interactions. Thus, it is necessary to analyze data for multiple disease-related molecular species to investigate complex functions or diseases. However, it is difficult to visualize multiple molecular species simultaneously with PET and SPECT. PET determines the position of PET probes by coincident detection of a pair of annihilation radiation events. PET detectors cannot distinguish different PET probes simultaneously, because the annihilation radiation energies are identical (511 keV) [2]. In SPECT, collimators are required in front of gamma-ray detectors to determine the traveling direction of gamma-rays. However, high-energy gamma rays pass through the collimator, resulting in a reduction in the quality of
SPECT images. Thus, the choice of radioisotopes for imaging is strictly limited in SPECT.

Our laboratory has developed a semiconductor Compton camera called Gamma-Ray Emission Imaging (GREI). GREI enables the simultaneous and nondestructive imaging of multiple species of radioisotopes by spectroscopically analyzing the gamma-ray energies for each detected gamma-ray photon [3-5]. Therefore, GREI potentially allows for the visualization of multiple species of molecules using different imaging probes labeled with radioisotopes emitting gamma-rays with different energies. However, the simultaneous imaging of multiple disease-related molecular species has not yet been achieved. Therefore, this study aimed to develop a simultaneous nuclear medical imaging method for multiple disease-related molecular species by GREI. Epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) were selected as imaging targets. They are representative target molecules for cancer treatment [6,7]. Simultaneous imaging of EGFR and HER2 in tumors was attempted using the $^{89}$Zr- and $^{111}$In-labeled monoclonal antibody (mAb) probes and GREI.
3.2 Materials and Methods

Cell culture

MDA-MB-468, BT474, and MDA-MB-361 were purchased from the American Type Culture Collection. MDA-MB-468 and BT474 were cultured in 500 mL of RPMI 1640 medium supplemented with 50 mL of fetal bovine serum, 10 U/mL penicillin, and 10 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. MDA-MB-361 was cultured in 500 mL of DMEM medium supplemented with 100 mL of fetal bovine serum, 10 U/mL penicillin, and 10 mg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Preparation of subcutaneous tumor model mice

Female BALB/c (nu/nu) nude mice (5–6 weeks old) were purchased from CLEA Japan Inc.. At least one day before tumor cell implantation, all mice were implanted with a 60-day-release 1.25-mg estradiol pellet (Innovative Research of America) subcutaneously. Two mice bearing two different tumor xenografts were prepared:
mouse 1 bore MDA-MB-468 and BT474 xenografts (n = 1), and mouse 2 bore MDA-MB-468 and MDA-MB-361 xenografts (n = 1). Mouse 1 was prepared by implantations of BT474 (HER2-positive and EGFR-negative) \((2.4 \times 10^7 \text{ cells})\) and MDA-MB-468 (HER2-negative and EGFR-positive) \((6.5 \times 10^6 \text{ cells})\) suspended in Matrigel (BD Biosciences) diluted 1:1 in phosphate-buffered saline (PBS) (-) under the skin near right and left rear feet, respectively. Mouse 2 was prepared by implantations of MDA-MB-361 (HER2-positive and EGFR-negative) \((9.0 \times 10^6 \text{ cells})\) and MDA-MB-468 \((7.0 \times 10^6 \text{ cells})\) suspended in Matrigel diluted 1:1 in PBS (-) under the skin near right and left rear feet, respectively. Investigations were initiated after receiving approval from the Committee on Animal Experiments of RIKEN and Okayama University.

\[ ^{89}\text{Zr-deferoxamine (DFO)-anti-EGFR mAb preparation} \]

