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Role for Glucose Transporter 1 Protein in Human Breast Cancer

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Glycolysis is increased in cancer cells compared with normal cells. It has been shown that glucose enters cells via a family of five functional glucose transporters (GLUT). However, GLUT expression appears to be altered in human breast cancer, which may serve as a selective advantage and facilitate the metastatic potential of these cells. The relationship of GLUT isoform expression and breast cancer cell invasiveness has not been adequately addressed. Thus, the purpose of this study was to investigate whether an association exists between GLUT expression and human breast cancer cell invasiveness. Invasiveness of the human breast cancer lines MCF-7, MDA-MB-435 and MDA-MB-231 was measured using an *in vitro* assay and compared with cellular GLUT isoform expression, assessed by Western blot analysis and verified by immunohistochemistry in a poorly differentiated human ductal breast cancer. Cell surface GLUT-1 expression was associated with the invasive ability of MCF-7 (2.0 ± 0.02%), MDA-MB-435 (6.4 ± 0.4%), and MDA-MB-231 (19.3 ± 2.0%). However, GLUT-2 and GLUT-5 were inversely associated with invasiveness; GLUT-3 expression was variable; and GLUT-4 was undetected. In a poorly differentiated human ductal breast cancer, *in situ* GLUT-1 staining was intense. GLUT-1 expression was associated with the *in vitro* invasive ability of human breast cancer cells which was validated *in situ*. If this relationship is found to exist in a larger number of human breast cancer tissues, it may be possible to develop diagnostic and therapeutic strategies based on targeted GLUT isoform expression. (Pathology Oncology Research Vol 4, No 2, 115–120, 1998)

Key words: breast cancer; invasion; GLUT expression

Introduction

Glycolysis has been shown to be increased in cancer cells compared with normal cells.[25] There is also evidence linking glycolysis and chemotaxis, which suggests the intriguing possibility that alterations in the glycolytic pathways of cancer cells may facilitate their migratory and invasive potential. In fact, a previous report has shown that in the presence of glucose, metastatic human melanoma cell motility depended on glycolysis as its principal source of energy.[2]

In a recently published study using highly invasive MDA-MB-468 breast cancer cells, glucose uptake was four times greater and fructose uptake was five times greater than in the less invasive MCF-7 cells.[27] This was the first study to infer an association between breast cancer cell invasive potential and glucose uptake.

In reviewing the biochemistry of glycolysis, glucose enters cells via a family of five functional glucose transporters (GLUT; *Table 1*).[3,14,15,24] The GLUT isoforms have different tissue distribution, function, and developmental regulation.[16,18,23] For example, GLUT-1 is expressed early in development. Increased GLUT-1 expression has been observed in cells transformed by virus or oncogenes,[4,10,11,17] and under hypoxic conditions in cancer cells[8] and in other tissues.[1,12,22] Most importantly, GLUT expression appears to be altered in human breast cancer. Specifically, GLUT-1, GLUT-2 and GLUT-5 have been demonstrated, with some evidence for GLUT-4, in MCF-7 and MDA-MB-468 human breast cancer cell lines.[27] In a study that focused only on GLUT-1, an increased expression was shown in

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Table 1. **Glucose transporter characteristics**

GLUT isoforms	*Type of transport*	*Km 2DG mM*	*Tissue distribution*
GLUT-1	basal	7	RBC, brain, placenta, ubiquitous
GLUT-2	glucose sensor	11–16	liver, kidney, pancreas, intestine
GLUT-3	basal	1–2	brain, placenta, testes
GLUT-4	insulin-responsive	5	fat, muscle
GLUT-5	fructose	NT	jejunum, testes, skeletal muscle, fat

NT = not transported; RBC = red blood cell; 2DG = 2-deoxyglucose

breast cancers with higher tumor grade and proliferative activity, but was undetectable in more than half the cases – leading the authors to conclude that glucose transport was also mediated by another unidentified isoform.[26] GLUT-5 has also been shown in human breast cancer tissue.[27]

The relationship of GLUT isoform expression and breast cancer cell invasiveness has not been well described. If GLUT expression correlates with breast cancer invasiveness, it may be possible to devise diagnostic and therapeutic strategies based on targeted GLUT isoform expression. Thus, the purpose of this study was to investigate the relationship between GLUT isoform protein expression and human breast cancer cell invasiveness. We hypothesized that GLUT isoform specific expression correlated with breast cancer cell invasiveness – a key step in the metastatic cascade.

