



A strategy for improving FDG accumulation for early detection of metastasis from primary pancreatic cancer: Stimulation of the Warburg effect in AsPC-1 cells



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ABSTRACT

Introduction: Early detection and/or prediction of metastasis provide more prognostic relevance than local recurrence. Direct spread into the peritoneum is frequently found in pancreatic cancer patients, but positron emission tomography (PET) with 2-deoxy-2-fluoro-D-glucose (FDG) is not useful for identifying such metastasis. We investigated a method to enhance FDG accumulation using AsPC-1 human ascites tumor cells.

Methods: ¹⁴C-FDG accumulation was assessed under the following conditions: 1) characteristics of ¹⁴C-FDG transport were examined using phloridzin, a Na⁺-free buffer, and various hexoses, and 2) accumulation of ¹⁴C-FDG was measured in cells that were pretreated with hexose for various time periods, and activity of 6-phosphofructo-1-kinase (PFK-1) was assayed.

Results: ¹⁴C-FDG transport into AsPC-1 cells was mediated primarily by a Na⁺-independent transport mechanism. Aldohexoses such as D-glucose, D-mannose, and D-galactose inhibited ¹⁴C-FDG transport. Cells pretreated with D-glucose, D-mannose, or D-fructose exhibited augmented ¹⁴C-FDG accumulation. Pretreatment with higher concentrations of D-glucose or D-fructose tended to increase PFK-1 activity.

Conclusions: Very little information has been published about the association between PFK-1 and FDG accumulation, and we confirmed the impacts of various hexoses on the activity of PFK-1 and FDG accumulation in AsPC-1 cells. Clarifying the relevance of PFK-1 in FDG accumulation will contribute to developing new features of FDG-PET, because PFK-1 is the main regulator of glycolysis.

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1. Introduction

Pancreatic cancer (PC) is highly aggressive, and its mortality rates have been approximately stable in many European countries, the USA, Japan, and Australia over the last few years [1]. Retrospective analysis has demonstrated that a dramatic change in surgical resectability and patient survival occurs as the size of the PC tumor increases from 20 mm to 30 mm [2,3]. Moreover, PC patient survival is dependent on distant metastasis rather than local recurrence, and therefore, early detection and/or prediction of metastasis provide more prognostic relevance than local recurrence [4].

Direct spread into the peritoneum is not uncommon in PC patients, but positron emission tomography (PET) with 2-deoxy-2-fluoro-D-glucose (FDG) is not useful for identifying such metastasis, especially microscopic peritoneal dissemination (<10 mm) and intraperitoneal seeding, because of the low concentration of cancer cells [5–7].

In acute hyperglycemia, FDG transport may decrease because of competition between FDG and D-glucose [8]. Numerous reports examining the relationship between FDG accumulation and D-glucose have been published [9–13]. In contrast, whether other hexoses are involved in FDG accumulation has yet to be determined, either *in vivo* or *in vitro*.

A future objective of ours is to be able to assess peritoneal metastasis with FDG-PET. Thus, in the present study, we investigated a method to enhance FDG accumulation using AsPC-1 human ascites tumor cells from a PC patient.

2. Materials and methods

2.1. Materials

Reagent grade D-glucose, L-glucose, and D-allose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and D-altrose, D-gulose, D-sorbose, and D-tagatose were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). D-mannose, D-galactose, D-fructose, L-fructose, D-fructose 6-phosphate (D-Fru-6-P), aldolase, α-glycerophosphate

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dehydrogenase-triosephosphate isomerase, and the other chemicals were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The radiolabeled compounds ^{14}C -FDG, ^{14}C -maltose, and ^{14}C -inulin were purchased from American Radiolabeled Chemicals Co. (St. Louis, MO, USA).

2.2. Cell culture

AsPC-1 human ascites tumor cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were incubated in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich Japan) supplemented with L-glutamine (2 mM final concentration) and fetal bovine serum (10% final concentration; Nichirei Biosciences Inc., Tokyo, Japan) at 37 °C in a 10% CO₂ atmosphere. Cells were maintained in 25-cm² cell culture flasks (Nalge Nunc International, Roskilde, Denmark) and were passaged every 4 days using 0.1% trypsin and 0.04% EDTA. AsPC-1 cells (5×10^5 cells per dish) were seeded in a plastic culture dish (60-mm diameter; Nalge Nunc International) and were used in the experiments described below 4 days after seeding. At the time experiments were initiated, the cell density was approximately 2.5×10^6 cells/dish. Before experiments, the culture medium was removed from the dish, and the cells were rinsed once with Dulbecco's phosphate-buffered saline (D-PBS; Sigma-Aldrich Japan).