DFO conjugation to anti-EGFR mAb and subsequent \(^{89}\text{Zr}\)-labeling were conducted using a method similar to that reported by Bhattacharyya S et al [8]. In brief, anti-human EGFR mAb (1 mg) in 0.1 M carbonate buffer (pH 9.0) was mixed with 15-fold molar excess of p-isothiocyanatobenzyl-DFO (Macrocylics, Inc.) in dimethyl
sulfoxide (DMSO) for one hour at 37 °C. DMSO concentration was below 5% in this reaction mixture. DFO-anti-EGFR mAb was purified by size exclusion chromatography using a PD10 column (GE Healthcare Life Sciences, Co., Ltd., Buckinghamshire, UK) and an Amicon Ultra 50K device (Millipore Co., Ltd., Billerica, MA, USA). For radiolabeling, $^{89}$Zr-oxalate in 1.0 M oxalic acid was neutralized using 2.0 M Na$_2$CO$_3$ (aq.). Neutralized $^{89}$Zr solution was added to 0.5 M 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (pH 7.2). Then, this solution was mixed with DFO-anti-EGFR mAb in 0.9% sodium chloride/gentisic acid 5 mg/mL (pH 5.2) was mixed. The reaction was incubated at 37 °C for 30 min. $^{89}$Zr-DFO-anti-EGFR mAb was purified by size exclusion chromatography using a PD10 column (GE Healthcare Life Sciences, Co., Ltd) and 0.9% sodium chloride/gentisic acid 5 mg/mL (pH 5.2) as eluent by spin-column centrifugation (Amicon Ultra 50K device, Millipore Co., Ltd.). The radiochemical purity of $^{89}$Zr-DFO-anti-EGFR mAb was confirmed by reversed-phase radio-thin-layer chromatography (TLC). This analysis was performed with a TLC aluminum sheet, RP-18 F254 S (Merck Chemicals Co., Ltd., Kenilworth, NJ, USA), and 20 mM citric acid (pH 4.9) was used as the mobile phase. TLC chromatograms were obtained by autoradiography (FLA-7000IR; GE Healthcare Co., Ltd.).
**111In-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-anti-HER2 mAb preparation**

DOTA-anti-human HER2 mAb was synthesized by mixing anti-human HER2 mAb (1.2 mg) in 0.1 M carbonate buffer (pH 9.0) with a 16-fold molar excess of p-isothiocyanatobenzyl-DOTA (Macrocyclics, Inc.) in DMSO for one hour at 37 °C. The DMSO concentration was below 5% in this reaction mixture. DOTA-anti-HER2 mAb was purified with a PD-10 column and an Amicon-Ultra 50K device.

111InCl₃ solution was purchased from Nihon Medi-Physics Co., Ltd. 111InCl₃ solution was evaporated, and 0.1 M HEPES buffer (pH 5.5) was added. The buffer solution of DOTA-anti-HER2 mAb was also replaced with 0.1 M HEPES (pH 5.5) by using an Amicon-Ultra 50K device. DOTA-anti-HER2 mAb was radiolabeled with 111In by incubating at 37 °C with 111InCl₃ in 0.1 M HEPES buffer for one hour. After this incubation, the buffer was replaced with 0.15 M acetate buffer (pH 5.5) by using the Amicon-Ultra 50K device to remove excess 111In. The buffer of the purified antibody solution was replaced with PBS (-) by using the Amicon-Ultra 50K device. The resultant solution was used for injections.

The radiochemical purity of 111In-DOTA-anti-HER2 mAb was confirmed by
reversed-phase radio-TLC. This analysis was performed with a TLC aluminum sheet, RP-18 F254 S (Merck Chemicals Co., Ltd.) and 4 mM ethylenediaminetetraacetic acid (EDTA)/PBS (-) was used as the mobile phase. TLC chromatograms were obtained by autoradiography (FLA-7000IR; GE Healthcare Co., Ltd.).

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to examine the number of chelators conjugated to mAbs. A 4800 Plus MALDI TOF/TOF Analyzer (AB SCIEX Co., Ltd., Framingham, MA, USA) was used for MALDI-TOF-MS. Non-conjugated- and chelator-conjugated mAbs were desalted with PD Spin Trap G-25 (GE Healthcare Co., Ltd.). Sinapinic acid (Wako, Inc.) at 10 mg/mL in 1:1 acetonitrile/H₂O with 0.1% trifluoroacetic acid was used as the MALDI matrix. For each sample, the measurements were performed ten times.

