Abstract. The high glucose consumption of tumour cells has been well known since Warburg’s investigations. Nevertheless, the reason for this phenomenon is still a matter of speculation. Glucose uptake of tumour cells is mainly mediated by GLUT1. In this study, the question of whether glucose uptake as well as expression of GLUT1 are both related to tumour cell proliferation was addressed. Three kinetically different cell lines of colorectal adenocarcinomas (HRT-18, HT29 and CX-2) were used for the experiments. All the cell lines expressed GLUT1 at a high level, being mainly localized within the cell membrane and the endoplasmic reticulum. Surprisingly, the highest expression of GLUT1 was found in the most slowly proliferating cell line, CX-2. Moreover, induction of cell cycle arrest increased both GLUT1 expression and glucose consumption, as well as global protein synthesis. These data suggest that the protein synthesis of tumour cells is much more glucose-consuming than proliferation and that both processes are inversely related.

Tumour cells have long been recognized as having a high glucose metabolism (1, 2). This phenomenon is the basis for the use of positron emission tomography (PET) in diagnostics after the injection of 18fluorine-2-fluoro-2-deoxy-D-glucose (FDG). This glucose analogue is a positron emitter that is taken up in normal and tumour cells by the same transport mechanisms as D-glucose. It is processed intracellularly by hexokinase without further metabolism (3-5). Thus, its accumulation within the cells allows for in vivo measurement of regional glucose uptake.

This glucose uptake in both normal and tumour cells is mediated by facilitative glucose transporters, an expanding family of transmembrane proteins named GLUT1-12 and HMIT1 (6). GLUT1 is the most ubiquitously distributed of the glucose transporter isoforms, being expressed in many foetal and adult tissues, particularly in erythrocytes and endothelial cells, and is the major glucose transporter isoform in most immortalized cell lines. This transporter was found to be expressed at high levels in a variety of human malignant tumours (7-15). Activation of glucose transporter gene transcription probably represents one of the earliest effects of oncogenesis (16-18), and the overexpression of GLUT1 seems to be crucial for tumour growth (19). Interestingly, the increased level of GLUT1 protein expression in malignant tumours is not induced by amplification or rearrangements of the gene, but up-regulation of transcription (13). The basal glucose uptake in cancer cells is mediated by GLUT1 and GLUT3, but only GLUT1 is responsible for an increased supply (6). There are some reports in the literature that indicate a worse prognosis for malignant tumours expressing high levels of GLUT1 (20-24). The increasing diagnostic use and the prognostic importance of data on glucose metabolism of malignancies highlight the need for understanding its biological background. One explanation for the high glucose consumption of malignant tumours is their glycolytic production of energy, consuming much more glucose compared to cells producing energy by oxidative phosphorylation. On the other hand, the high proliferation rates of tumour cells are thought to be energy-consuming, contributing to the increased glucose consumption. The present study investigates the relationship between proliferation of tumour cells, glucose consumption and the expression of glucose transporters.

Materials and Methods

Cell lines. The human colorectal cancer cell lines HRT-18 (obtained from ECACC, Salisbury, UK), CX-2 (kindly provided by the German Cancer Research Center, Heidelberg, Germany) and
Incubated with 2 ìCi/ml 3H-thymidine for 90 min, washed three times.

 Autoradiography was performed by covering 3H-thymidine incorporation of the supernatant at 595 nm in a spectrophotometer. Moreover, wallace, UK), and the amount of protein determined by measuring radioactivity was counted in a liquid scintillation counter (rackbeta 1214, LKB).

 Mitotic block. To induce growth arrest, cells were grown for 72 h in culture medium until the logarithmic growth phase was reached. Thereafter, 10-6 M methotrexate (MTX) was added for 17 h. The controls received an equivalent volume of phosphate-buffered saline (PBS). The glucose consumption of these cultures was investigated at the end of the incubation period. Cultures during MTX treatment were assessed by morphological examination, as well as by staining with ethidium bromide and acridine orange. The efficacy of growth arrest was monitored by MIB-1 immunostaining and 3H-thymidine incorporation. For the latter, cultures were incubated with 2 µCi/ml 3H-thymidine for 90 min, washed three times in 0.9% NaCl and lysed in 0.5 ml NaOH. Radioactivity was counted in a liquid scintillation counter (rackbeta 1214, LKB, wallace, UK), and the amount of protein determined by measuring absorbance at 595 nm in a spectrophotometer. Moreover, autoradiography was performed by covering 3H-thymidine-incubated cell cultures in Labtek chamber slides with photographic emulsion, followed by standard development. To investigate whether MTX treatment induces DNA damage, the comet assay was performed at the end of the incubation period.

 Immunohistochemistry. The cells were fixed with acetone, washed in PBS and incubated for 30 min in a 1% mixture of H2O2 and methanol to inhibit endogenous peroxidase. The slides were washed in PBS three times and incubated with the primary antibody MIB-1 (dianoia, Hamburg, Germany) for 60 min in a humid chamber at a dilution of 1:10. Following a further wash in PBS, the cells were incubated with secondary antibody (45 min) and DAB solution (5 min). Quantification of MIB1 reactivity was performed using an image analysis system.