2.3. Characteristics of ^{14}C -FDG transport into AsPC-1 cells

^{14}C -FDG accumulation experiments were performed according to previously reported methods, with modifications [14,15]. For time-course experiments, AsPC-1 cells were rinsed with D-PBS and then incubated with 2 ml D-PBS containing ^{14}C -FDG (^{14}C -FDG D-PBS; 9.25 kBq/dish) at 37 °C for a specific period (15 to 180 min). The ^{14}C -FDG D-PBS was then removed by aspiration, and the cells were immediately rinsed twice with 5 ml ice-cold D-PBS. Next, the cells were lysed overnight in 2 ml 0.2 M NaOH, and the radioactivity in the NaOH extract was assayed using a Tri-carb 2910TR liquid scintillation analyzer (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). Similar experiments using radiolabeled ^{14}C -maltose D-PBS (9.25 kBq/dish) or ^{14}C -inuline D-PBS (9.25 kBq/dish) were performed as described above.

To examine the characteristics of ^{14}C -FDG transport into AsPC-1 cells, we carried out experiments using ^{14}C -FDG D-PBS in 1 mM phloridzin or Na⁺-free buffer. Phloridzin is a specific inhibitor of the Na⁺-dependent glucose transporter family [16]. In experiments with the Na⁺-free buffer, NaCl and Na₂HPO₄ in the D-PBS were replaced with the same concentrations of choline chloride and K₂HPO₄, respectively. Cells were incubated in phloridzin-containing buffer or Na⁺-free buffer for 60 min, and then the radioactivity present in the cell extract was measured as described above. To suppress ^{14}C -FDG entry via transporters, cells were kept on ice during all incubations.

2.4. Competitive inhibition of ^{14}C -FDG transport

To confirm the competitive effects of hexoses on ^{14}C -FDG transport into AsPC-1 cells, the following modifications were incorporated into competitive inhibition experiments: 1) ^{14}C -FDG D-PBS contained one of the following 11 hexoses (D-/L-glucose, D-mannose, D-allose, D-galactose, D-altrose, D-gulose, D-/L-fructose, D-sorbose, or D-tagatose) at a concentration of 1 mM, and 2) ^{14}C -FDG D-PBS contained an indicated concentration (0.01 to 100 mM) of one select hexose (D-/L-glucose, D-mannose, D-galactose, or D-fructose). After incubation in ^{14}C -FDG D-PBS for 60 min, the radioactivity present in the cell extract was measured as described above.

2.5. ^{14}C -FDG accumulation in pretreated AsPC-1 cells

To examine the effect of pretreating AsPC-1 cells with hexoses on ^{14}C -FDG accumulation, the following modifications were incorporated into the pretreatment experiments: 1) D-PBS contained one of the

following 11 hexoses (D-/L-glucose, D-mannose, D-allose, D-galactose, D-altrose, D-gulose, D-/L-fructose, D-sorbose, or D-tagatose) at a concentration of 1 mM, and 2) D-PBS contained an indicated concentration (0.01 to 100 mM) of one select hexose (D-/L-glucose, D-mannose, D-galactose, or D-fructose). Cells were incubated in one of the above D-PBS solutions for 60 min, and then immediately rinsed twice with D-PBS and incubated with ^{14}C -FDG D-PBS for 60 min. Following cell lysis, the radioactivity present in the cell extract was measured as described above. A pretreatment time of 60 min was chosen because incubations less than 60 min produced no augmentation of FDG accumulation in our preliminary experiments.

2.6. 6-Phosphofructo-1-kinase activity in pretreated AsPC-1 cells

For the time-course experiments, AsPC-1 cells were exposed to 1 or 100 mM D-glucose or D-fructose for a specific period (15 to 60 min), after which the cells were immediately rinsed twice with D-PBS and removed from the culture dish with a rubber policeman.

