Rat C6 glioma cell growth is related to glucose transport and metabolism

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In order to establish whether growth of glioma cells is associated with glucose transport and metabolism, we investigated expression of the glucose transporter and hexokinase, as well as glucose transport and glucose phosphorylation in rat C6 glioma cells growing at different rates. Rat C6 glioma cells were subcloned to produce four different cell lines (CL1, CL2, CL3 and CL4) differing in growth, differentiation and morphology: CL1 cells were slow-growing with an astrocytic appearance whereas CL4 cells grew rapidly and were small and spindle-shaped. Immunocytochemical analysis using glial fibrillary acidic protein and galactocerebroside antibodies revealed that CL1 and CL4 cells differentiate to astrocytes and oligodendrocytes respectively. Both of these cell lines expressed GLUT1 mRNA predominantly, whereas little GLUT3 mRNA was evident by Northern-blot analysis. The GLUT1 mRNA level was much higher in CL4 than in CL1 cells, and the uptake of 2-deoxy-D-glucose and 3-*O*-methyl-D-glucose by CL4 cells was markedly higher than that by CL1 cells, indicating a correlation between the growth rate, glucose transporter (GLUT1) level and glucose-transport rate of C6 glioma cells. We then studied glucose metabolism by CL1 and CL4 cells by measuring their hexokinase activities and intracellular concentrations of glucose and ATP. The mitochondrial hexokinase activity of CL4 cells was about three times higher than that of CL1 cells, whereas the cytosolic hexokinase activity of CL4 cells was only about half that of CL1 cells. As the total amount of cellular hexokinase protein in CL4 cells was only slightly higher (about 20 %) than that in CL1 cells, the hexokinase protein of CL4 cells was considered to have moved from the cytosol to the mitochondrial membranes. Consistent with the increased mitochondrial hexokinase activity of CL4 cells, the intracellular glucose concentration was undetectable, and the ATP concentration was higher than that of CL1 cells, suggesting that glucose transport is the rate-limiting factor for overall glucose metabolism in rapidly growing C6 cells. Therefore the present data demonstrate that glioma cell growth is related to glucose transport, which is closely associated with glucose metabolism.

INTRODUCTION

Most tumour cells display increase glucose uptake and metabolic rates over untransformed cells [1–3]. An accelerated glucose-transport rate is one of the most characteristic biochemical features of neoplastic cells and transformed phenotypes [4]. As glucose transport is considered to be the first step of a rate-limiting reaction of overall glucose metabolism in cells [4], alterations in glucose transport and transporter levels may be closely related to the growth and/or proliferation of tumour cells. However, few studies showing that the growth rate of astrocytic tumours is related to the glucose-transport system have been reported. The transport of glucose into both normal and transformed cells is a facilitated-diffusion Na^+-independent process [4,5]. There are at least five different types of glucose-transporter genes in human, which have been designated GLUT1 to GLUT5 [6]. Recently, we found that human astrocytic tumours express GLUT1, GLUT3 and GLUT4, but we observed no correlation between tumour malignancy and the levels of glucose-transporter expression [7]. However, overexpression of the GLUT1 and GLUT3 genes in several human cancer tissues has been reported [8–10]. Thus the data on certain aspects of this subject are conflicting. It appears to be very difficult to perform precise quantitative studies to determine whether the growth rate and malignancy of human tumour cells are related to the level of glucose-transporter expression, particularly when human brain biopsy specimens are used.

In the present study, we explored the relationship between astrocytic tumour cell growth and the glucose-transport system using subcloned rat C6 glioma cells (CL1 and CL4) with different growth rates, and studied the levels of cellular hexokinase protein, mitochondrial and cytosolic hexokinase activities and intracellular concentrations of ATP and glucose.

