tors with shorter in-plane focal length and longer axial focal length can minimize the inadequate axial sampling while maintaining a gain in sensitivity. With the use of astigmatic HCB collimators, the in-plane focal length can be shortened to obtain better tradeoff between resolution and sensitivity. With triple-camera SPECT systems, another solution is to combine HCB, fan-beam and parallel-beam collimators to obtain adequate axial sampling. Simultaneous acquisition can be performed with triple-camera systems by using two HCB collimators and a single parallel-beam or fan-beam collimator (*21*). The loss in overall sensitivity (relative to the use of three HCB collimators) is only about 15%–20%.

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Effects of Hypoxia on the Uptake of Tritiated Thymidine, L-Leucine, L-Methionine and FDG in Cultured Cancer Cells

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We previously demonstrated in vitro that FDG uptake into viable cancer cells increases in the presence of hypoxic versus normoxic conditions. Since positron-emitter labeled thymidine and amino acids are being used for PET, we evaluated uptake into tumor cells of several tracers (thymidine, L-leucine, L-methionine and FDG) in the presence of either normoxic or hypoxic atmospheres in vitro. **Methods:** Uptake of tritiated thymidine, L-leucine, L-methionine and FDG into two human tumor cell lines (HTB 63 melanoma and HTB 77 IP3 ovarian carcinoma) was determined after a 4-hr exposure to each of three different oxygen atmospheres (0, 1.5 and 20% O_2) in vitro. **Results:** Under moderately hypoxic conditions (1.5% O_2), thymidine uptake decreased significantly from the 20% O_2 baseline for both melanoma and ovarian carcinoma cell lines (33% and 15%, respectively) and with anoxia, thymidine uptake declined from baseline by 43% and 21%, respectively. Leucine uptake decreased substantially in the melanoma cells, by 23% when exposed to 1.5% O_2 and 36% in the presence of 0% O_2, but only modestly or not at all in the IP3 cells. After 1.5% or 0% O_2 exposure, methionine uptake was not significantly different from 20% O_2 levels in either cell line. In contrast, FDG uptake in both cell lines increased significantly (23% and 38%, respectively) over normoxic (20% O_2) conditions when cells were exposed to moderate hypoxia. FDG uptake also increased over basal conditions after anoxia, by 11% and 30% for melanoma and ovarian carcinoma cells, respectively. **Conclusion:** Hypoxia decreases cellular uptake of thymidine and increases FDG uptake in two different malignant human cell lines. Leucine uptake decreases with hypoxia in the melanoma cell line but not markedly in the IP3 cell line, while hypoxia does not alter methionine uptake in either cell line significantly. Hypoxia has varying effects on metabolic tracers used for PET. The use of paired hypoxia-sensitive PET tracers has potential for noninvasively characterizing tissue oxygenation levels.

Key Words: fluorine-18-fluorodeoxyglucose; hypoxia; nucleotide uptake; amino acid uptake

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PET has an important and growing role in cancer imaging. The high sensitivity of PET allows more accurate staging of cancers than is possible with standard anatomic imaging methods (*1*) and permits quantitative assessments of metabolic responses to treatment (*2*). PET imaging using a variety of tracers has also made it possible to assess the biology of tumors noninvasively (e.g., metabolic activities such as glucose metabolism and synthesis of proteins and DNA), evaluate tumor morphology and gain insights on tumor aggressiveness and prognosis (*3*). When metabolic information is paired with anatomic data obtained from CT or MRI scans, images displaying both anatomy and physiology can be visualized as anatometabolic images (*4*) useful in defining the anatomic location of intense foci of PET tracer uptake. These locations are otherwise difficult to localize due to limited background activity.