To measure the 6-phosphofructo-1-kinase (PFK-1) activity in cells pretreated with various hexoses, cells were incubated at 37 °C for 60 min in D-PBS containing D-/L-glucose, D-mannose, D-galactose, or D-/L-fructose at concentrations ranging from 1 to 100 mM, and then removed from the culture dish with a rubber policeman.

The collected cells were homogenized on ice in extraction buffer comprised of 50 mM Tris-phosphate (pH 8.0), 0.2 mM ethylenediamine tetraacetic acid (EDTA), 90 mM potassium fluoride, 10 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride [17]. The homogenates were centrifuged at 29,000 × g for 30 min at 4 °C using a himac CS100FX ultracentrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan).

PFK-1 activity in the supernatants was measured as previously described [14]. The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM MgCl₂, 2 mM DTT, 5 mM (NH₄)₂SO₄, 1 mM D-Fru-6-P, 1.2 mM adenosine 5'-triphosphate, 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 0.8 U/ml aldolase, 0.8 U/ml α-glycerophosphate dehydrogenase, and 8.7 U/ml triosephosphate isomerase in a final volume of 1 ml. The reaction was initiated by addition of the extract (30 μl), and the absorbance at 340 nm was measured at 37 °C using a UV-2200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). PFK-1 activity was calculated as follows: 1 mol D-fructose 1,6-bisphosphate (D-Fru-1,6-P₂) = 2 mol NADH consumed. The NADH content in the homogenates was calculated using the absorption coefficient of NADH (6220 M⁻¹·cm⁻¹) [18].

2.7. Statistical analysis

Values obtained in each experiment are presented as the mean ± SD. Groups were compared using ANOVA followed by Dunnett's *post-hoc* analysis. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Characterization of ^{14}C -FDG accumulation in non-pretreated AsPC-1 cells

^{14}C -FDG transport into AsPC-1 cells was time dependent during the initial 60 min, and was saturated at approximately 35% of the administered dose by 120 min (Fig. 1a). No ^{14}C -inulin was detected in the cells, indicating that no error was present in the methodology that would have resulted in accumulation of radioactivity not associated with ^{14}C -FDG. In addition, radiolabeled ^{14}C -maltose did not accumulate in cells. Maltose must be enzymatically metabolized (e.g., maltase in the small intestine) before entry into cells.

The accumulation of ^{14}C -FDG after 60 min in various conditions is shown in Fig. 1b. Low temperature suppressed total ^{14}C -FDG accumulation to 35% of the control, suggesting that FDG transport is

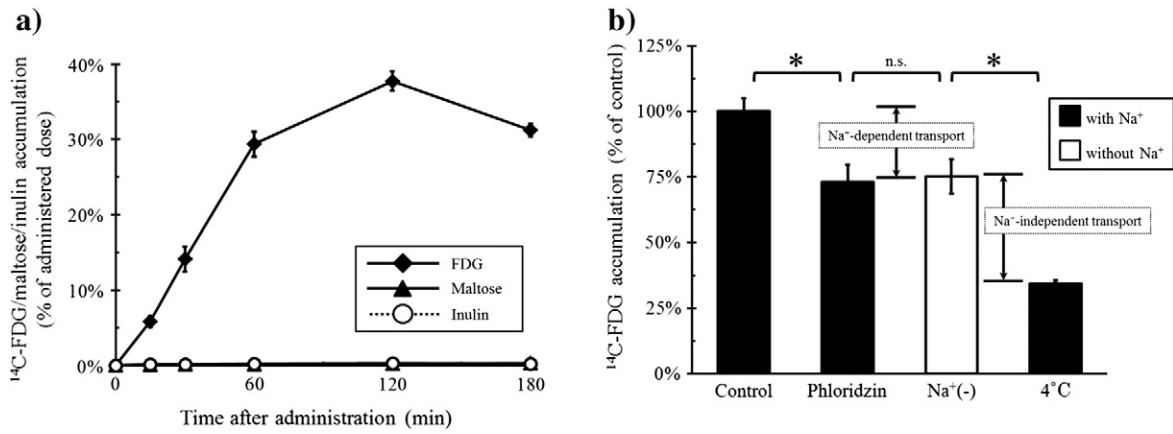


Fig. 1. Characteristics of ¹⁴C-FDG accumulation in AsPC-1 cells. (a) Time course of ¹⁴C-FDG (◆), ¹⁴C-maltose (▲), and ¹⁴C-inulin (○) accumulation. Accumulation (% of administered dose) is plotted against time after administration. (b) ¹⁴C-FDG transport in AsPC-1 cells under various conditions. Black columns and white columns represent ¹⁴C-FDG accumulation in cells incubated in D-PBS and in Na⁺-free buffer, respectively. Values represent the mean ± SD. n = 4–8. *P < 0.05; n.s., not significant.

