In Vitro Comparison of Cell Proliferation Kinetics and Uptake of Tritiated Fluorodeoxyglucose and L-Methionine in Squamous-Cell Carcinoma of the Head and Neck

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Estimation of tumor proliferative activity is important for the optimal management of head and neck cancer. Noninvasive metabolic imaging with PET may complement currently used cytopathologic methods to study tumor proliferation. **Methods:** Fluorodeoxyglucose (FDG) and L-methionine (MET) were studied for their potential to assess in vitro the growth characteristics of three squamous-cell carcinoma (SCC) cell lines established recently in patients with head and neck cancer. A time-course uptake of tritiated FDG and MET was measured over the complete growth cycle of one rapidly growing (UT-SCC-5) and two relatively slower growing (UT-SCC-1A and UT-SCC-9) cell lines. DNA flow cytometry was used for assessment of proliferative activity. **Results:** There was a strong linear relationship for both FDG and MET uptake versus the viable cell number although absolute MET uptake was consistently lower than that of FDG in the exponential and plateau phases of growth ($p < 0.01$), leading MET to underestimate cell number. The pattern of MET uptake followed the flow cytometric changes in the proliferation index (% of S + G2/M cells) in two of three cases (UT-SCC-1A and UT-SCC-5) while a similar, although clearly weaker, relationship was seen with FDG and flow cytometry findings in only one case (UT-SCC-5). **Conclusion:** In these three human SCC cell lines assessed in vitro, FDG is a better marker of cell viability than MET, whereas MET is superior for estimating proliferative activity. Extrapolations of these in vitro data to the interpretation of PET images should be made with caution since tumors may have confounding factors that may affect in vivo tracer uptake.

Key Words: fluorodeoxyglucose; L-methionine; flow cytometry; squamous-cell carcinoma; cell lines

J Nucl Med 1995; 36:252–258

Received Mar. 30, 1994; revision accepted Aug. 4, 1994.

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Squamous-cell carcinoma (SCC) of the head and neck is a challenge to oncologists aiming to select the most appropriate treatment to cure the disease. Optimal current management techniques often include a combination of surgery and radiotherapy, while some less-advanced cases may be amenable to treatment with a single modality (*1*). Neoadjuvant chemotherapy has been proposed for initial treatment of locally advanced SCC in an attempt to enable subsequent organ preserving surgery (*2*). However, the use of chemotherapy outside controlled clinical trials has been criticized (*3*). Clearly, the adequate implementation of various treatment modalities should be based on accurate staging and ideally on individually determined tumor- and patient-related characteristics that account for differences in tumor growth rate, metastatic potential and normal tissue tolerance.

Metabolic imaging with PET appears to complement the clinical information obtained from conventionally available methods to diagnose and stage SCC of the head and neck (*4*). In addition to the utility of detecting cancer in normal-sized lymph nodes (*5–6*) or undetermined residual masses (*7*), PET has been proposed to be useful for follow-up of treatment effects (*8–9*) and in vivo assessment of proliferative activity of tumor cells (*10–14*). Estimation of cell cycle distribution would indeed be valuable in terms of choosing fractionation schemes for therapeutic irradiation perhaps in combination with cycle-specific chemotherapeutic agents (*3,15–17*). Previous clinical studies where ^{18}F-fluorodeoxyglucose (FDG) uptake in SCC of the head and neck was compared to DNA flow cytometry supported a relationship between glucose metabolism and tumor proliferation (*10,12*) although experimental data from this and other laboratories using non-SCC tumor models have been at variance with these initial findings (*18–22*).

Besides FDG, there is limited knowledge on the differential potential of positron emitting radiopharmaceuticals

TABLE 1
Description of Individual Cell Line Growth Characteristics

Cell line	Origin	Grade of differentiation	Passages used	Time to confluency (d)	Doubling time (hr)*
UT-SCC-1A	Gingiva	Moderate–poor	41–43	12	56 ± 1
UT-SCC-5	Tongue	Moderate	34–36	8	31 ± 6
UT-SCC-9	Larynx	Well–moderate	24–26	11	51 ± 2

*± s.e.m.

to evaluate tumor proliferation. For this purpose, uptake of L-methionine, fluorodeoxyuridine and thymidine have been studied in experimental animals and cell cultures (*18–21*). It has been stated that uptake of radiolabeled nucleotide precursors may be used as an index of proliferative activity (*19–21*) and logically clinical studies have pursued to image cancer with positron emitting analogues of these agents (*11*). Presently, the experience is restricted to a few research-oriented facilities which have already shown the feasibility of in vivo imaging of neoplastic human tumors with [2-^{11}C]-thymidine (*11,23–24*).

