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Effects of antitumor agents on ^{3}H-2-deoxyglucose uptake in tumor cells and their relationship with the main targets of the antitumor agents

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To investigate the effects of antitumor drugs on ^{3}H-2-deoxyglucose (DG) uptake in tumor cells, we performed DG uptake studies of the short-term treatment of four kinds of antitumor drugs in a cell culture system. The antitumor drugs adriamycin (ADM) and cisplatin (cDDP), which affect on DNA synthesis, did not greatly affect DG uptake, but DG uptake was lowered by antitumor drugs, actinomycin D (AcD) and cycloheximide (CHX), which target the gene expression system. To investigate the mechanism of DG uptake changes, we also tested the effects of some glucose metabolic inhibitors on DG uptake. An inhibitor of glycolytic flow (iodoacetate) lowered DG uptake whereas mitochondrial inhibition increased DG uptake. These results on the inhibition of glucose metabolism indicated that there were two types of factors affecting DG uptake directly; one affects glycolysis and the other affects oxidative phosphorylation. The two antitumor drugs with effects on gene expression were thought to act by the former. The effects of the drug treatments for tumors on DG uptake could be divided into three groups; glycolysis inhibition, mitochondrial inhibition and no relation to glucose metabolism. With the further observations of FDG uptake changes based on this prediction, the biochemical relationship between treatment effects and FDG uptake changes will be clarified.

Key words: FDG, tumor cells-cultured, antitumor-drugs, glucose metabolism

INTRODUCTION

FDG-PET has been widely recognized to be useful for the diagnosis of tumors. Recent reports on the use of FDG-PET have indicated a relationship between FDG uptake and the grade of malignancy of tumors.[1–3] Based on their evidence, tumor diagnosis including the characterization of tumors is expected to be developed.

However, for adaptation of the FDG-PET information to the clinical situation, a problem concerning the effects of tumor treatment on FDG uptake in tumors should be pointed out. To use FDG-PET widely in clinical situations, diagnoses by FDG-PET for not only untreated tumors but also for tumors during treatments should be obtainable, but there is still little information about the effects of tumor treatments on FDG uptake in tumor cells.

In this study, we, therefore, investigated the effects of antitumor drugs with different reaction mechanisms or targets on the FDG uptake of tumor cells. The effects of the treatment of tumors on FDG uptake *in vivo* are thought to be complex. For example, there are several possible effects of treatment on FDG uptake; the effects on tumor cells and the blood vessels of tumors, and the inflammation induced by the treatments of tumors.[4] To understand the effects of antitumor drugs in tumor cells exactly, we therefore used tumor cell culture systems. Antitumor drugs which have different effects on tumors were used, because the effects on FDG uptake of antitumor drugs with different targets in cells are thought to be different. We also investigated DG uptake changes following treatment with inhibitors related to glucose metabolism. Based on these studies, the possible mechanisms of FDG uptake changes caused by chemotherapy are discussed.

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MATERIALS AND METHODS

Drugs used

The antitumor agents adriamycin (ADM) and cisplatin (cDDP) are known to affect DNA synthesis at the nucleus.[5,6] The concentrations of these drugs used in this study were 6 μM (ADM) and 400 μM (cDDP). Actinomycin D (AcD) and cycloheximide (CHX) inhibit RNA synthesis and protein synthesis, respectively.[17] AcD was used at 1 μg/m*l* and CHX at 20 mg/m*l*. Iodoacetate (IAA) is an inhibitor of glycolytic substrate flow at the point of 3-phosphoglyceraldehyde dehydrogenase.[16] IAA was used at 10 μM. 2,4-dinitrophenol (DNP) is an uncoupler of mitochondrial phosphorylation. DNP was used at 200 μM. Sodium azide (azide) is an inhibitor of oxidative phosphorylation in mitochondria. Azide was used at 5 μM.