carrier mediated. Transport of ¹⁴C-FDG via Na⁺-independent and Na⁺-dependent mechanisms was approximately 40% and 25%, respectively. In the presence of phloridzin, a Na⁺-dependent glucose transporter inhibitor, ¹⁴C-FDG accumulation was reduced by approximately 25% relative to the control. ¹⁴C-FDG transport into AsPC-1 cells is thus mediated primarily by a Na⁺-independent transport mechanism.

3.2. Competitive inhibition of ¹⁴C-FDG transport

The results of competitive inhibition experiments of ¹⁴C-FDG transport by D-/L-hexoses (1 mM) are shown in Fig. 2a. Three aldohexoses including D-glucose, D-mannose, and D-galactose inhibited ¹⁴C-FDG accumulation. As L-glucose is not transported via glucose transporter proteins and does not inhibit 2-deoxyglucose transport [19,20],

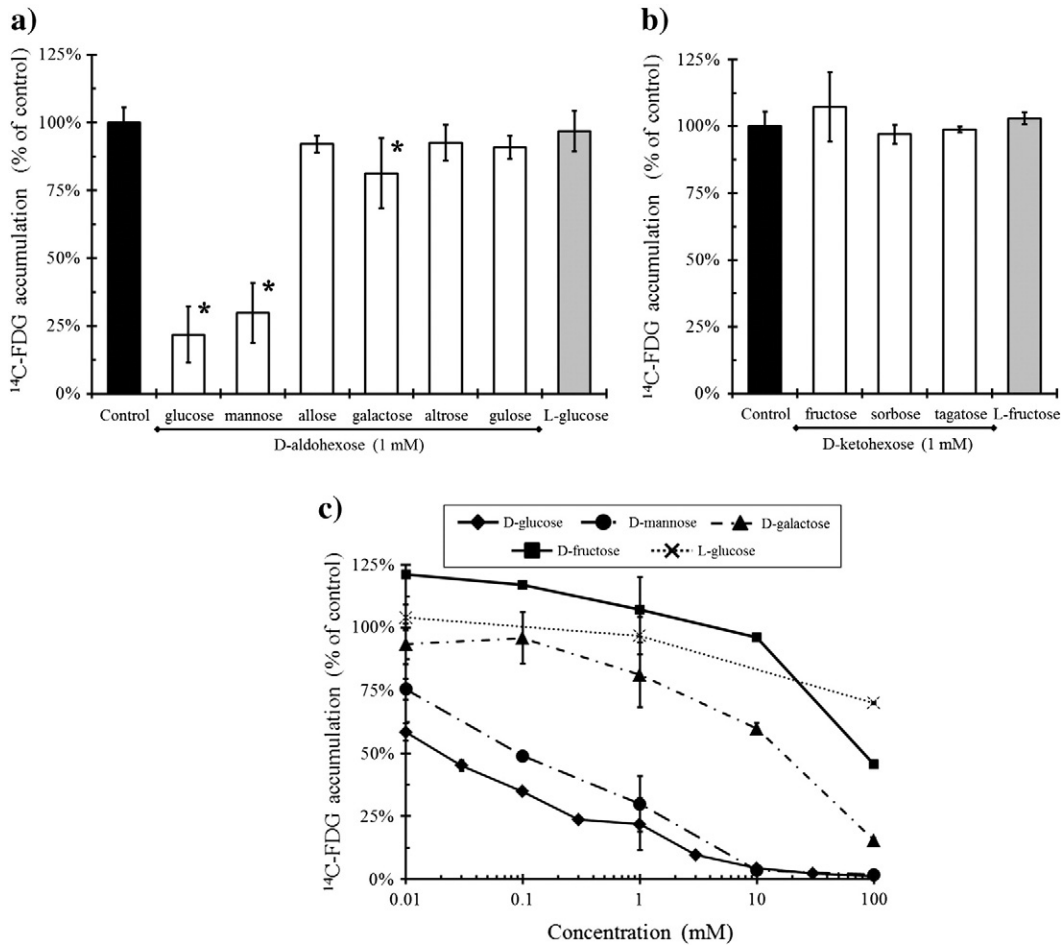


Fig. 2. Competitive inhibition of ¹⁴C-FDG transport by various hexoses. The effect of 1 mM aldohexose (a) or ketohexose (b). ¹⁴C-FDG accumulation was normalized to that of the control. (c) Effect of 0.01 to 100 mM D-glucose (◆), D-mannose (●), D-galactose (▲), D-fructose (■), or L-glucose (×) in ¹⁴C-FDG D-PBS. Values represent the mean ± SD. n = 4–8. *P < 0.05 vs. corresponding control.

^{14}C -FDG transport was not inhibited by L-glucose in AsPC-1 cells. None of the D-/L-ketohexoses we examined had any significant inhibitory effect (Fig. 2b). As shown in Fig. 2c, D-glucose and D-mannose strongly inhibited ^{14}C -FDG transport at all concentrations tested, and D-galactose had an inhibitory effect at concentrations above 1 mM. Lower concentrations (0.01 and 0.1 mM) of D-fructose augmented ^{14}C -FDG accumulation ($121.4 \pm 1.1\%$ and $117.1 \pm 0.5\%$ of control, respectively), and 100 mM D-fructose had an inhibitory effect ($45.7 \pm 1.4\%$ of control).

3.3. ^{14}C -FDG accumulation in AsPC-1 cells pretreated with hexoses