It is reasonable to expect that heterogeneity in tumor types and variances of methodology to measure cell kinetics and growth explain the current uncertainty in the choice of tracers for assessment of tumor proliferation. We used three SCC cells lines established recently from patients with head and neck cancer to study in vitro relationships between growth rate and metabolism with a focus on FDG and L-methionine, the two most widely used cancer imaging agents for PET (*25*). The in vitro model was thought to be most appropriate in obtaining basic information on growth characteristics without the confounding impact of non-neoplastic cells or competing plasma substrates (*26–27*).

MATERIALS AND METHODS

Cell Lines and Growth Conditions

Two cell lines derived from primary head and neck squamous cell carcinomas and one cell line obtained from a neck metastasis were included in the study. They were established at the University of Turku, Finland from previously irradiated tumors (UT-SCC series, (Table 1)), as described (*28–29*). All cells grew as a monolayer and were maintained in RPMI 1640 media (Sigma, St. Louis, MO) supplemented with 5% calf serum, 5% fetal bovine serum, L-Glutamine (2m*M*), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The glucose and L-methionine concentrations of this medium were 1.0 mg/ml and 15 μg/ml, respectively. All experiments were conducted in a humidified incubator containing 5% CO_2 at 37°C.

Cell Growth Study

Cells in the late exponential phase of growth (80%–90% confluency) were inoculated into 24 multi-well plastic plates with $3 * 10^4$ cells per aliquot in 1 ml of RPMI media. Cell growth curves were established by sequential replicate sampling from multiple wells (n = 4 per time point). A single cell suspension was obtained by harvesting cells with 0.05% trypsin and 1 m*M* EDTA (Gibco, Grand Island, NY) and the viable cell number was assessed visually by counting the cells excluding trypan blue using hemocytometer chambers and an inverted Olympus microscope. Cellular protein was determined by the colorimetric Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) which is based on the Bradford dye-binding procedure (*30*). Cell doubling times were calculated by making one exponential fits from the logarithmic phase of the growth as described (*16*). All wells were replaced with fresh medium every other day until conclusion of the study.

Radiopharmaceutical Uptake Study

Hydrogen-3-fluorodeoxyglucose [5,6-^{3}H] (FDG) and hydrogen-3-L-methionine [methyl ^{3}H] (MET) with specific activities of 1.48 TBq/m*M* (40 Ci/m*M*) and 3.145 TBq/m*M* (85 Ci/m*M*), respectively, were obtained (ARC Inc., St. Louis, MO) and diluted in complete RPMI to obtain a final concentration of 37 KBq (1 μCi/ml) in the solution used in the tracer uptake experiments. Preliminary uptake studies using FDG and MET in cell suspensions of different concentrations showed that tracer uptake per cell was not influenced by total cell number, confirming that both FDG and MET act as tracers at the previously mentioned media glucose and L-methionine concentrations. During the growth curve study, replicate wells (n = 4 for both tracers) were incubated with 37 KBq of FDG and MET in RPMI for 1 hr at 37°C and incorporation of radioactivity in cells was stopped by adding ice-cold Hank's Balanced Salt Solution (HBSS) and repeating the wash with HBSS two times. The cells were then dissolved for 30 min in 0.5 ml of 0.3 M NaOH and 1% sodium dodecyl sulphate. Solubilized cell extracts were diluted in vials containing 10 ml of scintillation agent (Hionic Fluor, Packard, Meridien, CT) for later measurement of ^{3}H-activity with a Packard 1600 TR liquid scintillation counter. The FDG or MET uptake per tumor cell protein mass in a tissue culture well was felt to be most analogous to in vivo tracer uptake per tumor.