EXPERIMENTAL

Materials

Minimal essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), streptomycin and penicillin were obtained from Gibco. [α-^{32}P]dCTP, [U-^{14}C]glucose, 3-*O*-methyl-D-[^{3}H]glucose and 2-deoxy-D-[1-^{14}C]-glucose were purchased from Amersham Corp. Kits for DNA labelling and RNA PCR and *Taq* DNA polymerase were purchased from TakaRa Shuzo Ltd. A Bio-Rad DC-protein assay kit was used. Nitrocellulose filters were obtained from Schleicher & Schuell. Cytochalasin B and 2-deoxy-D-glucose were purchased from Sigma. ATP, $NADP^+$, hexokinase (yeast) and glucose-6-phosphate dehydrogenase (yeast) were obtained

Abbreviations used: RT-PCR, reverse transcriptase PCR; GLUT, facilitated-diffusion glucose transporter; BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; GC, galactocerebroside; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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from Boehringer-Mannheim. Oligonucleotides were synthesized on an Applied Biosystems model 391 DNA synthesizer. All other reagents used were of the highest quality commercially available.

Colonial cloning of rat glioma C6 cells

The original strain of rat glioma C6 cells [11] used in this study came from the American Type Culture Collection (CLL107; Rockville, MD, U.S.A.). The cells were grown in MEM supplemented with 10% (v/v) heat-inactivated FBS, 100 μg/ml streptomycin and 100 units/ml penicillin, maintained in a humidified incubator under an atmosphere of 5% CO_2 and 95% air at 37 °C, and harvested with trypsin/EDTA solution (Gibco). A total of 1000 cells was plated on 150 mm culture dishes (Corning) and grown for 2 weeks under the above conditions. The resulting cell colonies were viewed and marked under a microscope, and each marked colony was scraped off the dish with a small piece (5 mm × 5 mm) of filter paper (Toyo, Tokyo, Japan) that had been immersed in trypsin/EDTA solution. Each single-cell colony was transferred to a 24-well culture dish (Corning) and cultured under the conditions described above. The growing cells were observed microscopically and four morphologically distinct clonal cell types, denoted CL1, CL2, CL3 and CL4, were established. The morphological appearances of these clones were astrocytic, fibroblastic, epithelioid and small spindle-shaped round cells respectively.

Determination of the labelling index

Cells from each clone suspended in 10% (v/v) FBS/MEM were seeded on to glass coverslips at about 2×10^4/cm^2, labelled with 10 mM bromodeoxyuridine (BrdU) for 30 min at 37 °C, fixed with 70% (v/v) ethanol for 10 min, washed twice with 0.01 M PBS, denatured with 4 M HCl for 10 min and then rinsed with 0.01 M PBS. After being blocked with 10% (v/v) normal goat serum for 30 min, the cells were incubated with a solution of anti-BrdU IgG (Caltag Laboratories), diluted 1:5000, for 1 h at room temperature, washed twice with PBS, then allowed to react with a solution of anti-mouse IgG conjugated with horseradish peroxidase (Dako), diluted 1:100, for 1 h at room temperature. After a wash with PBS, the cells were subjected to the peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride (Wako) in the presence of 0.03% (v/v) H_2O_2. The percentage of labelled nuclei on each coverslip (denoted as the BrdU labelling index) was estimated by counting a minimum of ten microscopic fields and at least 1000 cells.

Immunocytochemistry

Cells from each clone cultured on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min, washed three times with PBS containing 0.1 M glycine, and permeabilized with blocking buffer (PBS containing 10% normal goat serum and 0.05% Triton X-100) for 30 min. The cells were incubated with antibodies specific for either glial fibrillary acidic protein (GFAP) (diluted 1:100) or galactocerebroside (GC) (diluted 1:10) as described previously [12], washed three times, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Dako) diluted 1:1000 for 1 h. After three washes, the coverslips were processed by the avidin–biotin–peroxidase complex procedure described previously [12], and visualized.

RNA isolation and Northern-blot analysis

Total RNA was extracted by the acid guanidinium thiocyanate/phenol/chloroform method [13]. RNA was separated in a 1.5% agarose/0.66 M formaldehyde gel, blotted on to nitrocellulose filters, and hybridized with ^{32}P-labelled cDNA probes. The cDNAs of rat GLUT1 and rat GLUT3 were obtained as described previously [14]. The membranes were washed under high-stringency conditions [0.1 × SSC/0.1% SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate); 55 °C] and exposed to Kodak X-Omat AR film at −70°C.