As shown in Fig. 3a and b, ^{14}C -FDG accumulation was augmented in AsPC-1 cells pretreated with 1 mM D-glucose, D-mannose, or D-fructose ($147.8 \pm 18.6\%$, $129.0 \pm 4.3\%$, or $125.0 \pm 15.6\%$ of control, respectively). In contrast, pretreating cells with 1 mM D-galactose, D-allose, D-gulose, or L-glucose had no significant effect on ^{14}C -FDG accumulation. Pretreatment with D-allose resulted in a slight decrease in ^{14}C -FDG accumulation. Ketohexoses other than D-fructose (D-sorbose, D-tagatose, and L-fructose) did not have any effect on ^{14}C -FDG accumulation. The effect of pretreating cells with D-glucose, D-mannose, D-galactose, or D-fructose at concentrations ranging from 0.01 to 100 mM is shown in Fig. 3c. We chose to also include D-galactose because Miller demonstrated that D-galactose increased the velocity of hexose transport [21]. Significant effects were observed in cells pretreated with D-glucose (0.01 to 100 mM), D-mannose (1 to 100 mM), D-galactose (10 and

100 mM), and D-fructose (1 to 100 mM). Pretreating cells with 100 mM D-mannose led to a decrease in ^{14}C -FDG accumulation. Pretreatment with L-glucose had no effect on ^{14}C -FDG accumulation at any concentration tested.

3.4. PFK-1 activity in AsPC-1 cells pretreated with hexoses

Longer pretreatments resulted in greater enhancement of PFK-1 activity in control cells or in cells pretreated with D-glucose (1 mM) or D-fructose (1, 100 mM) (Table 1). An increase in PFK-1 activity relative to the control in cells pretreated with 100 mM D-glucose or D-fructose was observed at all time points examined.

To examine the effect of pretreatment with various concentrations of hexoses, we chose D-glucose, D-mannose, D-galactose, and D-fructose, which augmented ^{14}C -FDG accumulation. As shown in Fig. 4a and b, pretreatment with higher concentrations of D-glucose or D-fructose tended to increase PFK-1 activity. We found no difference in PFK-1 activity relative to the control in cells pretreated with L-glucose (1 mM), L-fructose (1 mM), D-mannose (1 to 100 mM), or D-galactose (1 to 100 mM).

