Distribution of the Glucose Transporters in Human Brain Tumors

Tatsuya Nishioka, Yoshifumi Oda, Yutaka Seino, Taizo Yamamoto, Nobuya Inagaki, Hideki Yano, Hiroo Imura, Ryuichi Shigemoto, and Haruhiko Kikuchi

Department of Neurosurgery [T. N., Y. O., H. K.], Department of Metabolism and Clinical Nutrition [Y. S., T. Y.], Second Department of Internal Medicine [N. I., H. Y., H. J.], and Department of Morphological Brain Science [R. S.], Kyoto University Medical School, Sakyo-ku, Kyoto 606, Japan

ABSTRACT

In the present study, we have investigated the expression of both the erythrocyte-type (GLUT1) and the brain-type (GLUT3) glucose transporter isoforms in primary human brain tumors. In situ hybridization made it possible to localize and semiquantify both GLUT1 and GLUT3 mRNAs of individual cells in all 18 samples examined. More signals for GLUT3 mRNA than for GLUT1 mRNA were found over astrocytoma cells, while the reverse was the case in all 6 meningiomas. In astrocytomas, for both mRNAs, the density of silver grains over tumor cells was well correlated with the malignancy of the cells. This correlation was, as was also confirmed by Northern blot analysis, more marked with GLUT3 mRNA than with GLUT1 mRNA. In 2 of 5 anaplastic astrocytomas and in all 3 glioblastomas, numerous tumor cells with large amounts of both mRNAs tended to surround the perivascular regions. "Tumor vessels" with endothelial proliferation, an almost pathognomonic feature of glioblastomas, expressed much GLUT3 mRNA but no significant GLUT1 mRNA, while a single- or a few-layered capillary endothelium expressed much GLUT1 mRNA. The distribution of both mRNAs was in good accordance with that of both proteins. Our results suggest that the expression of both glucose transporter isoforms may contribute to the maintenance of human brain tumors and that the expression of the GLUT3 isoform may be closely related to the malignant change of astrocytomas and particularly related to the aberrant neovascularization which accompanies glioblastomas.

INTRODUCTION

The major metabolic substrate of the brain is glucose, and the transport of glucose from blood to brain is tightly coupled with cerebral glucose utilization (1). The transport of this important molecule into brain tissue is accomplished by facilitated diffusion (2), which is mediated by a family of structurally related proteins (3-7). These facilitated glucose transporters consist of at least five isoforms, GLUT1-5, as designated by Bell et al. (8). Among them, both the erythrocyte-type (GLUT1) and the brain-type (GLUT3) glucose transporter isoforms are abundantly expressed in brain (8, 9) and may mediate basal glucose uptake, since they have a low Km for glucose (10). It has been suggested, however, that the expression of these two isoforms may be regulated by different mechanisms (11). The GLUT1 isoform is known to be quantitatively the most important, if not the only, glucose transporter isoform in brain capillary endothelium (12). In contrast, the precise location of the GLUT3 protein in the human brain is still unclear.

It is well known that neural neoplasms rely heavily upon glycolysis, rather than respiration, for the production of ATP (13-15). A consequence of aerobic glycolysis is that the metabolic activity of brain neoplasms must be assessed by glucose uptake, rather than by oxygen consumption (16). It has been demonstrated in both experimental (13, 17) and human tumors (using deoxyglucose methods of positron emission tomography) (18-20) that cerebral glucose uptake and consumption by tumor may be up to 3 times that of normal (21, 22).

It is also known that the proliferation of the transformed cells is accompanied by an elevation of glucose uptake and its metabolism (23). This phenomenon may be attributed in part to overexpression of facilitated glucose transporter genes, at least in the tumors of the digestive system (24, 25).

Astrocytomas are the most common primary intracranial tumors and may be classified, according to increasing malignancy, as low-grade astrocytomas, anaplastic astrocytomas, and glioblastomas (26). The malignant progression of astrocytomas is associated with dedifferentiation and increasing neovascularization (26, 27). The glioblastoma, which represents the most malignant variant of astrocytoma, is characterized microscopically by cellular pleomorphism, pseudopalisading, and endothelial proliferation (28). Meningiomas are tumors arising from meningothelial cells of mesodermal origin. Although benign, meningiomas often grow into a large mass and show increased vascularity (29).

