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Membranous expression of glucose transporter-1 protein (GLUT-1) in embryonal neoplasms of the central nervous system

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The human erythrocyte GLUT-1 is a transmembrane protein which facilitates transport of glucose in the cell in an energy-independent fashion. Neuroectodermal stem cells show strong membrane immunoreactivitry with this marker at early developmental stages in rodents. Membranous expression by undifferentiated neuroectodermal cells gradually decreases while GLUT-1 becomes confined to the endothelial cells, when these acquire blood–brain barrier function. We thus sought to determine whether GLUT-1 expression was limited to embryonal neoplasms of the central nervous system (CNS) which are presumably derived from developmentally arrested neuroectodermal stem cells. Archival material of 40 primary CNS neoplasms were examined for immunoreactivity with anti-GLUT-1. This included both non-embryonal neoplasms (18 astrocytic tumours, one ependymoma and three oligodendroglioma) and embryonal neoplasms (12 cerebellar medulloblastomas, four supratentorial PNETs and two atypical teratoid/rhabdoid tumours (AT/RhT)). In addition, cell lines and nude mice xenografts derived from both undifferentiated and differentiated tumours were assessed for GLUT-1 immunoreactivity by both immunohistochemistry and Western blotting. All embryonal tumours, MBs and PNET xenografts consistently showed GLUT-1 membrane staining. Non-embryonal neoplasms were negative except for vascular staining. Membrane protein fraction of embryonal tumours cell lines immunoreacted by immunoblot with GLUT-1, whereas the glioblastoma cell line was negative. Expression of GLUT-1 supports the stem cell nature of the cells of origin of MBs, supratentorial PNET and AT/RhTs. As a result, GLUT-1 is a useful marker to define the embryonal nature of CNS neoplasms.

Keywords: brain neoplasms, embryonal neoplasms, glucose transporter protein, medulloblastoma, PNET

Introduction

A family of glucose transporter proteins (GLUT) located on the surface of cells accomplishes transport of glucose into cells against its concentration gradient in an energy-independent fashion [1,18]. Among these, GLUT-1 is the major glucose transporter in erythrocytes and endothelial cells of tissue with blood–tissue barrier functions, including blood–brain barrier (BBB), blood–ocular barrier, blood–testis barrier and blood–placenta barrier [4,9,13]. All microvessels of the mature brain with the exception of the area postrema and the circumventricular organs located outside the blood–brain barrier express GLUT-1 [13]. However, there is evidence in rodents that

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loss of GLUT-1 expression parallels neuronal and glial commitment of embryonal neuroectodermal cells [12]. In the early stages of embryonic development, GLUT-1 is expressed by the plasma membranes of neuroepithelium in mice. By day 16, immunolocalization becomes restricted to the luminal aspect of brain microvessels whereas cells committed towards neuronal and glial lines no longer express it [4]. Glial fibrillary acidic protein (GFAP) intermediate filaments as well as synaptophysin begin to appear around days 14–16 and parallel morphological differentiation of neuroglial tissue [17,20]. Moreover, the shift of GLUT-1 from immature neuroectodermal cells to the intracerebral vessels in mature brain, which occurs at about the time of initial expression of GFAP in rat brain astrocytes, marks a precise stage of development, when barrier function is acquired by vascular endothelium [12]. These studies and a more recent observation that membranous staining for GLUT-1 was exclusively limited to the immature neuroectoderal component in ovarian teratomas [7] prompted us to determine whether GLUT-1 expression was limited to embryonal neoplasms of the central nervous system (CNS) which are presumably derived from neuroectodermal stem cells.

Material and methods

Formalin-fixed, paraffin-embedded material of 40 primary CNS neoplasms were retrieved from the files of the Brigham and Women's Hospital. Neoplasms were chosen to encompass a wide spectrum of tumours ranging from primitive to differentiated. According to the WHO classification [15,16] the neoplasms were classified as non-embryonal (13 glioblastomas, two anaplastic astrocytomas, one well differentiated astrocytomas, one pleomorphic xantoastrocytoma (PXA), one cerebellar pilocytic astrocytoma, one ependymoma) and embryonal (12 cerebellar medulloblastomas (MB), four supratentorial primitive neuroectodermal tumours (PNET) and two atypical teratoid/rhabdoid tumours (AT/RhT). The following cell lines and intracerebral and subcutaneous xenografts were tested: one human medulloblastoma cell line (Med 341 obtained from ATCC); one human supratentorial PNET cell line (PFSK, kindly provided by Dr D. Fults (Salt Lake City, Utah, USA) [8]; two PNET murine cell lines stably transfected with SV 40 large T antigen (TZ 870) and ras/myc (TZ 639) (kindly provided by Dr Otmar Wistler, Bonn, Germany) [22]; and one human glioblastoma multiforme (U87MG) (kindly provided by Dr D. Louis, Boston, USA).

