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Radio-copper-labeled Cu-ATSM: an indicator of quiescent but clonogenic cells under mild hypoxia in a Lewis lung carcinoma model

Myungmi Oh[a], Takeshi Tanaka[a], Masato Kobayashi[b], Takako Furukawa[c], Tetsuya Mori[d], Takashi Kudo[b], Shigeharu Fujieda[a], Yasuhisa Fujibayashi[b,*]

[a]*Department of Otorhinolaryngology, School of Medicine, University of Fukui, Matsuoka Shimoizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan*
[b]*Biomedical Imaging Research Center, University of Fukui, Matsuoka Shimoizuki, Eiheiji-cho, Yoshida-gun, Fukui 910-1193, Japan*
[c]*Diagnostic Imaging Group, Molecular Imaging Center, National Institute of Radiological Sciences, Anagawa 4-9-1, Chiba 263-8555, Japan*
[d]*Division of Radiological Sciences, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA*

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Abstract

The purpose of this study is to reveal characteristics of ^{64}Cu-labeled diacetyl-bis(N^4-methylthiosemicarbazone) ([^{64}Cu]Cu-ATSM) during cell proliferation and hypoxia by autoradiography imaging and immunohistochemical staining.

Methods: The intratumoral distributions of [^{64}Cu]Cu-ATSM and [^{18}F]-2-fluoro-2-deoxy-D-gloucose ([^{18}F]FDG) in mice implanted with Lewis lung carcinoma (LLC1) tumor cells according to dual autoradiography were compared with the immunohistochemical staining patterns of proliferating markers [Ki-67 and 5-bromo-2′-deoxyuridine (BrdU)] and a hypoxic marker (pimonidazole). A clonogenic assay was performed using the cells of LLC1 tumor-implanted mice, and it was compared with the distribution of [^{64}Cu]Cu-ATSM.

Results: [^{64}Cu]Cu-ATSM mainly accumulated at the edge of tumors, whereas [^{18}F]FDG was distributed inside the tumor and inside the [^{64}Cu]Cu-ATSM accumulation. The number of Ki-67-positive cells/area tended to increase with [^{18}F]FDG accumulation and decrease with [^{64}Cu]Cu-ATSM accumulation. On the other hand, the number of BrdU-positive cells/area was negatively correlated with [^{18}F]FDG accumulation and positively correlated with [^{64}Cu]Cu-ATSM accumulation. High [^{64}Cu]Cu-ATSM accumulation was found outside the high-[^{18}F]FDG-accumulation and pimonidazole-positive regions. Colony formation ability was significantly higher in the tumor cells obtained from high-[^{64}Cu]Cu-ATSM-accumulation regions than the cells from the intermediate- and the low-accumulation regions.

Conclusion: [^{64}Cu]Cu-ATSM accumulation regions in tumor cells indicate quiescent but clonogenic tumor cells under mild hypoxia. [^{64}Cu]Cu-ATSM could play an important role in planning appropriate tumor radiotherapy.

Keywords: [^{64}Cu]Cu-ATSM; [^{18}F]FDG; Proliferation; Hypoxia; Clonogenic assay

1. Introduction

Hypoxia is a key microenvironmental factor for tumor development; not only does it stimulate angiogenesis and glycolysis for tumor expansion, but it also induces cell cycle arrest and genetic instability with tumor progression [1]. In addition, hypoxic regions in solid tumors are known to be resistant to radiotherapy as well as chemotherapy [2]. Thus, precise detection of hypoxic regions in tumors is of importance to predict tumor malignancy and therapeutic outcome.

Radio-copper-labeled Cu-diacetyl-bis(N^4-methylthiosemicarbazone) (Cu-ATSM) has been developed as a positron emission tomography (PET) agent for hypoxia imaging [3–6] as well as an internal radiotherapy agent that allows selective delivery of β-emitting Cu nuclides [7,8]. Clinical study has indicated the usefulness of radio-copper-labeled Cu-ATSM for predicting the prognosis of radiotherapy in several types of cancer [9,10]. A basic comparative study of [^{64}Cu]Cu-ATSM and immunohistochemical staining revealed that high-[^{64}Cu]Cu-ATSM regions demonstrate fewer Ki-67-positive "proliferating" cells and lower vascularity, but a slight increase in apoptotic cells (although this

* Corresponding author. Tel.: +81 776 61 8431; fax: +81 776 61 8170.
E-mail addresses: sampo@u-fukui.ac.jp (M. Oh), yfuji@u-fukui.ac.jp (Y. Fujibayashi).

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was less than 1%), when compared with low-[^{64}Cu]Cu-ATSM regions [11]. These findings are consistent with the known characteristics of hypoxic tumor masses.

However, we also found that intratumor [^{18}F]-2-fluoro-2-deoxy-D-gloucose ([^{18}F]FDG) uptake, an indication of glycolysis, was not positively correlated with "hypoxia" as shown by [^{64}Cu]Cu-ATSM uptake [11,12]. In addition, the intratumor distribution of [^{64}Cu]Cu-ATSM is reported to be different from that of F-18-fluoromisonidazole ([^{18}F]F-MISO), a traditional hypoxia marker [13,14]. Considering these differing findings, radio-copper-labeled Cu-ATSM might visualize different aspects of hypoxia than traditional nitroimidazole compounds such as [^{18}F]F-MISO.