GREI imaging and biodistribution study
\(^{89}\text{Zr-DFO-anti-EGFR mAb}\) (100 µg, 4.30 MBq) and \(^{111}\text{In-DOTA-anti-HER2 mAb}\) (100 µg, 2.46 MBq) was intravenously administered to mouse 1 (n = 1) via the tail vein. In mouse 2, \(^{89}\text{Zr-DFO-anti-EGFR mAb}\) (100 µg, 4.02 MBq) and \(^{111}\text{In-DOTA-anti-HER2 mAb}\) (100 µg, approximately 2.46 MBq) were intravenously administered via the tail vein (n = 1). Twenty-four and seventy-two hours after administering the radiolabeled mAbs, the biodistribution of these probes in the tumor-bearing mice was assessed using the GREI apparatus. The imaging experiment by GREI was performed for 90 min. During imaging with GREI, the mice were anesthetized with isoflurane and placed in the prone position. After performing the imaging experiments using GREI at 72 h, the mice were euthanized and the organs and blood were removed. The organs and blood were weighed, and the radioactivity was estimated with high-purity germanium (Ge) detectors. Decay-corrected uptake was expressed as the percentage of injected dose per gram (%ID/g).
3.3 Results

*Chelator-conjugated mAb probe synthesis*

For radiolabeled mAb probe synthesis, anti-EGFR mAb and anti-HER2 mAb were conjugated to DFO or DOTA, respectively. Furthermore, MALDI-TOF-MS analysis was carried out to measure the average number of chelators that were conjugated to anti-EGFR mAb or anti-HER2 mAb (Table 5). The mass differences between anti-EGFR mAb and DFO-anti-EGFR mAb, and between anti-HER2 mAb and DOTA-anti-HER2 mAb were 1207 and 1001, respectively. The mass differences were divided by the mass value for single chelator conjugation (DFO: 752 mass units, DOTA: 551 mass units) and the resulting values represented the average number of chelators that were conjugated to anti-EGFR mAb or anti-HER2 mAb. From these calculations, approximately 1.6 DFO or 1.8 DOTA chelators on average were conjugated into a single molecule of anti-EGFR mAb or anti-HER2 mAb, respectively.
Table 5

Average molecular weights of original and chelator-conjugated mAbs, and estimated numbers of chelators per unit antibody.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Average molecular weight</th>
<th>Mass difference</th>
<th>The number of chelator per antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-EGFR mAb</td>
<td>151949</td>
<td>1207</td>
<td>1.6</td>
</tr>
<tr>
<td>DFO-anti-EGFR mAb</td>
<td>153156</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HER2 mAb</td>
<td>147659</td>
<td>1001</td>
<td>1.8</td>
</tr>
<tr>
<td>DOTA-anti-HER2 mAb</td>
<td>148660</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Demonstration of simultaneous imaging of $^{89}$Zr-DFO-anti-EGFR mAb and $^{111}$In-DOTA-HER2 mAb by GREI

$^{89}$Zr-DFO-anti-EGFR mAb and $^{111}$In-DOTA-anti-HER2 mAb were obtained with radiochemical purities of 96% and 95%, respectively.

The results of the imaging experiments with GREI suggested that, in mouse 1, $^{89}$Zr-DFO-anti-EGFR mAb clearly accumulated more in the MDA-MB-468 tumors than the BT474 tumors at 72 hours after probe administration (Fig. 18). The results also indicate the possibility that $^{111}$In-DOTA-anti-HER2 mAb exhibits slightly higher accumulation in the BT474 tumors compared with the MDA-MB-468 tumors. In mouse 2, the accumulation of $^{89}$Zr-DFO-anti-EGFR mAb in MDA-MB-468 was higher than in MDA-MB-361 tumors at 72 hours after probe administration, while $^{111}$In-DOTA-anti-HER2 mAb tended to exhibit higher accumulation in the MDA-MB-361 tumors than in the MDA-MB-468 tumors, but the difference in accumulation was small.
Figure 18

GREI images of $^{89}$Zr-DFO-anti-EGFR mAb and $^{111}$In-DOTA-anti-HER2 mAb in the mice bearing EGFR-positive and HER2-negative tumors (MDA-MB-468), and EGFR-negative and HER2-positive tumors (BT474 or MDA-MB-361).