Immunohistochemistry was performed with an anti-GLUT-1 polyclonal antibody raised against a synthetic peptide obtained from the highly conserved c-terminal sequence [2] (Chemicon, West Temecula, CA, USA). Five-μm paraffin-embedded tissue sections of tumours, as well as subcutaneous and intracranial xenografts of the cell lines, were used for immunohistochemical staining as described previously with a polyclonal rabbit anti-GLUT-1 antibody, dilution 1 : 1000 (Chemicon, West Temecula, CA, USA) and to GFAP, dilution 1 : 500 (Boehringer Mannheim, Indianapolis, IN, USA). The reaction was carried out in an automated immunohistochemistry instrument, the Ventana 320/ES (Ventana Medical Systems, Tuscon, AZ, USA). Antigen–antibody reactions were revealed with standardized development times by the instrument diaminobenzidine (DAB) as substrate. Positive controls of formalin-fixed paraffin-embedded sections of adult human cortex, which revealed positivity for GLUT-1 in endothelial cells, were run simultaneously. For negative controls the primary antibody was omitted.

In all embryonal tumours and cell line xenografts the immunophenotypic characterization was performed using the following additional antibodies: synaptophysin (1 : 500 dilution, Biogenex, San Ramon, CA, USA) vimentin (1 : 500 dilution, Dako, Milan, Italy), cytokeratins (1 : 50 dilution, Biogenex) and epithelial membrane antigen (EMA) (1 : 150 dilution, Dako).

Western blotting

Soluble proteins from the cell lines (prepared by pulverizing 250–500 mg of snap-frozen tumour samples) were immersed in ice-cold lysis buffer (20 mM Tris buffer, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 mM vanadate, 10 mM NaF, 1% NP40) including 250 mM sucrose and homogenized three times for 10 s with a Polytron (Brinkmann, Westbury, NY, USA) setting of 10. The homogenate was centrifuged at 50 000 ***g*** for 15 min at 4 °C. The supernatant was removed and stored at −80 °C. Membrane proteins from the cell lines were prepared in ice-cold lysis buffer, homogenizing three times for 10 s with a Polytron (Brinkmann, Westbury, NY, USA) setting of 10. After incubation in ice for 15 min, the homogenate was centrifuged at 15 000 ***g*** for 15 min at 4 °C. Fifty to 100 mg of

proteins were resolved on 12% SDS–polyacrylamide gels with standard conditions (bis to total monomer of 2.7%) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) with a Trans-Blot SD semidry electrophoretic transfer apparatus (Biorad, Hercules, CA, USA) with transfer buffer (48 mM Tris, pH 9.2, 39 mM glycine, 20% methanol and 1.3 mM (0.0375%) SDS). The filter was immunoblotted with anti-ERK1 antisera (0.5 μg/ml) or anti-MKP-1 (1 : 2000 dilution) antisera (Santa Cruz Biotech, Santa Cruz, CA, USA) in phosphate buffered saline containing 0.2% Tween 20, with an initial blocking buffer containing 10% non-fat dried milk. The immunodetection was performed with an ECL kit (Amersham, Arlington Heights, IL, USA).

Results

The results of GLUT-1 immunoreactivity are summarized in Table 1.

Embryonal neoplasms

Histologically, nine cases of MBs were of the classic type, one had a large cell component and one was desmoplastic. All cases except one showed focal but strong membranous positivity for GLUT-1. In most cases there were fields of neoplastic cells with membrane-bound reactivity adjacent to areas which were completely negative. Occasional single positive cells were also scattered among negative cells. The desmoplastic medulloblastoma showed only very focal GLUT-1 positivity in the internodular areas. In all cases except one, variable numbers of thin-walled blood vessels positive for GLUT-1 were observed. Six cases of classic MBs disclosed focal areas of immunoreactivity for synaptophysin. The desmoplastic tumour showed synaptophysin immunoreactivity within the nodular areas. All these cases showed no GFAP-positive neoplastic cells. The remaining four cases were negative for both GFAP and synaptophysin. All cases were negative for cytokeratin and EMA. In the six cases in which areas of synaptophysin immunoreactivity were present the comparison with the adjacent sections stained with GLUT-1 showed a different pattern of staining, indicating that most of GLUT-1-positive cells were negative for synatophysin.