Materials and Methods

Cell culture

The MCF-7 cell line was kindly supplied by Dr. F Miller (Michigan Cancer Foundation), and the MDA-MB-435 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD). The cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). Cell cultures were determined to be free of *Mycoplasma* contamination using the Gen Probe rapid detection system (Fisher, Itasca, IL).

Invasion assay

The membrane invasion culture system (MICS)[19,20] chamber was used to evaluate the degree of tumor cell invasion through a human laminin/collagen IV/gelatin-coated polycarbonate membrane containing 10 μm pore (Osmonics, Livermore, CA). The cells ($1 x 10^5$) were seeded into the upper wells of the MICS chamber in RPMI containing 1xMITO+ serum supplement (Collaborative Biomedical, Bedford, MA) to reduce protease inhibitors. After 24 hours of incubation at 37°C, the cells that had invaded the membrane were collected via the side-sampling ports, stained and counted, as previously described.[19] Percent invasion was corrected for proliferation and calculated as follows: (total number of invading cells/total number of cells seeded) x 100. Each cell line was tested in four wells per experiment and experiments were repeated twice.

Western blot analysis of GLUT proteins

Using an established technique for the evaluation of GLUT protein expression,[13] the cells were gently removed by cell scraping, centrifuged at 700g and the pellet stored at –70°C. The cells were lysed in 10 ml HES buffer (20 mM HEPES, 5 mM sodium azide, 250 mM sucrose) plus protease inhibitors (HESpi; 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin, 1 μM pepstatin A, 10 μM E-64) using ten strokes of a Teflon dounce homogenizer. The cell debris was removed by low speed centrifugation followed by the isolation of membrane bound proteins with high-speed centrifugation at 190,000g for 60 minutes at 4°C. The pellet was resuspended in HESpi buffer and the protein concentrations determined using the Bradford method (BioRad, Hercules, CA).[5]

GLUT protein was quantified using a modified protocol[22] in which 30 μg of protein were loaded per lane and resolved on 10% SDS-polyacrylamide gel electrophoresis, electrically transferred to HyBond C membrane (Amersham, Arlington Heights, IL). To ascertain even protein loading, immunoblots were stained with Ponceau S. Membranes were blocked by incubation in 10% non-fat dry milk in phosphate buffered saline with 0.1% Tween-20 (PBS-T; pH 7.4), rinsed in PBS-T, incubated with diluted primary antibody (polyclonal rabbit; anti-rat GLUT-1 (5 g/ml), anti-human GLUT-2 (1:1000), anti-human GLUT-3 (1:500), anti-rat GLUT-4 (5 μg/ml) or anti-human GLUT-5 (1:1000) for one hour at room temperature with agitation, washed twice with PBS-T, incubated at room temperature for 15 minutes with horseradish peroxidase-linked secondary antibody, anti-rabbit Ig from donkey (1:1000; Amersham), and then washed twice with PBS-T. Immunoblots were developed by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL), then exposed to autoradiography film (X-OMAT AR) for approximately one minute. All GLUT antibodies except GLUT-3 were purchased from Alpha Diagnostic (San Antonio, TX), and the antibody to GLUT-3 was kindly supplied by Dr. Gwyn Gould (University of Glasgow, Scotland).

Densitometric analysis was performed by scanning developed film using NIH Image software (version 1.52), a Mac I Scanner, and a Macintosh Power PC (Cupertino, CA).

Immunohistochemistry

Immunohistochemistry was performed on 8 µm sections prepared from archived tissue blocks. Tissue was deparaffinized in xylene, and rehydrated in a series of decreasing alcohols, and lastly in distilled water. Immunogold labeling (Aurion, Wageningen, Netherlands)[7,9] was performed using ultra small gold conjugates. Blocking of the tissues was accomplished by placement in phosphate buffered saline (PBS) with 50 mM glycine then in 5% bovine serum albumin (BSA) plus 5% normal goat serum, followed by two washes in incubation buffer (IB; 10 mM phosphate buffer, 150 mM NaCl, pH 7.4 plus 0.1% BSA-c© [Aurion, Wageningen, Netherlands]). Tissues were incubated overnight at 4°C in appropriate GLUT primary antibody diluted in IB using the same concentration as for the Western blots, followed by four washes in IB. A subsequent incubation occurred in a 1:75 dilution of secondary antibody consisting of goat anti-rabbit IgG ultra small (0.8 nm) gold particles in IB at room temperature for 3 hours. Four washes in IB were followed by 3 washes in PBS, then post fixation in 2.5% glutaraldehyde in PBS and a final wash in PBS and five washes in deionized water. To visualize the gold complexes, they were coated with silver using Aurion R-Gent Silver Enhancement© kit (Aurion, Wageningen, Netherlands). The sections were then stained with Harris hematoxylin and eosin (H&E), mounted in permount, and dried on a slide warmer for 24 hours. This staining technique enables visualization in the same microscopic field of both histology (using light microscopy to view the H&E stain) and GLUT isoform staining (using epi-polarization to view the silver-enhanced gold particles). To assess the amount of non-specific staining, control tissues were incubated with IB rather than the primary antibody; otherwise all steps were the same. A microscopic field with cancerous and non-cancerous tissue was identified with the H&E stain using a light microscope (Leitz Diaplan; Leica, Wetzlar, Germany). In the same microscopic field, silver-enhanced gold particles were visualized and photographed by switching to epi-polarization. The silver-enhanced gold particles depolarize and reflect the polarized incident light thereby showing up as bright spots against a dark background. GLUT protein staining was evaluated as absent, trace, mild, moderate, and intense.