Immunoblot analysis

Cells were disrupted by sonication in 0.5 ml of a solution of 40 mM Tris/HCl, pH 7.4, containing 250 mM sucrose, 1 mM PMSF and 1% Triton X-100, mixed with Laemmli sampling solution, and frozen at −20 °C until used. The amount of protein was determined using a DC-protein assay kit. Western blots were performed essentially as described previously [7]. The blots were probed with rabbit polyclonal antibodies raised against synthetic peptides of human GLUT1 (a gift from Dr. K. Takata, Gunma University, Maebashi, Japan) and rat hexokinase I. The specificities of these antibodies have already been well characterized [15,16]. The bands were visualized with alkaline phosphatase-coupled goat anti-rabbit IgG secondary antibody (Dako) using as substrates Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Wako). Intensities of the bands were determined with a densitometric analyser (TIAS-200; ACI Japan Co.)

Measurement of glucose transport

Glucose transport was assayed by measuring the uptake of 2-deoxyglucose or 3-*O*-methylglucose, essentially as described previously [17,18]. Cells (2×10^5 or 10^4) in Hanks buffer containing 0.02 mg/100 ml BSA, 136.9 mM NaCl, 5.6 mM KCl, 0.34 mM Na_2HPO_4,$7H_2O$, 0.44 mM KH_2PO_4, 1.27 mM $CaCl_2$ and 4.20 mM $NaHCO_3$, pH 7.4, were incubated on 35 or 10 mm plates at 37 °C for 4 h respectively. Uptake of 2-deoxyglucose was initiated by addition of 0.5 μCi of 2-deoxy-D-[1-^{14}C]glucose to 1 ml of Hanks buffer in the presence of 0.1 mM 2-deoxy-D-glucose in 35 mm plates, and, after 5 min at room temperature, it was terminated by rapid washing with ice-cold PBS. Uptake of 3-*O*-methyl-D-glucose was initiated by adding 0.5 μCi of 3-*O*-methyl[^{3}H]glucose to 10 mm plates, and terminated after 15 s at room temperature by rapid washing with ice-cold PBS containing 0.1 mM phleolentin. Although the data are not shown, 3-*O*-methylglucose uptake was linear until 60 s of incubation. The cells were solubilized in 2 ml of 0.2 M NaOH and the amount of radioactivity in 2 ml was determined using 10 ml of scintillation fluid. In order to correct for radioactivity associated non-specifically with the cells, uptake in the presence of the transport inhibitor cytochalasin B (50 μM) or phleolentin (0.1 mM) was also determined, and these values, which were 30% or less of those for the corresponding uninhibited plates, were subtracted from the values obtained in the absence of inhibitor. In each experiment, uptake was assayed in duplicate.

Measurement of glucose phosphorylation

The cytosolic fractions for enzyme assays were prepared as follows. Cells (5×10^6) were sonicated at 4 °C in 500 μl of a solution comprising 50 mM triethanolamine, 0.3 M sucrose and 1 mM EDTA, pH 7.2, centrifuged at 100000 ***g*** for 1 h, and the resulting supernatants used for the assays. The mitochondria-rich fraction was isolated basically by the method of Malaisse et al. [19,20], and the pellet was washed several times to eliminate any contamination from the cytosolic fraction. The final pellet (mitochondrial fraction) was resuspended in 300 μl of the above

buffer. The hexokinase activities in the cytosolic and mitochondrial fractions were assayed by measuring the conversion of [U-^{14}C]glucose to [U-^{14}C]glucose 6-phosphate, essentially as described by Kuwajima et al. [21] with some modifications. Assays were performed in the presence of 100 μg of the cytosolic and/or mitochondrial fraction and four glucose concentrations (0–0.5 mM) in a total volume of 50 μl of reaction mixture comprising 50 mM Hepes, pH 7.6, 500 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM ATP, 0.1% BSA and 0.125–6.25 μCi of [U-^{14}C]glucose. After incubation at 37 °C for 5 min, each sample was transferred to a tube containing 100 μl of 3% (v/v) methanol in 95% (v/v) ethanol to terminate the reaction; 20 μl of the mixture was applied to a DEAE-cellulose paper disc, which was dried, then washed five times with 15 ml of water, and the radioactivity retained on the paper was determined by liquid-scintillation counting. The recovery of authentic [^{14}C]glucose 6-phosphate added to the reaction mixture was more than 90%, which showed that virtually no free [^{14}C]glucose survived the washing procedure. The reaction rates were linear over the time period studied. The results were calculated as μmol/min per g of total protein.