4. Discussion

In this study, ^{14}C -FDG accumulation was augmented significantly compared to controls in AsPC-1 cells pretreated with 1 mM D-glucose, D-mannose, or D-fructose for 60 min. We previously reported that L-

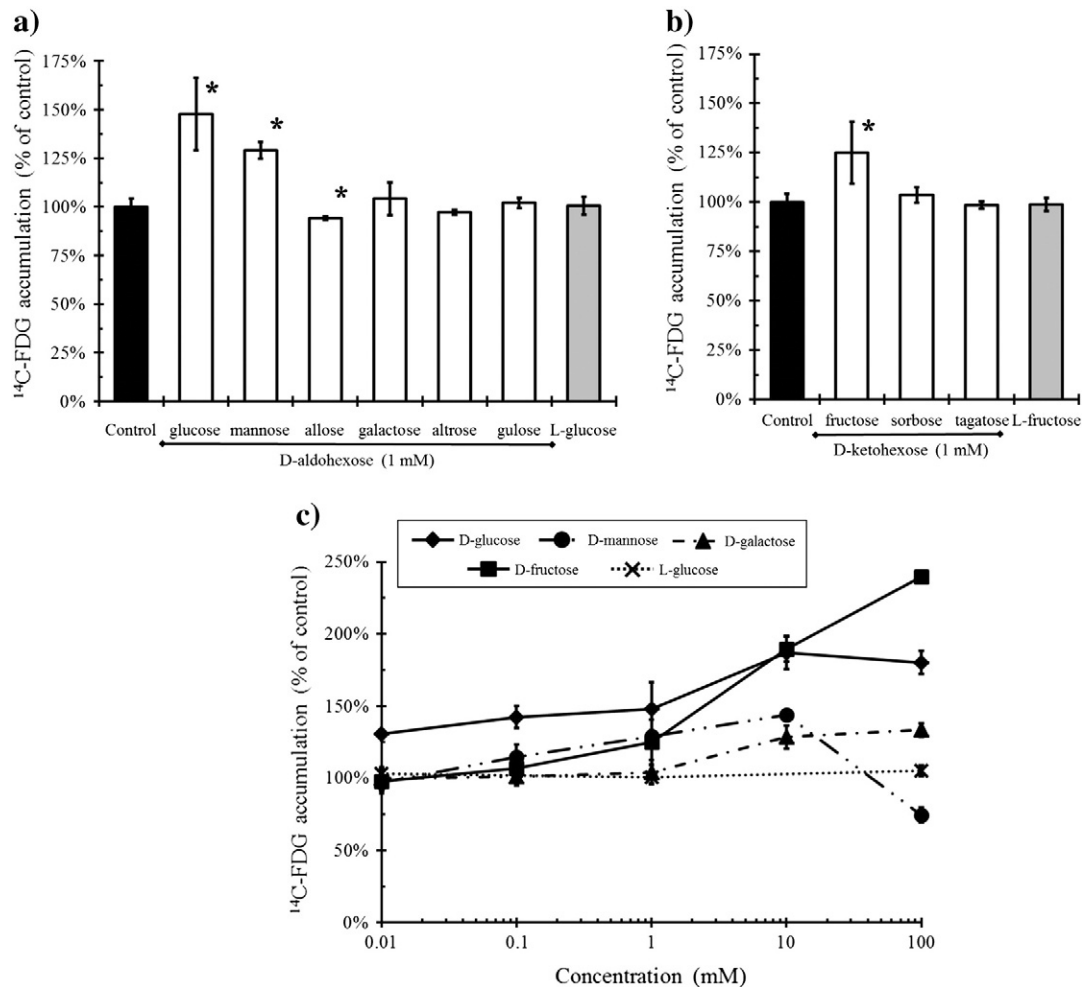


Fig. 3. ^{14}C -FDG accumulation in AsPC-1 cells pretreated with one hexose. Pretreatment with 1 mM (a) aldohexose or (b) ketohexose. ^{14}C -FDG accumulation was normalized to that of the control (black columns). * $P < 0.05$ vs. corresponding control. (c) Pretreatment with 0.01 to 100 mM D-glucose (◆), D-mannose (●), D-galactose (▲), D-fructose (■), or L-glucose (×). Values represent the mean \pm SD. $n = 4$ –8. Significant effects were observed with all of the hexoses we examined.

Table 1
PFK-1 activity in AsPC-1 cells pretreated with 1 or 100 mM D-glucose or D-fructose.

