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Reassessment of FDG Uptake in Tumor Cells: High FDG Uptake as a Reflection of Oxygen-Independent Glycolysis Dominant Energy Production

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ABSTRACT. To determine appropriate use of 2-[^{18}F]-fluoro-2-deoxy- D-glucose (FDG) in the diagnosis of malignant tumors, the mechanism of enhanced FDG uptake in tumor cells was reassessed using *in vitro* cultured cell lines and ^{3}H-deoxyglucose (DG), in combination with possible parameters of aerobic and anaerobic energy production. The high DG uptake in the tumor cells reflected the dependency of energy production on anaerobic glycolysis, and paradoxically on low levels of aerobic oxidative phosphorylation in mitochondria. We discuss here factors underlying anaerobic glycolysis in tumor cells. NUCL MED BIOL 24;7: 665–670, 1997. **© 1997 Elsevier Science Inc.**

KEY WORDS. Glucose metabolism, 2-deoxyglucose, Tumor cells-cultured, Cells-cultured, Oxygen consumption rate, Mitochondria

INTRODUCTION

Active glycolysis is generally recognized to be one of the typical characteristics of tumors (11, 30–32). Based on this characteristic, FDG-PET has been widely used in oncology. In clinical studies, high FDG uptake of tumors has been observed and good specificity and high sensitivity were reported (4). FDG-PET has thus been used for qualitative as well as quantitative diagnosis of characteristics, such as malignancy (22), differentiation, cell activity (8) and to forecast treatment efficacy (13, 27). In some cases such as brain tumors (22), lung tumors (16) and lymphoma (23), results obtained using FDG-PET have been reported to show a good correlation with the malignancy of tumors.

The term "malignancy" is used as a clinically useful scale for classification of tumors, and cells with high growth rate, high drug resistance, high metastatic ability, no contact inhibition, low differentiation levels etc. are considered to be malignant. However, it is not a simple matter to determine the direct relationship between "malignancy" and glucose metabolism, especially FDG uptake. In fact, Higashi et. al. (12), reported that FDG uptake showed no relation to proliferative activity one of the major parameters determining malignancy, in tumor cells *in vitro*.

For the appropriate use of FDG-PET for quantitative as well as qualitative diagnosis of malignant tumors, it is helpful to reassess what is indicated by high FDG uptake in malignant tumors. Glucose transporter, hexokinase and glucose 6-phosphate phosphatase are important proteins which directly contribute to the uptake and retention of FDG. This is an anaerobic step and high FDG uptake must be a result of enhanced activity of these proteins. However, it might be modified by conditions such as energy requirement, aerobic mitochondrial activity etc. In the present study, we selected hexokinase activity, dependence on anaerobic glycolysis, mitochondria content and function, growth rate, as direct and indirect parameters possibly affecting FDG uptake in tumors. Using three cultured cell lines including normal as well as tumor cells as model systems, the relationships between ^{3}H-2-deoxyglucose (DG) uptake and the parameters outlined above were investigated.

MATERIALS AND METHODS

Cell Culture

Human colon adenocarcinoma cells (LS180), human fibroblast cells (Fibro) and human microvascular endothelial cells (MvE) were used for all experiments. LS180 cells were maintained in culture dishes in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum. Fibro and MvE cells were purchased from Dai-Nippon Seiyaku Co. Ltd. and were maintained in their recommended culture media obtained from the manufacture. The three cell lines were grown at 37°C in an atmosphere of 95% air and 5% CO_2.

Cell Growth Assay

All three cell lines were seeded at a density of 2×10^4 / ml in 6-well plates (Falcon). From the next day (day 1) for 4 days, the cells were trypsinized and counted using the trypan blue dye exclusion test.

