Transporter Research Yields New Discoveries in Life Sciences

Review

Aberrant Uptake of a Fluorescent L-Glucose Analogue (fLG) into Tumor Cells Expressing Malignant Phenotypes

Katsuya Yamada

Department of Physiology, Hirosaki University Graduate School of Medicine; 5 Zaifu-cho, Hirosaki, Aomori 036–8562, Japan.

Received February 5, 2018

Glucose, one of the most fundamental sugar elements, has either D- or L-conformation. Of these, most cells preferentially take up D-glucose as an essential energy/carbon source. Such stereoselective uptake of glucose has been explored by fluorophore-bearing D- and L-glucose analogues. 2-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), the most widely used fluorescent D-glucose analogue, was abundantly taken up into living Escherichia coli cells, whereas no detectable uptake was obtained for 2-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-L-glucose (2-NBDLG), the antipode of 2-NBDG developed as a fluorescent L-glucose analogue (fLG). Interestingly, we found three-dimensionally accumulating tumor cell aggregates taking up 2-NBDLG when they expressed nuclear heterogeneity, one of the major cytological criteria for cells suspected of high-grade malignancy in clinical diagnosis. 2-NBDLG uptake was not detected in aggregates consisting of homogeneous cells and was specifically abolished by phloretin, a broad-spectrum inhibitor against transporters/channels. Preliminary studies have suggested that a combined use of 2-NBDLG, which emits green fluorescence, with 13-[4-[(2-deoxy-D-glucopyranose-2-yl)aminosulfonyl]-2-sulfonatophenyl]-4,5-trimethylene-7,8-trimethylene-1,2,3,4,6,9,10,11-octahydro-4-aza-6-oxa-8-azoniapentacene (2-TRLG), a membrane-impermeable fLG bearing a large red fluorophore, is effective for discriminating malignant tumor from benign cells both in living biopsy specimens endoscopically dissected from patients with early-stage gastric cancer and in ascites fluid of patients with gynecological cancers. Confocal endomicroscopic imaging of a carcinogen-induced cancer in bile duct of hamsters indicated that the fLG uptake pattern well correlated with pathological diagnosis for carcinoma. Safety tests according to Good Laboratory Practice regulations have been successfully completed so far. fLGs are unique fluorescent glucose analogues for identifying and characterizing living cancer cells based on derangements in their transport function.

Key words tumor imaging; fluorescent marker; glucose transport; cancer detection; stereoselectivity; L-glucose

1. INTRODUCTION

In the present review, I discuss the possibility of visualizing the stereo-preference for glucose transport of cells using fluorophore-attached, D- and L-glucose analogues, with a particular focus on the specific uptake of the latter analogue into tumor cells showing malignant phenotypes.

Glucose has long been a target of active studies as the most common monosaccharide (minimum sugar element) found in nature. Nonetheless, cellular transport of glucose is yet to be fully understood. According to German chemist Emil Fischer’s nomenclature, spatial structure of glucose is either D- or its mirror image L-form (Fig. 1). Whereas the former, which we know as the building block of starch, is readily taken up by most if not all cells, the latter is thought unnatural and useless. Indeed, most cells, except for some Gram-negative bacteria, catabolize only D-, and not L-, glucose. Due to its inability to enter into cells, L-glucose was used even as a marker for extracellular space and an index of cellular integrity in both in vitro and in vivo studies.

Nonetheless, looking at evidence for the uptake preference more closely might be worthwhile. Indeed, compared to D-glucose transport, only limited publications could be found for L-glucose transport. In the next section, I summarize the literature on the stereo-preference of cells for glucose transport and mechanisms underlying the selective uptake of glucose into cells.

2. HISTORICAL BACKGROUND

2.1. Early Twentieth Century

For more than 100 years, investigators have focused on elucidating specific mechanisms through which glucose is taken up into cells. In 1914, Shuzo Kozawa, a Japanese scientist at the University of Kiel, reported on permeability of red blood cells for glucose. Kozawa measured changes in hematocrit caused by swelling of red cells taking up hexose. He concluded that glucose concentration in red cells never exceeded that in the medium, suggesting a passive nature of the glucose transport. He also mentioned an important observation that the ability of individual hexose passing through the red cell membrane differed considerably depending on species.

By the mid-twentieth century, these results were confirmed and expanded by many researchers using radio-labelled glucose tracers. Using mostly erythrocytes, LeFevre and Widdas contributed particularly to define basic charac-
Inhibitory effect of D- and L-glucose entry was much lower than that of D-glucose, the especially high rate of glucose uptake compared to saturable changes in the extracellular space of most organs but is excluded from tumors discriminating from sites of inflammation, since both these tissues accumulate FDG abundantly. In 1993, Meng et al. synthesized 2-[18F]fluoro-2-deoxy-L-glucose (referred to as 1-FDG), the mirror image isomer of FDG. They reported distribution of 1-FDG in monkeys by PET imaging, describing, “this sugar analog distributes in the extracellular space of most organs but is excluded from the CNS.” Further, they discussed, “2-[18F]fluoro-2-deoxy-L-glucose may be a useful tracer for quantifying the extracellular space of most organs by PET. Currently, a kinetic model, which will use PET measurements with 1-FDG to quantitate extracellular space in both normal tissues and tumors is under development in our laboratory. This data could be of great value in determining the effect of non-specific mechanisms on glucose accumulation in numerous tissues.”