DNA Flow Cytometry

DNA flow cytometry was performed to estimate the cell cycle distribution and proliferation index (the percentage of tumor cells in S + G2/M phase). The cells from several replicate wells (total number approximately $5 * 10^5$) cultured as above were trypsinized, washed with HBSS and fixed in 70% ethanol. The samples were stored at 4°C until staining which was typically performed within 2 wk of the growth study. On the day of the flow cytometric analyses the samples were washed before treating them with 0.5 ml RNase (54.4 μg/ml) for 30 min at 37°C and subsequent 1 hr staining with 0.5 ml of propidium iodide (50 μg/ml). Flow cytometry was performed using a FacStar Elite (Beckton-Dickinson Immunocytometry systems, Mountain View, CA) flow cytometer. Histograms from the Elite printout were used to evaluate the cell fractions in the G0/1, S, and G2/M phases

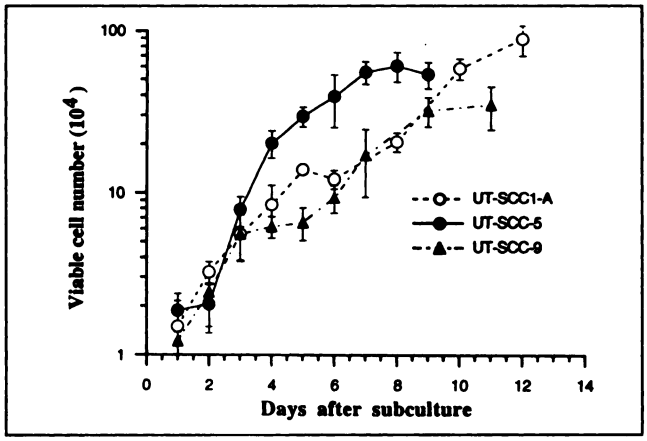

FIGURE 1. Growth curves (mean cell number ± s.d.) of three human SCC (UT-SCC-series) cell lines. The curves for the two more slowly growing cell lines almost coincide while UT-SCC-5 (solid circles) clearly shows a faster growth rate.

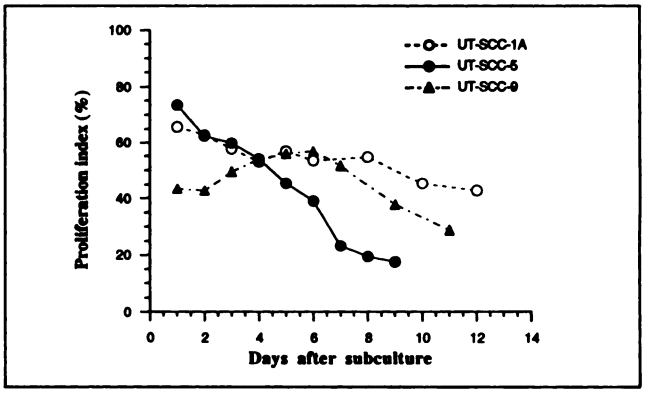

FIGURE 2. Proliferation index (percentage of cells in S + G2/M phase) as measured by DNA flow cytometry in the UT-SCC-series. Each circle represents $5 * 10^5$ cells sorted from several replicate wells per each time point. Note the slightly different pattern of change in cell cycle distribution for the UT-SCC-9 cells (solid triangles) in comparison to UT-SCC-1A and 5, which show a steady decline in the proliferative fraction throughout the growth cycle.

using commercial software (Phoenix Flow Systems, San Diego, CA).

Statistical Analysis

All measurements are expressed as mean ± s.d. Paired t-test was used for comparison of group means and linear regression analysis for assessment of relationship between tracer uptake measurements and cell growth characteristics. All p values are two-tailed.

RESULTS

All three cell lines progressed through a characteristic cell growth cycle including lag, exponential and plateau phases (Fig. 1, (*20*)). The lengths of time to reach confluency as well as mean cell doubling times are given in Table 1. UT-SCC-1A and UT-SCC-5 entered the exponential phase of growth on days 2–3 after passage. In contrast, UT-SCC-9 progressed slowly to the exponential phase which was not readily detectable before Day 5. In flow cytometry all cell lines had consistently a diploid DNA histogram. The proliferative activity showed a similar pattern of progressive decrease of percentage of cells in S + G2/M phase for UT-SCC-1A and five while UT-SCC-9 showed a slightly different cell cycle distribution in the early part of the study (Fig. 2). The proportion of UT-SCC-9 cells in the S-phase remained constant until mid-exponential phase which, together with increase in percentage of G2/M-phase cells on days 3–5, caused a concomitant overall increase in the proliferative fraction through days 3–6. From this day onward, all three cell lines had a constant drop in the fraction of S + G2/M cells. The rate of decrease was in accordance with the cell doubling times, resulting in a low percentage of S-phase in cells reaching confluency.