An important characteristic of tumors is that they are often poorly perfused and have low oxygenation regions. Such hypoxic regions can prove resistant to standard external beam radiation therapy and some forms of chemotherapy (*5–12*). We recently reported in vitro studies showing that hypoxia increased FDG uptake into human melanoma and adenocarcinoma cancer cell lines (*13–15*). While the mechanisms responsible for producing the increased FDG uptake in these tumor cells during hypoxia are not certain, increased expression of the Glut-1 glucose transporter and increased membrane glucose transport during hypoxia certainly contribute (*15*). Identifying hypoxic cells in vivo is potentially important, as they are less responsive to radiation therapy and chemotherapy. Since tracers of tumor protein and DNA synthesis are also being used for PET (*16–19*), we assessed in vitro whether tumor cell uptake of other metabolic tracers were also affected by hypoxia. Such data are important for a more complete understanding of the significance of PET images and for planning future multitracer studies to determine if PET can differentiate between hypoxic and normoxic tissues with tracers other than the nitroimidazole-based compounds used to date (*20,21*).

MATERIALS AND METHODS

Cell Lines

The human melanoma, HTB 63, and ovarian carcinoma, HTB 77, cell lines were obtained from ATCC (Rockville, MD) and handled as previously described (*15*). HTB 63 and HTB 77 IP3 cells were grown in either McCoy's 5a or RPMI 1640 medium (Gibco, Gaithersburg, MD) and maintained in a 5% CO_2-humidified atmosphere at 37°C until ready for use. All media were supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, 50 IU/ml penicillin G and 50 μg/ml streptomycin sulfate. D-(+)-glucose (10% w/v) was added to glucose-free RPMI to obtain a final concentration of 100 mg/dl glucose (5.5 m*M*). The final concentrations in the media for L-leucine, L-methionine and thymidine were 5, 1.5 and 0.185 mg/dl, respectively. Additives (antibiotics, serum and supplements), enzymatic dispersing agents and buffers, such as HBSS (Hanks' Balanced Salt Solution, without Ca^{+2} or Mg^{+2}) and PBS (phosphate-buffer, saline), were obtained either from Gibco or Sigma (St. Louis, MO). Feeding of the cultures was conducted on alternate days with a complete change of media. Tumor cell viability was assessed by the Trypan blue dye exclusion technique using an Olympus IMT-2 (Lake Success, NY) inverted microscope.

Oxygen Studies

Medical gas mixtures containing different oxygen concentrations (0, 1.5 or 20% O_2:5% CO_2:balance N_2) were obtained from AGA Gas (Cleveland, OH) and further tested in an ABL-30, CO Oximeter 282 (Radiometer, Copenhagen, Denmark) blood gas analyzer for accuracy of oxygen concentrations. The gasses were delivered into sterile tubes using a N_2 CGA 580 regulator (Victor Equipment Co., Denton, TX) to provide a constant low pressure of 86.2 kPa (1 psi = 6.895 kPa) as previously described (*15*).

TABLE 1
Tritiated Tracer Accumulation in a Human Melanoma Cell Line

	Oxygen concentration			
	1.5%		0%	
Tracer	% change ± s.d.	p value	% change ± s.d.	p value
FDG	22.9 ± 5.3†	<0.002	11.0 ± 5.4†	<0.005
Leucine	22.9 ± 4.6‡	<0.015	36.4 ± 12.6‡	<0.04
Methionine	9.4 ± 4.9†	ns	17.6 ± 7.8‡	ns
Thymidine	32.5 ± 5.4‡	<0.0005	43.2 ± 12.8‡	<0.09

Percentage change (increase [†] or decrease [‡]) in tracer uptake in a melanoma (HTB 63) cell line as compared to basal (20% O_2) conditions; ns = not significant.