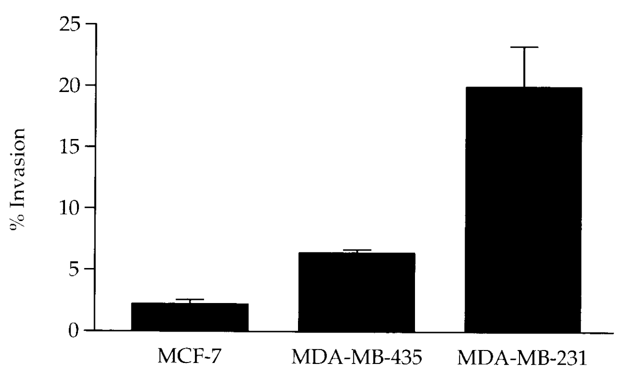

Figure 1. *Invasive potential of human breast cancer lines, measured* in vitro *using the membrane invasion culture system (MICS) assay. Invasive ability was calculated as the percentage of cells capable of invading a human laminin/collagen IV/gelatin-coated polycarbonate membrane over a 24-hour period within the MICS chamber, and compared with the total number of cells seeded (± SE determined; n = 4 wells/measurement and run in duplicate experiments).*

Statistics

Results are presented as mean and standard error. For comparison of the percent invasion and densitometry data among the three groups, a one way non-parametric Kruskal-Wallis H test was performed, and $p<0.05$ was considered significant.

Results

Invasive potential of human breast cancer cell lines

The ability of three human breast cancer cell lines to invade a human basement membrane extracellular matrix was measured using an *in vitro* invasion assay, as shown in *Figure 1*. Statistical analysis of percent invasion revealed three categories of invasive potential: MCF-7 cells were poorly invasive (2.0% ± 0.02%); MDA-MB-435 cells were moderately invasive (6.4% ± 0.4%); and, MDA-MB-231 cells were highly invasive (19.3 ± 3.0%). When all three cell types were included in the analysis, the groups were significantly different ($p<0.05$).

GLUT protein analysis

Western blot analysis of GLUT isoforms was made using the MCF-7, MDA-MB-435 and MDA-MB-231 cells to perform a comparative analysis of GLUT expression versus invasive potential *(Figure 2)*. Overall, the data revealed that GLUT-1 expression increased with increasing invasiveness of human breast cancer cells *(Figure 2A)*. GLUT-1 protein was moderately expressed in the poorly invasive MCF-7 cells; was more pronounced in the moderately invasive MDA-MB-435 cells; and was intensely expressed in the highly invasive MDA-MB-231 cells. GLUT-2 was barely detectable in the poor-