^{3}H-DG Uptake

Normal cells have growth control systems, one of which is contact inhibition. In this study, to exclude the effects of contact inhibition we carefully regulated seeding numbers of cells: cells were trypsinized and seeded at a density of 20×10^4 (LS180), 10×10^4 (Fibro) or 5×10^4 (MvE) in 24-well plates (Falcon). The uptake experiments were performed after 24 h. As consideration of difference of glucose concentration in each medium, the medium used for the assay was serum-free RPMI-1640 including glutamine at 37°C. After 30 min incubation in the assay medium, 2 μCi of ^{3}H-2-deoxy-D-glucose (DG, Amersham International) was added followed by a further incubation for 60 min. The medium was then removed and cells were washed twice with ice-cold phosphate-buffered saline

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(PBS). Lysis was performed by incubation at room temperature for 2 h with 500 μl of 0.2 normal NaOH. Then, the whole lysates were used for measurement of the radioactivity. Lysates were mixed with ACSII (Amersham International) and counted with a scintillation counter (LSC5000, Aloka, Japan). To estimate the numbers of cells in the wells, those in another 3 wells not treated with NaOH were trypsinized and counted by the trypan blue dye exclusion test. For unification of cell number including the lysates, the number of cells measured was used.

Total Hexokinase Activity

Total hexokinase activity was measured essentially by the method of Viñuela et. al. (29) with some modifications. Briefly, 10^7 cells attached to dishes were scraped off in solution I (0.05 M triethanol amine-0.3M $MgCl_2$. HCl) and then the lysates were homogenized with a Dounce homogenizer. The homogenates were centrifuged at 1,500rpm at 4°C, and the supernatants were assayed. Briefly, 2.5 ml of solution I, 0.05 ml of 15 mM NADP, 0.2 ml of 75 mM ATP (pH 7.0), 0.2 ml 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 aliquots of the solution were sampled and absorption was measured at 340 nm. The differences in the absorbance were calculated and hexokinase activity was determined from the standard curve.

Lactate Production

For measurement of 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. The conditioned medium were centrifuged at 1000 rpm for 10 min, incubated at 80°C for 5 min and then used for measurement of lactate production. Lactate levels were measured with a lactate assay kit obtained from Böehringer Mannheim according to the manufacturer's protocol.

Compensation of Mitochondrial ATP Depletion by Glycolysis

To investigate the compensation of mitochondrial ATP depletion by glycolysis, DG uptake was assayed when cells were treated with the uncoupler 2,4-dinitrophenol (DNP). In addition to DG uptake assay, cells were incubated with DNP at a final concentration of 200 μM. Cells were incubated with DNP from 0 h to 4 h, and the DG uptake for 1h were measured at each time from 1h to 4 h. Then cells were harvested and counted as described above.

Mitochondria Content and Membrane Potential

Mitochondria content and membrane potential of cells were investigated by flow cytometry (Shyowa Denko Co., Japan). To determine mitochondria content, the cells were stained by the standard 10-N nonyl-acridine orange (NAO) method as described previously (20). Cell suspensions (5×10^5 cells / ml) in RPMI-1640 were incubated in the presence of 5 μM NAO for 15 min. at room temperature and directly analyzed by flow cytometry. Under these conditions, the staining remained stable for 1 h. To measure membrane potential, the cells were stained by the standard rhodamine 123 (Rh123) method as described previously (19). Cell suspensions (5×10^5 cells / ml) were incubated in the presence of 25 μM Rh123 for 10 min and then washed and resuspended in RPMI-1640 medium. The suspensions were analyzed by flow cytometry and the staining was stable for at least 1 h.