Tritiated FDG Tracer Uptake

Cells were seeded at an initial density of 1×10^6 cells per 150 cm^2 tissue culture flasks fed with a complete change of media on the second and fifth days and used on the sixth day (*15*). At confluency, cells were dissociated with either trypsin (0.25%) or trypsin-EDTA (0.05% trypsin; 0.02% EDTA), their concentration adjusted to 0.25×10^6 per ml, aliquoted in sterile, no additives, Vacutainer™ (Becton Dickinson, Rutherford, NJ) tubes at 2 ml per tube and exposed to the desired O_2 tension for 4 hr at 37°C. The radiolabeled compounds leucine (L-[4,5-^{3}H(N)]), methionine, (L-[methyl-^{3}H]), thymidine ([methyl-^{3}H]) and FDG (2-deoxy-2-fluoro-D-[5,6-^{3}H] glucose) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Radioactive tracer was diluted in RPMI, added to cells (2 μCi in 100 μl of growth media) and incubation continued for 60 min longer at 37°C. Cells were spun at 250 × g for 10 min. Cell pellets were resuspended, washed three times with 4.5 ml of ice-cold HBSS and immediately lysed with 0.3 *M* NaOH:1% SDS (incubated 30 min at room temperature). Whole-cell extracts were transferred to scintillation vials containing 10 ml of Hionic Fluor (Packard, Meridien, CT) and vials were incubated overnight, in the dark, at 4°C. Bound radioactivity was measured by beta counting the following day in a 1600 TR Packard Liquid Scintillation Analyzer (Meriden, CT). Tracer uptake was expressed as percent basal levels and represent the mean of 8 to 16 individual determinations ± s.e.m., unless otherwise stated. Statistical comparisons were based on unpaired Student's t-tests and ANOVA.

RESULTS

For all tracer studies and incubation conditions, cell viability was preserved at nearly 100% (as assessed by Trypan blue). Tables 1 and 2 illustrate the results obtained for the HTB 63 melanoma and for the HTB 77 IP3 ovarian carcinoma cell lines. Basal conditions are normalized to uptake at the 20% oxygen atmosphere (100%) for all findings.

Effect of Hypoxia on Thymidine Uptake

We observed a significant decrease in thymidine uptake versus basal (20% O_2) in both the melanoma and the ovarian carcinoma tumor lines when cells were incubated for 4 hr in the presence of either hypoxia or anoxia (1.5% or 0% oxygen). Exposure to a moderate hypoxic environment (1.5% O_2) produced a decrease in thymidine uptake versus basal conditions of 32.5% ± 5.4% ($p < 0.0005$) and 15% ± 8.5% ($p < 0.02$), respectively; while with 4 hr of anoxia (0% O_2), the decrease in

TABLE 2
Tritiated Tracer Accumulation in a Human Ovarian Carcinoma Cell Line

	Oxygen concentration			
	1.5%		0%	
Tracer	% change ± s.d.	p value	% change ± s.d.	p value
FDG	37.7 ± 5.3†	<0.0001	29.9 ± 4.6†	<0.0001
Leucine	17.2 ± 7.0‡	<0.02	0.7 ± 10.5†	ns
Methionine	1.1 ± 3.0†	ns	3.1 ± 3.8‡	ns
Thymidine	15 ± 8.5‡	<0.02	20.8 ± 4.7‡	<0.005

Percentage change (increase [†] or decrease [‡]) in tracer uptake in an ovarian carcinoma (HTB 77 IP3) cell line as compared to basal (20% O_2) conditions; ns = not significant.

thymidine accumulation was 43.2% ± 12.8% ($p < 0.09$) for the melanoma and 20.8% ± 4.7% ($p < 0.005$) for the ovarian carcinoma cells.