Supply of oxygen as well as nutrition is limited to the range of 100 to 200 μm from the vessel, and outside this range, it should become necrotic. Positron emission tomography with 3- to 5-mm resolution cannot visualize such microenvironment; hence, the hypoxic region in the PET image should be evaluated as a mixture or an average of heterogenous phenotypes.

In the present study, we compared the intratumor distribution of [^{64}Cu]Cu-ATSM with [^{18}F]FDG, a marker of glycolysis that is known to be enhanced under hypoxic conditions, macroscopically. Pimonidazole staining was also performed as a "low-oxygen, tension-specific" probe, at macroscopic as well as microscopic levels. The intratumor distribution of the three "hypoxia"-seeking probes with different aspects was compared with immunohistochemical staining of Ki-67 and 5-bromo-2′-deoxyuridine (BrdU) to elucidate the regional proliferation status, which is considered as an important feature of tumor cells. Regional clonogenicity was also examined as another aspect of tumor cells. Based on these results, possible interpretation of PET images obtained with radiolabeled Cu-ATSM, FDG and nitroimidazole probes was discussed.

2. Materials and methods

2.1. Radiopharmaceutical synthesis

^{64}Cu was produced in a small biomedical cyclotron at the Biomedical Imaging Research Center at the University of Fukui, Japan, according to a published method [12]. [^{64}Cu]Cu-ATSM was synthesized by mixing 200 mM of glycine buffer containing ^{64}Cu and H_2ATSM in dimethyl sulfoxide (1:100 by mole ratio), as described previously [6]. The radiochemical purity of synthesized [^{64}Cu]Cu-ATSM was >99%, as evaluated by high-performance liquid chromatography (LC-10ADVP; Shimadzu, Kyoto, Japan) using a reversed-phase column (Cosmosil 5C18-AR, 4.6×50 mm+4.6×150 mm; Nacalai Tesque, Kyoto, Japan) [15]. [^{18}F]FDG was synthesized by the method of Hamacher et al. [16] with an automated [^{18}F]FDG synthesizing system (JFE, Tokyo, Japan). The specific activity of [^{64}Cu]Cu-ATSM was 56 GBq/μmol, and that of [^{18}F]FDG was 20 to 50 GBq/μmol.

2.2. Animal model

Mice were treated in accordance with the animal treatment guidelines of the University of Fukui throughout the experiments. Male C57BL/6 mice (10 weeks old, weighing 20–25 g) were obtained from Japan SLC (Shizuoka, Japan). Approximately 10^7 Lewis lung carcinoma (LLC1) cells suspended in phosphate-buffered saline (PBS) were subcutaneously implanted into the right flank of mice.

2.3. Autoradiographic study

At 3 weeks after the implantation of tumor cells, each mouse was injected intravenously with 92.5 MBq (2.5 mCi) of [^{18}F]FDG, 463 kBq (12.5 μCi) of [^{64}Cu]Cu-ATSM and BrdU (10 μg/g of body weight; Sigma-Aldrich, St Louis, MO, USA) or pimonidazole hydrochloride [1-([2-hydroxy-3-piperidinyl]propyl)-2-nitroimidazole hydrochloride] (60 μg/g of body weight, HP1-100, Hypoxyprobe-1 Kit for the detection of tissue hypoxia; CHEMICON, Temecula, CA, USA). Sixty minutes after the injection, the mice were sacrificed and the tumors were removed. The removed tumors were immediately covered with optimal cutting temperature compound and frozen in methanol cooled with dry ice. They were divided into two sections and frozen, and the cutting surfaces were flattened with a cryostat (Cryocut 1800; Leica, Wetzlar, Germany) and subjected to dual-tracer autoradiography [5]. [^{18}F]FDG images were acquired over 3 min by exposing the frozen sections to an imaging plate (BAS-MP 2040S; Fuji Photo Films, Japan) in a freezer. The imaging plate was scanned with a bioimaging analyzer (BAS-1500, Fuji Photo Films). After waiting 40 h for ^{18}F decay, [^{64}Cu]Cu-ATSM images were acquired over 45 h under frozen conditions, and the imaging plate was scanned. The distributions of [^{18}F]FDG and [^{64}Cu]Cu-ATSM were visualized by Mac-BAS v2.52 software (Fuji Photo Films). The contribution of ^{64}Cu radioactivity to the FDG image (the first autoradiography) was estimated to be around 1%, and the contribution of ^{18}F radioactivity to the Cu-ATSM image (the second exposure) was thought to be less than 0.1%. In each tumor section, the most photostimulated luminescence region was classified as 100%, and the background was defined as 0%. The 0% to 100% range was divided into four parts and colored red (75–100%), orange (50–75%), green (25–50%) and blue (0–25%), while the background was covered black. The colored image was saved in true color TIFF format.

2.4. Immunohistochemical staining with Ki-67 and BrdU

The frozen blocks used for the double tracer autoradiography were thawed, fixed in 10% neutral buffered formalin and embedded in paraffin. The sections used for the immunohistochemical staining were taken from a region 50 μm from the surface exposed for autoradiography. After ^{64}Cu decay, immunohistochemical staining was carried out to detect proliferating cells, using 4-μm-thick serial paraffin