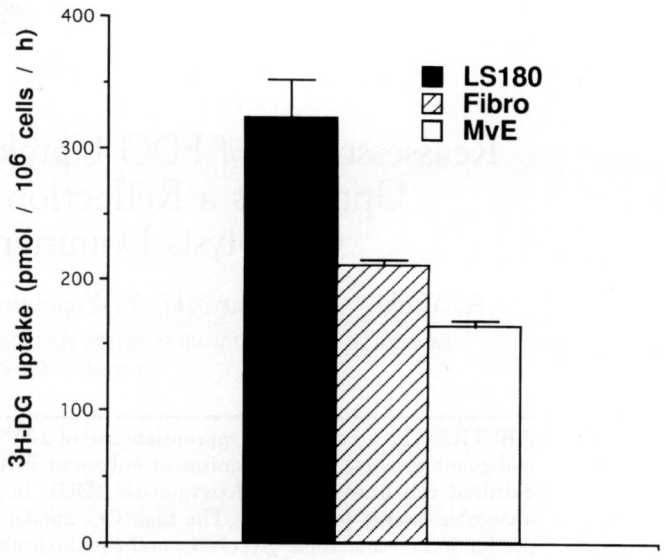

FIG. 1. DG uptake for 1 h in human normal and tumor cell lines. Tumor cells, human colon adenocarcinoma cells showed 1.5–2-fold higher uptake of DG than normal cells. Data represent ^{3}H-DG uptake per 10^6 cells (n = 4).

Oxygen Consumption Rate

The rate of oxygen consumption was measured in sealed chambers containing 700 μl of HEPES-buffered RPMI1640 medium (37°C) saturated with air. The electrode output, initially adjusted to a 100% reading, was monitored for 15 min (YSI-53, Yellow Spring, Ohio). The decline in oxygen tension was linear during this period. Suspensions of 10^7 cells in a volume of 200 μl were added to the oxygen-saturated medium, then oxygen tension of the medium was measured for 15 min. The oxygen consumption rate was calculated from the decreases in oxygen tension.

Electron Transport in Mitochondria as Reduction of Alamar Blue Dye

Alamar Blue (American Biotechnology Laboratory) is an indicator of oxidation-reduction potential, and the dye is reduced at a site in mitochondrial respiration downstream of MTT. We used Alamar Blue dye to monitor mitochondrial electron flow in place of MTT and XTT dye because of their toxicity. A 1/10 volume of Alamar Blue dye was added to culture medium and cells were incubated for 3 h at 37°C. The medium was resuspended and absorption at 570 nm was measured. As a negative control, culture medium only was treated in the same way and the differences in the two absorbance values were calculated as the potential of the electron flow of the cells.

RESULTS

Figure 1 shows DG uptake of the three cell lines for 60 min. DG uptake of the tumor cells was the highest among the three cell lines examined, followed by Fibro, and MvE showed the lowest uptake. There was a difference in DG uptake of about twofold between tumor cells and

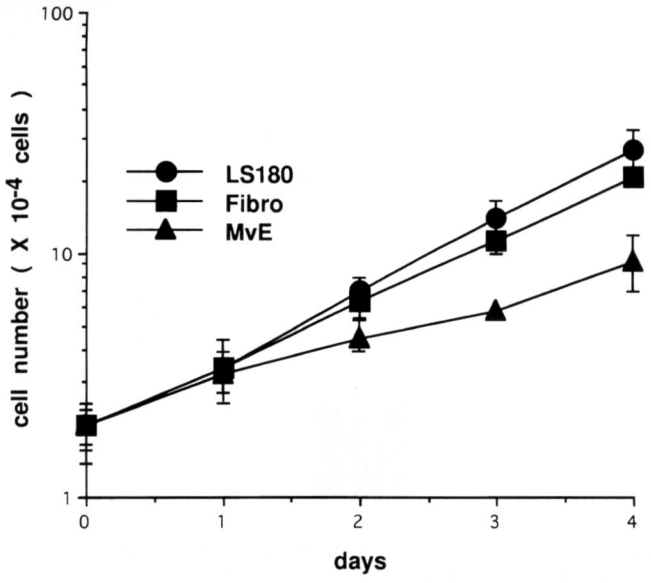

FIG. 2. Growth curves of human normal and tumor cell lines. Tumor cells (LS180) and fibroblast cells (Fibro) showed similar growth rates, but that of microvascular endothelial cells (MvE) was slow. Data represent mean cell number ± SD ($n = 4$).