Figure 3 shows FDG and MET uptake per microgram of tumor cell protein through the various phases of the growth cycle. FDG had highest uptake per microgram protein during the first few days, after which it declined only slightly and remained rather unchanged throughout the rest of the exponential and plateau phases. In UT-SCC-9 cells with no distinctive changes in FDG uptake were seen over the complete cycle. MET uptake in the lag phase was, however, notably higher than during the late exponential and plateau phases. This was particularly contrasted when comparing relative changes in FDG and MET uptake where MET showed a mean decrease of 78% ± 10% from lag to late-exponential phase compared to only a decrease of 30% ± 17% in FDG uptake.

The uptake of both tracers was highly related to viable cell number throughout the growth cycle (Table 2). In the logarithmic and plateau phases MET uptake remained lower than that of FDG and MET uptake measurement that appeared always to underestimate cell number. In contrast, the relative increase of FDG was higher in UT-SCC-9, parallel in UT-SCC-1A and lower in UT-SCC-5 in comparison to the increase in cell number (Fig. 4). The comparison of tracer uptake to the cell doubling time indicated that the fast growing UT-SCC-5 cells had a higher rate of uptake of FDG and MET than the two more slowly growing UT-SCC-1A and nine cell lines (Fig. 5).

The relationship between proliferative activity and tracer uptake was different among the three cell lines. For UT-SCC-5 cells both FDG and MET were positively correlated to the fraction of cells in S + G2/M-phase ($r = 0.66$, $p < 0.05$ for FDG and $r = 0.89$, $p < 0.001$ for MET, $n = 9$). In case of UT-SCC-1A only MET uptake and proliferative index showed a fair correlation ($r = 0.74$, $p < 0.03$, $n = 8$) while no significant relationship was found for FDG uptake and flow cytometry assays. Neither FDG nor MET uptake could be related to the fraction of S + G2/M-cells in UT-SCC-9, where the pattern of progression through the various phases of the cell cycle during lag and early exponential phases was exceptionally slow (Fig. 2). The correlations of tracer uptake to S-phase fraction showed similar, although weaker relationships: $r = 0.68$, $p < 0.05$ for UT-SCC-1A and MET; $r = 0.86$, $p < 0.004$ for UT-

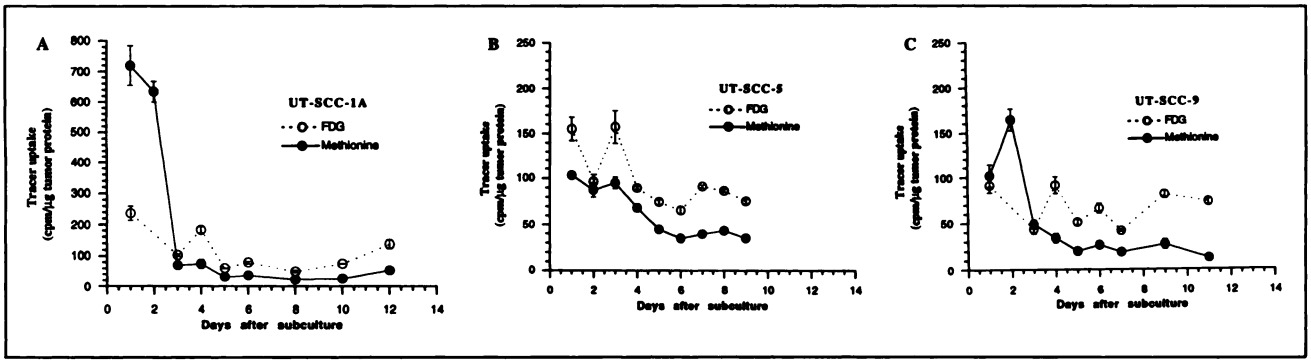

FIGURE 3. Uptake of tritiated FDG and L-methionine in relation to tumor cell protein concentration in the UT-SCC-series (A: UT-SCC-1A; B: UT-SCC-5; C: UT-SCC-9). Each point represents an average of measurements from four replicate wells where 37 KBq (1 µCi) of radiolabeled FDG or methionine were added as indicated in Methods. With the exception of the first three days following subculture, total methionine uptake is lower than that of FDG. There is a tendency of methionine uptake to decline over the growth cycle, while such a pattern is not as obvious for FDG.

