Glut-1 and Hexokinase Expression: Relationship with 2-Fluoro-2-deoxy-D-glucose Uptake in A431 and T47D Cells in Culture

Luigi Aloj,1 Corradina Caracó,2 Elaine Jagoda, William C. Eckelman, and Ronald D. Neumann3


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MATERIALS AND METHODS

Cell Culture. A431 cells were originally obtained from Dr. G. Todaro (NIH). This is a human epidermoid carcinoma cell line that has been shown to yield s.c. tumors with high efficiency in immunologically incompetent mice (11). T47D cells were obtained from Dr. T. Moody (NIH). This cell line is derived from a pleural effusion of a patient with metastatic breast cancer (12).

Two steps are required to accumulate FDG in cancer cells: (a) facilitated diffusion through a glucose transport protein; and (b) subsequent phosphorylation by one of the hexokinase isoforms, FDG-6-P. FDG-6-P is not transported out of cells nor undergoes glycolytic breakdown; it is metabolically trapped inside cells. Identification of which of these two steps is rate limiting in the process of FDG uptake should provide a better understanding of how glucose metabolism is regulated in cancer cells. This information may also be useful for developing therapeutic strategies aimed at blocking increased glucose consumption in tumors.

Several studies have focused on the expression of glucose transporters and hexokinase activity to define the role of these two classes of genes in the regulation of FDG uptake. Five subtypes of the human facilitative glucose transporters have been described (6). Although most of these subtypes have, to some extent, been detected in different human cancers and cancer cell lines, Glut-1 is the only subtype that has been detected in nearly all cell lines tested (6) and has also been shown to be overexpressed in many human cancers (7). The overexpression of Glut-1 in cancers has lead to speculation that this protein may be regulating glucose metabolism and FDG uptake in cancer cells. On the other hand, several hexokinase subtypes have also been described. Hexokinase I and II have been found to be expressed in tumors. Some authors suggest that these proteins, hexokinase II in particular, are indeed regulating glucose metabolism in cancer cells (8), and some clinical studies show that the FDG phosphorylation step may be rate limiting in the FDG uptake of cancer (9). Some studies also suggest that the lack of glucose-6-phosphatase activity in tumors plays a role in determining FDG retention by preventing dephosphorylation of FDG-6-P to FDG (10).

To shed more light on this issue, we have taken a comprehensive approach to studying the role of the Glut-1 protein and of hexokinase activity in FDG uptake in cultured cancer cells. We have characterized the expression of all of the known facilitative and sodium-dependent glucose transporters in six different cell lines. Among these, we have chosen two cell lines expressing only the Glut-1 isoform; one cell line has severalfold higher levels of Glut-1 mRNA than the other. These two cell lines have been tested for their ability to: (a) express Glut-1 protein as determined by Western blot; (b) accumulate DG when grown in nude mice as xenografts; (c) accumulate FDG in culture in the presence or absence of glucose; (d) transport 3-OMeG, a glucose analogue that is transported into the cell but not appreciably phosphorylated; (e) synthesize mRNA for hexokinase subtypes I and II; and (f) phosphorylate FDG in mitochondrial extracts. Such an approach has not been used in previous studies on this issue. Furthermore, experiments conducted in cultured cancer cells allow us to control potentially confounding variables, such as blood flow and necrosis, which are present when studying these biochemical parameters in animal tumor models or in human tumors.
After UV cross-linking, the membrane was hybridized with 32P-labeled membrane using a Turboblotter apparatus (Schleicher and Schuell, Keene, (Sunnyvale, CA), and the gel contents were subsequently transferred to a nylon control.

Both of these cell lines were grown in a humidified atmosphere containing 5% CO2, and 95% air at 37°C in DMEM with 4.5 g/l glucose, supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY), HepG2, CaCo II, MCF7, and MDMA-MB-231 were originally obtained from the American Type Culture Collection (Rockville, MD) and were cultured according to the instructions provided by the supplier. For all cells, the medium was exchanged every 2 to 3 days, and cells were passaged after trypsinization when confluent. Cells that were overconfluent (i.e., confluent for more than 2 days) were discarded, and fresh cultures were reestablished by thawing cells stored in liquid nitrogen. All experiments herein described were repeated on at least three batches of frozen cells to ensure reproducibility.