MvE. The growth curves of the three cell types are shown in Fig. 2. Tumor cells and Fibro showed almost the same growth rate, while that of MvE was significantly slower ($p < 0.01$). All experiments in this study were designed such that the contact inhibition observed in normal cells could be ignored (described in materials and methods), so the growth rates of Fibro and MvE were not influenced by this phenomenon. Tumor cells showed the highest level of hexokinase activity, followed by Fibro, and that of MvE was the lowest (Fig. 3). This order was the same as that of DG uptake.

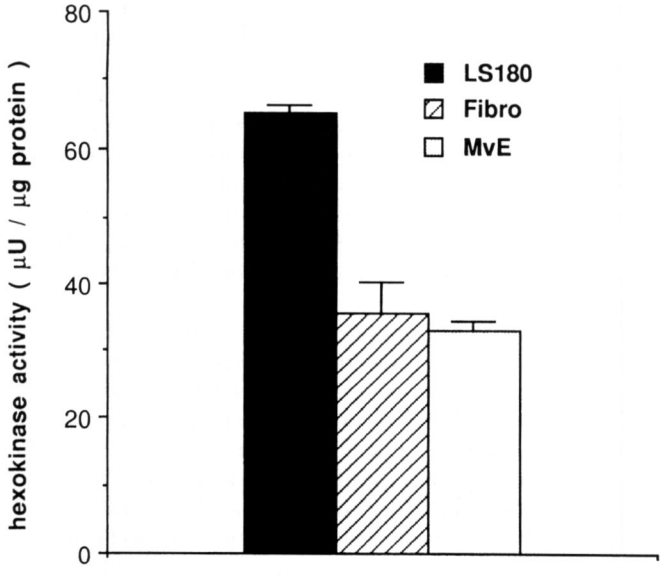

FIG. 3. Hexokinase activities of normal and tumor cells. Tumor cells (LS180) showed the highest hexokinase activity of the three cell types examined. The order of activity was the same as that of DG uptake. Data represent mean hexokinase activity per mg protein ± SD ($n = 4$).

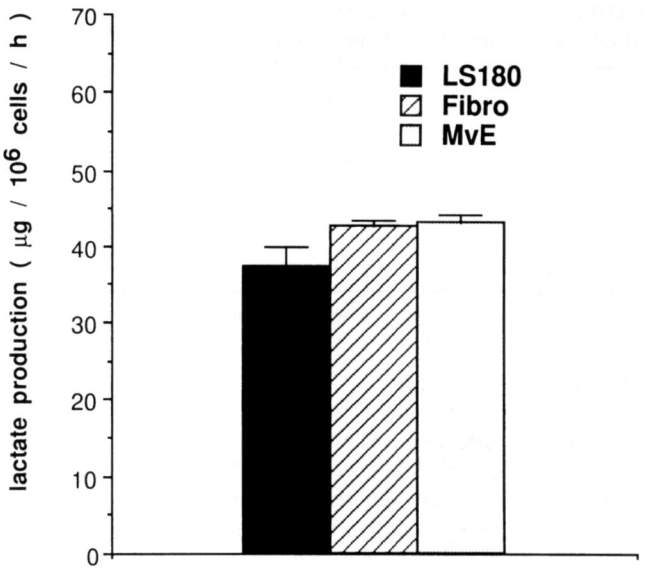

FIG. 4. Lactate production by the three types of cells. No significant differences in lactate production were seen between normal and tumor cells. Data represent mean of lactate production per 10^6 cells per h ± SD ($n = 4$).