A. GREI image of the mAb probes in the mouse 1 bearing MDA-MB-468 and BT474 tumors. B. GREI image of the mAb probes in the mouse 2 bearing MDA-MB-468 and MDA-MB-361 tumors. $^{89}$Zr-DFO-anti-EGFR mAb emitted 511 keV of annihilation radiation and 909 keV of gamma-rays, while $^{111}$In-DOTA-anti-HER2 mAb emitted 245 keV of gamma-rays.
Moreover, actual radioactivity accumulation of the tumors was examined by a high-purity Ge detector (Fig. 19). In mouse 1, $^{89}$Zr-DFO-anti-EGFR mAb accumulated more in the MDA-MB-468 tumor than the BT474 tumor at 72 hours after probe administration (32.57 %ID/g vs. 13.49 %ID/g, respectively), while $^{111}$In-DOTA-anti-HER2 mAb exhibited higher accumulation in the BT474 tumor than in the MDA-MB-468 tumor (76.12 %ID/g vs. 12.01 %ID/g, respectively). In mouse 2, $^{89}$Zr-DFO-anti-CTLA-4 mAb accumulated more in the MDA-MB-468 tumor than the MDA-MB-361 tumor (37.63 %ID/g vs. 15.95 %ID/g, respectively). On the other hand, $^{111}$In-DOTA-anti-HER2 mAb showed higher accumulation in the MDA-MB-361 tumor, compared with the MDA-MB-468 tumor (71.32 %ID/g vs. 5.56 %ID/g).
Figure 19

Accumulation of $^{89}$Zr-DFO-anti-EGFR mAb and $^{111}$In-DOTA-anti-HER2 mAb in EGFR-positive and HER2-negative tumors (MDA-MB-468), and EGFR-negative and HER2-positive tumors (BT474 or MDA-MB-361).

A. Accumulation of the mAb probes in MDA-MB-468 and BT474 tumors in mouse 1.

B. Accumulation of the mAb probes in MDA-MB-468 and MDA-MB-361 tumors in mouse 2.
3.4 Discussion

This research aimed to demonstrate imaging of multiple molecular species using GREI and two different nuclear medical imaging probes. To accomplish this goal, highly selective nuclear medical imaging probes for target molecules must be synthesized. Therefore, the nuclear medical imaging probes were made of mAb. mAbs have high selectivity and binding activity for its target molecule; thus, a large number of mAbs have been approved for cancer treatment [9]. EGFR and HER2 are well-researched target molecules for cancer treatment, and an EGFR-positive and HER2-negative tumor cell line (MDA-MB-468), and EGFR-negative and HER2-positive tumor cell lines (BT474 or MDA-MB-361) are available [10, 11]. Thus, anti-EGFR mAb and anti-HER2 mAb were used for the preparation of mAb probes. Furthermore, previous studies of nuclear medical imaging including PET have shown that the radiolabeled anti-EGFR mAb selectively accumulates in MDA-MB-468, whereas the radiolabeled anti-HER2 mAb highly accumulates in the HER2-expressing tumors (BT474 or MDA-MB-361) [12-15].

To enable nuclear medical imaging of intact mAbs, they should be labeled with radioisotopes with relatively long half-lives, because mAb accumulation in tumors is a
slow process [16]. Therefore, $^{89}\text{Zr}$ ($T_{1/2} = 78.4 \text{ h}$) and $^{111}\text{In}$ ($T_{1/2} = 67.3 \text{ h}$) were selected as radioisotopes to label mAbs for molecular imaging. In addition, a clinical trial of $^{89}\text{Zr}$-labeled anti-EGFR mAb and $^{111}\text{In}$-labeled anti-HER2 mAb has been conducted [17, 18]. In this study, anti-EGFR mAb and anti-HER2 mAb were conjugated to DFO and DOTA, respectively, because previous studies have shown that DFO and DOTA are suitable for $^{89}\text{Zr}$-labeling and $^{111}\text{In}$-labeling, respectively [19]. In this study, $^{89}\text{Zr}$-DFO-anti-EGFR mAb and $^{111}\text{In}$-DOTA-HER2 mAb with high radiochemical purities could be obtained.