These PET studies using labeled D- and L-glucose or its analogues are important because they are functional imaging studies of glucose uptake in living human beings. On the other hand, the interpretation of PET data is made macroscopically apart from spatially and temporally divergent actual transports occurring at the single cell level, tempting us to visualize microscopic events.

2.4. The Era of Molecular Cloning

The advancement of glucose uptake into cells such as carrier-mediated (transport starts when the substrate binds to a specific binding site of a transporter or a “carrier,” causing movement of the carrier protein, ultimately allowing the substrate to pass through the membrane), saturable nature (transport activity saturates along with an increase in the substrate concentration), facilitative transport (transport activity is facilitated by the entry of substrate), and competitive inhibition (inhibition of transport activity by a large amount of substrates) by various hexoses.

2.2. Late Twentieth Century

In the early 1970s, Tavera and Langdon reported inhibition of D-glucose transport through human erythrocyte membrane by cytochalasin B with a detailed stoichiometry of the cytochalasin binding. Lin and Spudich soon reported a large difference (>50 times) in inhibitory effect of D- and L-glucose on high affinity binding of cytochalasin B to red cell ghosts. The erythrocyte glucose transporter system is postulated to have a binding site to cytochalasin B in intracellular moiety of the molecule. As such, cytochalasin B has long been used as an effective inhibitor against glucose transport, although it also binds to F-actin filament.

In 1981, Baldwin et al. published a seminal paper in which they compared uptake of D- and L-glucose through a monosaccharide transporter, which was purified from human erythrocytes then reconstituted into phospholipid vesicles. They reported time-dependent inhibitory effect of cytochalasin B on the uptake of D-[14C]glucose, but not on that of L-[3H]glucose. The authors reported also time-dependent changes in the entry of L-[3H]glucose into the vesicles. Although L-glucose entry was much lower than that of D-glucose, the difference became smaller over time due to a linear increase in the L-glucose uptake compared to saturable changes in the D-glucose uptake, suggesting that distinct mechanisms may underlie uptake of these mirror image isomers.

2.3. Investigations with Positron Emission Tomography (PET)

Apart from the cellular level studies, radiolabeled tracers made it possible to use them in vivo. In the late 1970s, Sokoloff, Reivich, Phelps, and others established a method to use 2-deoxy-D-glucose tracers such as 2-[18F]fluoro-2-deoxy-D-glucose (FDG) to image glucose uptake in living subjects by PET. FDG is a radiolabeled glucose analogue wherein the hydroxyl group at the 2-position of D-glucose is replaced with 18F atom. After injection into the bloodstream, FDG is supposed to move from the vascular space to interstitial or perivascular space, then glucose transporters expressed on cells recognize and transport FDG into cells. Similarly to glycolysis of D-glucose, FDG can be phosphorylated by hexokinase to form FDG-6-phosphate. In contrast to D-glucose, however, FDG-6-phosphate is not further metabolized due to the absence of hydroxyl group at 2-position. As a result, in cells wherein phosphatase activity is negligible and the phosphorylated D-glucose is not rapidly degraded nor exported, the labeled tracer FDG-6-phosphate tends to accumulate in the cell. This would make whole body imaging possible depending on import/metabolism/export rates.

FDG-PET has been used successfully for various studies, particularly for identifying certain types of tumors in clinical settings, since it is known that tumors generally have a relatively high rate of glucose uptake compared with normal tissues. Incidentally, as mentioned below, one of the unsettled issues in tumor detection by FDG-PET is how to identify tumors discriminating from sites of inflammation, since both these tissues accumulate FDG abundantly.

Fig. 1. Structures of the Mirror Image Isomers of Glucose and Corresponding Fluorescent Glucose Analogues Bearing Fluorophore at Carbon Position 2

Glucose has four chiral centers at positions 2, 3, 4, and 5. The D or L nomenclature is made according to the orientation of the hydroxyl group at position 5 in Fischer’s projection of glucose in an open chain form. (Color figure can be accessed in the online version.)
in molecular technology through the 1970s enabled researchers to discuss or predict protein function from sequence data. In 1985, Mueckler et al. eventually deduced the amino acid sequence and structure of human glucose transporter protein, the first molecular identity for the glucose transporter.25 Then, Wright's laboratory reported another type of transporter.26 The era of cloning glucose transporters started as summarized in many excellent reviews.1,23–26 In short, in mammals, D-glucose can enter into cells either passively through one of the GLUT family proteins, which have 12 membrane-spanning domains, down a concentration gradient (facilitative transport),26 or actively through Na⁺/glucose cotransporters (symporters) the SGLT family proteins, which have 14 membrane-spanning domains, along with the sodium gradient maintained by Na⁺/K⁺ pump.25

As for SGLTs, apparent sugar affinities (Kₘ) for the cloned transporters were estimated electrophysiologically by recording Na⁺ current by Wright et al.25 They reported that the relative affinity of L-glucose and that of D-glucose to human SGLT1 differed by over two orders of magnitude, suggesting that L-glucose is a very poor substrate for SGLTs.