Table 1. GLUT-1 membranous expression in human neoplasms and human and murine cell lines and xenografts

Tumour histotype	*GLUT-1 positivity*
Embryonal tumours	
Medulloblastoma	11/12
Supratentorial PNET	4/4
Atypical teratoid/rhabdoid tumour	2/2
Non-embryonal tumours	
Glioblastoma	0/13
Anaplastic astrocytoma	0/2
Astrocytoma	0/1
Pleomorphic xantoastrocytoma	0/1
Pilocytic astrocytoma	0/1
Ependymoma	0/1
Oligodendroglioma	0/3
Cell lines and xenografts	
Human medulloblastoma murine	2/2*
PNET	2/2*
Human PNET	0/1*
Human glioblastoma	1/1

*Immunohistochemistry and Western blot analysis.

A strong membranous focal GLUT-1 immunoreactivity was also observed in all cases of supratentorial PNET and AT/RhT (Figure 1). All four cases of PNET showed glial differentiation with areas focally positive for GFAP. No neuronal differentiation as assessed by synaptophysin positivity was detected in any of the cases. The two cases of AT/RhT were composed of sheets of neoplastic cells interrupted by fibrovascular septa. Cytologically both lesions were composed of large, pale cells. Some cells had centrally or eccentrically placed nuclei and prominent cytoplasms. A few cells had a 'rhabdoid' appearance with prominent nucleoli and eosinophilic cytoplasmic masses. (Figure 1**a**). Both cases had a complex immunophenotype characterized by immunoreactivity for vimentin, GFAP, EMA and cytokeratin. Both cases were negative for synaptophysin.

Non-embryonal neoplasms

No membranous cellular positivity for GLUT-1 was observed in this group of neoplasms except for pseudopalisading areas adjacent to areas of necrosis in glioblastomas.

GLUT-1 positivity was limited to intratumoural vessels. Interestingly, the number of negative vessels was higher in the glioblastomas and anaplastic astrocytoma compared to low-grade astrocytoma. GLUT-1-negative vessels were only rarely detected in pilocytic astrocytoma, PXA, ependymoma and oligodendrogliomas. Vessels with endothelial proliferation were negative. Adjacent brain tissue serving as internal control showed strong GLUT-1 immunoreactivity only in the vasculature.

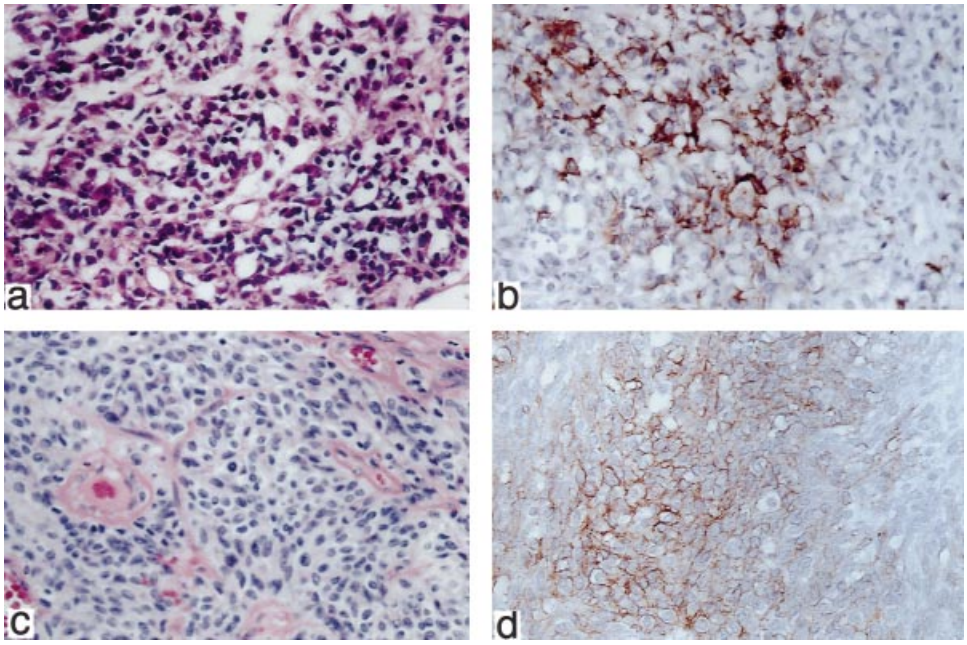

Figure 1. Embryonal neoplasms. Atypical teratoid/rhabdoid tumour (**a**) and supratentorial PNET (**c**) showing focal membranous immunoreactivity for glucose transporter protein 1 (GLUT-1) (**b** and **d**). Vasculature is negative for GLUT-1.