Determination of intracellular ATP and glucose concentrations

Cells (2×10^6) were washed with PBS twice on ice and mixed well with 1 ml of ice-cold 0.9 M perchloric acid using plastic pipette tips. The mixture was centrifuged at 12000 ***g*** for 5 min at 4 °C and the supernatant obtained was neutralized with $KHCO_3$. After most of the evolved CO_2 had dissipated, the $KClO_4$ precipitate was removed by centrifugation at 2 °C at 12000 ***g*** for 5 min, and the resulting supernatant was used for determination of the intracellular ATP and glucose concentrations. The ATP and glucose assays were performed essentially as described by Lowry and Passonneau [22], with slight modifications. Briefly, the reaction mixture for the ATP assay comprised 50 mM Tris/HCl buffer, pH 8.1, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μM $NADP^+$, 100 μM glucose, 0.5 μg/ml hexokinase (yeast), 1.25 μg/ml glucose-6-phosphate dehydrogenase and the cell extract, and that used for the glucose assay was the same except that it contained 250 μM ATP instead of glucose. The reactions were started by adding the cell extract and allowed to proceed for 20 min at room temperature, after which the increase in the fluorescence of NADPH was measured with a Ratio-2 System Fluorometer (Optical Technology Devices), and the ATP and glucose concentrations were calculated by reference to standard curves.

Statistical analysis

The data were expressed as means ± S.E.M. and were analysed using one-way analysis of variance. Differences at P values less than or equal to 0.05 were considered to be significant.

RESULTS

C6 glioma cells were subcloned to produce CL1, CL2, CL3 and CL4 cells, in accordance with their morphological differences, as described in the Experimental section. Figure 1 shows the cellular morphology of each clone: CL1, astrocytic-shaped cells with long cellular processes; CL2, fibroblastic-shaped cells; CL3, epithelioid-like cells; CL4, small spindle-shaped cells. The growth rates of these clones differed from each other. As shown in Figure 2(A), the growth of CL4 C6 cells was about ten times faster than that of CL1 cells. The BrdU labelling index, which represents the S phase of DNA synthesis during mitosis [23,24], for CL4 cells was also higher than that for CL1 cells (Figure 2B). Therefore CL4 cells grew rapidly with a concomitant increase in DNA synthesis in comparison with CL1 cells.

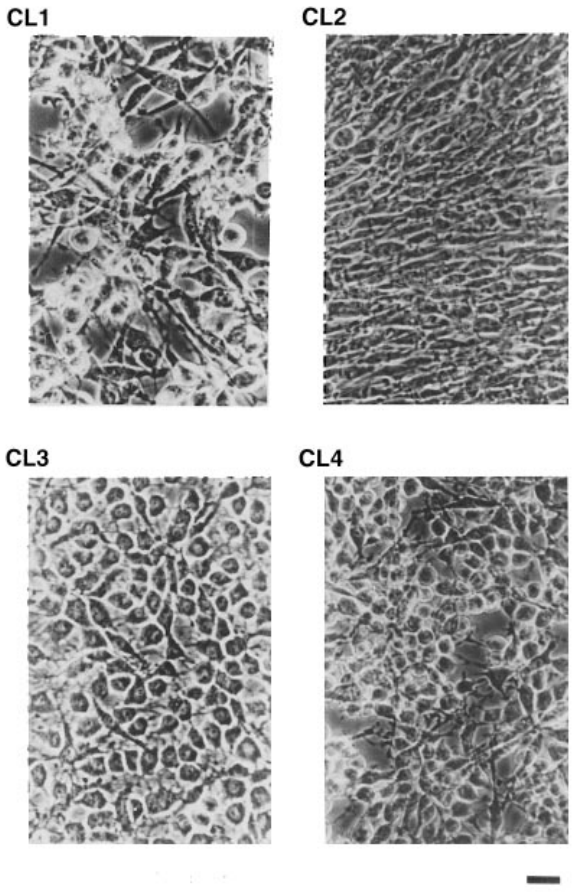

Figure 1 Phase contrast microscopic images of C6 glioma cell morphology

The clonal cells were grown for 3 days in DMEM supplemented with 10% (v/v) FBS. (Bar = 10 μm.)