Effects of Hypoxia on Amino Acid Uptake

Leucine uptake values varied somewhat between the melanoma and the ovarian carcinoma cell lines exposed to hypoxia. Moderately hypoxic HTB 63 (melanoma) cells exhibited a significant decrease in leucine uptake of 22.9% ± 4.6% (1.5% O_2; $p < 0.015$) and anoxic cells a decline of 36.4% ± 12.6% (0% O_2; $p < 0.04$) as compared to normoxic conditions (20% O_2). HTB 77 IP3 cells displayed only a mild 17.2% ± 7% (1.5% O_2; $p < 0.02$) decrease in tracer uptake with moderate hypoxia and essentially no change with anoxia (0.7% ± 10.5% increase at 0% O_2; p = ns) when compared to normoxic (20% O_2) conditions. The response of methionine uptake to hypoxia did not differ significantly between the two cell lines. Either a nonsignificant increase in uptake for both melanoma and ovarian carcinoma cells (9.4% ± 4.9% and 1.1% ± 3%, respectively with 1.5% O_2) or a nonsignificant decrease of 17.6% ± 7.8% for the melanoma and 3.1% ± 3.8% for the ovarian cancer cells with the 0% O_2 condition was observed. Thus, the hypoxic conditions we explored did not significantly alter methionine uptake versus basal 20% oxygenation levels in either cell line.

Effects of Hypoxia on Glucose Analog Uptake

We observed a significant increase in FDG uptake over basal uptake (20% O_2) in both the melanoma and the ovarian carcinoma tumor lines when cells were incubated for 4 hr in the presence of a low oxygen atmosphere (either 1.5% or 0% oxygen) (*14,15*). Exposure to a moderately hypoxic environment (1.5% O_2) produced a net increase in FDG uptake over basal conditions of 22.9% ± 5.3% ($p < 0.002$) and 37.7% ± 5.3% ($p < 0.0001$) respectively; while with 4 hr of anoxia (0% O_2), the increase was 11% ± 5.4% ($p < 0.005$) and 29.9% ± 4.6% ($p < 0.0001$) for melanoma and ovarian carcinoma cells, respectively.

DISCUSSION

It has long been recognized that hypoxic cells, which exist in many tumors and are resistant to standard radiation therapy fractionation and possibly to some other forms of treatments such as chemotherapy or hyperthermia, can adversely modify the outcome of such treatments (*5–12,22–24*). The hypoxic microenvironment a tumor cell experiences creates a cellular stress that activates many new genes (*25*). This gene activation or induction by hypoxia results in increased gene expression and therefore increased production of hypoxia stress proteins such as glucose and oxygen-regulated proteins (GRPs and ORPs) and heat-shock proteins (HSPs). In fact, ORP 80 kD and ORP 100 kD, two of the five originally reported ORPs, share complete peptide homology with GRP 78 and GRP 94 (*26*).

At present, most approaches to detect hypoxia in vivo have involved direct invasive measurements, including polarographic electrodes. Such data indicate, for example, that hypoxic cervical cancers respond less well to radiation therapy than well-oxygenated tumors (*24*). Noninvasive methods of assessment for hypoxia have included magnetic resonance spectroscopy (MRS), PET using [^{18}F]fluoromisonidazole and SPECT with agents which accumulate in hypoxic cells in vitro and in vivo (*27,28*).

The influence of hypoxia on metabolic tracers may be of practical importance given that PET is being increasingly applied to tumor imaging. We recently reported that FDG uptake is increased by moderate or severe hypoxia in several human cell lines in vitro (*13–15*). This increase in FDG uptake during hypoxia has been shown to be mediated in part by increased expression of the Glut-1 glucose transporter (*15*). Since metabolic tracers other than FDG can be used to image varying aspects of tumor physiology, we assessed the influence of hypoxia on the in vitro uptake of three metabolic tracers (thymidine, L-methionine, and L-leucine) which, when labeled with positron emitters, have been successfully used for PET. Thymidine has been used to assess DNA synthesis by several groups, and a significant correlation between proliferative activity and prognosis has been found with the thymidine labeling index (TLI) in human breast, colorectal, blood, skin, head and neck, non-small-cell lung and gastric cancers (*29–37*). Methionine and leucine have more recently been used to assess protein transport/synthesis in brain and other tumors (*38–43*).