Radioactive Glucose Analogues. [18 F]FDG was prepared in the PET Department by standard means (13). All batches were quality controlled to assess purity of the compound and were suitable for patient use (∼1000 Ci/mmol, end of synthesis). [14 C]3-OMeG (50 mCi/mmol) and [3 H]DG (30 Ci/mmol) were obtained from DuPont NEN (Boston, MA).

DG Uptake in A431 and T47D Xenografts. Before being implanted in nude mice, cells were trypsinized, washed in culture medium, and resuspended in PBS at a density of 2 × 10⁶/ml. Approximately 100 μl of the cell suspensions were s.c. injected in the flank of Balb-c athymic nude mice (4–6 weeks of age; weight, 17–23 g). Xenografts were allowed to grow for 4–6 weeks; they were harvested, weighed, and counted in a gamma counter that had been calibrated with quenched [3 H]standards. Uptake was expressed as %ID/g of tumor tissue normalized to a 20-g mouse.

RNA Analysis. Total RNA was extracted from cells grown in culture and, in the case of A431 and T47D cells, xenografted tumors using RNAzol B (Tel-Test, Inc., Friendswood, TX), following the manufacturer’s suggested procedure. Fifteen μg of total RNA were denatured by heating at 65°C for 15 min and loaded onto a 1% agarose formaldehyde gel containing ethidium bromide (14). The samples were electrophoresed for 2 h at 100 V. The ethidium signal was captured using a Molecular Dynamics 595 Fluorimager (Sunnyvale, CA), and the gel contents were subsequently transferred to a nylon membrane using a Turboblotter apparatus (Schleicher and Schuell, Keene, NH). After UV cross-linking, the membrane was hybridized with 32P-labeled probes obtained by random priming of the following cDNA templates. Plasmids containing the sequences for the human Glut-1, -2, -3, -4, and -5 isoforms were kindly provided by Dr. Charles Burant (University of Chicago, Chicago, IL). The human SGLT 1 and 2 isoform cDNA plasmids (15) were kindly provided by Dr. Matthias Hediger (Harvard University, Boston, MA). The human β-actin and hexokinase 1 cDNA plasmids were purchased from the American Type Culture Collection (Rockville, MD). A 461-bp fragment of the human hexokinase II mRNA sequence (GenBank accession number Z46376), was amplified by RT-PCR from commercially available poli-A RNA of human myocardium (Clontech, Palo Alto, CA) and cloned into a pCR2 vector (Invitrogen, San Diego, CA). After hybridization, the membranes were washed twice in 2× sodium chloride-sodium citrate with 0.1% SDS at 70°C, exposed overnight on imaging plates, and analyzed using a FUJI BAS-1500 Phosphorimager ( Stamford, CT). All probes were tested on commercially available Northern blots (Clontech) prior to use in the experiments reported. The intensities of the different bands were normalized to the β-actin signal or to the 18S band values of the ethidium stain.