For GLUTs, in contrast, the measurement of transport activity was much more difficult, because the electrophysiology is not applicable for glucose itself. In 1990, Johnson et al.27 evaluated Kₘ of glucose transporters using acutely dispersed pancreatic islet cells. They compared time-dependent changes in the uptake of of L-[1-³H]glucose with radiolabeled 3-O-methyl-D-glucose, an unmetabolizable D-glucose analogue, demonstrating approximately 10 times difference in the uptake between in the islet cells. Here again, it is noted that the uptake of L-[1-³H]glucose appeared to increase linearly except the initial uptake period for a few seconds. Apparent Kₘ for the L-glucose was not shown possibly due to such a property.7

In this way, radiolabeled glucose tracers seem to provide a strong measure for evaluating the transport when applied to acutely dissociated cells, providing that the cells of interest express transporters homogeneously. However, in actual living organisms, a single cell may express spatially and temporally divergent transport systems that change depending on environmental cues, intercellular signaling, and the site on the single cell.23,28 Moreover, even the substrate preference of GLUT1 is drastically altered by other membrane proteins associating with this transporter.31 In such situations, live imaging could be a powerful tool. However, PET has a limitation for evaluating the dynamism of glucose transport at the cellular/subcellular level, since its spatial resolution is at millimeter range.22

3. MEASUREMENT OF D-GLUCOSE UPTAKE WITH A FLUORESCENT D-GLUCOSE ANALOGUE

At the end of the twentieth century, a new imaging technique emerged for monitoring glucose uptake at the single-cell level. In 1996, Matsuoka25 synthesized 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) as a green fluorescence-emitting tracer of D-glucose for counting living microorganisms (Fig. 1). In contrast to 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (6-NBDG), a pioneering molecule reported by Speizer et al.,33 2-NBDG can be phosphorylated at carbon 6-position like D-glucose, assuring effective trapping of the tracer within the cell after entrance with no further glycolytic process.34

Collaborating with Matsuoka, we have demonstrated that 2-NBDG is taken up into single living mammalian cells through GLUTs in a concentration-, time-, and temperature-dependent manner, showing Kₘ values similar to those reported by Johnson et al.7 for the non-metabolizable D-glucose analogue 3-O-methyl-D-glucose.35 The uptake of 2-NBDG was inhibited by cytochalasin B, which acts as a GLUT inhibitor when used in small doses for a short period, and was competitively inhibited by a large amount of D-glucose.35

4. DEVELOPMENT OF FLUORESCENT L-GLUCOSE ANALOGUES (FLGS)

For the last 20 years, 2-NBDG has been effectively used as a standard fluorescent D-glucose tracer for monitoring D-glucose uptake into living cells36–39 However, fluorescence intensity is an arbitrary measure. Quantification therefore requires an accurate procedure.36 Indeed, precise evaluation of 2-NBDG uptake into living cells is a challenging issue, especially when applied to delicate preparations including excitable cells such as brain cells, wherein the cellular and intercellular activity, cell metabolism, and membrane integrity may change rapidly depending on the experimental conditions.40

To overcome these difficulties, we developed 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-L-glucose (2-NBDLG), the antipode of 2-NBDG, as a control substrate41 (Fig. 1). Our original aim was to examine whether loss of membrane integrity as well as occurrence of non-specific uptake/binding of 2-NBDG could be evaluated by 2-NBDLG, which was expected to have the same physical properties as 2-NBDG except for its chirality. In other words, we thought that 2-NBDLG could be a control substrate that would have a value only when compared with 2-NBDG.41,42

When applied to Escherichia coli, whereas these cells abundantly took up 2-NBDG, no uptake was detected for 2-NBDLG in the same experimental conditions.42 In this case, no detectable 2-NBDLG uptake assures that the experiment was conducted in a condition where the stereospecific uptake of 2-NBDG can be successfully detected. Even when some increase in the fluorescence was detected for 2-NBDLG, the net stereo-specific uptake could well be obtained by subtracting the fluorescence induced by 2-NBDLG from that induced by 2-NBDG.

It is of note, however, that these D- and L-glucose analogues bearing a fluorophore are apparently not the same as D- and L-glucose, respectively. 2-NBDG and 2-NBDLG are 2-deoxy-glucose derivatives. In addition, the fluorophore NBD is much larger than glucose itself.

