

The expression of the epitope H recognized by the monoclonal antibody H is higher in astrocytomas compared to anaplastic astrocytomas and glioblastomas

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Summary. The epitope H contains an O-linked N-acetylglucosamine residue in a specific conformation and/or environment recognized by the monoclonal antibody H (mAbH). mAbH stains two bands with Mr $\times 10^{-3}$ of 209 and 62 in lysates of cultured rat astrocytes. In normal human brains epitope H is absent from the overwhelming majority of normal astrocytes and only sparse reactivity is observed, confined mostly to fibrous astrocytes. Upregulation of the epitope H takes place in reactive astrocytes. In the present study we used the mAbH to investigate the immunohistochemical expression of the epitope H in 41 cases of astrocytic tumors including 19 cases of astrocytomas, 8 cases of anaplastic astrocytomas and 14 cases of glioblastomas. Seven out of 19 cases (37%) of astrocytomas showed weak staining, 10 cases (53%) moderate staining and 2 cases (10%) intense staining. Two out of 8 cases (25%) of anaplastic astrocytomas appeared negative, 3 cases (37.5%) showed weak staining and 3 cases (37.5%) moderate staining. Four out of 14 cases (28.5) of glioblastomas appeared negative, 7 cases (50%) showed weak staining, 2 cases (14%) showed moderate staining and only one case (7.5%) showed intense staining. There was a statistically significant elevation of the expression of the epitope H in astrocytomas compared to anaplastic astrocytomas and glioblastomas ($p=0.047$). These results indicate that the expression of the epitope H decreases in parallel with the increase of the grade of astrocytic tumors from low to higher grade neoplasms. This could be of interest for predicting the progression of an astrocytic tumor since it is documented that astrocytomas progress to tumors of higher grade of malignancy. Further investigation of the antigens bearing the epitope H might help to gain further insight into the mechanisms which regulate the progression of astrocytic

tumors and to examine the relevance of the mAbH staining with respect to the prognosis of these neoplasms.

Key words: Epitope H, Monoclonal antibody H, Astrocytoma, Glioblastoma

Introduction

Tumors of the central nervous system often have a wide morphological spectrum and classification depends on the recognition of areas with the characteristic morphology for a particular tumor type (Kleihues and Cavenee, 2000; Collins, 2004). The most common brain tumors in adults are the diffuse astrocytic tumors which include the astrocytomas, the anaplastic astrocytomas and the glioblastomas (Kleihues and Cavenee, 2000; Collins, 2004). Glioblastomas are the most common form and are divided into those that develop *de novo* and those that develop from a previously diagnosed tumor of lower malignancy grade (Kleihues and Cavenee, 2000; Collins, 2004). The astrocytomas and the anaplastic astrocytomas have been documented to progress to tumors of higher grade (Kleihues and Cavenee, 2000; Collins, 2004).

Astrocytic tumors are biologically heterogeneous neoplasias (Gladson, 1999; Collins, 2004; Iwadate et al., 2004); various genetic changes have been detected, mainly in glioblastomas, including aberrations of the p53 and Rb1 pathways, amplification of the epidermal growth factor receptor gene and mutations of the PTEN tumor suppressor genes (Collins, 2004). In addition to genetic changes, cell-cell and cell-extracellular matrix (ECM) interactions are thought to play an important role during malignant progression in human astrocytic tumors (Gladson, 1999; Zamecnik et al., 2004). Neoplastic cells of astrocytic tumors are embedded in a network of protein-protein and protein-carbohydrate interactions mediated by a wide variety of glycoproteins,

glycolipids, lectins and proteoglycans (Zanetta, 1998; Gladson, 1999; Yates et al., 1999; Zamecnik et al., 2004). Neoplastic cells of astrocytic tumors are capable of remodeling their ECM through synthesis of ECM proteins and proteoglycans, as well as through upregulation of integrin receptors and proteoglycans of their cell surface (Gladson, 1999). Among the diverse glycoprotein families of the ECM of the normal brain, which include laminins, fibronectin, collagens, tenascins, etc, several members serve as ligands for integrins (Gladson, 1999; Zamecnik et al., 2004). While integrins use protein-protein interactions with ECM components, galectins, a family of nonintegrin laminin-binding mammalian lectins, use protein-carbohydrate interactions with ECM glycoproteins based on the sugar code (Perillo et al., 1998; Rabinovich, 1999; Gabius, 2000; Reuter and Gabius, 2000; Gabius et al., 2002). Galectins are defined by their affinity to poly-N-acetylglucosamine enriched glycoconjugates and sequence similarities in the carbohydrate-recognition domain (Rabinovich, 1999). The expression of galectins is relevant for modulation of tumor cell invasion and migration in astrocytic tumors (Gordower et al., 1999; Camby et al., 2001a,b; Rorive et al., 2001; reviewed in Danguy et al., 2002). In addition to galectins, Lewis antigens and gangliosides may also play a role in astrocytoma glycopathobiology (Yates et al., 1999; Dall'Olio and Chiricolo, 2001; Kannagi et al., 2004). Indeed, Lewis antigen [such as sialyl Lewis (a) and sialyl Lewis (x)] are Fucosyl-related LacNac ligands that bind to selectins (Dall'Olio and Chiricolo, 2001; Kannagi et al., 2004) and experimental findings suggest that Lewis (x) glycolipids may be involved in cellular differentiation and initiation of cellular growth in human glioma cells (Ariga et al., 1996). Moreover, the expression profiles of gangliosides, which are sialyl-acid containing glycosphingolipids (Dall'Olio and Chiricolo, 2001), exhibit alterations in astrocytic tumors and correlate with tumor grade (Comas et al., 1999; Wagener et al., 1999). Furthermore, the CD15 epitope (fucosyl-N-acetyl-lactosamine), which is an adhesive oligosaccharide functioning as a ligand for selectins, is present in normal human astrocytes but human glioma cells express little or no CD15 on their surface (Martin et al., 1995; Gocht et al., 1996).