Pretreatment	Concentration (mM)	Pretreatment span (min)	PFK-1 activity (nmol NADH/min/mg protein)	% of PFK-1 activity (Pretreatment/each Control)
Control	0	15	20.88 ± 0.16	–
D-glucose	1	15	22.78 ± 0.34	109 *
	100	15	24.39 ± 0.20	117 *
D-fructose	1	15	23.71 ± 0.34	114 *
	100	15	23.85 ± 0.48	114 *
Control	0	30	24.90 ± 0.87	–
D-glucose	1	30	26.21 ± 0.17	105
	100	30	27.63 ± 0.51	111 *
D-fructose	1	30	25.86 ± 0.40	104
	100	30	27.70 ± 0.04	111 *
Control	0	60	25.66 ± 3.92	–
D-glucose	1	60	23.99 ± 0.35	94 *
	100	60	32.02 ± 0.16	125 *
D-fructose	1	60	28.32 ± 0.27	110
	100	60	37.11 ± 0.94	145 *

The activity of PFK-1 was expressed as the amount of NADH consumed (nmol/min/mg protein). Values represent the mean ± SD. n = 3–6.

* P < 0.05 vs. corresponding control.

tyrosine esters effectively enhance transfer of a radiolabeled L-tyrosine analog, which prompted us to examine effects on FDG accumulation in cells pretreated with hexoses [15]. Similar induction of sugar transport was reported by Miller, who observed accelerated sugar out-flow [21]. Such an acceleration of substrate transport is an obligatory exchange mechanism in amino acid transport [15,22]. Our preliminary experiments, however, did not show acceleration of the out-flow of ¹⁴C-FDG when hexose was present outside the cell, and we hypothesized that augmentation of FDG accumulation is due to elevation in the levels of intracellular glycolytic enzymes, not acceleration in the obligatory exchange.

Control of glycolysis is usually explained in terms of the allosteric properties of three enzymes: hexokinase, PFK-1, and pyruvate kinase. Many (or all) of the glycolytic enzymes in cancer cells have been suggested to be overexpressed [23,24]. Although numerous studies investigating the association between FDG accumulation and glucose transporters or hexokinase have been published, the effects of the main regulator of glycolysis, PFK-1, on FDG accumulation have not been thoroughly explored [25–28].

We focused our investigation on PFK-1, because its phosphorylation step is considered the major rate-limiting reaction and surmounts the regulatory roles of hexokinase and pyruvate kinase [29]. Consistent with the results obtained from Malaisse et al. [30] and Colomer et al. [31], our results demonstrated elevation of PFK-1 activity in AsPC-1 cells pretreated with D-glucose or D-fructose. The roles of these hexoses in the metabolism of cancer cells have been thoroughly investigated. In the early 20th century, D-fructose was shown to be converted into lactic acid more rapidly than D-glucose, and cancer cells were shown to rely on aerobic glycolysis, a phenomenon known as “the Warburg effect” [32,33]. PFK-1 mediates the phosphorylation of D-Fru-6-P to D-Fru-1,6-P₂ using ATP, and is regulated allosterically by the level of D-fructose 2,6-bisphosphate (D-Fru-2,6-P₂) [34]. The level of the allosteric regulator D-Fru-2,6-P₂ is controlled by a bifunctional enzyme, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase). PFK-1 and PFK-2/FBPase share the same substrate, and PFK-2/FBPase is capable of phosphorylating D-Fru-6-P to D-Fru-2,6-P₂ or dephosphorylating D-Fru-2,6-P₂ to D-Fru-6-P [35].

Human PFK-1 is expressed as three isozymes, type-M, -L, and -P, which undergo random tetramerization to produce various homo- and heterotetrameric isozymes, each of which shows different kinetic properties [36]. Active investigations continue to elucidate alterations in gene expression and enzymatic activity of these isozymes regarding high energy production in cancer [29,35,37]. Colomer et al. have reported that PFK-1 from cancer cell extracts is more sensitive to an activation effect by D-Fru-2,6-P₂ than normal cells, although the intracellular levels of D-Fru-2,6-P₂ are equal in both normal and cancer cells [31]. Sanchez-Martinez et al. demonstrated that ascites tumor cells mainly express PFK-1 type-P, whereas type-L is the more abundant isozyme in normal mammary glands [38]. Wang et al. recently reported that enhancement of glycolytic activity depends on the conversion of PFK-1 isozyme from type-L to type-P during the development of cancer [39]. Consequently, PFK-1 type-P may contribute to maintaining a high glycolytic status in cancer cells [40]. Another report, however, showed that PFK-1 activity is negligible for glycolytic flux control in hepatoma cells [41]. The characteristics of PFK-1 should be further clarified in future studies.