Interestingly, for example, a 2-deoxy-glucose derivative FDG is, unlike D-glucose, not reabsorbed well in the renal tubules expressing SGLTs.22,23 However, 2-NBDG may have a broader applicability, since it appears to pass through SGLTs.44–47 Also, these results raise a possibility to use this fluorescent glucose analogue for imaging of renal, small intestine, and other SGLT-expressing sites as a complementary imaging technique for PET, although the stereo-preference of SGLTs is yet to be clarified for such analogues.48

4.1. Aberrant 2-NBDDLG Uptake into Tumor Cell Aggregates Showing Nuclear Anomalies To compare the uptake of 2-NBDG and 2-NBDDLG, initially we used neurons
Fig. 2. Confocal Microscopic Images Showing Specific Uptake of Fluorescent L-Glucose Analogue 2-NBDLG into Three-Dimensionally Accumulating Mouse Insulinoma MIN6 Cells, When They Showed Nuclear Heterogeneity

A, Differential interference contrast image of MIN6 cells forming thick spheroids merged with 2-NBDLG fluorescence. Only cells in the upper spheroid (a) exhibited remarkable fluorescence of 2-NBDLG. B, A single optical section of nuclear image with 4′,6-diamidino-2-phenylindole (DAPI) in live-cell condition. Note that upper spheroid (a) exhibited considerable nuclear heterogeneity, whereas the lower one consisted of evenly arranged cells with small nuclei (b). C, Merged image of (B) and the 2-NBDLG fluorescence. Note that small cells in the upper spheroid (asterisk) bearing small nuclei took up 2-NBDLG, indicating that aberrant function and morphological anomalies are not necessarily co-expressed. Images were reproduced with permission from Sasaki *et al.*, *Human Cell*, 29(1), 37–45 (2016).

Fig. 3. Confocal Microscopic Images of 12 Days *in Vitro* (DIV) MIN6 Spheroids Subjected to 100 μM of 2-NBDLG (Green) and 20 μM of 2-TRLG (Red) Mixture for 3 min Followed by Washout

a, Nuclear staining with DAPI in live cell condition. The central core region of spheroids appears necrotic (see also d). b and c, Fluorescence images taken at 2 min after starting washout of the tracers in the green (b, 500–580 nm) and the red (c, 580–740 nm) channel, reflecting entrance of 2-NBDLG and 2-TRLG, respectively. d, Differential interference contrast (DIC) image. e, Overlay of the green, red, and DIC images. f, Overlay of (a) and (e). Cellular heterogeneity is clearly seen by a combination of the two fluorescent colors. Cells indicated by arrows exhibited yellow color at 2 min (e), turned red at 4 min (k). This is because green 2-NBDLG was lost (h, b), possibly due to partial loss of membrane integrity, while red 2-TRLG remained (c, i). If one saw a single 2-NBDLG image (b), cells indicated by arrows would have been misinterpreted as similar to nearby cells. f, DAPI image was merged to (e). g–l, Similar to a–f, but images taken at 4 min after starting washout, demonstrating marked cellular heterogeneity within the tumor spheroids. Numbers of green cells with no red fluorescence, seen in the area surrounding the central core (h, e, h, k), suggesting cells with no apparent uptake at this stage. Bars are common to all panels. Descriptions and images reproduced with permission from Sasaki *et al.*, *Human Cell*, 29(1), 37–45 (2016) and from Sasaki *et al.*, *Human Cell*, 29(1), 138–139 (2016).
acytically dissociated from midbrain nucleus substantia nigra pars reticulata of adult mice, while recording the spontaneous firing activity by patch clamp recording. We chose the GABAergic principal neurons in the nucleus, since they are the key output neurons in the basal ganglia and their firing rate is among the highest in the central nervous system. Since we have reported that the spontaneous firing activity of these neurons changes depending on extracellular oxygen and glucose concentration, our aim was to understand whether these neurons take up D-glucose according to their firing activity using 2-NBDG as a tracer. 2-NBDLG was thus essential for us to exclude a possibility of non-specific entry. After considerable trial using 2-NBDG and 2-NBDLG, however, we noticed that these neurons have more extremely delicate membrane than expected while we recorded only electrically. Indeed, these tracers are very sensitive to a transepidermal flux, and GLUTs. From these and other evidence, we speculate that the 2-NBDLG uptake into the tumor cells occurs by a non-transporter-type mechanism participates in the uptake of 2-NBDLG into the tumor cells.

Consistently with this idea, the 2-NBDLG uptake linearly increased with its concentration (unpublished observation). Interestingly, phloretin almost completely abolished the 2-NBDG uptake. Phloretin, an aglycone of an apple tree polyphenol, is a broad-spectrum inhibitor of such as water channels and GLUTs. From these and other evidence, we speculate that the 2-NBDLG uptake into the tumor cells occurred through a phloretin-inhibitable, non-GLUT, non-SGLT, possibly non-transporter-mediated, mechanism.