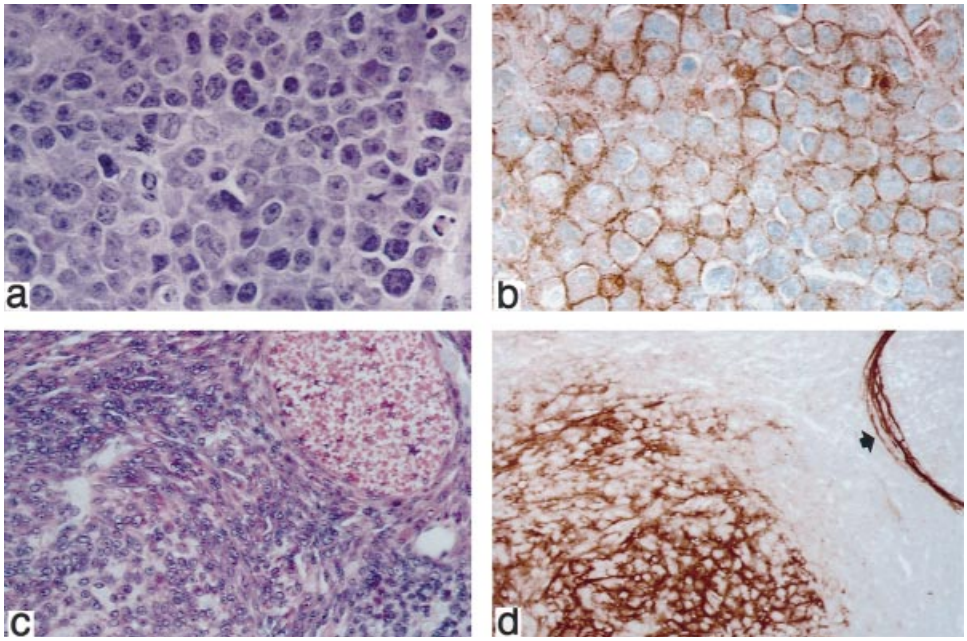

Figure 2. Intracerebral xenograft of human medulloblastoma Med 341 (**a**) and subcutaneus xenograft of murine PNET TZ 639 cell line (**c**) showing intense membranous immunoreactivity for GLUT-1 (**b** and **d**). In **d**, the perineural immunoreactivity (arrow) is expression of the blood–nerve barrier.

Cell lines and nude mice xenografts

Intracerebral and subcutaneous xenografts of human medulloblastomas (Med 341) and human (PFSK) and murine (TZ870 and TZ 639) PNET showed focal but strong membranous positivity similar to the human embryonal tumours (Figure 2). The immunophenotype of xenografts was as follows: Med 341 showed focal immunoreactivity for synaptophysin and negativity for GFAP; PFSK was negative for both GFAP and synapto-

physin; TZ870 showed focal positivity for both GFAP and synaptophysin; TZ639 had only focal immunoreactivity for GFAP.

Western blot on whole protein lysates (not shown) and membrane fraction (Figure 3) of these lysates confirmed this positivity, although only faint positivity for GLUT-1 was observed in TZ639. In contrast no band corresponding to GLUT-1 was identified on the human glioblastoma cell line U87MG.

Discussion

Studies of GLUT-1 expression in the early stages of CNS embryonal development in rodents have demonstrated that GLUT-1 is expressed by the cytosolic or cytoplasmic membrane of primitive neuroepithelium [4,16]. However, by day 16 immunolocalization becomes restricted to the luminal aspect of brain microvessels whereas embryonal cells committed toward neuronal or glial lines no longer express it [9]. GFAP intermediate filaments as well as synaptophysin begin to appear around days 14–16 and parallel morphological differentiation of neuroglial tissue [17,20].