The imaging experiments using GREI were conducted at 24 and 72 h after probe administration. However, the radioactivity of $^{111}\text{In}$-DOTA-anti-HER2 mAb was too strong at 24 h; consequently, the GREI image could not be obtained at this time. Therefore, only the GREI image acquired at 72 h is presented. GREI experiments and ex-vivo biodistribution studies with high-purity Ge detectors were carried out for this demonstration. In both mouse 1 and mouse 2, GREI could be used to visualize the selective accumulation of $^{89}\text{Zr}$-DFO-anti-EGFR mAb into the EGFR-positive tumor (MDA-MB-468). The results indicated a similar tendency to that observed in ex-vivo biodistribution studies. Biodistribution studies of $^{89}\text{Zr}$-DFO-anti-EGFR mAb also indicated that the accumulation of $^{89}\text{Zr}$-DFO-anti-EGFR mAb in EGFR-positive tumors
was 2.36–2.41 times higher than in EGFR-negative tumors. Although ex-vivo biodistribution analysis indicated that the accumulation of $^{111}$In-DOTA-anti-HER2 mAb in HER2-positive tumors was 6.34–12.84 times higher than that in HER2-negative tumors, the difference in accumulation was small in the GREI image. In general, Ge semiconductor detectors have excellent energy resolution, when compared to other gamma-ray detectors such as the NaI(Tl) scintillator [20]. This characteristic allows the separation of the many closely spaced gamma-ray energies. Since GREI uses planar Ge semiconductor detectors for the detection of gamma-rays, GREI has extremely high resolution and can easily discriminate between gamma-rays from $^{89}$Zr and $^{111}$In. Therefore, the gamma-rays from $^{89}$Zr were unlikely to negatively affect the detection of gamma-rays from $^{111}$In. Low-contrast images are probably due to relatively low energy resolution of low-energy gamma-rays in the Ge detectors of GREI in comparison to high-energy gamma-rays. Low energy resolution generates errors in Compton scattering angles ($\theta$). These errors cause a decrease in the overlap of circular cones, which indicate the direction of the radiation source, resulting in low contrast. Therefore, novel image reconstruction methods that correct for errors in Compton scattering angles should be developed for improved image contrast. In addition, it should be noted that only one
animal was used for each tumor model. Therefore, reproducibility should be quantitatively evaluated in the future experiments.

In conclusion, although further improvement is required for the image reconstruction method of the $^{111}$In-labeled probe data, multiple and simultaneous nuclear medical imaging of disease-related molecules was achieved in this study for the first time. These results indicated that the nuclear medical imaging methods developed in this study may lead to more accurate evidence-based medical care and the elucidation of disease mechanisms.
3.5 Reference


labeled panitumumab: a potential immuno-PET probe for HER1-expressing

Imaging of Positron Emission Tomographic Radiotracers Using Cerenkov

Measuring the Pharmacodynamic Effects of a Novel Hsp90 Inhibitor on HER2/neu

Micro-SPECT/CT with 111In-DTPA-pertuzumab sensitively detects
trastuzumab-mediated HER2 downregulation and tumor response in athymic mice
bearing MDA-MB-361 human breast cancer xenografts. J Nucl Med 50:
1340-1348.

tomography: antibodies for molecular imaging in oncology. J Clin Oncol 30:
3884-3892.


Summary and conclusion

Nuclear medical imaging modalities are valuable technologies for early detection, characterization, and real time monitoring of human diseases as well as animal models. However, the number of clinically usable probes targeting disease-related molecules is extremely low. Furthermore, it is extremely difficult to simultaneously visualize multiple disease-related molecular species using the existing nuclear medical imaging modalities such as PET and SPECT. Thus, the ultimate objective of my study was to develop a nuclear medical imaging method allowing to acquire the information on multiple molecular species relevant to IBD and cancer.

Four stages were envisioned for accomplishing the ultimate objective: (1) Exploration of imaging targets or selection of the targets from previously reported disease-related molecules, (2) Development of novel nuclear imaging probes for visualizing the target molecules, (3) Development of a novel nuclear imaging technique allowing simultaneously visualizing multiple molecular species. The research presented in this thesis covers stages (1) to (3).

In Chapter 1 I described the target molecules for IBD imaging. I have successfully explored target molecules for the nuclear medical imaging of IBD. I found that IL-6,
IL-1β, and S100A8 were particularly promising target molecules for IBD imaging because the expression levels of these molecules are altered early during the inflammation and depend on the extent of inflammation. Hence, the utilization of nuclear medical imaging probes targeting these inflammation-related molecules would be helpful for grasping in greater detail the inflammatory conditions of IBD early during its development, providing the appropriate guidelines for treatment.