In our in vitro studies, exposure of two human cancer cell lines to moderate or severe hypoxia for 4 hr resulted in significant decreases in thymidine accumulation versus tracer uptake seen under basal oxygenation conditions. By contrast, methionine uptake after hypoxia for this period of time did not significantly change in either cell line. Changes in leucine uptake were variable, declining significantly with moderate hypoxia in both cell lines and with severe hypoxia in one line. Consistent with our previous reports, FDG uptake increased with hypoxia in both cell lines, although the magnitude of increase was somewhat lower than previously reported. Therefore, our observations for acute/subacute hypoxia in cancer cells are generally consistent with results obtained over the past several years by various groups studying several levels and durations of hypoxia. It appears that prolonged hypoxia generally will decrease DNA synthesis, the growth rate of cells, the proportion of cells synthesizing DNA and will increase the fraction of cells in the G-1 phase of the life cycle (*44–46*). Similarly, hypoxia (3.5% O_2) has been shown to decrease the proliferation rate of Ehrlich ascites cells by 50% (*47*). The reduction we observed in thymidine uptake is consistent with these past observations and compatible with thymidine being an antihypoxic marker (i.e., tracer uptake is lower in hypoxic than in well-oxygenated tumor cells). The decline in L-leucine uptake with hypoxia in the melanoma cell line and with moderate hypoxia in the adenocarcinoma line was also expected, since hypoxia has been reported to reduce protein synthesis in cells of many types (*48*).

It has been suggested that the tumor microvasculature in vivo is quite variable in terms of the extent of blood flow to different areas of tumors over time. This being the case, it is then quite

likely that regions of tumor hypoxia are, in fact, somewhat transient and variable, so that the effects of acute and subacute hypoxia we studied are physiologically relevant (*12,25*). The duration of hypoxia affects the extent of tracer uptake in vivo, with one study showing that brief hypoxia decreased leucine uptake, whereas intermediate term hypoxia (12–24 hr) increased tracer uptake in brain tissue (*49,50*). In addition, in situations of transient hypoxia, posthypoxia brain tissues can have increased rates of protein synthesis (although rates are generally decreased during hypoxia), suggesting that effects of hypoxia can vary, depending on the exact timing of the exposure (*50*). Nonetheless, our data and those of others (*48*). Both in vitro and in vivo, support the importance of hypoxia on tumor uptake of leucine.

The lack of change in tumor cell L-methionine uptake during the 4-hr exposure to hypoxia in our studies was of considerable interest. This lack of decline in L-methionine uptake may be due to the fact that L-methionine is not only used for protein synthesis, but also in auxiliary biochemical processes, specifically transmethylation pathways. Thus, while protein synthesis might be expected to ultimately decline in hypoxia if cell growth slowed, methionine uptake in our studies of tumor cells did not change after up to 4 hr of anoxia.

Information on the metabolic characteristics of individual tumors before treatment (*51*), and identification of hypoxic tumors in particular (*10,12,52*), may aid in planning the best schedule of radiation therapy for a particular patient. Procedures now available to detect tumor hypoxia (*12*) are of an invasive nature (biopsy, microelectrodes), in various stages of development or generally not feasible for clinical use. Clearly, nuclear medicine methods are promising in this regard (*53–55*). Availability of a noninvasive method to detect tumor oxygenation would provide information needed to design effective anticancer strategies and find a correlation between tumor hypoxia and treatment outcomes (*56*).

Our in vitro data support the concept that relatively increased FDG uptake and relatively decreased thymidine accumulation (with or without decreased L-leucine uptake) are metabolic phenotypes of hypoxic cells. It is possible that such a metabolic phenotype of disproportionately greater FDG than thymidine uptake might also be true of hypoxic tumor tissue in vivo. If this were proven, and of sufficient magnitude, dual-isotope PET imaging with FDG and either thymidine or leucine tracers, might identify hypoxic portions of tumors noninvasively. Similarly, such a dual-tracer method might help to define the effects of treatment for tumor hypoxia. A portion of this hypothesis has been supported experimentally, as studies with high-resolution PET devices and microelectrodes have shown that glucose utilization can be maintained or increased in vivo in areas of hypoxia (*57*). Clearly, further studies are necessary to determine if hypoxia can be detected in vitro by paired metabolic tracers and perhaps used to monitor anti-hypoxic tumor therapy.