Recently, we have shown strong GLUT-1 membranous immunoreactivity restricted to the immature component of neuroectodermal tissue in human ovarian teratomas [7]. These observations suggested that GLUT-1 could be expressed in primitive embryonal neoplasms of the CNS which are postulated to originate from precursor cells of the developing brain [6,21]. Here we demonstrate GLUT-1 expression on the cell membrane of neoplastic cells only in the embryonal neoplasms such as MBs, supratentorial PNET and ATh/RT. Membranous expression was also observed in subcutaneous xenografts of both human and murine medulloblastoma and PNET cell lines. Interestingly, the two GLUT-1 positive PNET murine cell line TZ870 and TZ 639 are obtained by transfection of embryonal brain cell derived from embryos at 13–14 days of gestation with a retroviral vector encoding SV40 large T and ras/myc oncogens, respectively [22]. On the basis of GLUT-1 expression alone, it is tempting to speculate that both human and murine embryonal CNS neoplasms might be derived from cells whose development has arrested before the equivalent of rodent day 16 of gestation. Fractionated immunoblots further confirmed GLUT-1 expression within the membrane protein fraction of these cell lines. Conversely, no immunoreactivity, except for vascular staining, was observed in non-embryonal glial neoplasms and no GLUT-1 was detected by immunoblotting in the human gliobastoma cell line.

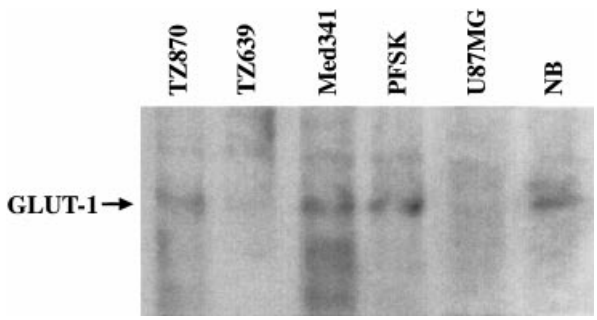

Figure 3. Western blot analysis of GLUT-1 expression on membrane fraction of cell lines. Human medulloblastoma (Med341) and human (PFSK) and murine (TZ870 and TZ639) PNET show variable expression of GLUT-1. No band is present on the human glioblastoma cell line (U87MG). Normal mouse brain (NB) is the positive control.

In our series of non-embryonal neoplasms, which included the full spectrum of astrocytic neoplasms, the number of GLUT-1-positive vessels decreased with increasing grades of anaplasia. This finding has interesting implications pertaining to the integrity of the blood–brain barrier and has been largely documented in previous studies [10,11,14,19]. Neoplastic astrocytes, ependymal cells and oligodendroglia were GLUT-1-negative, with the exception of tumour cells located adjacent to areas necrosis in glioblastomas. These findings confirm GLUT-1 upregulation under hypoxic conditions in tumour cells [5].

GLUT-1 membranous immunoreactivity in embryonal neoplasms and xenografts was focal. GLUT-1-positive cells were morphologically indistinguishable from cells positive for GFAP or synaptophysin. The comparison of adjacent sections of the six cases of classic MBs positive for both synaptophysin and GLUT-1 indicated that most of the GLUT-1 positive cells were negative for synaptophysin. Similarly, in desmoplastic medulloblastoma GLUT-1 was only focally present in the internodular undifferentiated cells and no positivity was observed within the synaptophysin-positive nodules. This suggests that precursor cells in medulloblastoma represent only part of the whole neoplasm and differentiation occurs even after transformation.

In our series of embryonal tumours there were two cases of ATh/RT. This recently described tumour entity occurs in young children and affects the cerebrum and

cerebellum with similar frequency, and less frequently the cerebellopontine angle and brain stem [3,21]. Histologically it has a complex pattern with an undifferentiated PNET-like component associated with a proliferation of large rhabdoid cell and a variable amount of mesenchymal and/or epithelial tissue. Similarly ATh/RT has a complex immunophenotype with expression of glial, neuronal, epithelial and mesenchymal markers, suggesting a multi-lineage differentiation [3,21]. The histogenesis of ATh/RT is poorly understood [23]. The presence of focal anti-GLUT-1-positive large tumour cells, some with rhabdoid features, may suggest that such highly malignant cells may be the transformed progenitor cells with the potential to differentiate along multiple lineages.

This study confirms the PNET concept according to which undifferentiated neoplasms of CNS probably originate from early precursor cells of the developing CNS. This common histogenesis, however, is not in conflict with their characterization in distinct clinicopathological entity [6,16]. GLUT-1 may thus be considered a useful marker to define the embryonal nature of primitive undifferentiated small cell neoplasms of CNS.

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