Cell culture

The human colon adenocarcinoma cell LS180 cells were maintained in a 5% CO_2-humidified atmosphere at 37°C. The cells were grown and maintained in RPMI-1640 medium (GIBCO BRL, NY, USA) supplemented with 10% fetal bovine serum and 50 μg/m*l* streptomycin sulfate throughout the experiment. At confluency, cells were dissociated with trypsin-EDTA (0.05% trypsin; 1 mM EDTA). Tumor cell viability was assessed by the trypan blue dye exclusion test with an Olympus inverted microscope (Tokyo, Japan).

^{3}H-DG uptake

Cells were trypsinized and seeded at a density of 20×10^4 in 24-well plates (Nippon Becton Dickinson, Tokyo, Japan). After 24 h, uptake experiments were performed. The medium used for the assay was serum-free RPMI-1640 including glutamine at 37°C. The medium glucose concentration was 2,000 mg/*l*. After 30 min incubation in the assay medium with or without a drug, 2 μCi of ^{3}H-2DG (Amersham, Tokyo, Japan) was added at the appropriate time followed by further incubation for 60 min. The medium was then removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS). Lysis was performed by incubation at room temperature for 2 h with 500 μ*l* of 0.2 normal NaOH. The whole lysates were then used for measurement of the radioactivity. Lysates were mixed with ACSII (Amersham, Tokyo, Japan) and counted with a scintillation counter (LSC5000, Aloka, Tokyo, Japan). To estimate the number of cells in the wells, those in another 3 wells not treated with NaOH, but were trypsinized and counted by the trypan blue dye exclusion test. The number of cells measured was used for unification of the number of cell including the lysates.

Lactate production

To measure lactate production, cells seeded on 6-well plates were washed twice in ice-cold PBS and then incubated in serum-free RPMI-1640 for 2 h at 37°C, with or without the drugs DNP and IAA. The conditioned medium was centrifuged at 1,000 rpm for 10 min and incubated at 80°C for 5 min, and the medium was then used for the measurement of lactate production. Lactate was measured with the lactate assay kit obtained from Böehringer Mannheim (Mannheim, Germany) according to the manufacturer's protocol.

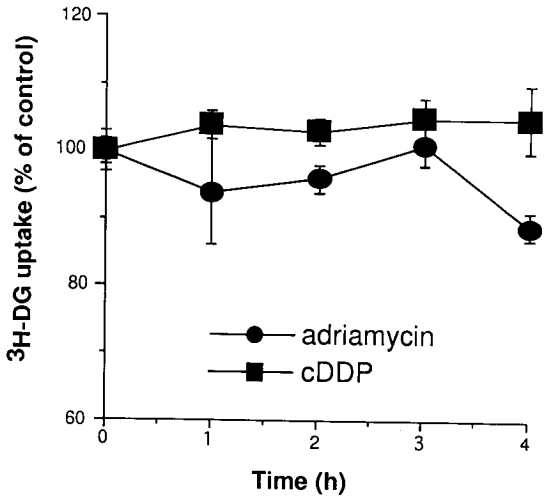

Fig. 1 Effects of antitumor drugs targeting DNA synthesis, ADM and cDDP, on DG uptake in tumor cells; time course study. Both ADM and cDDP didn't affect on DG uptake largely. DATA represent mean percentages of control ± s.d (n = 4).

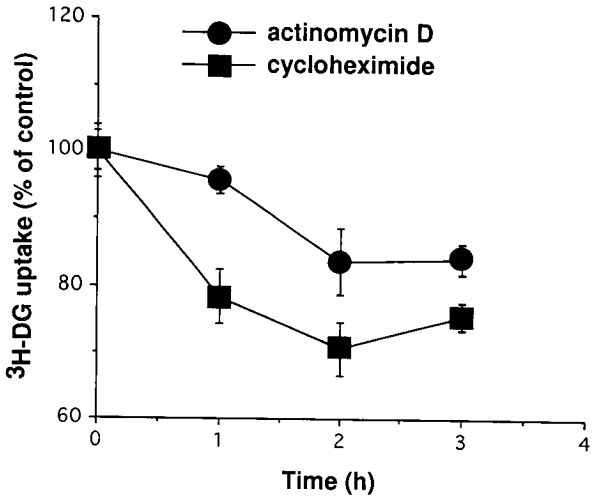

Fig. 2 Effects of antitumor drugs targeting gene expression system, AcD and CHX, on DG uptake; time course study. Both AcD and CHX decreased DG uptake. DATA represent mean percentages of control ± s.d (n = 4).