The three cell types showed almost the same levels of lactate production (Fig. 4). Treatment with the classical uncoupler DNP is known to cause depletion of ATP derived from mitochondria, and thus induce compensation reaction by glycolysis. We estimated the level of this reaction by changes in DG uptake level compared to the controls. MvE showed the highest level of compensation reaction in this study, and that of tumor cells was the lowest (Fig. 5). This order was the inverse of that of DG uptake. This reaction

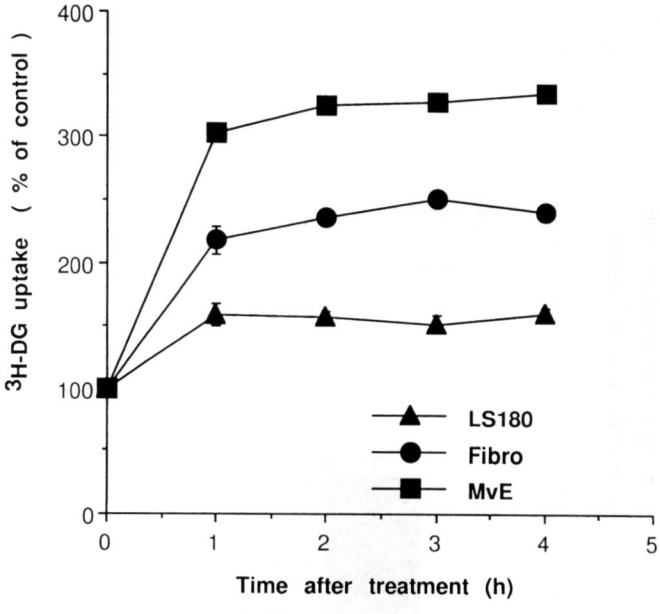

FIG. 5. Compensation reaction against ATP depletion evoked by 2,4- dinitrophenol (DNP). The reaction of tumor cells (LS180), which was 1.6-fold higher than control, was the lowest of the three cells. MvE cells showed 3.3-fold higher reaction than controls at 3 h after treatment with DNP. Data represent mean percentages ± SD ($n = 4$).

TABLE 1. Comparison of Mitochondrial Parameters Among the Three Cell Types Examined

Cell	Relative fluorescence intensity*	
	NAO	Rh123
LS180	99	73
Fibro	15	55
MvE	105	69

* Fluorescence intensity was measured with the range of linear phase.

continued for at least 4 h.

MvE showed the highest number of mitochondria per cell, followed by tumor cells. Fibro showed the lowest number of mitochondria, and the difference between MvE and Fibro was about sevenfold (Table 1). The differences in membrane potential of the three cell types were small, but that of tumor cells was larger than those of normal cells. Tumor cells showed the lowest oxygen consumption rate and electron flow in mitochondria detected as reduction of Alamar Blue dye (Figs. 6, 7). The compensation reaction of mitochondrial ATP depletion and oxygen consumption rate were similar (Figs. 5 and 6). The order was MvE > Fibro > tumor cells.

DISCUSSION

Generally, level of FDG uptake in tumor tissues is higher than that in normal tissues, and tumors have higher growth activity than normal tissues. Thus, it has been suggested that high growth rate as a malignant phenotype might induce high rates of glucose metabolism (i.e., high FDG uptake). However, in the present study, the growth rates of the cells did not always correspond with DG uptake; fibroblasts which have a growth rate as high as that of LS180 tumor cells showed rather low DG uptake similar to that of MvE cells which have a low growth rate. Singh et. al. (26) reported that transformation of a cell line induced higher DG uptake than that in