### 4.2. Simultaneous Evaluation of 2-NBDLG Uptake and Cellular State
When 2-NBDLG uptake is detected in a cell, it is critical to determine whether the uptake is due to loss of membrane integrity. A combined use of 2-NBDLG with 13-[4-[(2-deoxy-D-glucopyranose-2-yl)aminosulfonyl]-2-sulfonatophenyl]-4,5-trimethylene-7,8-trimethylene-1,2,3,4,6,9,10,11-octahydro-4-aza-6-oxa-8-azoniapentacene (2-TRLG), a membrane-impermeable fLG bearing Texas Red (sulfurphodamine 101 acid), has made this possible. 2-TRLG (MW 768) is a unique red fLG that we developed to monitor to what extent loss of membrane integrity occurs in real time, when used in combination with green 2-NBDLG (or 2-NBDG) (Tables 1A, B). Texas Red is a highly lipophilic, large fluorophore bearing positive and negative charg-
3). As demonstrated, one can identify 2-NBDLG-positive cells with cell states in graded green to red fluorescence (Fig. 3). Heterogeneous 2-NBDLG uptake was visualized simultaneously with 2-TRLG to tumor cells with a varied extent of membrane integrity (Fig. 3). Combination with 2-TRLG to tumor cells with a varied extent of membrane integrity. 2-TRLG, a membrane-impermeable fLG bearing a large red fluorophore Texas Red, is used for monitoring such non-specific uptake due to loss of membrane integrity. 2-TRLG easily enters into living cells when the plasma membrane is damaged regardless of whether they are tumor or non-tumor cells. Typically, administration of the mixture of 2-NBDLG and 2-TRLG makes fluorescent color of membrane-damaged cells yellow due to entry of red 2-TRLG as well as green 2-NBDLG (see also Table 1 for details).

On the other hand, 2-NBDLG (MW 342) bears a relatively small, neutral fluorophore NBD, making it soluble in aqueous solution. Such features of 2-NBDLG allow it to permeate through damaged plasma membrane much faster than 2-TRLG. As a result, various time-dependent changes in fluorescent colors appear when 2-NBDLG is administered in combination with 2-TRLG to tumor cells with a varied extent of membrane integrity. Another feature of 2-TRLG is that l-glucose moiety would minimize interaction of the fluorescent analogue with the l-glucose binding site in conventional glucose transporters or with other glucose-binding proteins followed by subsequent internalization via substrate-protein complex. Practically, by administrating 2-NBDLG and 2-TRLG in a mixture (2-NBDLG : 2-TRLG=5 : 1) to MIN6 cell aggregates, heterogeneous 2-NBDLG uptake was visualized simultaneously with cell states in graded green to red fluorescence (Fig. 3). As demonstrated, one can identify 2-NBDLG-positive green cells that maintain cellular integrity, discriminating from 2-TRLG-positive, yellow to red cells (Figs. 3e, k).

In a slightly damaged cell, the fluorescent color turned to yellow soon after administration, since both green 2-NBDLG and red 2-TRLG were still maintained in the cell after entrance (Fig. 3e, Fig. 4). For a cell with more damaged membrane, the color was red, because small, neutral, and water-soluble green 2-NBDLG had already exited, while large, charged, and water-insoluble red 2-TRLG remained in the cell (Fig. 3e). Along with time after washout of the mixture, even the yellow cells subsequently turned to red due to faster exit of 2-NBDLG compared with that of 2-TRLG (Figs. 3b, h, k, arrows).

For a totally collapsed cell, not only 2-NBDLG but also 2-TRLG rapidly exited, resulting in colorless appearance (Fig. 3k). This demonstrates, unlike the dead cell markers DAPI and propidium iodide (PI), that 2-TRLG is not a marker for dead cells, but for a cell in an intermediate state between healthy and completely dead.

Taken together, the combined use of 2-NBDLG and 2-TRLG provides a unique tool for classifying living tumor cells based on functional expression of aberrant 2-NBDLG uptake, while evaluating the cellular integrity through dynamic changes in the entry and washout of these tracers (Fig. 4, Table 1).

5. APPLICATION OF FLGS TO PRE-CLINICAL AND CLINICAL SETTINGS

To explore the applicability of FLGs in clinical settings, a mixture of 2-NBDLG and 2-TRLG was administered to living cancer cells acutely extracted from abdominal fluid of gynecological cancer patients, and to biopsy specimens such as dissected endoscopically from the stomach of patients with early-stage gastric cancer. All procedures performed were approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine and were in accordance with the 1964 Helsinki Declaration and its later amendments. In preliminary studies, pathological examinations were conducted for the same cells after fluorescence imaging, demonstrating an effectiveness of a mixture of 2-NBDLG and 2-TRLG for identifying cancerous cells, although these results should be verified in a quantitative manner by different groups in strictly controlled conditions.