Intracellular ATP level

Intracellular ATP was extracted with trichloroacetic acid (TCA) and ATP extracted from cells was measured by an ATP assay (Toyo Ink, Tokyo, Japan). Briefly, 10^7 cells were incubated with or without the drugs DNP and IAA, following the time course, and cells were washed and then

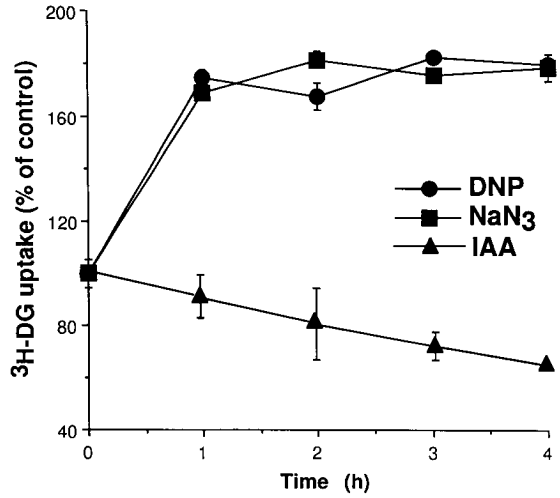

Fig. 3 Effects of three kinds of inhibitors of glucose metabolism on DG uptake. IAA decreased DG uptake in tumor cells linearly, but mitochondrial inhibitors, DNP and azide increased DG uptake till 180% of control. DATA represent mean percentages of control ± s.d (n = 4).

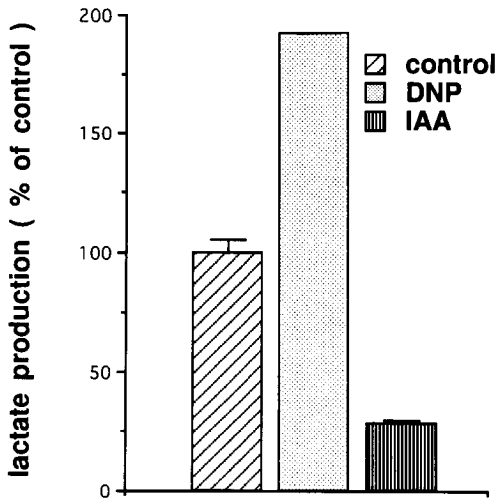

Fig. 4 Lactate production at treatment of DNP and IAA for 2 h. DNP increased lactate production till about twice, but IAA decreased largely. DATA represent mean percentages of control ± s.d (n = 4).

lysed with a final concentration of 1% of TCA for 10 min. Then the lysate was neutralized with 1 M Tris-acetate, pH 7.8. The ATP obtained in the lysate was then assayed by the method of luciferase-luciferin with the kit for the ATP assay. Biological luminescence of the reaction was measured with the luminescence mode of the liquid scintillation counter (LSC5000, Aloka, Tokyo, Japan). The strength of luminescence was calculated with the standard curve between the strength of luminescence and absolute mole of ATP.