Protein Analysis. Cells grown in 15-cm diameter plates were rinsed five times in ice-cold PBS, scraped off the plate, and collected in centrifuge tubes. After centrifugation, the pellet was resuspended in 1 ml of 20 mM Hepes, 1 mM EDTA, and 255 mM sucrose (HES, pH 7.4) containing protease inhibitors (Complete; Boehringer Mannheim, Indianapolis, IN). The suspension was homogenized with 20 strokes in a Teflon homogenizer on ice, and aliquots were either processed to obtain plasma membrane fractions or total extracts were stored at −70°C prior to use. Plasma membrane preparations were obtained by centrifugation over a sucrose cushion as described (16). Protein concentrations were determined using a Coomassie-Plus kit (Pierce Chemical Co., Rockford, IL). SDS-PAGE was performed as described (17) on precast 10% acrylamide Tris-glycine gels (Novex, San Diego, CA). Samples were not boiled prior to loading. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes using a Novex X-cell II apparatus. The membranes were incubated with dilutions of the following antibodies, according to the technical sheets supplied with the products, in Tris buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h. Polyclonal antibodies raised against the NH2 terminus of the human Glut-1 protein were purchased from Santa Cruz (Santa Cruz, CA). A mouse monoclonal antibody (M7-PB-E9) against the α1 subunit of the human Na+-K+-ATPase (Affinity Bioreagents, Golden, CO) was used as a control. After several washes in TBS-T, the membranes were incubated with a dilution of an appropriate fluorescein-labeled secondary antibody (Amersham, Arlington Heights, IL) for an additional hour, and washed several times in TBS-T; finally, the fluorescent signal was detected with a Molecular Dynamics 595 Fluorimager.

Cell Uptake Experiments. For experiments with FDG, cells were plated at a density of 1–200,000 cells/well in 12-well multwell plates (Costar, Cambridge, MA) 2 or 3 days prior to the uptake experiments. These conditions allowed for the cells to be almost confluent at the time of the assay. On the day of the experiment, the wells were rinsed once in cold uptake medium; subsequently, 1 ml of uptake medium containing 20 μCi of [18 F]FDG was added to each well. Uptake medium consisted of DMEM supplemented with 0.5% FBS with or without 5.5 mM glucose in the medium. FDG was allowed to accumulate into cells in the incubator, with times ranging from 10 to 40 min. Triplicate wells were rinsed twice in cold PBS, and the cells were lysed with 1 ml NaOH to collect cell-associated radioactivity. Uptake of FDG and 3-OMeG between 5 s and 3 min was performed under similar conditions. Cells were plated in 35-mm-diameter dishes. Each plate received ∼20 μCi of

| Glut-1 | α Na+K+ATPase
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Fig. 2. Western blot analysis of total and plasma membrane-enriched fractions of A431 and T47D cells. Total and plasma membrane Glut-1 levels are higher in A431 extracts than in T47D extracts. The plasma membrane marker, α1 subunit of the human Na+K+-ATPase, is enriched in the plasma membrane fractions of both cell lines. Comparable amounts of total protein are loaded in each lane, as shown by amido black staining of the blot.
RESULTS

Expression of mRNA for Glut-1, -2, -3, -4, and -5 and for the SGLT types I and II was assessed by Northern blot. A431, T47D, and HepG2 cells expressed detectable levels of Glut-1 only. Glut-1 and Glut-3 mRNAs were detected in MDA-MB-231 and MCF-7 cells. In CaCo II cells, Glut-1, Glut-3, Glut-5, and SGLT-2 were detectable. A431 cells expressed the highest amount of Glut-1 mRNA of all cells tested. The intensity of the Glut-1 band was 11.8 ± 5.6 (mean ± SD, n = 3) times higher when normalized to the β-actin signal and 15.7 ± 7.6 times higher when normalized to the 18S rRNA band on the ethidium stain than in T47D cells, which had the lowest level. These two cell lines were, therefore, selected for further analysis (Fig. 1). The level of Glut-1 protein expression was determined in total extracts and in plasma membrane preparations of A431 and T47D cells (Fig. 2). The Glut-1 band shows higher intensity in A431 than in T47D in both total extracts and plasma membrane-enriched fractions. In plasma membrane fractions, the cell surface marker α1 subunit of the human Na⁺K⁺-ATPase is enriched for both cell lines, and levels of this protein appear higher in A431 in both fractions. The total amount of protein loaded is very similar for all samples, as shown by the amido black staining of the blot. Total Glut-1 protein levels in A431 were 4.4 ± 1.2 (mean ± SD, n = 3) times higher than in T47D cells, similar to the results obtained for the mRNA. Plasma membrane-enriched fractions showed similar differences in Glut-1 protein content, with A431 extracts containing 3.8 ± 1.0 times higher levels than T47D.