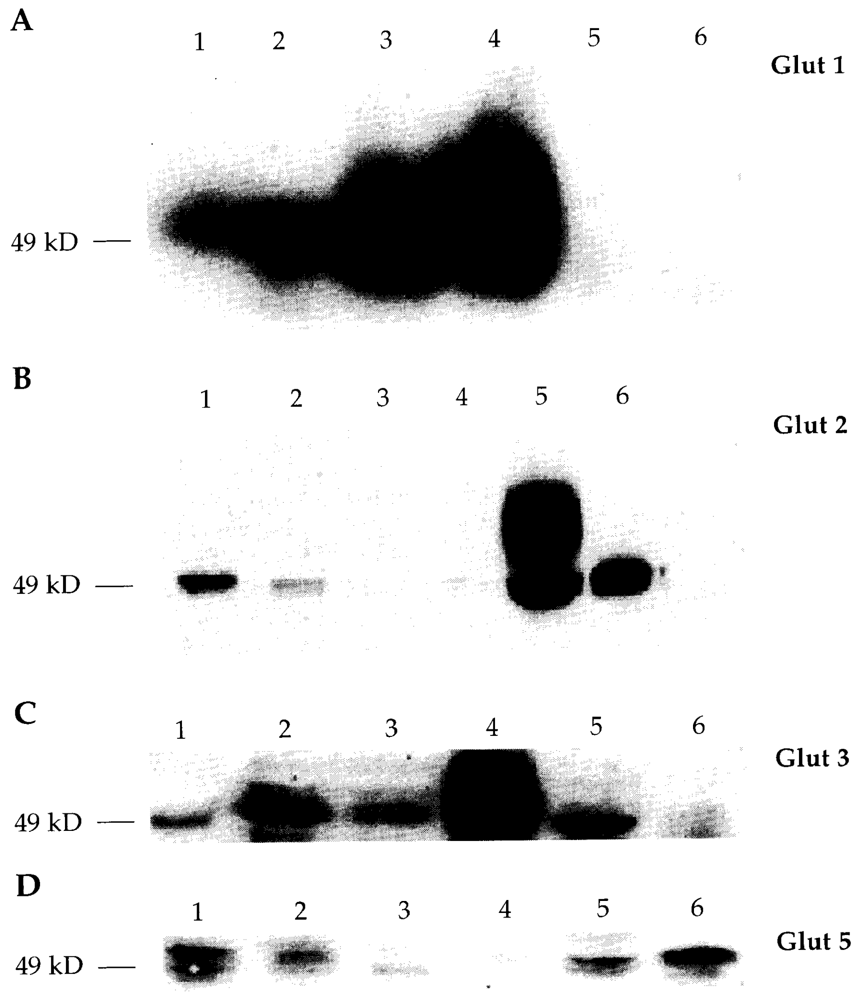

Figure 2. *Expression by Western blot analysis of GLUT-1 (A); GLUT-2 (B); GLUT-3 (C); GLUT 5 (D) in breast cancer cell lines of differing invasive potential. Equal amounts of protein was loaded onto a 10% SDS PAGE gel, electroblotted to Hybond C membranes, probed with the appropriate anti-GLUT antibody and detected using enhanced chemiluminescence. Lane 1) MCF-7; 2) MDA-MB-435; 3) MDA-MB-231; 4) human brain; 5) human liver; 6) rat skeletal muscle. Lanes 4 to 6 served as a positive or negative control depending on the GLUT isoform.*

ly and moderately invasive cells, and decreased as invasiveness increased – showing an inverse relationship *(Figure 2B)*. GLUT-3 was present in all three cell types, but did not correlate with invasiveness; the amount of GLUT-3 was greatest in the moderately invasive cells *(Figure 2C)*. GLUT-4 was not detectable in these three cell types (data not shown). GLUT-5 also decreased as invasiveness increased, revealing an inverse relationship *(Figure 2D)*. Densitometric analysis for GLUT-1 confirmed and quantified the visual interpretation ($n = 3$; normalized to brain): MCF-7 = 18 ± 9%; MDA-MB-435 = 26 ± 9.5%; MDA-MB-231 = 147 ± 2,8%. The value for GLUT-1 expression in MDA-MB-231 cells was significantly different than for MCF-7 ($p<0.05$) and MDA-MB-435 cells ($p<0.05$), whereas the difference between MCF-7 and MDA-MB-435 was not (p = NS).

Immunohistochemistry in human breast cancer tissue

Immunohistochemistry was performed for each GLUT isoform on serial slides from the same archived tissue block. Coordinate microscopic fields containing cancerous and non-cancerous tissue (stained with H&E) were viewed with light microscopy (*Figure 3A*) and correlated with epipolarization for detection of silver-enhanced gold particles, which indicate the presence of the GLUT isoform (*Figure 3B*). In a poorly differentiated human ductal breast carcinoma, GLUT-1 staining was intense in the cancerous but not adjacent tissue (*Figure 3B*). As expected, red blood cells also demonstrated intense GLUT-1 staining. GLUT-3 staining was moderate; GLUT-2 and GLUT-5 were detected in trace amounts only; and GLUT-4 was not detected (data not shown).