 Preparative ultracentrifugation. The cells were washed twice with TES buffer containing 20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 225 mM sucrose and 200 µM phenylmethylsulfonylfluoride (PMSF) and were scraped off with a rubber policeman. For homogenization, a 30-ml Potter-Elvehjem (thomas scientific, swedesboro, NJ, USA) was used, employing 15 handstrokes. The homogenate was centrifuged at 12,000 x g for 15 min at 4°C, the resulting pellet layered on a sucrose cushion (38.5%, 20 mM Tris, 1 mM EDTA, pH 7.4) and centrifuged for 60 min at 100,000 x g. Plasma membranes collected from the interface between the buffer and sucrose cushion were centrifuged again for 60 min at 100,000 x g in TES buffer containing PMSF. The pellet was resuspended and homogenized with a 5-ml Potter-Elvehjem at 30 handstrokes and 1,500 rpm of the pestle. The first supernatant was spun for 30 min at 40,000 x g and the resulting pellet was homogenized, as described previously, and collected as high-density microsomes. The supernatant from this step was centrifuged at 200,000 x g for 75 min, so that the low-density pellet was obtained. Aliquots were taken from all three membrane fractions and stored at –80°C until use.

 Immunoblot. Samples containing 10 µg protein were mixed with Laemmli buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters. An antiserum against GLUT1, isolated from human erythrocytes, was purified with protein A-sepharose (amersham pharmacia, buckinghamshire, UK) and the resulting IgG fraction was used. The antibody was visualized by 125I-protein A-assay. To quantify the protein amounts, the corresponding bands were cut out of the nitrocellulose membrane. Their radioactivity was evaluated in a scintillation counter.

 Measurement of protein synthesis. Three x 104 cells/ml medium were cultured for 24 h, then washed with medium (without serum). A leucin-free RPMI 1640 medium was supplemented with Leu-[4,5-3H]-leucine. The cells were incubated in this medium and growth-arrested, as described above. At the end of the incubation period, the cells were washed three times with PBS followed by lysis using 500 µl NaOH/well. One hundred µl of the lysate were mixed with 4.5 ml scintigel rotiszint 2000 (roth, karlsruhe, Germany). The incorporation of leucine was measured with a liquid scintillation counter (rackbeta 1214, LKB).

 Statistics. Data were evaluated by Student’s t-test, p-values less than 0.05 were considered to be statistically significant.

 Results

 Expression and subcellular distribution of GLUT1 in cell lines HRT-18, HT29 and CX-2. The cell line CX-2 has a population doubling time (PDT) of 26.5 h and expresses the

 Glucose consumption. The cells were seeded at a density of 5x105. After day 3, the concentration of glucose was investigated in the supernatant at 24-h intervals by standard clinicochemical procedure until day 6. The difference between the initial glucose concentration within the medium (10.7 mmol/l) and the measured value within the supernatant gives the glucose consumption per past time. The cell number was determined in parallel to calculate the glucose consumption per cell.
glucose transporter GLUT1 most intensely, whereas the fast proliferating cell lines HRT-18 and HT29, with a PDT of 20.2 h and 16.5 h, respectively, had lower levels of GLUT1 protein (Figure 1). To study the subcellular distribution of GLUT1, a preparative ultracentrifugation was performed. CX-2 cells showed the strongest expression of the transporter in the plasma membrane fraction (containing the cell membrane and the smooth endoplasmic reticulum). In HRT-18 and HT29, GLUT1 was distributed equally in both the plasma membrane and high-density microsome fraction (containing rough endoplasmic reticulum). The low-density microsome fraction (representing Golgi-vesicles) contained the least amount of GLUT1 of all cell lines examined (Figure 1). It is also remarkable that the molecular weight of GLUT1 differed to some extent in the three cell lines, probably due to different glycosylation patterns of the transporter.

**Evaluation of growth arrest.** The cell lines were cultured for 72 h and subsequently incubated with MTX for 17 h. The viability of the cell cultures was examined visually and by vital staining. Viability was greater than 95% in all cultures and no differences in cell viability were found between MTX-treated cultures and controls (data not shown). Thymidine incorporation was reduced more than 7-fold in cell cultures treated with MTX (Figure 2), while MIB1 was found in less than 1% of the nuclei of the arrested cells (Figure 3). Because MTX treatment has been shown to induce DNA strand-breaks in a variety of cells (25), a Comet assay was performed to clarify this issue. Using MTX at a concentration range from 1-20 μM for 17 h, no significant difference between controls (value 5.15) and MTX (mean value over the concentration range 6.62) was detected.

Figure 4. Glucose consumption within 89 h (black) and protein synthesis (empty) in unrestricted proliferating cells. The mean and SD of three independent experiments are shown. The star symbol marks significant differences (glucose consumption: p<0.01; protein synthesis: p<0.001).