Hexokinase activity

Hexokinase activity was measured essentially by the method of Viñuela et al.[7] with some modifications. Briefly, 10^7 cells were incubated with or without DNP for 2 h and washed three times with PBS (−), cells attached to dishes were scraped off in solution I (0.05 M triethanol amine-0.3 M $MgCl_2 \cdot HCl$) and then the lysates were homogenized with a Dounce homogenizer. The homogenates were centrifuged at 15,000 rpm at 4°C, and the supernatants were assayed. Briefly, 2.5 m*l* of solution I, 0.05 m*l* of 15 mM NADP, 0.2 m*l* of 75 mM ATP (pH 7.0), 0.2 m*l* of 7.5 mM glucose and 6 μl of glucose 6-phosphate dehydrogenase (Böehringer Mannheim) were mixed and preincubated in a water bath at 20°C for 5 min. The cell homogenates were added to the mixture and incubated at 20°C for 20 min. After 5 and 20 min, 100 μl of the solution was sampled and absorption was measured at 340 nm. The differences in the absorbance were calculated and hexokinase activity was determined from the standard curve.

^{3}H-3-o-methyl glucose (3-OMG) uptake as levels of glucose transport function

For evaluation of the level of glucose transport function, we used 3-OMG uptake because of the difficulty of measuring the glucose transporter (GLUT) expression level directly only on plasma membrane by other methods such as western blot. 3-OMG is known to bind to cell membrane non-specifically, and the net uptake of 3-OMG into cells is generally estimated as cytocharasin B (CB)-inhibitable 3-OMG uptake. The medium used for this assay was serum-free RPMI-1640 including glutamine at 37°C. After 1 h incubation of cells with or without 200 μM DNP, 3-OMG was incubated for 1 min with or without 50 μM CB in 5×10^5 cells. Then the cells were washed three times with ice-cold PBS and lysed and counted as described above. The net uptake of 3-OMG was calculated as the difference between the radioactivity with or without CB.

RESULTS

We first investigated the effects on DG uptake of ADM and cDDP which target DNA synthesis (Fig. 1). cDDP did not affect DG uptake at all until the 4 h point. ADM treatment did not influence DG uptake until the 3 h point, and only a small but significant decrease in DG uptake was seen by 4 h ($p < 0.05$, compared to the control).

Figure 2 shows the effects of AcD and CHX, inhibitors of gene expression, on DG uptake. Both AcD and CHX produced lowered DG uptake within 1 h of treatment. The DG uptake decreased to about 80% of the control value at 1 h after CHX treatment, and the low level was maintained at 3 h. The amount of DG uptake decrease in CHX treatment was somewhat larger than that in AcD treatment. The differences between DG uptake in AcD and CHX treatment from 1 h to 3 h were significant ($p < 0.01$).

The effects of inhibitors related to glucose metabolism on DG uptake were also investigated (Fig. 3). IAA, which

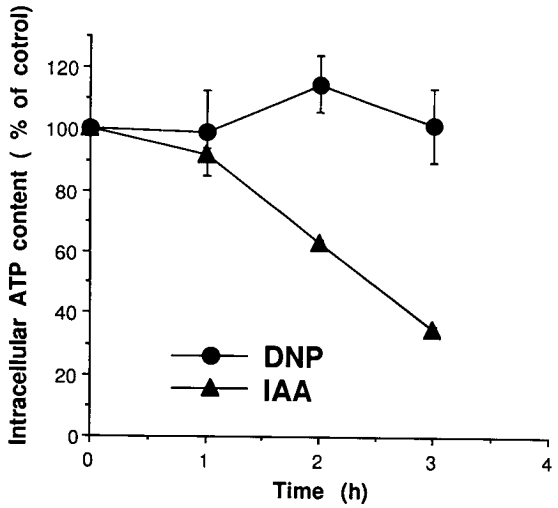

Fig. 5 Intracellular ATP content at treatment of DNP and IAA; time course study. DNP didn't affect intracellular ATP content, but IAA decreased largely. DATA represent mean percentages of control ± s.d (n = 4).

Table 1 Effect of 2,4-dinitrophenol on rate of 3OMG uptake in LS180 cells

	3OMG uptake (dpm/min/100 μg protein)	
	control	2,4-dinitrophenol
Without cytochalasin B	3525 ± 307	4763 ± 669
With cytochalasin B	1511 ± 231	1303 ± 229
Δ	2014 (1.00)	3460 (1.71)

is known to be an inhibitor of glycolytic flow, significantly decreased DG uptake linearly from 1 h to about 60% of the control at 4 h after treatment ($p < 0.01$, compared to control). Conversely, azide and DNP, which are a mitochondrial inhibitor and a mitochondrial uncoupler, respectively, increased DG uptake significantly to about 170% of the control within 1 h, and the high levels of uptake were maintained at 4 h ($p < 0.01$, compared to the control).