In the present study, we have examined in vivo both the expression and the distribution of these two glucose transporter mRNAs and their protein products in primary human brain tumors, in order to clarify the molecular events associated with the glucose metabolism of these tumors.

MATERIALS AND METHODS

Materials. For Northern blot analysis, samples were prospectively collected immediately after surgical removal and were frozen in liquid nitrogen before being stored in a −80°C freezer. Of the 31 specimens investigated by Northern blot analysis, normal human brain was derived from a patient with temporal epilepsy, 11 were from patients with astrocytomas, and 19 were from patients with meningiomas. All 30 brain tumors were primary cases. Samples of human hepatoma cell line (Hep G2), bovine cortex, and a human esophageal cancer (histologically diagnosed as squamous cell carcinoma) were also used. Eighteen samples were used for in situ hybridization (Table I). One of them, a case of pontine astrocytoma, was not examined by Northern blot analysis, because of the scarcity of available material. Samples obtained from the same 18 patients were also immediately fixed in Bouin’s fixative for immunocytochemical study. The microscopic slides from all 18 specimens were reviewed, and astrocytomas were categorized into one of three grades, i.e., low-grade astrocytoma, anaplastic astrocytoma, or glioblastoma multiforme, according to the method of Burger and Vogel (26). Tissue samples of an esophageal cancer and the normal brain obtained from a patient with temporal epilepsy were also used for both in situ hybridization and immunocytochemistry.

Northern Blot Analysis. Total RNAs were isolated by the guanidinium thiocyanate-cesium chloride procedure (30). Twenty μg of total RNA were denatured with formaldehyde, electrophoresed on 1% agarose gels, blotted onto nylon filters, and then hybridized to 32P-labeled GLUT1 or GLUT3 cDNA probes. Hybridization was carried out at 42°C in a solution of 50% formamide, 5x SSC (1x SSC is 0.15 M NaCl, 0.1 M sodium citrate, pH 7.0), 0.1% SDS, and 10% dextran sulfate at 42°C in a solution of 50% formamide, 5x SSC (1x SSC is 0.15 M NaCl, 0.1 M sodium citrate, pH 7.0), 0.1% SDS, and 10% dextran sulfate.

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To whom requests for reprints should be addressed, at Department of Neurosurgery, Kyoto University Medical School, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan.

The abbreviations used are: GLUT1-5, glucose transporter isoforms 1-5; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; cDNA, complementary DNA; ISH, in situ hybridization.
Table 1 Summary of 18 patients analyzed by in situ hybridization and immunocytochemistry

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* Case numbers of astrocytomas correspond to lane numbers in Fig. 1. A sample of case 9 was derived from a patient with pontine astrocytoma and was not examined by Northern blot analysis due to the scarcity of available material. Meningioma cases 1 and 2 and normal brain correspond to lanes M1, M2, and N in Fig. 1, respectively. Moreover, astrocytomas are categorized into one of three grades, according to the system of Burger and Vogel (26): astrocytoma cases 1, 2, and 4 are graded as low-grade astrocytomas, cases 5, 6, 7, 8, and 9 are anaplastic astrocytomas, and cases 10, 11, and 12 are glioblastomas.

c Expression of both proteins is classified as follows: +, positive; -, negative; ND, not determined.

f Vascular cells, single- or a few-layered capillary epithelium.

0.015 M sodium citrate, pH 7.0), 2× Denhardt’s solution (1× Denhardt’s solution is bovine serum albumin, polyvinylpyrrolidone, Ficoll, all at 0.2 mg/ml), 20 mM sodium phosphate buffer (pH 7.4), 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate. After this, the filters were washed twice in a solution of 0.1× SSC and 0.1% SDS at room temperature for 30 min, followed by a wash at 50°C for 30 min, and autoradiography was performed at ~80°C for 3 days, with an intensifying screen. The probes for the present studies included GLUT1 cDNA (a 1396-base pair EcoRI-HindIII insert from phGT2-I) (3, 31) and GLUT3 cDNA (a 2190-base pair EcoRI insert from ph-MGT-3I) (5).