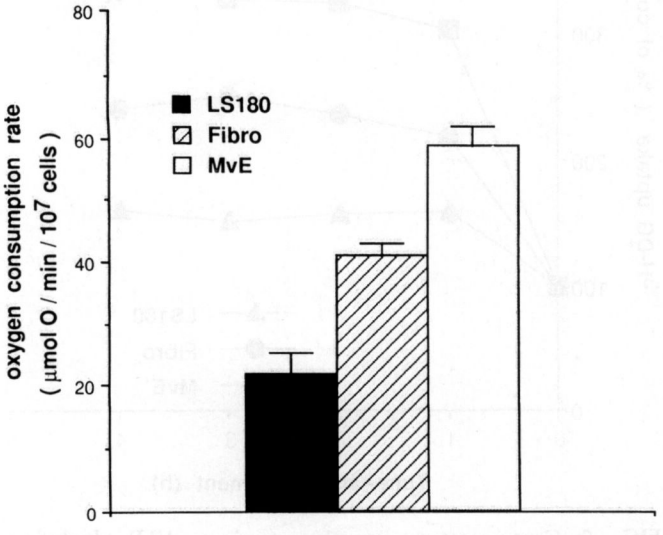

FIG. 6. Oxygen consumption rates of the three human cell lines. Oxygen consumption of MvE cells was about 3-fold higher than that of tumor cells. The order of oxygen consumption rate was the inverse of that of DG uptake. Data represent mean oxygen consumption rate ± SD (n = 3).

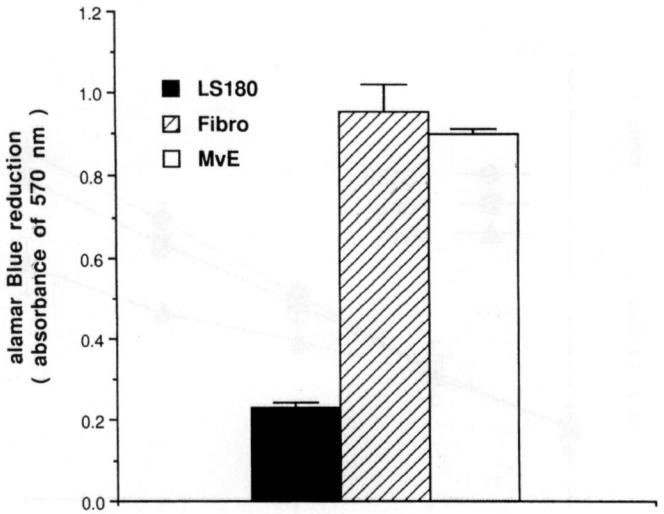

FIG. 7. Electron flow in mitochondria detected as Alamar Blue reduction. Alamar Blue reduction in tumor cells was much lower than that in normal cells. Data represent mean Alamar Blue reduction ± SD (n = 4).

the untransformed cells, while both cell types showed similar growth rates. As mentioned above, Higashi et. al. (12) also reported that FDG uptake had no relation to proliferative activity of tumor cells. Thus, DG uptake is not related to the growth rate of the cells. On the other hand, hexokinase activity, a direct and anaerobic parameter of DG uptake, correlated well with DG uptake in the three cell types examined here. Increments in level of expression of hexokinase were also reported in transformed cells compared to normal cells (15, 17, 24).

Lactate production, another anaerobic parameter, was not correlated with DG uptake. The lactate production levels in all three cells were very similar. Lactate production is known to be increased in proliferating cells compared to quiescent cells (1, 6), but the differences among various kinds of proliferating cells are not clear. Lactate is generally thought to be a product of anaerobic glycolysis. However, DG uptake may not necessarily be correlated with lactate production because several amino acids and nucleic acids are also known to be made from glucose (9). Most studies indicating high lactate production in tumors have been performed in tissues *in vivo*, so we cannot simply compare these results to those of our *in vitro* cell study. Malignant tumor tissues commonly have poor perfusion and areas of low oxygenation. As a result, tumor tissues may show high lactate production and low pH. Lactate production, at least at the cellular and not the tissue level, is not considered to be an index of DG uptake.