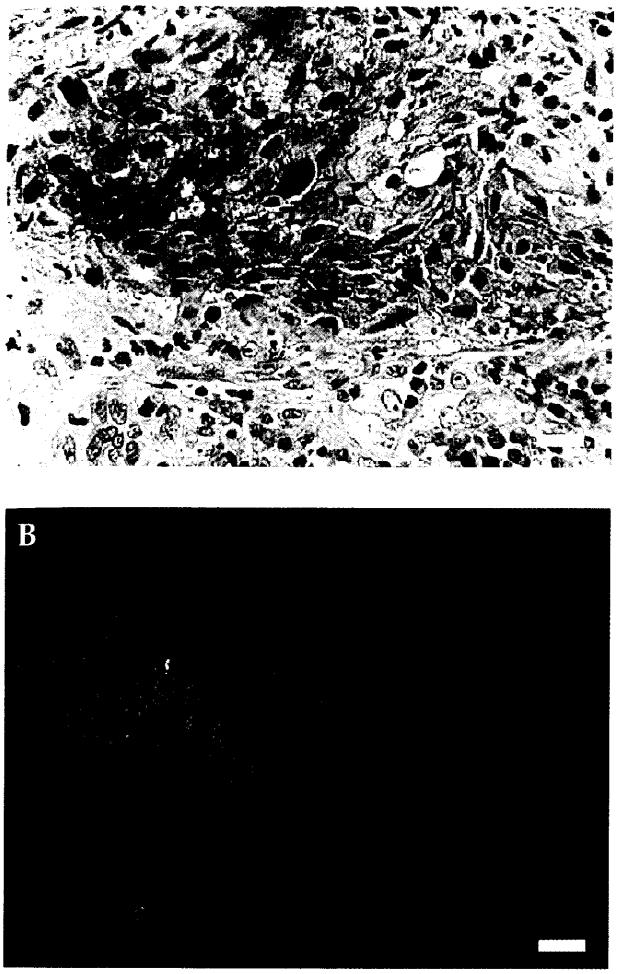

Figure 3. Immunohistochemical analysis of GLUT-1 localization in histologic sections of a human ductal breast cancer. (A) Hematoxylin and eosin stain demonstrating a poorly differentiated ductal breast cancer. Bar = 10 μm. (B) Immunogold-conjugated localization of GLUT-1 expression. Intense GLUT-1 staining appears to occur at the cell surface and in the cytoplasm, but not in the nuclei of the cancerous tissue. Red blood cells present in the adjacent tissue stained intensely, in contrast to the adjacent tissue that did not. Bar = 10 μm.

Discussion

The present study demonstrated a strong and direct association between GLUT-1 protein expression and breast cancer cell invasive ability – a key step in metastasis formation. An inverse relationship was shown between the protein expression of GLUT-2 and GLUT-5 and invasiveness. GLUT-3 expression was variable, and GLUT-4 was not detected in the cell lines tested. Verification of these *in vitro* observations was provided by *in situ* immunohistochemical staining of a poorly differentiated human ductal breast carcinoma.

A previous study has shown GLUT-1, GLUT-2 and GLUT-5 in human breast cancer cell lines MCF-7 and MDA-MB-468.[27] In addition to these GLUT isoforms, we also observed GLUT-3. In fact, we believe that our unique observation of GLUT-3 may be due to a putatively different affinity of the GLUT-3 antibody used in our study compared with a previous report.[21] In the highly invasive MDA-MB-231 cells, GLUT-1 expression was predominant with lesser or negligible amounts of the other GLUT isoforms. Thus, our study has extended the previous results reported by Zamora-Leon and colleagues[27] and has focused on the unique correlation between GLUT specific isoform expression and breast cancer cell invasive potential.

The reports of GLUT expression in human breast cancer tissues have been inconsistent. GLUT-1 has been shown to be variably present in breast cancer tissue specimens, and staining was more intense than that found in adjacent benign mammary epithelium.[6,27] In a study that investigated only GLUT-1, expression of this isoform was correlated with proliferative activity and histologic score, but was expressed in only 42% of breast tumors, thus raising the question of the role of other GLUT isoforms.[26] GLUT-2 expression has been shown to be comparable in malignant and benign breast tissues; whereas, clusters of GLUT-4 staining have been shown near the cell nucleus in some breast cancers; and GLUT-3 and GLUT-5 were not detected in breast cancer.[6] By comparison, GLUT-5 expression has been reported in another study to be present in human breast cancer tissues; however, the pathologic staging of these tissues is unknown.[27]

The significance of these collective biological observations is that with increasing invasiveness, human breast cancer cell lines progressively express more GLUT-1 and less of the other GLUT isoforms, which may serve as a selective advantage and facilitate the metastatic potential of these cells. Future investigation of all GLUT isoforms and correlation of expression with invasive and metastatic activity is warranted to provide a more in depth analysis of GLUT isoform function in human breast cancer tissue and its potential utility as a diagnostic marker or target for therapeutic intervention.

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