ISH. ISH was carried out essentially as described (32). Cryostat sections (10 μm) were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min, rinsed in phosphate-buffered saline, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, 0.9% NaCl, for 10 min. After dehydration in an ascending ethanol series, the sections were air dried and kept at ~80°C until hybridization. Hybridization was carried out in 50% formamide, 2× SSC, 10 mM Tris-HCl (pH 7.5), 1× Denhardt’s solution, 10% dextran sulfate, 0.2% SDS, 100 μg/ml dithiothreitol, 500 μg/ml sheared single-stranded salmon sperm DNA, 250 μg/ml yeast tRNA. The riboprobes were preheated at 80°C for 3 min in 1 μl dithiothreitol.
GLUCOSE TRANSPORTERS IN HUMAN BRAIN TUMORS

Fig. 2. Localization of GLUT1 mRNA by hybridization of a 35S-labeled antisense cRNA probe to cryostat tissue sections of low-grade (A) and anaplastic (B and C) astrocytoma. A and B, bright-field micrographs; C, corresponding dark-field micrograph of the section shown in B. In A, sparse silver grains are found over astrocytoma cells. In B and C, many hybridization-positive astrocytoma cells are distributed around densely hybridized capillary endothelium. A, low-grade astrocytoma; AA, anaplastic astrocytoma. Cresyl violet counterstain. A, x 750; B and C, x 250.

The sections were treated with hybridization solution, covered with a coverslip and sealed with rubber cement. After incubation at 57°C for 8 h, the slides were immersed in 2× SSC, to remove the coverslips, and were washed in 2× SSC, 10 mM 2-mercaptoethanol, at room temperature for 1 h and at 60°C for 1 h. The slides were then treated with 20 μg/ml of RNase A in 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, followed by an additional wash performed at a final stringency of 0.1× SSC at 60°C for 1 h. After dehydration in ascending ethanol, the slides were air dried and hand-dipped in Kodak NTB2 emulsion, exposed at 4°C for 4 weeks, developed, and stained with cresyl violet.

Semiquantitative evaluation of relative grain densities on the individual cell bodies per slide was accomplished by comparison with densities on the capillary endothelial cells of human normal brain obtained from a patient with temporal epilepsy. The control sections were placed on the same slide and processed under identical conditions (Table 1).

The hybridization reaction contained 5 × 10⁶ cpm of 35S-labeled antisense or sense riboprobes per slide. The riboprobes for GLUT1 and GLUT3 were prepared by subcloning a 669-base pair EcoRI-HincII fragment (nucleotides 280–948, clone phGT2-l) (3) and a 625-base pair BamHI-EcoRI fragment (nucleotides 116–740, clone phMGT-31) (5), respectively, into pGEM3Z (Promega Biotec, Madison, WI). Antisense riboprobes were synthesized with SP6 and T7 RNA polymerase following linearization of the plasmid with EcoRI and BamHI, respectively, and were labeled by incorporating [35S]CTP in the polymerase reaction. The corresponding sense riboprobes for GLUT1 and GLUT3 were synthesized with T7 and SP6 DNA polymerase, following linearization of the plasmid with HincII and EcoRI, respectively. The RNA was subsequently purified by digestion with RNase-free DNase I, followed by phenol–chloroform extraction. After removal of unincorporated nucleotides by two rounds of ethanol precipitation with 4 M ammonium acetate, riboprobes were fragmented to ~150-base lengths by alkaline hydrolysis. The specificity of the probes for in situ hybridization was controlled by Northern blot analysis and by hybridization of serial sections with sense RNA probes.

Immunocytochemistry. Fixed samples were embedded in paraffin and sectioned. The 4-μm-thick tissue sections were reacted with the appropriate antibody using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) (33). The specificity of the immunostaining was tested by replacing the primary antibody with normal rabbit immunoglobulin. The following specific antisera were used in these studies: polyclonal antibody against the carboxyl terminus of the GLUT1 sequence (AB 104; Chemicon International Inc., Temecula, CA) (3, 34, 35) and polyclonal antibody against the carboxyl terminus of the human GLUT3 sequence (RatGlut-3-AP; East Acres Farm Inc., Southbridge, MA) (5).