We then focused on the differences in DG uptake produced by DNP and IAA treatments to assess the effects of these two drugs on lactate production and intracellular ATP content. Figure 4 shows that DNP treatment induced a significant increase in lactate production to a value about 200% of the control ($p < 0.01$) and IAA treatment produced a decrease to about 30% of the control ($p < 0.01$) (Fig. 4). DNP treatment did not influence the amount of intracellular ATP (Fig. 5). In contrast, IAA treatment significantly decreased lactate production, to about 40% of the control at 3 h ($p < 0.01$).

To investigate the cause of the increase in DG uptake produced by DNP, 3-OMG uptake was measured following DNP treatment as a parameter of GLUT function and hexokinase activity as another parameter of glycolysis. After incubation with DNP, 3-OMG uptake was increased to 171% of the control value (Table 1). In contrast hexokinase activity was not changed after DNP treatment (Fig. 6).

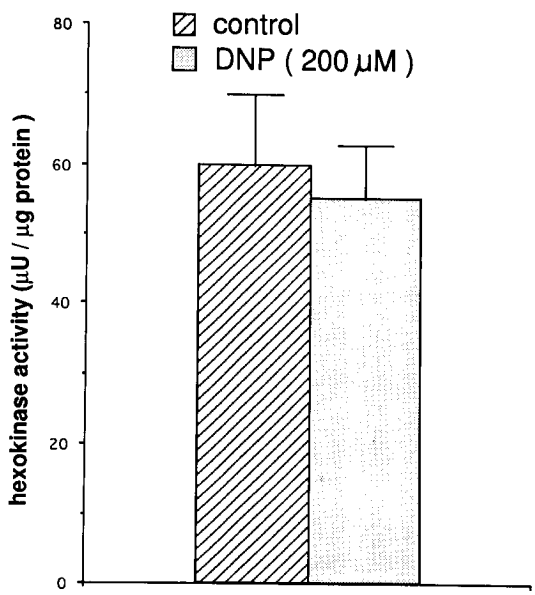

Fig. 6 Effect of DNP on hexokinase activity in tumor cells. DNP didn't affect on hexokinase activity. DATA represent mean hexokinase activity ± s.d (n = 4).

DISCUSSION

FDG-PET study is thought to be the most appropriate assessment of glucose metabolism in nuclear medicine.[8] In the present study, we focused on the effects of some chemotherapeutic agents on DG uptake in tumor cells as an *in vitro* cell culture system. Drug concentrations in interventional studies are known to be an important factor in evaluating the effects of the drugs, especially antitumor drugs, and we therefore determined the concentrations of drugs by IC_{90} tests with 3 days' treatment (for antitumor drugs) or by the concentration generally used.[17]

Two major antitumor drugs, ADM and cDDP, did not largely affect DG uptake. These two drugs are known to be intercalaters of DNA and inhibitors of DNA synthesis. There, therefore, seemed not to be a relationship between DG uptake and DNA synthesis in a short-term treatment with these drugs. Other reports have also indicated that short-term treatment with antitumor drugs targeting DNA did not affect DG uptake.[9,10] In contrast, two antitumor drugs known as inhibitors of gene expression, AcD and CHX, decreased DG uptake. In view of this result, it is thought that antitumor drugs targeting gene expression influence DG uptake. The effect of CHX on DG uptake was greatly than that of AcD. AcD and CHX were generally known to be the inhibitors of RNA synthesis and

protein synthesis, respectively. This is speculated to be because the process of protein synthesis is a final part of gene expression rather than that of RNA synthesis.