SCC-5 and MET, while S-phase fraction and FDG uptake had a significant correlation in none of the cases.

DISCUSSION

The proliferation kinetics of head and neck SCC has important implications for therapeutic irradiation using conventional or hyperfractionated schemes (*17*). Bearing in mind the notion derived from clinical protocols, we carried out the current series of cell culture experiments to expand previous studies by Higashi et al. (*20*) on a human ovarian carcinoma cell line HTB77IP3 to a panel of cell lines established from patients with head and neck cancer. Our in vitro model may thus be seen as a guide to a rational use of PET in the management of patients with head and neck cancer. We felt that comparison of uptake characteristics of FDG and MET was important since both radiopharmaceuticals have been shown to possess comparable clinical potential to image head and neck cancer (*4*) by the fact that their pattern of uptake in relation to cell proliferation, at least after treatment of experimental brain tumors, seems to be different (*19*).

We found that FDG uptake was strongly related to the number of viable cancer cells (cf. 20) although in the most rapidly growing cell line (UT-SCC-5) an underestimation of the total cell number was observed (Fig. 4). MET uptake followed a rather similar pattern with the exception of the a tendency of a relatively larger decline towards the late exponential and plateau phases of the growth cycle and a smaller absolute uptake in terms of counts per tumor cells during log and plateau phases. These differences make FDG a stronger marker of cell viability than MET in this SCC in vitro model. This may not, however, translate to a higher specificity or sensitivity in detection of head and neck tumors with FDG as compared to MET using PET. Autoradiographic techniques have indicated that tumor-infiltrating inflammatory cells may, in some instances, have a higher rate of glucose metabolism than true neoplastic cells whereas MET uptake in experimental inflammation has reportedly been low (*31–32*). Still, findings from this laboratory lend support to the fact that the contribution of FDG signal is predominantly from malignant cells in several experimental tumors (*33*). The impact of immunoreactive cells (e.g., macrophages) is different in syngeneic and nude mice which may explain the apparent discrepancy between the observations in the above-mentioned autoradiographic studies. Other important factors that modify FDG and MET uptake and, presumably, tumor appearance in a PET scan, include anatomic location (*4*),

TABLE 2
Pearson Rank Correlation Coefficients and Slopes of Linear Regression Plots with Viable Cell Number as X-axis and FDG or MET Uptake as Y-axis in Three SCC Lines (UT-SCC-series)

	FDG uptake		MET uptake		FDG/MET uptake ratio†
Cell line	r value*	Slope	r value*	Slope	
UT-SCC-1A	0.983	1.34	0.959	0.16	8.4
UT-SCC-5	0.994	0.51	0.983	0.33	1.5
UT-SCC-9	0.962	2.93	0.937	0.50	5.9

*$p < 0.001$ in each case.
†Ratio between slopes of the FDG and MET regression lines.

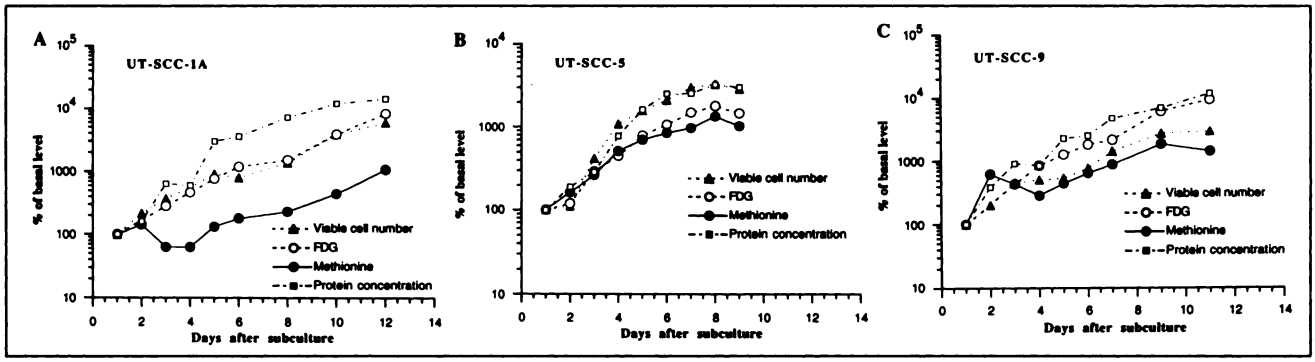

FIGURE 4. FDG and methionine uptake per total number of UT-SCC cells in a tissue culture well (A: UT-SCC-1A; B: UT-SCC-5; C: UT-SCC-9). Each point represents an average of measurements from four replicate wells. Error bars have been omitted to enhance clarity of display. Although the relative increases in FDG and methionine uptake show distinct differences there is a strong relationship between FDG and methionine uptake ($r = 0.995$, $p < 0.001$ for UT-SCC-1A, $r = 0.983$, $p < 0.001$ for UT-SCC-5, and $r = 0.876$, $p < 0.005$ for UT-SCC-9, respectively).

