Does FDG Uptake Measure Proliferative Activity of Human Cancer Cells? In Vitro Comparison with DNA Flow Cytometry and Tritiated Thymidine Uptake

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The relationship between ^{3}H-2-fluoro-2-deoxy-D-glucose (FDG) uptake and the proliferative rate of a human ovarian adenocarcinoma cell line (HTB77IP3) was examined in vitro. HTB77IP3 cells were plated and allowed to grow through lag, exponential and plateau phases. Proliferative rate assessed by DNA flow cytometry and ^{3}H-thymidine incorporation was highest in the lag phase and fell significantly as the cells progressed from the exponential through plateau phases. By DNA flow cytometry, the proliferation index (% of S+G2/M phase cells) fell from 65% to 23%. Thymidine uptake per cell also declined, by 82%, from lag to plateau phase. By contrast, ^{3}H-FDG uptake per cell was largely unchanged as the cells progressed through the cell growth cycle. Total ^{3}H-FDG uptake was strongly correlated with the number of viable cancer cells present ($r = 0.957$). Total thymidine uptake, however, substantially underestimated the number of viable cancer cells present. These in vitro differences in tracer uptake suggest that in this adenocarcinoma cell line, FDG measures a substantially different parameter (viable cell number) than thymidine (proliferative rate) and that these differences may result in disparate findings on PET imaging of cancers using these two tracers. Our data for this in vitro system indicate that FDG uptake does not relate to the proliferative activity of cancer cells. However, FDG uptake is strongly related to the number of viable tumor cells.

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The increased rate of glucose utilization seen in most cancers and recognized many years ago as relatively typical of cancers (*1*) is being applied in an increasingly greater extent in nuclear medicine (*2–5*). Fluoro-2-deoxy-D-glucose (FDG) uptake is increased in many malignancies, and this has allowed for the detection of a variety of cancers by PET scanning (*6–18*). Much clinical experience using FDG in vivo comes from studies in brain tumors (*9–13*), lung tumors (*14*), malignant lymphoma (*15–18*) and musculoskeletal tumors (*17–18*) where there appears to be a general correlation between FDG accumulation and the malignant potential of cancers.

There also are data from head and neck cancers in which a relatively clear relationship was seen between the proliferative rate of cells as measured by flow cytometry and the intensity of FDG uptake (*18*). Minn et al. found that a higher proportion of cells in the S+G2/M phases (dividing cells) were seen in patients with high FDG uptake on planar FDG scans versus those with lower uptake (*19*). In addition, a recent report by Haberkorn has suggested that, at least in a subset of head and neck cancer patients, there is a relationship between the proliferative rate of the tumor as defined by flow cytometry and the extent of tumor FDG uptake, with the more rapidly proliferating tumors having greater levels of FDG uptake. Haberkorn's data did not, however, show that there was a relationship between FDG uptake and the proliferative rate in patients with head and neck cancer (*20*). In animal studies, Sweeney and associates have shown that the degree of glycolysis acceleration correlated with the rate of tumor growth in a series of Morris minimum deviation hepatomas (*21*). In rat brain tumors, the high glucose utilization area correlated well with the distribution of BUdR-positive nuclei (*22*). In in vitro studies, it was shown that an accelerated rate of glucose transport is among the most characteristic biochemical markers of cellular transformation induced by the sarcoma virus (*23*). It was also shown that elevated levels of glucose transport and of transporter messenger RNA were induced by ras or src oncogenes (*24*). However, additional evidence also indicated that the change in glucose transport seen in transformed cells was independent of tumor growth rate and transformation specific (*25*).

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Our study was designed to partly evaluate in human tumor cells the question: Is glucose utilization related to the proliferative rate of tumor cells in vitro? It would seem reasonable to expect that this would be true in vitro if it is true in vivo, but the data from Haberkorn and Weber suggest that this may not be consistently true (*20*). Thus, our goal was to determine if FDG uptake measures the proliferative activity of human adenocarcinoma cells in vitro. Proliferative rate was assessed by comparison to relatively standard assays: DNA flow cytometry and ^{3}H-thymidine uptake in tumor cells, while FDG uptake was directly quantified.