FDG uptake in the two cell lines is shown in Fig. 3. Both in the absence (Fig. 3a) or presence (Fig. 3b) of 5.5 mM glucose in the medium, uptake of FDG between 10 and 40 min after addition of the compound is linear for both cell lines and higher in T47D cells than in A431. Absolute uptake values are 17–20 times higher when cells are incubated in medium without glucose as expected. Uptake of FDG
in the two cell lines at early time points and in the presence of 5.5 mM glucose are shown in Fig. 4. A431 cells show more rapid initial uptake of FDG, as values are higher for these cells up to the 40-s time point. T47D cells show higher uptake at later times. Time uptake curves for 3-OMeG are shown in Fig. 5. Both cell lines show uptake of this compound that rapidly reaches equilibrium. Initial transport rates of 3-OMeG are more rapid in A431 (0.96 ± 0.30, percentage of available tracer/mg protein/min, mean ± SD) than in T47D cells (0.42 ± 0.12), calculated assuming linear uptake for the first 20 s after addition of the compound.

Total RNA was extracted from A431 and T47D xenografts. Glut-1 mRNA levels in the xenografts were determined and compared with levels found in cultured cells (Fig. 6a). Levels of Glut-1 mRNA in A431 xenografts are higher than in T47D cells, similar to results obtained in cells grown in culture. Uptake of DG was determined 60 min after injection (Fig. 6b). Average uptake in T47D xenografts was significantly higher than in A431 (4.35 ± 1.09, %ID/g ± SD, n = 14 versus 3.58 ± 1.10, %ID/g ± SD, n = 19; unpaired t test, P < 0.05).

Northern blot analysis for hexokinase subtypes I and II is shown in Fig. 7. Levels of hexokinase I mRNA are 5.2 ± 2.0 (mean ± SD, n = 3) times higher in A431 than in T47D cells grown in culture, when normalized for β-actin, and 7.0 ± 2.8 times when normalized for 18S rRNA. Hexokinase II mRNA levels are 7.5 ± 0.2 (mean ± SD, n = 3) times higher in A431 than in T47D cells, when normalized for β-actin, and 10.0 ± 0.4 times when normalized for 18S rRNA. FDG phosphorylating activity, however, is higher in mitochondrial extracts of T47D cells (Fig. 8), because these preparations show similar affinity (\(K_m\) is 109 ± 49 μM in T47D versus 328 ± 145 μM in A431; unpaired \(t\) test, \(P > 0.1\)), similar to previously published values for FDG using yeast hexokinase (21), and higher \(V_{max}\) (62 ± 4 versus 30 ± 3 nmol/min/mg protein; unpaired \(t\) test, \(P < 0.0001\)) than in A431.

**DISCUSSION**

Among the cell lines tested, A431 and T47D cells were chosen because: (a) both cell lines only express Glut-1 mRNA at detectable levels and no other facilitative or SGLTs, therefore Glut-1 appears to be the isoform mainly being used by these cells to transport FDG; and (b) the level of expression of Glut-1 is very different in these two cells, with the A431 expressing 10–20 times more Glut-1 mRNA than T47D, a condition that should favor uptake of FDG in A431 cells if Glut-1 levels were indeed the rate-limiting step. The level of Glut-1 protein detected by Western blot (Fig. 2) is consistent with results obtained at the mRNA level, with A431 cells expressing ~4 times more total protein than T47D. Furthermore, the amount of Glut-1 associated with the plasma membrane preparations, which should be

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**Fig. 5.** Initial transport rates of 3-OMeG in A431 and T47D cells. Uptake of 3-OMeG rapidly reaches equilibrium in both cell lines. Initial rates are higher in A431 cells (n ≥3 per time point; bars, SD).