To test whether FLGs are effective for in vivo imaging of cancer, Yokoyama et al. topically administered FLGs to
bile duct of hamsters. Extrahepatic cholangiocarcinoma was established in hamsters by administering the carcinoen \( N \)-nitrosobis(2-oxopropyl)amine (BOP) for 9 weeks after cholecystoduodenostomy (surgical anastomosis of the gallbladder and the duodenum) with ligation of the extrahepatic bile duct in the distal end of the common duct (CDDB). A probe-based confocal laser endomicroscopy (pCLE) and subsequent pathological examination demonstrated characteristic fLG fluorescence pattern (consisting of bright spots and dark clumps) correlated well with the area diagnosed as carcinoma \textit{in situ} or adenocarcinoma\(^6\) (Fig. 5). Bile duct exhibiting hyperplastic atypia as well as that of control animals showed no such fluorescence pattern.

Compared to the case when a conventional \( d \)-glucose analogue 2-NBDG was applied, 2-NBDLG would achieve a higher signal-to-background ratio at the same dosage due to lower 2-NBDLG entrance into non-cancerous cells surrounding the lesion.\(^5\) All animal studies above were performed in accordance with and approved by the Animal Care and Use Committee of Hirosaki University Graduate School of Medicine and Animal Care and Use Committee of RIKEN Kobe Institute.

Fortunately, safety tests including Ames mutagenicity tests and extended single-dose toxicity studies have successfully been completed both for 2-NBDLG and 2-TRLG, according to Good Laboratory Practice regulations. Result of animal studies and these safety tests suggest the possibility of using 2-NBDLG and 2-TRLG in human \textit{as in vivo} contrast agents to image cancerous cells while minimizing toxicity.

6. FUTURE PROSPECTS

As described in the historical background, \( L \)-glucose is expected to show minimum interaction with living body. Our findings might therefore encourage use of \( L \)-glucose as a drug delivery system. Whereas most fLGs, including 2-TRLG, could not specifically enter into tumor cells in phloretin-inhibitable manner, some fLGs bearing small fluorophores other than NBD could show properties similar to those found for 2-NBDLG.\(^6\) Incidentally, no detectable fluorescence was obtained when fluorophore NBD alone was applied to MIN6 cells (data not shown). These results suggest that a combination of parent \( L \)-glucose moiety and the fluorophore is of essential importance for uptake to occur.

In this review, we introduced unexpected properties of fluorescent \( L \)-glucose analogues for detecting and characterizing various types of cancer cells. Although these analogues and \( L \)-glucose itself differ considerably, it would be a promising approach to develop a cancer detection method based on aberrant preference for sugars.\(^6\) To understand stereo-preference for sugar is a long-standing issue common to living organisms. To our surprise, Shimizu \textit{et al.}\(^5\) reported an \( L \)-glucose-utilizing bacterium. A catabolic pathway including D/L conversion, which is distinct from usual glycolysis, has been proposed in this organism. It is still unknown whether \( L \)-glucose itself can be used as a fuel by malignant tumor cells. Molecular mechanisms underlying the fLG uptake should be clarified as well.

In clinical settings, manipulating cancer cells in live-cell conditions is a challenging task, particularly when fluorescence of individual cells is the measure to be evaluated. Technical issues include a rapid and accurate method for quantifying uptake of individual cells in three-dimensional multicellular structure of cancer.\(^6\) Development of fluorescence endomicroscopy for 2-NBDLG and 2-TRLG might also accelerate the \textit{in vivo} effectiveness of the fLG method. Intensive studies may provide new insights to our fight against cancer.

Acknowledgments This research was supported by Science and Technology Incubation Program in Advanced Regions, Support for Increasing the Value for University Patents, Collaborative Research Based on Industrial Demand, and A-STEP from JST/AMED, Grant-in-Aid for Scientific Research on Priority Areas (20019003, 20056001), Research Funds from Research Foundation for Opto-Science and Technology, and Grant for Hirosaki University Institutional Research.

I am grateful to Drs. Toshihiro Yamamoto, Yuji Otsuka, and Tadashi Teshima (Peptide Institute) for their extensive contribution on synthesizing fLGs, Profs. Hideaki Matsuoka.
Conflict of Interest The author declares the following competing financial interests: The author received Grants from the Japanese government for developing potential cancer diagnostic agents, including Science and Technology Incubation Program in Advanced Regions from JST, Collaborative Research Based on Industrial Demand and A-STEP from JST/AMED, and is an applicant for multiple patents including WO2012/133688 with Peptide Institute. The author assigned ownership of the patent to Hirosaki University. A collaboration was done with Mauna Kea Technologies for use of fLG in fluorescence endomicroscopy. A research fund was provided also from Nihon Medi-Physics and Hamamatsu Photonics.