MATERIALS AND METHODS

Cell Growth Study

The cell line chosen for examination was HTB77IP3, a human ovarian carcinoma cell line that was originally subcloned from a parental line (HTB77) for its characteristics of intraperitoneal growth in nude mice. This cell line also grows well in vitro as a monolayer (*26*). These IP3 cells were inoculated into 24 multiwell plastic plates with 4×10^4 cells per aliquot. These were then cultured in 1 ml RPMI 1640 media supplemented with 7.5% calf serum, 2.5% fetal calf serum, L-glutamine (2 n*M*), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The incubations were conducted at ambient oxygen concentrations with the presence of 5% CO_2 at 37°C in an incubator (Forma) and the pH of the culture media was 7.4. Cell growth curves were established by sequential replicate samplings from multiple wells (n = 6 per time point) and cell viability was determined daily by trypan blue exclusion analysis after trypsinization of the cells to remove them from the monolayer. To remove attached cells, 0.05% trypsin and 0.02% EDTA were used (Sigma). The trypan blue examination was conducted visually using an inverted Olympus microscope.

Hydrogen-3-FDG Uptake Study

Hydrogen-3-FDG [5.6-^{3}H] (specific activity 30 Ci/m*M* [1.1 TBq/m*M*]) (ARC, St. Louis, MO) was used in the uptake studies. Replicate wells of human ovarian carcinoma IP3 (n = 3) were incubated in 1 ml RPMI media with a glucose concentration of 0.8 mg/ml, a thymidine concentration of 1.85 mcg/ml and 1 μCi (37 KBq) of ^{3}H-FDG. In our experiments, the FDG uptake per cell was measured at many time points (Days 1–7, 9, 11 and 13) when cell number differed significantly. Therefore, we initially studied whether FDG uptake per cell was influenced by cell number. Varying numbers of viable cells (6×10^4 or 6×10^5 cells) were placed in each test tube with the glucose-free medium or the medium with a glucose concentration at 0.8 mg/ml. To these were added 1 μCi of FDG. Triplicate samples of the cells were incubated at 37°C for 1 hr, washed and FDG incorporation was determined.

When cells were incubated with FDG in the glucose-free medium, the FDG uptake per cell was less in the test tubes with a large number of cells (6×10^5 cells) than with smaller number of cells (6×10^4 cells) ($p < 0.001$) (Fig. 1A). Thus, FDG uptake per cell was influenced by the total number of cells incubated in glucose-free medium. Probably, ^{3}H-FDG of a high specific activity (30 Ci/m*M*) accounts for this phenomenon. In the medium with a glucose concentration of 0.8 mg/ml, however, FDG uptake per cell was not influenced by the total number of cells, while absolute values for FDG uptake per cell were reduced significantly by an increasing quantity of glucose in the medium (Fig. 1B). In this situation, 1 μCi of ^{3}H-FDG (3.33×10^{-11} *M*) acted as a radioactive tracer. The glucose concentration (0.8 mg/ml) is equal to normal blood glucose levels for fasting humans. For these reasons, FDG uptake was measured in the medium with a glucose concentration of 0.8 mg/ml. The uptake did not reach equilibrium after 1 hr incubation, with FDG uptake per cell linearly increasing through at least 80 min of incubation.

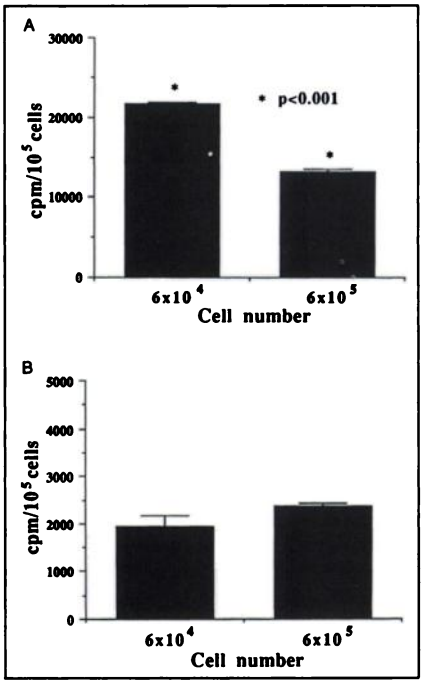

FIGURE 1.
(A) When cells were incubated with FDG in the glucose-free medium, there was a statistically significant difference in FDG uptake between samples with a large number of cells (6×10^5 cells) and those with a smaller number of cells (6×10^4 cells). (B) In the medium with a glucose concentration of 0.8 mg/ml (equivalent to normal human blood sugar levels), FDG uptake per cell was not influenced by the total number of cells that were incubated with FDG, while absolute values for FDG uptake per cell were reduced significantly by increasing the quantity of glucose in the medium (data not shown).