RESULTS

Expression of GLUT1 and GLUT3 mRNAs. Northern blot analyses for both GLUT1 and GLUT3 mRNAs in human brains and primary brain tumors are shown in Fig. 1. As previously described, the GLUT1 and GLUT3 mRNAs consisted of a single transcript of 2.8 kilobases (3) and two transcripts of 4.1 and 2.7 kilobases (5), respectively. Moderate amounts of both GLUT1 and GLUT3 mRNAs were detected in the normal human brain. In all 11 astrocytomas, the level of GLUT1 mRNA was lower than that in the normal brain. In contrast, the level of GLUT3 mRNA in astrocytomas varied widely, depending upon their histological grading. The levels of GLUT3 transcripts in all 4 low-grade astrocytomas were below the sensitivity of our RNA blotting assay. In 3 of 4 anaplastic astrocytomas, GLUT3 mRNA was expressed only faintly. In 1 of 4 anaplastic astrocytomas and all 3 glioblastomas, moderate or high GLUT3 mRNA levels were detected. In all 19 meningiomas except 1, the level of GLUT3 mRNA was below that in the normal brain. On the other hand, moderate amounts of GLUT1 mRNA were detected in 8 of 19 meningiomas. Two representative cases of the 19 meningiomas are presented in Fig. 1.

ISH using antisense RNA probes permitted us to semiquantitatively evaluate hybridization signals over individual cell bodies (Table 1). The results presented here were obtained from 8–10 serial sections of each tissue. The control data with ISH of
Fig. 3. Photomicrographs of cresyl violet-stained frozen sections of glioblastoma tissue. A, B, and C, bright-field views of sections hybridized to an anti-sense 35S-labeled GLUT1 RNA probe; D, dark-field view of the same section shown in C. In A, while little accumulation of silver grains is seen over tumor cells in the perivascular region, large amounts of silver grains are found over numerous pseudopalisading tumor cells. In the vicinity of the pseudopalisading cells, necrosis is seen (arrow). In B, a single-layered capillary epithelium contains positive hybridization signals. In C and D, no significant hybridization signals are found over tumor vessels with endothelial proliferation. Hybridization-positive tumor cells are distributed around the tumor vessel. GM, glioblastoma multiforme. Cresyl violet counterstain. A, C, and D, x 250; B, x 500.

serial sections using sense RNA probes, moreover, did not demonstrate significant expression of either mRNA. In estimating hybridization signals, only the highest-grade areas of anaplastic astrocytomas and glioblastomas were considered, since histological diagnosis is usually based on the highest-grade area observed. In astrocytomas, more silver grains for both mRNAs tended to be seen over individual tumor cells as the histological malignancy increased (Table 1). This tendency was, in addition, more prominent with GLUT3 mRNA. In one esophageal cancer, in contrast, extremely dense signals for GLUT1 mRNA were found over almost all tumor cells, while dense signals for GLUT3 were seen over only a few tumor cells (data not shown).

Localization of GLUT1 mRNA in Human Brain Tumors. In 3 of 4 low-grade astrocytomas and 3 of 5 anaplastic astrocytomas, tumor cells with faint hybridization were distributed diffusely (Fig. 2A). In 2 of 5 anaplastic astrocytomas, the number of silver grains over hybridization-positive tumor cells clearly increased, except over the perivascular tumor cells. Capillary endothelial cells within the tumor tissue always displayed positive hybridization signals for GLUT1 mRNA (Fig. 2, B and C).

The expression of GLUT1 mRNA was highest in glioblastoma cells, and the distribution of densely hybridized tumor cells had a prominent feature: while little accumulation of silver grains was seen over tumor cells in the perivascular regions, strong hybridization signals were found over numerous tumor cells which surrounded these regions (Fig. 3A). In some areas, moreover, the necrosis was distributed around these "pseudopalisading" tumor cells with a dense accumulation of grains. Although a single- or a few-layered capillary endothelium usually showed positive hybridization signals (Fig. 3B), "tumor vessels" with endothelial proliferation did not show any significant expression (Fig. 3, C and D). These tumor vessels were recognized in 2 of 5 anaplastic astrocytomas and all 3 glioblastomas (Table 1).

In all 6 meningiomas, densely hybridized tumor cells tended to form an aggregation. Positive hybridization signals were usually seen over capillary endothelial cells (data not shown).