hypoxia (*34*), and host nutritive status (*35–36*), which may further affect the choice of an optimal tracer for a PET study of head and neck cancer.

Significant correlations between proliferative fraction and tracer uptake were found in two cell lines for MET and a borderline significant correlation in one cell line for FDG. The lack of significant relationship between MET uptake and proliferative index in the UT-SCC-9 may at least be partly due to the low initial S-phase fraction, with a slow progression of cells to S-phase in these particular cells. Furthermore, the relatively low passage number of these cells may indicate a shorter period of in vitro adaptation as compared to the other two cell lines. A unique variation in metabolic phenotype may also account for this somewhat atypical finding.

The role of MET in estimating proliferative activity is reflected in a concurrent study by Sato et al. (*19*) who indicated that chemotherapy caused similar changes in MET uptake and bromodeoxyuridine labeling index in rat KEG-1 glioma. Furthermore, Kubota et al. (*18*) found in a multitracer study of rat AH109 hepatoma that radiation-induced changes in [^{14}C]MET uptake paralleled those of [^{3}H]thymidine. These rat tumor studies and an in vitro study by Slosman et al. (*21*) indicate more delayed responses to treatment in terms of change in deoxyglucose or FDG uptake as compared to nucleotide precursor or MET uptake. On the other hand, Haberkorn et al. (*22*) could not establish a relationship between proliferative index and FDG uptake in several rat tumor models. Indeed, MET metabolism in tumor cells seems to be more sensitive to cytotoxic treatment effects than FDG, perhaps owing to the important role of MET not only in protein synthesis but also in pathways involving nucleotide and lipid metabolism (*37*).

The current study demonstrated higher overall uptake of FDG and MET per cell and tumor protein in the more rapidly growing cell line UT-SCC-5 than in the two more slowly growing lines UT-SCC-1A and nine (Fig. 5). Accordingly, it is tempting to think that rapidly growing tumors have a higher rate of metabolism to maintain energy requirements for cell build-up and passage through the cell cycle than their more slowly growing counterparts. Indeed, supportive findings have been reported in several experimental tumors and human meningiomas when FDG uptake has been related to grade of differentiation and volume doubling times (*32,38–40*). To expand these findings to clinical PET is, however, premature because of the above mentioned multiple factors affecting in vivo tumor metabolism. The growth fraction and cellular hierarchy of tumor cell subpopulations with differential capacity to proliferate strongly affect clinical growth of human tumors (*41*). How this heterogeneity, which results from genetic instability,

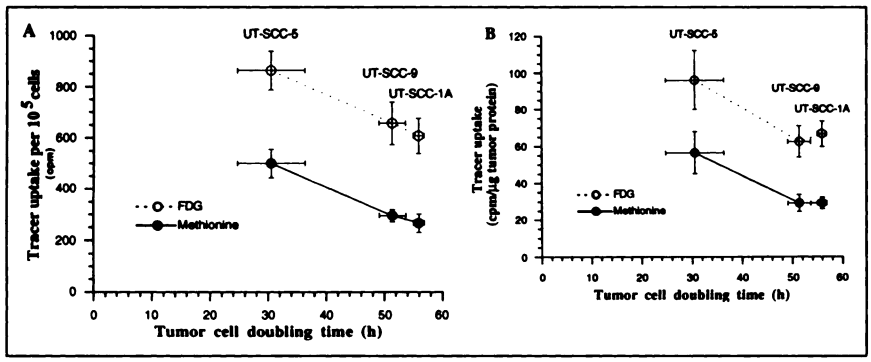

FIGURE 5. Comparison of tumor cell doubling times for the UT-SCC-series versus mean FDG and methionine uptake rates as expressed per cell (A) and per microgram tumor protein mass (B) in the exponential phase of growth. The rapidly growing UT-SCC-5 cells have a notably higher uptake of both radiopharmaceuticals than UT-SCC-1A and 9 which have a relatively long cell doubling time, supporting a relationship between the rate of FDG and methionine uptake and growth.

relates to the metabolic needs of tumor and, hence, PET signal is poorly understood.