Glucose consumption, expression of GLUT1 and protein synthesis in normal and arrested cells. The expression of GLUT1 (Figure 1) was compared with glucose consumption in all three cell lines. Our data indicated that there might be a correlation between the two (Figure 4), which is
particularly true for CX-2 cells, that had the highest glucose consumption and the strongest expression of GLUT1. Both HRT-18 and HT29 cells showed a significantly lower expression of GLUT1 and the glucose consumption of HT29 cells was as low as expected. However, HRT-18 cells, which expressed GLUT1 at a level comparable to HT29, had a significantly higher rate of glucose consumption than HT29 cells.

After induction of growth arrest, an increase of glucose consumption was observed in CX-2 and HT29 cells, but not in HRT-18 cells (Figure 5). To investigate whether this increase in glucose consumption was due to an up-regulation of GLUT1 protein, extracts of CX-2 cells were blotted. The data showed that there was a significant increase in GLUT1 protein in the plasma membrane, but no reduction in the other subcellular fractions (Figure 6). To identify other cellular processes that consume glucose, global protein synthesis of the cells was also investigated (Figure 4). Interestingly, after induction of growth arrest, an increased protein synthesis was notable in all the arrested cells (Figure 5).

Discussion

Carcinomas utilize much more glucose than their normal tissues of origin. This occurs in parallel with the enhanced expression of glucose transport proteins like GLUT1, that has been described by immunohistochemical studies for a variety of carcinomas. It is believed that enhanced tumour cell proliferation is responsible for both high glucose utilization and overexpression of GLUT1. Some studies have shown a parallel increase in the expression of proliferation markers and GLUT1, e.g. in carcinomas of the lung and the colorectum (15, 23). Moreover, the expression of GLUT1 in fibroblasts is higher in proliferating cells when compared to contact-inhibited ones (17, 26), and can be stimulated by various growth factors (e.g. EGF, PDGF, FGF), as well as by activation of various oncogenes like ras, src and c-myc (13, 16, 27). On the other hand, in breast cancer only a weak correlation between expression of GLUT1 and Ki-67 (MIB-1) was found (28), and it has already been suggested that, in carcinoma cells, the enhanced glucose transporter expression is independent of the growth rate (26). This is reasonable as the expression of the GLUT1 gene is influenced by the hypoxia inducible factor-1. Therefore, oxygen tension might regulate GLUT1 expression as well, and any correlative study using immunohistochemistry should consider this fact which is frequently ignored.

Since no in vitro study investigating the relationship between proliferation, GLUT1 expression and glucose consumption is available, this issue was studied in three kinetically different cell lines of colorectal adenocarcinomas. Standard cell culture conditions disclosed hypoxic stress. Our data indicate that the cells under investigation constitutively overexpress GLUT1 and that there might be an inverse relationship between the expression of GLUT1, glucose consumption and cell proliferation. Moreover, the increased protein synthesis of arrested cells suggests that protein synthesis and glycosylation require more glucose than cell division. In agreement with our findings are data described by Harris et al., where GLUT3 and GLUT5 were increased in Caco-2 cells in the post-confluent state (29).
Moreover, the subcellular distribution of GLUT1 was studied. In normal cells, GLUT1 is distributed evenly on the plasma membrane and low-density microsomes (30). This situation changes in carcinoma cells, where the expression seems to be dependent on cellular differentiation. It has been suggested that, in undifferentiated cells, GLUT1 is mainly localized in the cell membrane (31, 32). However, we found GLUT1 to be evenly distributed in the cell membrane and high-density microsomes in both HT29 and HRT-18 cells, which show a lesser degree of differentiation than CX-2 cells, that had double the amount of GLUT1 in the plasma membrane fraction than in high-density microsomes. Although plasma membranes are difficult to separate completely from the endoplasmic reticulum in cell culture samples, the distribution differences between CX-2 cells and both HRT-18 and HT29 cells is too distinct to be a separation problem.

The functional activity of glucose transporters in tumour cells is regulated by its maximal velocity (intrinsic activity) and the $K_m$ of 2-20 mM (33). Therefore, GLUT1 is saturated under physiological glucose concentrations. However, the intrinsic activity depends on structural modifications like glycosylation or alternative splicing. GLUT1 is a heterogeneous glycosylated protein (34). The lower molecular weight found in CX-2 and HT29 cells (around 5-10 kD less than the expected size of 50 kD that was found in HRT-18 cells) is probably the result of a different glycosylation pattern. Different glycosylation patterns may explain the different glucose uptake of HRT-18 and HT29 cells, despite comparable expression levels of GLUT1. Since alternative splicing variants of GLUT1 exist, it is necessary to investigate whether isoforms are expressed in tumour cells.

The biological significance of GLUT1 expression in carcinomas is not yet clear. Some evidence suggests that GLUT1 expression in colorectal adenocarcinomas is associated with a high incidence of lymph node metastasis (20) and a poor prognosis in lung cancer (15). In brain tumours and breast cancer, a correlation was found between expression of GLUT1 and the grade of malignancy (28, 35).

As a whole, our data suggest that high glucose consumption is needed for synthesis of various products like glycoproteins – either as a source of energy or as a structural component – rather than for proliferation. Therefore, even in tumour cells, an indirect correlation exists between proliferation and metabolic activity.

References