For uptake studies, HTB77IP3 cells were thus preincubated with the RPMI medium in a glucose concentration of 0.8 mg/ml, a thymidine concentration of 1.85 μg/ml and then incubated with the RPMI medium containing 1 μCi (37 KBq) of ^{3}H-FDG in replicate wells (n = 3). Incubations were conducted for 1 hr at 37°C for 1–7, 9, 11 and 13 days after subculture. Thus, varying numbers of cells following subculture were studied for FDG uptake. Incorporation of ^{3}H-FDG was stopped by adding ice-cold Hank's balanced saline solution (HBSS), washing the monolayer three times with HBSS and dissolving the cells in 0.5 ml of 0.3 normal sodium hydroxide plus 10% sodium laurel sulfate. Cell extracts were mixed in 10 ml of scintillation fluid (Hionic Fluor, Packard) and bound ^{3}H activity was measured using a liquid scintillation counter. Calibration standards were also used. Tracer uptake was expressed as cpm per 10^5 viable cells or cpm per total number of cells in a tissue culture well. The FDG uptake per total number of cells in a tissue culture well was felt to be most analogous to FDG uptake per tumor. Cell viability was typically >90%.

Hydrogen-3-Thymidine Uptake Study

The ^{3}H-thymidine uptake studies were performed because measurements of DNA synthesis are often taken as representative of cell proliferative rates. Thus, this assay was performed to estimate DNA synthesis. Again, HTB77IP3 cells in replicate wells (n = 3) were incubated in 1 ml RPMI (glucose concentration 0.8 mg/ml, thymidine concentration 1.85 mcg/ml) containing ^{3}H-thymidine [methyl ^{3}H], 1 μCi (37 KBq), specific activity 45 Ci/m*M* (1.665 TBq/m*M*) for 1 hr at 37°C for 1–7, 9, 11 and 13 days after subculture. Incorporation was stopped by adding ice-cold HBSS, washing the cells three times with HBSS, treating the cells with 2 ml ice-cold 10% trichloroacetic acid (TCA) for 10 min and repeating TCA washes twice (5 min each) (*27*). Subsequently, the TCA insoluble fraction was dissolved in 0.5 ml of 0.3 normal sodium hydroxide plus 1% sodium lauryl sulfate. Samples of the solutions were solubilized in 10 ml scintillation fluid (Hionic Fluor, Packard) and ^{3}H-thymidine concentration was measured using a liquid scintillation counter. Tracer uptake, as cpm per 10^5 viable cells, was determined. In addition, cpm per total number of cells in a tissue culture well was measured.

DNA Flow Cytometry Study

DNA flow cytometry was also performed to independently estimate the proliferation index (the percentage of proliferative cells, S+G2/M phase cells). IP3 cells (approximately 2 × 10^6 cells) cultured as above were trypsinized and washed with PBS at 1–7, 9, 11 and 13 days post-subculture. This pellet was resuspended in 70% ethanol and kept at −20°C until staining. The samples were then treated with 0.5 ml RNase (54.4 mcg/ml) for 30 min at room temperature and stained with 0.5 ml propidium iodide (50 mcg/ml) for 1 hr at 4°C. Flow cytometry was performed with a Coulter Epics C Flow Cytometer. Histograms from the Epics C printout were used to estimate the cell fractions in the GO/1, S and G2/M phases using commercial software (Dean's model). The proliferation index was calculated as [(S+G2+M)/GO/1+S+G2+M)] × 100%.

RESULTS

Initial studies were performed to determine the cell growth curve shape (Fig. 2). Following subculture, IP3 cells progressed through a characteristic growth cycle of lag phase (Days 0–2), exponential phase (Days 3–10) and plateau phase (from Day 11 onward). The lag phase was the time following subculture and replating during which there was little evidence of an increase in cell number. The exponential phase was the period of exponential increase in cell number following the lag phase. Toward the end of the exponential phase, the culture became confluent, and entered the plateau phase.

The proliferation index was determined by DNA flow cytometry. The proliferation index was highest at Day 1 after the cells were plated (Fig. 3). This measure of cell proliferative activity by DNA flow cytometry showed that there was a progressive decline in the proliferation index as the cells went from lag to exponential to plateau phase. Of interest is that even during part of the exponential phase, the proliferative index had declined substantially from that present immediately after the cells were plated. A continuing fall in the proliferative index was seen through the plateau phase.