In order to examine the differentiation of each clone, immunocytochemical studies were carried out using anti-GFAP and anti-GC antibodies. As shown in Table 1, the CL1 clone expressed GFAP but not GC, whereas the CL4 clone expressed GC but not GFAP. This suggested that CL1 cells differentiated to astrocytes, and CL4 to oligodendrocytes. Thus the CL1 and CL4 clones had different characteristics. In the present study, we chose these clones for further examination because their growth rates were the slowest and fastest respectively of the four. The rates of uptake of 2-deoxyglucose and 3-*O*-methylglucose by C6 cells are shown in Table 2. As it was not known whether glucose transport was the rate-limiting step in these clones, uptake of both glucose analogues was measured. The CL4 cells took up 2-deoxyglucose about ten times faster than the CL1 cells. In agreement with this result, CL4 cells also took up 3-*O*-methylglucose about three times faster than CL1 cells.

In order to determine whether the glucose-uptake rates were associated with glucose-transporter expression, we first examined the facilitated glucose-transporter isoforms expressed in CL1 and CL4 cells by reverse transcriptase (RT)-PCR using degenerate oligonucleotide primers based on the sequences of various members of the human glucose-transporter family, as

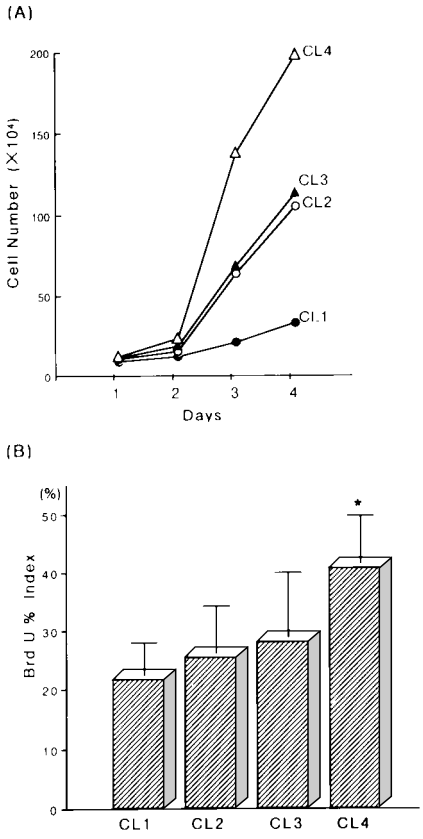

Figure 2 Growth of subcloned C6 glioma cell lines

(**A**) The cells were grown in DMEM supplemented with 10% (v/v) FBS for the incubation periods indicated and the numbers of cells were counted. (**B**) The BrdU labelling indices of subcloned C6 glioma cell lines (CL1–CL4). Cells seeded on to glass coverslips were pulse-labelled (30 min) with 10 mM BrdU and the labelling indices were determined as described in the Experimental section $^{*}P < 0.001$ compared with CL1.

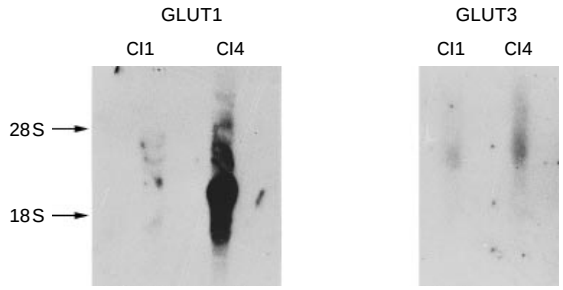

Figure 3 Expression of GLUT1 and GLUT3 mRNAs in CL1 and CL4 C6 glioma cell lines

Total cellular RNAs isolated from CL1 and CL4 C6 cells were electrophoresed, transferred to nitrocellulose filters and hybridized with ^{32}P-labelled cDNA probes of rat GLUT1 and rat GLUT3. A representative autoradiograph is shown of RNA-blot analysis of three separate experiments demonstrating a 2.7 kb GLUT1 and little evidence of a GLUT3 transcript in 10 μg of total RNA from CL1 and CL4 C6 cells. Positions of 28 S and 18 S rRNAs are indicated.