DNA synthesis is a major target of many antitumor drugs. Radio-labeled thymidine uptake, which was reported to have a relationship to DNA synthesis, is known to be altered by bleomycin.[10] ^{11}C-thymidine uptake is thought to be affected mainly by antitumor drugs targeting DNA synthesis. Nevertheless, based on the present finding that ADM and cDDP did not largely alter DG uptake in tumor cells, FDG is thought to be one of the tracers that do not have much effect on antitumor drugs.

IAA is thought to be an inhibitor of glycolytic substrate flow at the point of 3-phosphoglyceraldehyde dehydrogenase.[8] The substrates of glycolysis are then accumulated, and the enzymes of the upper part of the glycolysis are thought to be inhibited allosterically. In the present experiment, DG uptake following IAA treatment was decreased linearly up to the 3 h point. This indicated that the DG uptake decrease due to IAA treatment occurred because glycolytic flow was stopped by IAA. The lactate production measurement in this study also indicated that in IAA treatment, the substrate flow of glycolysis was decreased. In contrast, both the mitochondrial inhibitor, azide, and the uncoupler of the electron transport system in mitochondria, DNP, noticeably increased DG uptake. The lactate production measurement also indicated that in DNP treatment the activity of glycolysis was increased. These glycolysis reactions were thought to be a kind of compensation reaction for the inactivation of mitochondria by glycolysis. Intracellular ATP depletion caused by the treatments with DNP and other mitochondrial inhibitors is reported to increase glucose metabolism, especially in a stage of the glycolysis in normal cells.[11,12] In these studies, after an increase in intracellular ATP, the concentration soon returns to normal. In the DNP treatment of the present study, the same phenomenon occurred in tumor cells. In normal cells, GLUT expression level is reported to be increased as a mechanism of the increase in glycolysis.[11] In tumor cells, however, glucose transport is generally known to be already more active than that in normal cells, based on their high levels of GLUT expression.[11,12] Thus in the present study, not only glucose transport activity but also hexokinase activity following treatment with DNP was measured. The 3-OMG uptake, indicating GLUT expression on the plasma membrane, was increased by DNP treatment, but hexokinase activity was not increased by DNP treatment. The increase in DG uptake caused by the compensation reaction was therefore speculated to be due to the increase in the GLUT expression level, as in normal cells. In other words, hexokinase activity in this tumor cell line (LS180) was thought to have the ability to adapt to the increase the level of glucose metabolism.

Considering these findings concerning the inhibitors of glucose metabolism, it can be said that there are two types of factors in the mechanism of DG uptake changes: one is a type like that produced by IAA treatment and another is a type like that produced by DNP treatment. The former factor affects glycolysis and the latter affects oxidative phosphorylation at the mitochondria. Both factors influence the intracellular ATP concentration, at least for in short-term drug treatment. However, the effects of these two drugs on DG uptake were quite different, since DNP caused the compensation reaction by glycolysis but IAA did not. It can therefore be proposed that the decrease in DG uptake due to drug treatments indicates lowered glycolysis activity, and the increased DG uptake does lowered mitochondrial activity. The effects of the drugs AcD and CHX might be to lower glycolysis activity. The effects observed following treatment with these two drugs may be because of a lowered gene expression of proteins related to glycolysis. The two antitumor agents ADM and cDDP that did not greatly affect DG uptake might have had no effect on the whole glucose metabolism system, at least in short-term treatment, but, MIBG, an inhibitor of mitochondria, is also reported to increase FDG uptake.[10] In hypoxia, the rate of glucose uptake and also FDG uptake in tumor cells is known to increase.[15] In this situation, the decrease in oxygen consumption is also thought to cause the mitochondrial activity to decrease.

By dividing the main effects of the antitumor drug treatments on glucose metabolism into three types, namely glycolysis inhibition, mitochondrial inhibition, and no effect on glucose metabolism, the DG uptake changes induced by the treatments might be predictable. With further observations of FDG uptake changes based on this prediction, the biochemical relationship between treatment effects and FDG uptake changes will be clarified.

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