**Fig. 6.** a, Northern blot analysis of A431 and T47D cells grown in culture (c) and as xenografts (x). b, relative levels of β-actin normalized Glut-1 mRNA (n = 3; bars, SD) in A431 and T47D cells grown in culture and as xenografts, as well as DG uptake values 60 min after injection in xenografts (n ≥13 xenografts; bars, SD).

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higher $V_{\text{max}}$ (nmol/min/mg mitochondrial protein) values in T47D cells of 3-OMeG that shows that this compound, which is not appreciably phosphorylated by cells, very rapidly reaches equilibrium between extracellular and intracellular space in both cell lines. A431 cells show initial transport rates of 3-OMeG that are slightly higher than in T47D cells (0.96 ± 0.30 vs. 0.42 ± 0.12% of available tracer/min/mg protein; Fig. 5), consistent with the different amounts of Glut-1 expressed, again indicating that the ability to transport is not the rate-limiting step in FDG retention.

Another interesting aspect of the differences in transport rates between the two cell lines is that they are not as different as one would expect, given the very different levels of the protein in the two cells. Although we did not measure kinetic properties of glucose transport in A431 and T47D cells, our results suggest that the Glut-1 protein may, under different conditions (i.e., in the two different cell lines), be able to transport glucose analogues at different rates. Differences in the intrinsic activity of Glut-1 under some experimental conditions have been demonstrated in other cell systems (22).

The extracellular tracer concentration in the experiments in cells in culture is virtually constant for the duration of the experiment. This situation is very different from that found in the setting of a PET study, where tracer availability (i.e., the concentration of tracer in the blood) decreases with time. The experiments in xenografts show that under experimental conditions, which are similar to those found in human studies, uptake of the glucose analogue DG still appears to be independent of the level of Glut-1 expression (Fig. 6). Under these conditions of tracer delivery, membrane transport does not appear to be rate determining, and uptake of DG is significantly higher in T74D xenografts than in A431.

Thus far, all of the evidence presented points away from Glut-1 levels as the controlling factor on FDG uptake. It appears that flux of FDG, 3-OMeG, and DG across the plasma membrane is not the rate-limiting factor in determining how much FDG is retained in A431 and T47D cells. Indeed, it appears that even with relatively low levels of Glut-1 protein in the plasma membrane, T47D cells are able to sustain higher FDG retention values than A431 cells. Both hexokinase I and II mRNA levels are lower in T47D cells (Fig. 7); however, mitochondrial extracts of these cells, where active hexokinase is presumed to be located (23), show that T47D cells have a higher activity than A431 cells, indicating that: (a) mRNA levels of the two hexokinase isoforms do not necessarily correlate with the actual activity to phosphorylate; and (b) phosphorylating activity in the mitochondria plays a more important role in determining how much FDG is retained by cells and perhaps glucose metabolic rates.

Other studies have focused on the relationship of glucose transporter expression and the uptake of FDG. Most data gathered thus far have focused on vast evidence that the Glut-1 protein is overexpressed in many human tumors (7). In some instances, there appears to be a correlation between the level of expression of Glut-1 and FDG uptake (24); however, there is no direct evidence linking the amount of
Glut-1 protein to the level of FDG uptake. The idea that glucose metabolism in tumors may be controlled by the level of Glut-1 expression is, therefore, appealing because it may provide a target for the development of alternative therapeutic strategies. Recently, Torizuka et al. (25) have shown, by kinetic modeling of clinical PET-FDG studies, that the phosphorylation step appears to be rate determining in the uptake of FDG in primary breast cancers but not so in primary lung cancers, suggesting that there may be differences among cancers derived from different tissues in how FDG uptake and perhaps glucose metabolism is controlled by the cells. Our data, although limited to the comparison of two cell lines, indicate that the amount of Glut-1 does not regulate how much FDG, a surrogate marker for glucose metabolism, is retained by cancer cells.

In principle, higher levels of glucose transporter protein do not guarantee increased FDG uptake by cancer cells, and in our two cell systems, metabolic trapping via phosphorylation of FDG appears more likely as the rate-determining step in FDG uptake.

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