Because of the inhibitory effects of PFK-2/FBPase, which regulates the D-Fru-2,6-P₂ level and leads to a reduction in PFK-1 activity, antimetabolic agents have been investigated [29,42,43]. Expression of the PFK-2/FBPase gene is markedly elevated in cancer cells, resulting in stimulation of glycolysis [44]. Clem et al. concluded that their novel class of antimetabolic agents selectively suppresses glucose uptake by

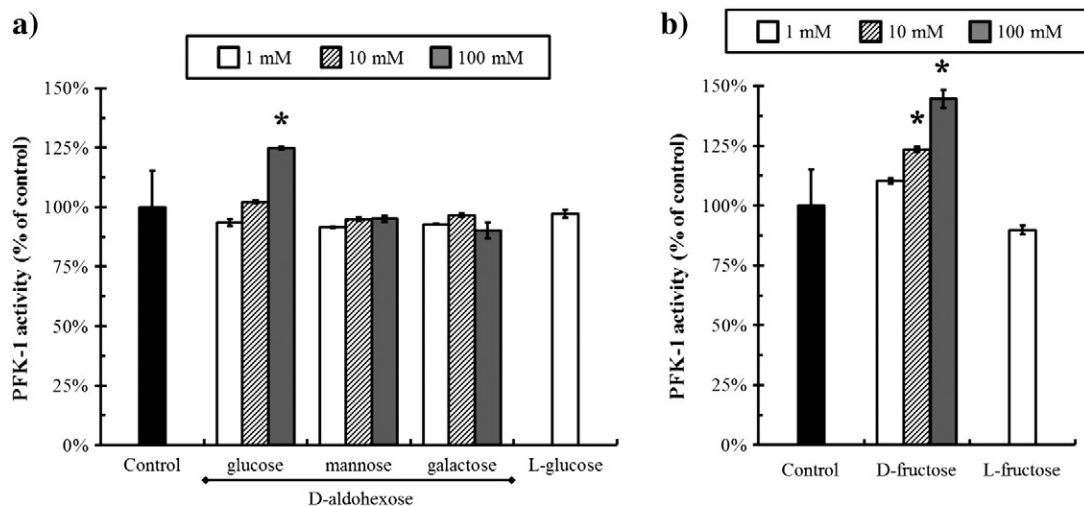


Fig. 4. PFK-1 activity in homogenates of AsPC-1 cells pretreated with one hexose. Pretreatment with 1 mM (white columns), 10 mM (hatched columns), or 100 mM (gray columns) aldohexose (a) or ketohexose (b). PFK-1 activities were normalized to the activity of the control (black columns). Values represent the mean ± SD. n = 3–6. *P < 0.05 vs. corresponding control.

cancer *in vivo* and that glucose uptake measurements by FDG scans can be used as pharmacodynamic endpoints in clinical trials [42]. We expect that FDG-PET will play a role as a predictive modality for the therapeutic effects of PFK-1 inhibitors.

Because the only curative treatment for PC patients is surgical resection, detection and/or assessment of dissemination or seeding in the peritoneum by FDG-PET are important for selecting patients who will benefit from PC resection and for avoiding unnecessary surgery (surgical resection benefits only patients with localized disease) [45]. In this study, we demonstrated that pretreating cells with hexoses augments FDG accumulation, and D-fructose was the most effective at stimulating an increase in FDG accumulation among the hexoses we examined. Nutrient treatment before injection of metabolic imaging agents may effectively enhance tracer accumulation by cancer cells [22]. Oral administration of D-fructose leads to transient elevation of the level of D-fructose in the blood [46].

Very little has been published in the literature about the association between PFK-1 and FDG accumulation, and we confirmed the impact of various hexoses on the activity of PFK-1 and FDG accumulation in AsPC-1 cells. Although PFK-1 in cancer cells has been thoroughly investigated, further studies are needed to clarify the PFK-1 characteristics that are relevant to FDG accumulation. Such investigations will identify new features of FDG-PET; e.g., the detection of microscopic ascites tumor cells and prediction of the effect of novel metabolic agents. In future work, we will use *in vivo* studies to investigate how each treatment with each hexose impacts FDG accumulation in various cancer cells.

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