REFERENCES

1) Chen LQ, Cheung LS, Feng L, Tanner W, Frommer WB. Transport of sugars. *Annu. Rev. Biochem.*, **84**, 865–894 (2015).
2) Fischer E. Syntheses in the purine and sugar group. *Nobel Lecture*, December 12, 1902.
3) Breslow R, Cheng Z-L. α-Amino acids catalyze the formation of an excess of α-glyceraldehyde, and thus of other D sugars, under credible prebiotic conditions. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 5723–5725 (2010).
4) Sasajima K, Sinskey AJ. Oxidation of L-glucose by a pseudomonad. *Biochim. Biophys. Acta*, **5723–5725** (2010).
5) Shimizu T, Takaya N, Nakamura A. An L-glucose catabolic pathway in *Paracoccus wayi* in carbohydrate assimilation, distribution, metabolism, and homeostasis. *J. Cell Biol.*, **91**, 231–256 (1980).
6) Rudney H. The utilization of L-glucose by mammalian tissues and bacteria. *Science.*, **122**, 113–140 (1949).
7) Johnson JH, Newgard CB, Milburn JL, Lodish HF, Thorens B. The high Km glucose transporter of islet of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence. *J. Biol. Chem.*, **265**, 6548–6551 (1990).
8) Meng J, Elmaleh DR, Jayeowski S, Weiss S, Alpert NM, Babich JW, Tompkins RG, Fischman AJ. Synthesis of 2-[18F]fluoro-2-deoxy-L-glucose and positron emission tomography studies in monkeys. *Annu. Neurol.*, **23**, 683–693 (2012).
9) Kozawa S. Beiträge zum arteigenen Verhalten der rothen Blutkörnchen. *Biochem. Z.*, **60**, 231–256 (1914).
10) Lefevre PG. Sugar transport in the red blood cell: structure–function relationships in substrates and antagonists. *Pharmacol. Rev.*, **13**, 1–90 (1961).
11) Widdas WF. Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. *J. Physiol.*, **118**, 23–39 (1952).
12) Taverna RD, Langdon RG. Reversible association of cytochalasin B with the human erythrocyte membrane. Inhibition of glucose transport and the stoichiometry of cytochalasin binding. *Biochim. Biophys. Acta*, **233**, 207–219 (1973).
13) Lin S, Spudich Q. Biochemical studies on the mode of action of cytochalasin B. *Cytochalasin B binding to red cell membrane in relation to glucose transport. J. Biol. Chem.*, **249**, 5778–5783 (1974).
14) Carruthers A. Facilitated diffusion of glucose. *Physiol. Rev.*, **70**, 1135–1176 (1990).
15) MacLean-Fletcher S, Pollard TM. Mechanism of action of cytochalasin B on actin. *Cell*, **20**, 329–341 (1980).
16) Brown SS, Spudich JA. Mechanism of action of cytochalasin: evidence that it binds to actin filament ends. *J. Cell Biol.*, **88**, 487–491 (1981).
17) Baldwin JM, Gorga JC, Lienhard GE. The monosaccharide transport of the human erythrocyte. Transport activity upon reconstitution. *J. Biol. Chem.*, **256**, 3685–3689 (1981).
18) Sokoloff L, Reivich M, Kennedy C, Des Rossiers MH, Patlak CS, Pettigrew KD, Sakurada O, Shinohara M. The [14C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. *J. Neurochem.*, **28**, 897–916 (1977).
19) Reivich M, Kuhl D, Wolf A, Greenberg J, Phelps M, Ido T, Casella V, Fowler J, Hoffman E, Alavi A, Som P, Sokoloff L. The [18F]fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man. *Circ. Res.*, **44**, 127–137 (1979).
20) Phelps ME, Huang SC, Hoffman EF, Selim C, Sokoloff L, Kuhl DE. Tomographic measurement of local cerebral glucose metabolic rate in humans with [18F]fluoro-2-deoxy-o-glucose: validation of method. *Ann. Neurol.*, **6**, 371–388 (1979).
21) Ido T, Wan C-N, Casella V, Fowler JS, Wolf AP, Reivich M, Kuhl DE. Labeled 2-deoxy-o-glucose analogs. 18F-labeled 2-deoxy-2-fluoro-o-glucose, 2-deoxy-2-fluoro-o-mannose and 2-C2-deoxy-2-fluoro-o-glucose. *J. Labelled Compounds Radioisotopes.*, **14**, 175–186 (1978).
22) Gambhir SS. Molecular imaging of cancer with positron emission tomography. *Nat. Rev. Cancer.*, **2**, 683–693 (2002).
23) Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ, Lienhard GE, Lodish HF. Sequence and structure of a human glucose transporter. *Science.*, **229**, 941–945 (1985).
24) Hediger MA, Coody MJ, Ikeda TS, Wright EM. Expression cloning and cDNA sequencing of the Na+/glucose co-transporter. *Nature*, **330**, 379–381 (1987).
25) Wright EM, Loo DDF, Hirayama BA. Biology of human sodium glucose transporters. *Physiol. Rev.*, **91**, 733–794 (2011).
26) Cura AJ, Carruthers A. Role of monosaccharide transport proteins in carbohydrate assimilation, distribution, metabolism, and homeostasis. *Compr. Physiol.*, **2**, 863–914 (2012).
27) Pessin JE, Bell GI. Mammalian facilitative glucose transporter family: structure and molecular regulation. *Ann. Rev. Physiol.*, **54**, 911–930 (1992).
28) Thorens B, Mueckler M. Glucose transporters in the 21st Century. *Am. J. Physiol. Endocrinol. Metab.*, **298**, E141–E145 (2010).
29) Simpson JA, Carruthers A, Venucci SJ. Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J. Cereb. Blood Flow Metab.*, **27**, 1766–1791 (2007).
30) Conde C, Silva P, Agasse A, Tavares RM, Delrot S, Geros H. An Hg-sensitive channel mediates the diffusional component of glucose transport in olive cells. *Biochim. Biophys. Acta*, **1768**, 2801–2811 (2007).
31) Montel-Hagen A, Kinet S, Manel N, Mongellaz C, Prohaska R, Batini JL, Delaunay J, Sitbon M, Taylor N. Monge Erythrocyte Glut1 Hg-sensitive channel mediates the diffusional component of glucose transport in olive cells. *Biochim. Biophys. Acta*, **132**, 329–341 (1994).
32) Yoshio K, Takahashi H, Homma T, Saito M, Oh KB, Nemoto Y, Matsuoka K. A novel fluorescent derivative of glucose applicable to human erythrocytes. *Biochim. Biophys. Acta*, **1289**, 5–9 (1996).
33) Speijer L, Haugland R, Kutchai H. Asymmetric transport of a fluorescent glucose analogue by human erythrocytes. *Biochim. Biophys. Acta*, **1289**, 5–9 (1996).
Sodium-glucose transporter-2 (SGLT2; SLC5A2) enhances cellular uptake of aminoglycosides. *PLOS ONE*, **9**, e108941 (2014).