On the other hand, the expression of TNF-α increased in the DSS-induced IBD model mice early on, while no significant changes were found regarding the IND-treated mice. These results suggest that not all cytokines and cytokine receptors are fundamental to the pathogenesis of this inflammatory disease. Therefore, IBD imaging targeting cytokines or cytokine receptors can under-detect the inflammation if the probe is targeting the molecules that are not fundamental for the pathogenesis. To avoid such under-detection of IBD, multiple molecular species should be imaged rather than single molecules. Therefore, a diagnosis of multiple molecular species using GREI would be useful in searching for disease-related molecules that are fundamental to the pathogenesis.

In this study, I have evaluated the genetic-level expression changes of cytokines and cytokine receptors. In the future, it will be necessary to develop nuclear medical
imaging probes targeting the molecules described above, and to investigate whether the probes can be used for visualizing the target molecules in IBD models and patients.

In Chapter 2 I described the development of a novel antibody probe targeting CTLA-4, a target molecule for cancer immunotherapy. The results of PET imaging and ex-vivo biodistribution analyses suggested that although $^{64}$Cu-DOTA-Control IgG exhibited slight accumulation in the CT26 tumor tissues likely owing to EPR effects, the accumulation of $^{64}$Cu-DOTA-anti-CTLA-4 mAb was more pronounced. Furthermore, $^{64}$Cu-DOTA-anti-CTLA-4 mAb exhibited significantly higher tumor-to-blood and tumor-to-muscle ratios compared with $^{64}$Cu-DOTA-Control IgG. These results suggest that $^{64}$Cu-DOTA-anti-CTLA-4 mAb can be used for evaluating the CTLA-4 expression in tumors.

On the other hand, although it appears that the difference between the accumulations of $^{64}$Cu-DOTA-anti-CTLA-4 mAb and $^{64}$Cu-DOTA-Control IgG was owing to the CTLA-4 expression in the CT26 tumor tissues, this fact was not demonstrated in my study. To demonstrate this, it will be necessary to consider a binding competition assay or to perform an autoradiography analysis. Moreover, in my experiments, $^{64}$Cu-DOTA-anti-CTLA-4 mAb was obtained using the anti-mouse CTLA-4 mAb. In the future, biodistribution analysis in humans using $^{64}$Cu-DOTA-anti-human CTLA-4
mAb and determination of the cutoff value for radioactivity accumulation of the mAb probe, such as SUV, will be necessary for developing the methods for clinical evaluation of CTLA-4 expression in tumors.

Chapter 3 presented the results of imaging studies for multiple molecular species, performed by using GREI. In this study, ^{89}\text{Zr}-\text{DFO}-\text{anti-EGFR} mAb and ^{111}\text{In}-\text{DOTA}-\text{anti-HER2} mAb were intravenously administered to tumor-bearing mice, and biodistributions of these probes in the tumor-bearing mice were visualized by using GREI. The results suggest that, although the number of samples has to be increased and the quality of ^{111}\text{In}-labeled probe data remains to be improved, the GREI method can be used for noninvasively visualizing multiple disease-related molecular species in tumors. Recent research has revealed that many pathological conditions are not characterized by single molecules but rather by multiple molecular interactions. It will be necessary to analyze the data on multiple disease-related molecular species for investigating more complex functions or diseases. Therefore, the GREI method can be used for evaluating the expression of multiple disease-related molecular species in vivo, helping to develop more accurate evidence-based medical care methods.

As mentioned above, these studies provide the fundamental information necessary for developing the methods for evaluating the expression of multiple disease-related
molecular species relevant to IBD and cancer. In the future work, the achievements of different research stages will need to be combined. For instance, for establishing multiple diagnoses of disease-related molecules in IBD, it will be necessary to develop nuclear medical imaging probes targeting disease-related molecules of IBD, such as the molecules that were considered in Chapter 1, and to investigate whether these molecules could be visualized by using GREI. In addition, in Chapters 2 and 3, the mAb probes targeting CTLA-4, EGFR, and HER2 have been successfully developed. Diagnosis performed using GREI and these multiple mAb probes is likely to enable the selection of most suitable molecular drug for cancer therapy among CTLA-4, HER2, and EGFR-targeted drugs. In future experiments that will aim at establishing multiple diagnoses of disease-related molecules relevant to cancer, it will be necessary to investigate whether the target molecules other than EGFR and HER2 can be visualized by using GREI. These studies are likely to help guide the development of more accurate evidence-based medical care methods.
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Okayama, Japan, January 2015

Kei Higashikawa