Minn et al. (*10*) found a strong association between S + G2M cells and FDG uptake in 13 patients with head and neck tumors imaged with a specially collimated gamma camera. Although a larger series by Haberkorn et al. (*13*) using PET could not demonstrate a similar linear relationship between flow cytometry and FDG uptake, a concern was raised of the existence of two separate tumor groups with a postulated differential oncogene activation and proliferation characteristics still related to FDG-PET findings. In addition to these studies where proliferation was assessed with flow cytometry, the question of relating growth to glucose metabolism has been repeatedly addressed in different settings. These studies of patients with gliomas (*42*), soft-tissue sarcomas (*43*) and lymphomas (*13*) have pointed to the utility of FDG-PET to assess tumor growth characteristics independent of histopathologic and radiologic findings.

Although MET metabolism in relation to tumor proliferation and growth has been studied less extensively, the initial findings of Leskinen-Kallio et al. (*14*) in breast tumors and a recent report on patients with non-small-cell lung cancer (*44*) have seen a significant association of MET uptake with percentage of S-phase cells as studied by flow cytometry. In fact, Kubota et al. (*18,45*) underline the feasibility of MET for both diagnostic and follow-up evaluations with PET based on its favorable uptake changes during treatment possibly reflecting the linkage of amino acid metabolism to DNA synthesis in the process of radiation injury and repair. The same authors (*46*) found, however, no significant difference between the sensitivity and specificity of FDG and MET to detect lung tumors which is in accordance with a study by Lindholm et al. (*4*) where FDG and MET uptake were compared in patients with head and neck cancer. In soft-tissue sarcomas both FDG and MET imaging compared favorably with anatomic imaging modalities; FDG showed superior accuracy in tumor diagnosis in comparison to both MET and CT or MRI (*47*). A slightly differential uptake pattern has been found in non-Hodgkin lymphomas (*48*) where MET could better detect low-grade tumors than FDG which seemed to allow differentiation of malignancy grade based on its relatively large difference in uptake between low- and high-grade lesions. It is conceivable from all these studies that the pattern of uptake of FDG and MET manifests differently over a large variety of malignant tumors and is affected not only by cancer cells themselves but by host tissues, their metabolism and previous treatment.

Estimates of potential tumor doubling time (t_{pot}) which take into account cell loss and cycle time have been shown to yield more realistic views on growth potential of head and neck SCC than measurements of pure volume doublings (*15–17*). Presently employed cytopathologic methods to assess t_{pot} use monoclonal antibodies against 5-bromo- or 5-iododeoxyuridine which label cells actively synthesizing DNA (*15,49*). The labeled cell cohort is then followed as it passages through the cell cycle using flow cytometry. These methods have been suggested to be useful, based on actual t_{pot} measurements, for classifying head and neck tumors best suited for treatment with either conventional or hyperfractionated radiotherapy (*17,50*). However, a biopsy of tumor after intravenous injection of the nucleotide precursor is required and the most rapidly proliferating cell fraction may bias the information (*16*). Therefore, metabolic imaging of cancer tissue with PET should be evaluated for its possible role in complementing cytopathologic methods in estimating tumor growth.

In conclusion, we found that uptake characteristics of FDG and MET over progression through growth cycle of three human SCC cell lines showed different patterns with MET uptake consistently lower than those of FDG in the exponential phase of growth ($p < 0.01$) in every case. FDG uptake more closely reflected the number of viable cells in culture than MET uptake which tended to underestimate the viable cell population. In vitro uptake of MET was strongly related to the proliferating cell fraction in two lines and that of FDG was weakly related in one case. The uptake of both tracers was highest in the rapidly growing cell line which is in accordance with previous studies pointing to the potential utility of FDG and MET to assess tumor growth rate. Validation of the applicability of these in vitro observations to clinical situation requires in vivo evaluation of patterns of glucose and amino acid metabolism in experimental and human tumors with differential growth characteristics.

ACKNOWLEDGMENTS

This study was supported in part by grants CA 53172, CA 52880 and CA 56731 awarded by the NCI/PHS and grants from the Academy of Finland and Southwestern Finland Cancer Foundation.

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