Hydrogen-3-thymidine uptake was also assessed as a measure of DNA synthesis. Much like the proliferation index assessed by DNA flow cytometry, thymidine uptake per 10^5 viable cells peaked at lag phase and declined rapidly through the exponential phase (Fig. 4). Thus, changes in proliferation index and DNA synthesis assessed by ^{3}H-thymidine showed that the proliferative rate was highest at lag phase with a rapid decline as the cells progressed through the growth cycle. The proliferation index showed a significant positive correlation with thymidine uptake per 10^5 viable cells ($r = 0.958$, $p < 0.01$), as would be expected.

FDG uptake was assessed as described in the Methods section and as shown in Figure 5. The pattern of the FDG uptake per 10^5 viable cells was much different than that seen for thymidine uptake per 10^5 viable cells or the proliferation index. In contrast to proliferative activity, FDG uptake per 10^5 viable cells was lowest in the lag

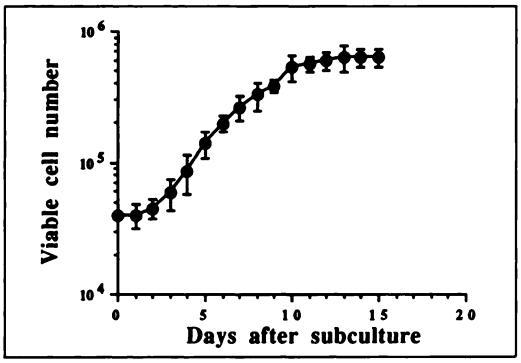

FIGURE 2. Cell growth curve. Following subculture, IP3 cells progressed through a characteristic growth cycle of lag phase (Days 0–2), exponential phase (Days 3–10) and plateau phase (from Day 11 onward) (n = 6 per time point). Note the log scale y-axis.

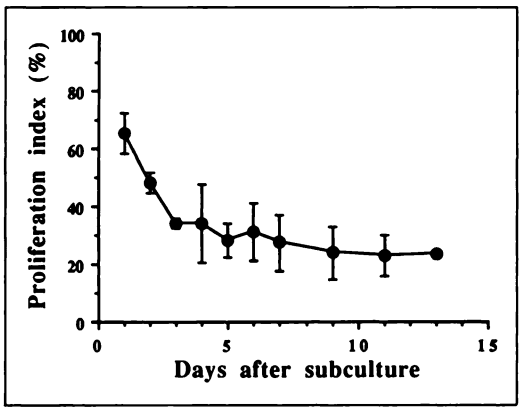

FIGURE 3. Proliferation index measured by DNA flow cytometry (n = 2 per time point) was highest at lag phase and showed a rapid decline as the cells progressed through the growth cycle.

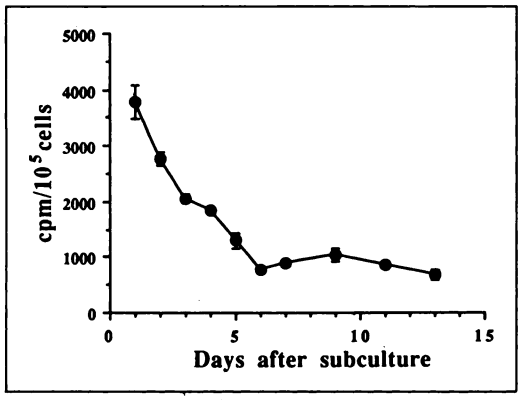

FIGURE 4. Thymidine uptake per 10^5 viable cells (n = 3 per time point) peaked at the lag phase and declined rapidly. Plateau phase ^{3}H-thymidine uptake was significantly less than that in the exponential phase ($p < 0.05$). Note the expected similarity to the proliferation index as assessed by DNA flow cytometry.

phase and increased slightly during the exponential phase. After the exponential phase, there appeared to be a slight decrement in FDG uptake rate in the plateau phase. Overall, the changes in FDG uptake were relatively modest and not clearly related to proliferative activity in malignant cells, at least as determined by DNA flow cytometry or ^{3}H-thymidine uptake, the two most common measures of proliferative rate.