The uncoupler 2,4-dinitrophenol causes selective depletion of ATP derived from mitochondria, and increased cytosolic AMP induces allosteric enhancement of the activity of glycolytic enzymes (14, 21). As a result, DNP is considered to raise glycolytic activity until ATP concentration reaches the normal level (5, 25). In the present study, the degree of the increment was significantly different between each of the cell lines examined; high in normal MvE cells and low in LS180 tumor cells. In addition, intracellular ATP content was not changed in LS180 or Fibro during the 4 h period of treatment with DNP (data not shown). Therefore, the differences in the amount of DG uptake among the three cells was thought to indicate respiration activity under normoxic conditions. That is, high DG uptake in tumor cells is thought to be an indication of glycolysis-dominant ATP production.

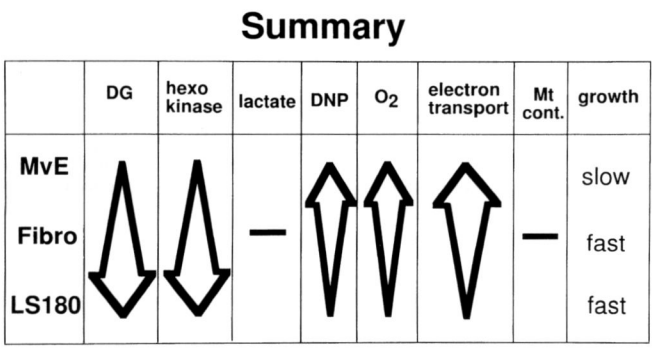

FIG. 8. Summary.

Therefore, our focus shifted to mitochondria. Most tumor cells have reduced numbers of normal mitochondria (2, 18). In our study, although the mitochondria content in tumor cells was larger than that in fibroblasts, oxygen consumption rate and electron transport activity, which indicate mitochondrial activity, were extremely low in LS180 tumor cells. Thus, the mitochondrial function in tumor cells was somewhat damaged or changed when compared to the normal proliferating cells. High DG uptake is a direct indicator of accelerated anaerobic glycolysis, but is also considered to be an indirect indication of suppressed oxidative phosphorylation in mitochondria.

The results of this study (summarized in Fig. 8) indicated that the high DG uptake in the tumor cells reflected the dependency of energy production on anaerobic glycolysis, and paradoxically on the low levels of aerobic oxidative phosphorylation. The balance in tumor cells, and to an extent also in Fibro, was shifted toward glycolysis rather than oxidative phosphorylation. Such changes are general findings in hypoxic tissue, but this was not the case in the present study. Hypoxic cells are commonly seen within tumors *in vivo*, and hypoxia was reported to change FDG uptake *in vitro*, accompanied with increased expression of GLUT (7). Also, in our DNP treatment study (considered as "chemical hypoxia"), increased DG uptake could be seen in tumor cells. However, the higher DG uptake in tumor cells than in normal cells under normoxic culture conditions cannot be considered to be a result of hypoxia. From these considerations and the differences in DG uptake along with oxygen consumption rate even in the two normal cell types, the degree of utilization of glycolysis, related to DG uptake, could be thought to be determined by degree of oxidative phosphorylation, irrespective of whether cells are normal or tumor cells. Although the tumor cells had the same number of mitochondria as normal cells, the dysfunction of mitochondria observed in tumor cells was considered to be one of causes of this shift. In cases where this shift occurred, it is natural that levels of many proteins related to glycolysis, hexokinase as well as GLUT, should be increased. The types of hexokinase expressed in tumor cells are known to be different from those in normal tissues (24). For example, hexokinase of mitochondrial binding type is found in many kinds of tumor cells and highly proliferating normal cells. However, the relationships between the proportions of several types of hexokinase and characteristics of tumor cells have not yet been characterized.

In addition to anaerobic glycolysis-dominant energy production under normxic conditions in tumor cells, FDG uptake could be thought to be increased by hypoxia *in vivo*. Therefore, FDG uptake in tumor cells is considered paradoxically to indicate a high oxygen consumption rate. Low oxygen consumption rates are known to cause expression of malignant phenotypic characteristics such as fragmentation of genomic DNA (3, 10, 28). Therefore, the malignancy indicated by FDG uptake might be related to oxygen consumption rate in tumors.

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