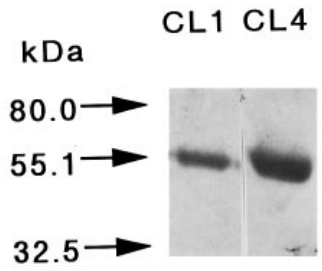

Figure 4 GLUT1 immunoblots of CL1 and CL4 C6 glioma cells

The total cell lysates of CL1 and CL4 C6 cells were separated by SDS/PAGE (10% gels) and immunoblotted with anti-GLUT1 serum. The mobilities of prestained molecular mass markers are shown on the left. Each determination was carried out at least three times and a representative result is presented.

Table 1 Immunocytological profiles of subcloned C6 glioma cell lines

	CL1	CL2	CL3	CL4
GFAP	+	+	+	−
GC	−	−	+	+

Table 2 Glucose uptake by the subcloned C6 glioma cell lines CL1 and CL4

The uptake of 2-[^{14}C]deoxyglucose and 3-*O*-methyl-[^{3}H]glucose was measured at room temperature as described in the Experimental section in 5 min and 15 s incubation periods respectively. $^{*}P < 0.0001$ compared with CL1.

	Glucose uptake	
Glucose	CL1	CL4
2-Deoxyglucose (c.p.m./5 min per 10^5 cells)	450 ± 58	3120 ± 210*
3-*O*-methylglucose (c.p.m./15 s per 10^4 cells)	750 ± 130	1920 ± 185*

described elsewhere [7]. Although the RT-PCR procedure detected both GLUT1 and GLUT3 mRNAs in CL1 and CL4 C6 cells (results not shown), Northern-blot analysis of total RNA (10 μg) detected only GLUT1 mRNA, and showed little evidence of GLUT3 mRNA (Figure 3), indicating that C6 cells predominantly expressed the GLUT1 glucose transporter. In agreement with the glucose-uptake results, as shown in Figure 3, the level of GLUT1 mRNA was markedly increased in CL4 over CL1 cells. In order to examine the level of GLUT1 protein, immunoblot analysis was performed using total cell lysates of CL1 and CL4 cells with anti-GLUT1 serum. Representative GLUT1 immunoblots of CL1 and CL4 cells, showing that the molecular masses of their proteins were approximately 50–60 kDa, are presented in Figure 4. Densitometric analysis of the bands revealed that the amount of GLUT1 in CL4 cells was about three times higher ($320 \pm 23\%$; $P < 0.001$) than that in CL1 cells.

In order to establish whether differences in glucose metabolism were also attributable to the different growth rates of the glioma cells, we examined the hexokinase activities of the mitochondrial and cytosolic fractions prepared from CL1 and CL4 cells using the radiometric glucose-phosphorylation assay, and then determined the level of hexokinase protein in the total cell lysate by

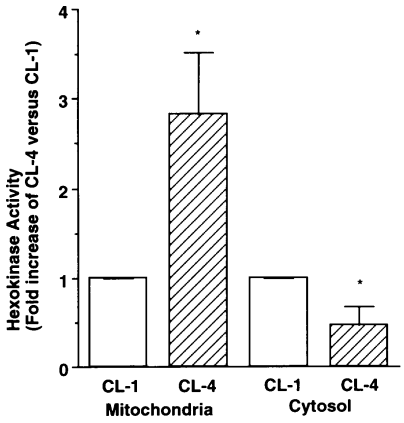

Figure 5 Hexokinase activity of mitochondrial and cytosolic fractions of CL1 and CL4 C6 glioma cells

Cytosolic and mitochondrial fractions were prepared from CL1 and CL4 C6 cells and radiometric enzyme assays in the presence of various concentrations (0–0.5 mM) of [^{14}C]glucose were performed, as described in the Experimental section. The hexokinase activity is expressed as the fold increase in activity relative to that of the CL1 sample. The data represent the mean $\pm$ S.E.M. values obtained from three or four independent plates of CL1 and CL4 cells. *$P < 0.001$ compared with CL1.