Statistical assessment of the proliferation index during the exponential (35.6% ± 4.8%) versus the plateau phase (26.0% ± 2.7%) showed that there was a substantial difference in proliferation index and a corresponding and significant difference in thymidine uptake/cell between exponential and plateau phase cells. There was, however, no significant difference in FDG uptake per 10^5 viable cells between the exponential (0.243% ± 0.039%) and plateau phases (0.204% ± 0.068%). There was, however,

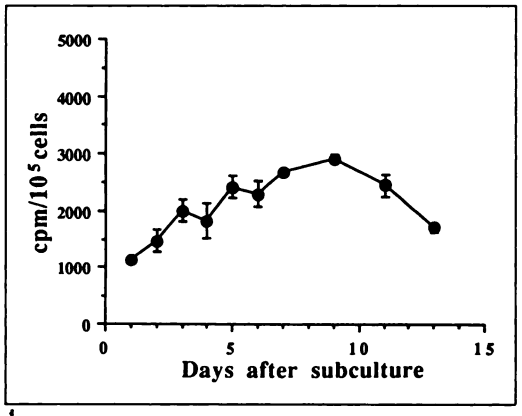

FIGURE 5. FDG uptake per 10^5 viable cells (n = 3 per time point) was lowest during the lag phase, increased slightly during the exponential phase and is much different in appearance than thymidine uptake. Overall, the changes in FDG uptake per 10^5 viable cells were relatively modest, with no significant difference seen between exponential and plateau phase tracer uptakes.

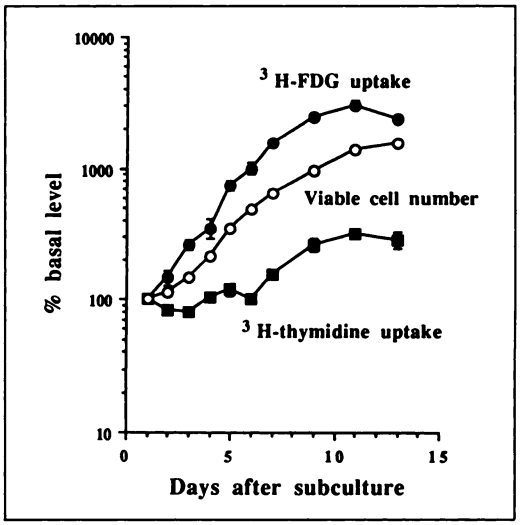

FIGURE 6. FDG uptake per total number of cells in a tissue culture well (n = 3 per time point) increased in parallel with the increase in cell number (n = 6 per time point) following subculture. In contrast, the magnitude of change in total cell uptake seen with ^{3}H-thymidine (n = 3 per time point) was not as great as that with ^{3}H-FDG. Note that the y-axis is expressed as percent basal level and has a logarithmic scale. ● = ^{3}H-FDG uptake; ■ = ^{3}H-thymidine uptake; ○ = viable cell number.

a substantial difference between exponential and plateau phase uptake of thymidine (Fig. 4).

When FDG uptake was examined as cpm incorporated per total number of cells in a well and expressed as a percentage of the basal uptake levels (Day 1), a continuing increase was seen in parallel with an increase in viable cell number following subculture (Fig. 6). Indeed, the morphology of the uptake curve is quite similar to that of the cell growth curve. By analogy, this indicates that FDG uptake is related to viable cell numbers. When FDG uptake per total number of cells in a well was plotted against viable cell numbers, there was a strong positive correlation ($r = 0.957$, $p < 0.01$), which suggests that FDG uptake in HTB77IP3 cells is directly related to viable cell number. By contrast, the magnitude of the change in total cell uptake seen with thymidine was not as great as that with FDG following subculture (Fig. 6). Thymidine uptake per total number of cells in a well showed only a 294% increase from Day 1 to Day 13 following subculture, while the number of viable cells showed a 1577% increase. Thus, increases in total thymidine uptake appeared to underestimate the increase in the number of viable cancer cells present in this system.

DISCUSSION

These in vitro studies demonstrate that a characteristic growth curve can be established with the HTB77IP3 ovarian carcinoma cell line consisting of lag, exponential growth and plateau phases. The changes in the proliferation index and ^{3}H-thymidine uptake show that the pro-

liferative rate is highest during lag phase and declines rapidly through the exponential phase. Indeed, there is roughly an 82% reduction in thymidine uptake per cell during the cell growth cycle in comparing lag phase to plateau phase uptake. In contrast, a lesser variation in FDG uptake was seen during the cell growth cycle, and the changes in FDG uptake were not clearly related to the changes in proliferative activity. However, FDG uptake per total number of cells correlated almost directly with the total number of viable cells in the culture. This result coincides with the finding of Minn et al. who found that FDG uptake was related to intracellular ATP content in vitro (*28*). ATP content was also significantly associated with cell number and viability as measured by the trypan blue exclusion test (*29*). In contrast, the magnitude of the change in thymidine uptake per total number of cells was not as great as that with FDG uptake due to a progressive decline in thymidine uptake per cell during the cell growth cycle.