Localization of GLUT3 mRNA in Human Brain Tumors. In 3 of 4 low-grade astrocytomas, only a few tumor cells were hybridized strongly. In 1 of 4 low-grade astrocytomas and 2 of 5 anaplastic astrocytomas, strongly hybridized tumor cells were interspersed among hybridization-negative tumor cells (Fig. 4, A and B). In 2 of 5 anaplastic astrocytomas, many hybridized tumor cells tended to be dispersed diffusely, except in the perivascular region (Fig. 4, C and D). The silver grains over capillary endothelial cells within the tumor tissue tended to increase in number as the histological grading increased (Table 1).

In all glioblastomas, the distribution of the hybridization signals for GLUT3 over tumor cells was similar to that of GLUT1
mRNA, but more strongly hybridized tumor cells were found for GLUT3 mRNA than for GLUT1 mRNA. Each hybridization-positive tumor cell, moreover, showed an extremely dense accumulation of silver grains (Fig. 5A). In contrast, the distribution of hybridization signals over capillary endothelial cells was distinct; not only did a single- or a few-layered capillary epithelium, being increased in number, usually have high levels of signals (Table 1; Fig. 5B), but tumor vessels with endothelial proliferation also showed dense hybridization signals (Fig. 5C).

In all meningiomas, only a few tumor cells showed a strong expression of GLUT3 mRNA. A few hybridization signals were usually seen over capillary endothelial cells (data not shown).

Expression of GLUT1 and GLUT3 Proteins. Immunocytochemistry of human brain tumors showed the expression of both GLUT1 and GLUT3 proteins in individual cells. Similar to the trend in hybridization properties using ISH, the presence of GLUT3 proteins in individual tumor cells was found in 1 of 4 low-grade astrocytomas, 3 of 5 anaplastic astrocytomas, and all 3 glioblastomas. On the other hand, the presence of GLUT1 proteins in tumor cells was observed in only 2 of 5 anaplastic astrocytomas and all 3 glioblastomas (Table 1). Positive staining for the GLUT3 protein but negative staining for the GLUT1 protein were also seen in tumor vessels with endothelial proliferation (Fig. 6, A and B).

The presence of GLUT1 protein in tumor cells was found in 2 of 6 meningiomas (Fig. 6, C and D). Positive staining for the GLUT1 protein in capillary endothelium was observed in 3 of 6 meningiomas, although that for the GLUT3 protein was seen in only 1 of 6 meningiomas (Table 1).

DISCUSSION

The present study has demonstrated the in vivo expression of both GLUT1 and GLUT3 mRNAs and their proteins in a number of primary human brain tumors. ISH showed the expression of the two glucose transporter genes in the individual cells of all 18 primary human brain tumors examined. In 2 of 5 anaplastic astrocytomas and all 3 glioblastomas, only a few hybridization signals for both mRNAs were seen over tumor cells comprising the perivascular regions. These regions, in contrast, surrounded by many tumor cells with a dense accumulation of silver grains for both mRNAs (Figs. 3A and 5A). This conspicuous feature was also recognized by immunocytochemistry (Fig. 6, A and B). With respect to the glucose supply, it is reasonable that, with increasing distance from the capillaries, the glucose uptake by tumor cells becomes more difficult. In primary cultures of rat brain glial cells, the GLUT1 isoform is regulated by the extracellular
GLUCOSE TRANSPORTERS IN HUMAN BRAIN TUMORS

Fig. 5. In situ hybridization for GLUT3 mRNA in glioblastoma tissue. Bright-field micrographs. In A, pseudopalisading tumor cells are characterized by an extremely dense accumulation of silver grains. In B, large amounts of silver grains are seen over a single- or a few-layered capillary epithelium. In C, tumor vessels with endothelial proliferation are densely hybridized by GLUT3 mRNA probes. GM, glioblastoma multiforme. Cresyl violet counterstain. A, B, and C, × 500.

glucose concentration (36). This feature of astrocytomas suggests that the similar mechanisms for regulation of both isoforms by the extracellular glucose concentration may be preserved in human astrocytoma cells in vivo.