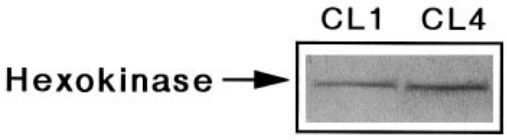

Figure 6 Hexokinase immunoblots of the clonal C6 glioma cells (CL1 and CL4)

Immunoblot analysis with anti-hexokinase serum was performed, as described in the legend to Figure 4. Hexokinase was identified as a protein of about 110 kDa.

immunoblot analysis with anti-hexokinase antibody. As shown in Figure 5, the mitochondrial hexokinase activity of CL4 cells was about three times higher than that of CL1 cells, but densitometric analysis of the hexokinase protein band obtained from the immunoblot showed that the total amount of immunodetectable hexokinase protein in CL4 cells was only slightly higher ($122 \pm 9.0\%$; $P < 0.001$) than that in CL1 cells (Figure 6). Therefore the increased mitochondrial hexokinase activity of CL4 cells appears to be attributable, in part at least, to the movement of cytosolic hexokinase protein from the cytosol to the mitochondria. Indeed, the cytosolic hexokinase activity of CL4 cells was about half that of CL1 cells (Figure 5). In agreement with the high mitochondria hexokinase activity of CL4 cells, their intracellular glucose concentration was undetectable, whereas that of CL1 cells was 0.19 ± 0.06 nmol/mg of protein (Table 3), indicating that most of the glucose taken up by CL4 cells was rapidly converted into glucose 6-phosphate. In contrast, the intracellular ATP concentration in CL4 cells (10.29 ± 1.65 nmol/mg of protein) was higher than that in CL1 cells (7.69 ± 0.36 nmol/mg of protein; $P < 0.05$). Taken together, these results indicate that glucose uptake by rapidly growing C6 cells (CL4) was accelerated by increased GLUT1 expression, with subsequent efficient glucose metabolism.

Table 3 Intracellular levels of glucose and ATP in the C6 glioma cell lines CL1 and CL4

Glucose and ATP concentrations were determined as described in the Experimental section. *$P < 0.05$ compared with CL1.

	CL1	CL4
Glucose (nmol/mg of protein)	0.19 ± 0.06	Undetectable
ATP (nmol/mg of protein)	7.69 ± 0.36	10.29 ± 1.65*

DISCUSSION

In a previous study, we demonstrated that there was no apparent correlation between the histological grading of human astrocytic tumour cells, based on the World Health Organization classification, and their levels of glucose-transporter expression [7]. However, there is some controversy about this finding, because other investigators have reported that the biological aggressiveness of astrocytic tumours is related to the level of expression of glucose transporters [9,10], and clinical studies using positron-emission tomography have demonstrated that metabolic utilization of glucose is higher in brain tumours than in normal cerebral tissue [25,26]. The discrepancy between the present results and previous findings probably reflects the fact that the histological grading of astrocytic tumours does not always correspond to their growth rates *in situ* [27]. As mentioned in the Introduction, it is very difficult to perform such a detailed study using human brain biopsy samples. Therefore rat C6 glioma cells were used in the present study to enable us to investigate whether glioma cell growth is associated with the glucose-transport system and glucose metabolism. First, we subdivided rat C6 glioma cells into four different clones (CL1–CL4), which not only differed morphologically, but also grew at different rates (CL4 > CL3 > CL2 > CL1). As the CL4 clone showed both the fastest growth rate and highest BrdU labelling index, we decided to use it for a comparative study with CL1, the slowest growing clone.