Measurements of DNA synthesis are often taken as representative of the rate of cellular proliferation. Radioactive thymidine is the usual precursor to estimate DNA synthesis and proliferative rate (*27*). It has been shown that thymidine incorporation into DNA was related to the growth rate of hepatoma as estimated by generation time in animal studies (*30*). In contrast to thymidine, FDG uptake/cell seems to be relatively comparable whether or not the proliferative rate of the cell is high. This apparent disparity may well be due to fundamental differences in the ways that glucose and thymidine are used. It is conceivable that glucose is necessary for basal levels of cellular metabolism whether or not the cell is dividing. By contrast, thymidine utilization appears to be much higher when DNA synthesis is occurring rapidly. Thus, cellular thymidine uptake and FDG uptake can be quite different from each other, depending upon the timing of the cell growth cycle.

Since tumor cell FDG uptake in our study does not appear to be strongly related to the proliferative rate of tumor cells but rather to the number of viable tumor cells, it is somewhat difficult to understand fully how this relates to some of the findings reported by Minn (*19*), Haberkorn (*20*) and DiChiro (*9,10*). It must be realized that our in vitro study does not address the in vivo relationship between the proliferative rate of tumors and FDG uptake. It should also be noted that cellular debris and sampling heterogeneity may make flow cytometry of samples from human tumor biopsies more difficult to perform than flow cytometry of human cancer cell lines. It is certainly possible that in vivo additional factors in growing tumors may cause FDG uptake to be greater in the faster growing tumors than in the slower growing tumors. This area will need additional study since preliminary data recently presented by Minn and Haberkorn suggest that FDG uptake may not be as closely related to tumor cell proliferative rate as initially believed, at least in some tumors (*20,31*). This area clearly needs additional study and similarly designed studies in other cell lines and in vivo may prove informative.

Thus, our data in this human adenocarcinoma cell line suggest that tumor FDG uptake is an excellent indicator of the total number of viable tumor cells, but it is not a good, instantaneous indicator of the rate of growth of the tumor cells. FDG could, however, be used as an indicator of the rate of growth of tumor cells if one had sequential scans showing an increase in FDG uptake. In contrast, ^{3}H-thymidine uptake appears to be reasonably good as an instantaneous indicator of the proliferative rate of tumors, at least if the number of viable cancer cells is known. Extrapolation to in vivo studies suggests that thymidine uptake could underestimate the total amount of tumor present if a substantial portion of the tumor was not growing rapidly. Similarly, ^{3}H-thymidine uptake might overestimate the amount of tumor present if there were relatively few tumor cells that were proliferating rapidly. It is conceivable that the combined assessment of FDG and thymidine uptake at a single time point may provide unique information. This would be possible with PET. This area will need substantial additional study because thymidine is complex to use in vivo due to the possibility of metabolism (*32*) and lower absolute targeting to tumors (*3,4,33,34*).

In summary, in a human adenocarcinoma cell line in vitro, FDG uptake is strongly related to the number of viable cancer cells but is not clearly associated with the proliferative rate of the cells as determined by ^{3}H-thymidine uptake or DNA flow cytometry. In contrast, thymidine uptake is less directly related to viable cell numbers but does reflect tumor proliferative rate. These differences between the tracers may prove useful in future clinical applications. They also suggest that additional clinical and laboratory study is essential to better define the relationship between tumor FDG and thymidine uptake and the proliferative rate of tumors as defined in vitro and in vivo in animal models and in vivo in man. Clearly, our data, in which FDG uptake appears to directly measure the number of viable cancer cells, suggest that PET scanning should be able to provide unique clinical information currently unavailable by other imaging methods.

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EDITORIAL

FDG Accumulation in Tumor Tissue

GLYCOLYSIS AND CANCER CELLS

Enhanced glycolytic rate of cancer cells was first demonstrated more than a half century ago. Originally, decreased respiration and both aerobic and anerobic increased glycolysis were considered to form the most important and specific characteristics of cancer cells (*1*). Considerable efforts have been devoted to elucidate the role of increased glycolysis in malignant cell proliferation. Studies using Morris hepatoma cell lines revealed that the degree of increased glycolysis and the activity of key enzymes in glycolysis such as hexokinase correlated with the rate of tumor growth (*2*). However, none have been conclusive to determine whether a high glycolytic rate is es-

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