Positive hybridization signals for GLUT1 mRNA were seen over a single- or a few-layered capillary endothelium in all 12 astrocytomas examined (Table 1; Fig. 2, B and C). In 2 of 5 anaplastic astrocytomas and all 3 glioblastomas, moreover, a dense accumulation of silver grains for GLUT3 mRNA was found over tumor vessels with endothelial proliferation (Fig. 5C). These tumor vessels lacked any significant expression of GLUT1 mRNA (Fig. 3, C and D). Although both isoforms are thought to mediate basal glucose uptake (10), this marked difference in the distribution between the two, as was also confirmed by immunocytochemistry (Fig. 6, A and B), suggests that each has a unique role in the tumorigenesis of astrocytomas.

The presence of tumor vessels with endothelial proliferation is known to be a harbinger of anaplastic change in cerebral astrocytomas, as well as an almost pathognomonic feature of glioblastomas (27, 37, 38). This unique expression of the GLUT3 isoform over tumor vessels suggests that this isoform may be closely involved in anaplastic changes of astrocytomas. At the same time, our observation is quite in harmony with a previous study which showed that the expression of the GLUT1 protein was high in blood vessels of low-grade astrocytomas and anaplastic astrocytomas but that the expression of the protein decreased significantly in blood vessels of glioblastomas (39).

It is often observed that low-grade astrocytomas evolve into anaplastic astrocytomas or glioblastomas (27). In 1 of 4 low-grade astrocytomas and 2 of 5 anaplastic astrocytomas, many tumor cells with dense hybridization signals for GLUT3 mRNA were scattered among tumor cells with no significant signals (Fig. 4, A and B). This characteristic distribution of tumor cells may reflect multicentric development, sometimes seen in the malignant phase of astrocytomas as well as being frequently found in glioblastomas (27).

In all 12 astrocytomas except 1, many silver grains for GLUT1 mRNA, and even more for GLUT3 mRNA, were found over tumor cells. The presence of the GLUT1 protein was also confirmed by immunocytochemistry in 4 of 12 astrocytomas (Table 1). The staining properties for the GLUT1 protein in human brain tumors have already been described. According to Guerin et al. (39), in contrast to our results, no tumor cells were stained by the GLUT1 antiserum in 30 astrocytomas or 3 metastatic brain tumors. While those authors fixed tissue samples using 10% neutral buffered formalin, the tissue samples used in our study were all freshly resected and immediately fixed in Bouin’s fixative. This discrepancy in the results between these two studies may be due to the differences in fixatives, as well as the antibodies used. In fact, any tumor cells in a sample obtained from the same patients as shown in Fig. 6A, fixed in 10% neutral formalin, were unstainable for the GLUT1 protein by using the same GLUT1 antibody as used in our study (data not shown). In the brain, the GLUT1 isoform is known to be selectively localized to the microvascular endothelium, with minimal, if any, expression of GLUT1 mRNA in neurons and glial cells (12). Our data demonstrate that not only the GLUT3 isoform but also the GLUT1 isoform is certainly expressed in tumorous states of neuroglial cells, that is, in astrocytomas.

In astrocytomas, the density of hybridization signals for both mRNAs in individual tumor cells tended to be elevated with increasing tumor grade (Table 1). In 2 of 5 anaplastic astrocytomas and all 3 glioblastomas, a dense accumulation of signals for both mRNAs was found over numerous tumor cells. Consistent with mRNA overexpression, more cases with both isoforms were detected by immunocytochemistry as malignancy advanced. By Northern blot analysis, however, the level of GLUT1 mRNA did not correlate obviously with the histological grading of astrocytomas, while that of GLUT3 mRNA was well correlated (Fig. 1). This is probably because the elevation of GLUT1 mRNA in tumor cells is not so marked as that of...
GLUT3 mRNA (Table 1) and, additionally, the expression of GLUT1 mRNA in tumor vessels with endothelial proliferation is not significant (Fig. 3, C and D). In a series of patients with cerebral astrocytomas studied with positron emission tomography using $^{18}$F-fluorodeoxyglucose, all glioblastomas and anaplastic astrocytomas contained zones of high metabolic activity, varying from large and occasionally multiple areas to barely discernible foci. In contrast, regions of distinctly elevated metabolism were not observed within low-grade astrocytomas (18). Our data, which closely parallel these observations, suggest that the overexpression of both isoforms, particularly that of the GLUT3 isoform, may be involved in elevated glucose metabolism of both anaplastic astrocytomas and glioblastomas.

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