In agreement with the results of previous studies on other cell lines [2,28–30], the rate of glucose uptake could be correlated with growth rates. An increased glucose-transport rate is considered to be attributable either to a change in the transport mechanism or increased phosphorylation by intracellular kinases. First, we studied the levels of glucose transporter expressed in C6 glioma cells. Since the type of glucose transporter isoform they express is not known, we carried out RT-PCR using degenerative oligonucleotide primers to pick up all the glucose-transporter isoforms, as described previously [7]. It was found that the C6 glioma cells predominantly expressed GLUT1. In contrast, human astrocytic tumours *in situ* express GLUT3 predominantly [9,10] and rat glial cells do not usually express appreciable amounts of GLUT1 and GLUT3 [14,31]. The predominant expression of GLUT1 in C6 cells may be due to tumorigenic change or the tissue culture conditions. Indeed, FBS is known to stimulate transcription of the GLUT1 gene in NIH3T3 cells [32]. Nevertheless, consistent with the glucose-uptake results, the expression levels of GLUT1 mRNA and protein were higher in the rapidly growing CL4 cells than in the slowly growing CL1 cells, indicating that the accelerated glucose uptake in the former resulted from increased GLUT1 expression. The magnitude of the increase in 3-*O*-methylglucose-transport activity in CL4 cells (about 3-fold relative to CL1) also correlated with the

increased level of GLUT1 protein (about 3-fold relative to CL1). On the other hand, the CL1 and CL4 clones showed differences in differentiation (Table 1); CL1 expressed only GFAP, whereas CL4 expressed only GC. Since GFAP and GC are markers for astrocytes and oligodendrocytes respectively [12], it is concluded that CL1 cells differentiate to astrocytes, and CL4 cells to oligodendrocytes. As Flier et al. [2] have reported that glucose-transport activity differs between differentiated and non-differentiated cells, the difference in GLUT1 activity observed between CL1 and CL4 cells might also be due to the difference in cell differentiation.

Next, we studied glucose metabolism in C6 cells by determining the activity and level of expression of hexokinase. As hexokinase levels have been reported to be higher in rapidly growing cells than in slowly growing cells [33,34], we expected to see higher mitochondrial and cytosolic hexokinase activities in CL4 than in CL1 cells. Mitochondrial hexokinase activity in CL4 cells was about three times higher than that in CL1 cells, but, in contrast, the cytosolic hexokinase activity of CL4 cells was only about half that of CL1 cells. As the total amount of immunodetectable hexokinase protein in CL4 cells was only slightly higher (about 20%) than that in CL1 cells, the increased mitochondrial hexokinase activity of the former was considered to be attributable to the movement of hexokinase protein from the cytosol to the mitochondria. Hexokinase binding to mitochondria is believed to increase the affinity of the enzyme for ATP and reduce its sensitivity to glucose 6-phosphate [35]. Furthermore, since mitochondrial hexokinase efficiently utilizes ATP generated in the mitochondria either by the adenylate kinase reaction or oxidative phosphorylation [36], the participation of mitochondrial hexokinase in glucose phosphorylation might favour the maintenance of a high level of cytosolic ATP. Therefore our data support the previous proposal that mitochondrial hexokinase participates in the high glycolytic rate of rapidly growing tumour cells [37]. Indeed, intracellular glucose in CL4 cells was undetectable, suggesting that mitochondrial membrane-bound hexokinase metabolized the glucose efficiently. These results indicate that glucose transport is rate-limiting for overall glucose metabolism in rapidly growing CL4 cells but not in slowly growing CL1 cells. Unexpectedly, the magnitude of the increase in the intracellular ATP concentration in CL4 cells was small, perhaps because of rapid utilization of the ATP produced by the cells for their high energy requirement. Eventually, the steady-state amount of ATP in CL4 cells may not differ much from that in CL1 cells.

In summary, after rapidly growing C6 cells have taken up glucose at a fast rate because of their high levels of GLUT1 mRNA and protein, they rapidly metabolize the glucose to ATP, probably using mitochondrial-bound hexokinase. Therefore our data demonstrate that the glucose-transport system, coupled with glucose metabolism, is closely related to the growth of glioma cells.

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