CONCLUSION

In vitro exposure of tumor cells to 4 hr of hypoxia decreased tumor uptake of thymidine (a marker of DNA synthesis) in two different malignant human tumor cell lines but increased FDG uptake in both lines. L-Leucine (a marker of protein synthesis) uptake declined in both cell lines with moderate hypoxia, consistent with reduced protein synthesis. Methionine uptake was not significantly changed with hypoxia in either cell line during this duration of hypoxia. Based on these in vitro observations, it is probable that local tissue oxygen concentrations affect the metabolic signals observed in vivo in human tumors using PET. Paired tracer studies with FDG and thymidine, for example, may warrant study in vivo, as a noninvasive approach to either detect the presence of tumor hypoxia or to monitor methods that modify it.

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Quantification of Serial Tumor Glucose Metabolism

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We developed a method to improve the quantitative precision of FDG-PET scans in cancer patients. The total-lesion evaluation method generates a correlation coefficient (r) constrained Patlak parametric image of the lesion together with three calculated glucose metabolic indices: (a) the total-lesion metabolic index ("K_{T_tle}", ml/min/lesion); (b) the total-lesion voxel index ("V_{T_tle}", voxels/lesion); and (c) the global average metabolic index ("K_{V_tle}", ml/min/voxel). **Methods:** The glucose metabolic indices obtained from conventional region of interest (ROI) and multiplane evaluation were used as standards to evaluate the accuracy of the total-lesion evaluation method. Computer simulations and four patients with metastatic melanoma before and after chemotherapy were studied. **Results:** Computer simulations showed that the total-lesion evaluation method has improved precision (%s.d. < 0.6%) and accuracy (~10% error) compared with the conventional ROI method (%s.d. ~ 5%; ~25% error). The K_{T_tle} and V_{T_tle} indices from human FDG-PET studies using the total-lesion evaluation method showed excellent correlations with the corresponding values obtained from the conventional ROI methods and multiplane evaluation (r ~ 1.0) and CT lesion volume measurements. **Conclusion:** This method is a simple but reliable way to quantitatively monitor tumor FDG uptake. The method has several advantages over the conventional ROI method: (a) less sensitive to the ROI definition, (b) no need for image registration of serial scan data and (c) includes tumor volume changes in the global tumor metabolism.

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Recent studies have established the potential of FDG-PET imaging as a tumor localization procedure. These studies produced interesting insights into the prognostic and therapeutic implications of FDG uptake in tumors (*1–6*). The conventional method to estimate the net FDG phosphorylation rate constant K (ml/min/g), proportional to the glucose metabolic rate, is accomplished by three-compartmental model fitting (*7,8*) using a nonlinear regression routine or using estimates from the Patlak graphical analysis (*9*). The nonlinear regression route or Patlak graphical analysis method (*7–9*) estimates the lesion glucose metabolic rate using a time-activity curve (TAC) generated from a region of interest (ROI) in a chosen image (single-plane-single-ROI method). Glucose metabolic rate is defined by:

$$MR = [K_1^* \cdot k_3^*/(k_2^* + k_3^*)] \cdot C_p/LC = K \cdot (C_p/LC),$$

where $K = K_1^* \cdot k_3^*/(k_2^* + k_3^*)$, K_1^* and k_2^* refer to forward and reverse capillary transport of FDG, k_3^* and k_4^* refer to phosphorylation of FDG and dephosphorylation of FDG-6-PO_4, respectively, C_p is the plasma-glucose concentration and the lumped constant is a calibration term based on the difference in transport and phosphorylation kinetics between glucose and FDG (*7,8*).

Our study in patients with metastatic melanoma showed that