Hato T, Friedman AN, Mang H, Plotkin Z, Dube S, Hutchins GD, Territo PR, McCarthy BP, Riley AA, Pichumani K, Malloy CR, Harris RA, Dagher PC, Sutton TA. Novel application of complement-mediated imaging techniques to examine in vivo glucose metabolism in the kidney. *Am. J. Physiol. Renal Physiol.*, **310**, F717–F725 (2016).

Yamada K, Ji JJ, Yuan H, Miki T, Sato S, Horimoto N, Shimizu T, Seino S, Inagaki N. Protective role of AP-sensitive potassium channels in hypoxia-induced generalized seizure. *Science*, **292**, 1543–1546 (2001).

Yamada K, Inagaki N. Neuroprotection by K<sub>ATP</sub> channels. *J. Mol. Cell. Cardiol.*, **38**, 945–949 (2005).

Yuan H, Yamada K, Inagaki N. Glucose sensitivity in mouse substantia nigra pars reticulata neurons in *vivo*. *Neurosci. Lett.*, **355**, 173–176 (2004).

Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K. Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology*, **127**, 126–132 (1990).

Sasaki A, Nagamoto K, Ono K, Yamamoto T, Otsuka Y, Teshima T, Yamada K. Uptake of a fluorescent t-glucose derivative 2-NBDLG into three-dimensionally accumulating insulinoma cells in a phloretin-sensitive manner. *Hum. Cell*, **29**, 37–45 (2016).

Yamamoto T, Tanaka S, Suga S, Watanabe S, Nagamoto K, Sasaki A, Nishiyama Y, Teshima T, Yamada K. Syntheses of 2-NBDG analogues for monitoring stereoselective uptake of t-glucose. *Bioorg. Med. Chem. Lett.*, **21**, 4088–4096 (2011).

Yamada K, Onoe H, Teshima T, Yamamoto T. PCT Publication WO2012/133688A1 (2012), China Patent 201280015126.5 (2013), Japan Patent 26976–26978 (2008).

Yamada K, Sasaki A, Ono K, Tone K. PCT Publication WO2016/047676A1 (2016).

Nimmerjahn A, Kirchhoff F, Kerr JN, Helmchen F. Sulforhodamine 101 as a specific marker of astroglia in the neocortex in *vivo*. *Nat. Methods*, **1**, 31–37 (2004).

Levi J, Cheng Z, Ghysens O, Patel M, Chan CT, Wang Y, Nama vari M, Gambhir SS. Fluorescent fructose derivatives for imaging breast cancer cells. *Bioconjug. Chem.*, **18**, 628–634 (2007).

Yamada K. PCT Publication WO2015/115781A1 (2015).

Yokoyama H, Sasaki A, Yoshizawa T, Kijima H, Hakamada K, Yamada K. Imaging hamster model of bile duct cancer in *vivo* using fluorescent t-glucose derivatives. *Hum. Cell*, **29**, 111–121 (2016).

Otsuka Y, Sasaki A, Teshima T, Yamada K. Synthesis of α-glucose derivatives emitting blue fluorescence through Pd-catalyzed C–N coupling. *Org. Lett.*, **18**, 1338–1341 (2016).

Yamada K, Sato D, Nakamura T, Amano H, Morimoto Y. Unknown biological effects of t-glucose, ALA, and PUFA. *J. Physiol. Sci.*, **67**, 539–548 (2017).

Yamada K, Ono K, Kojima Y, Takamatsu T. PCT Publication WO2017/170805A1 (2017).