In the context of brain glycobiology, we have recently generated the monoclonal antibody H (mABH) which stains two bands with Mr $\times 10^{-3}$ of 209 and 62 in lysates of cultured rat astrocytes and recognizes the epitope H consisting of an O-linked N-acetylglucosamine (O-GlcNAc) and neighboring amino acids (Arvanitis et al., 2001). Modification of Ser and Thr residues by the attachment of O-linked N-acetylglucosamine {Ser (Thr)-O-GlcNAcylation to nuclear and cytosolic proteins is a dynamic process and possibly as abundant as Ser (Thr) phosphorylation (Hart, 1997; Zachara and Hart, 2002, 2004). O-glcNAcylated proteins include cytoskeletal proteins, transcription factors, heat-shock proteins, tumor suppressor proteins,

oncoproteins and chromatin proteins (Jackson and Tjian, 1989; Privalsky, 1990; Chou et al., 1995; Shaw et al., 1996; Haltiwagner et al., 1997; Hart, 1997; Kreppel et al., 1997; Van den Steen et al., 1998; Miller et al., 1999; Chou and Hart, 2001; Zachara and Hart, 2002, 2004). The widespread distribution of O-linked N-acetylglucosamine on proteins and its regulatory function on basic biological processes, suggests that it may play a role in the pathology of many diseases, including cancer (Chou and Hart, 2001; Zachara and Hart, 2004). In this respect, we have used the mAb to investigate by immunostaining the expression of the epitope H in infiltrating ductal breast carcinomas, fibroadenomas, normal human brains and human brains with a variety of lesions (Arvanitis et al., 1995, 2001; Havaki et al., 2003). In normal human brains the epitope H was absent from the overwhelming majority of normal astrocytes and only sparse reactivity was observed, confined mostly to fibrous astrocytes (Arvanitis et al., 2001). Upregulation of the expression of the epitope H was found in reactive astrocytes observed in pathological specimens from a variety of brain lesions, including anisomorphic and isomorphic gliosis (Arvanitis et al., 2001).

The above mentioned findings prompted us to investigate the expression pattern of the epitope H in human astrocytic tumors (Kleihues and Cavenee, 2000; Collins, 2004). Therefore, in the present study we used the mAbH to investigate the immunohistochemical expression of the epitope H in forty-one cases of astrocytic tumors including nineteen cases of astrocytomas, eight cases of anaplastic astrocytomas and fourteen cases of glioblastomas. In addition, some cases were immunostained for glial fibrillary acidic protein (GFAP) in order to use them as an internal positive control for the staining procedure.

Materials and methods

Forty-one cases of human astrocytic tumors including nineteen cases of low-grade astrocytomas, eight cases of anaplastic astrocytomas and fourteen cases of glioblastomas were classified according the WHO classification 2000 (Kleihues and Cavenee, 2000). For the immunodetection of the epitope H, the indirect immunoperoxidase procedure was applied as described in details previously (Arvanitis et al., 2001). Briefly, tissue sections about 4 μ m were cut from formalin fixed paraffin-embedded tumor blocks. After deparaffinization and blocking of endogenous peroxidase activity by immersing the sections in 3% H₂O₂ in Tris-Saline buffer pH 7.6, the sections were incubated in 10% normal rabbit serum in buffer for 30 minutes in order to inhibit the non-specific binding of antibodies. Then the sections were incubated in undiluted supernatant containing the mouse monoclonal antibody H (Arvanitis et al., 2001) for 2 hours at room temperature.

After washing 3x10 minutes in buffer the sections were incubated with peroxidase conjugated rabbit anti-

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mouse antibody diluted 1:50 in buffer for 1 hour. After washing 3x10 minutes the color was developed by incubating the sections in DAB-H₂O₂ in buffer for 8 minutes, then after washing counter staining in Hematoxylin, and dehydrating, the sections were covered with permount. In the negative control sections the primary antibody was omitted. The same indirect immunoperoxidase procedure was applied for the GFAP immunostaining. The anti-GFAP antibody was a polyclonal rabbit antibody (DAKO), which was diluted in 1:50 as well as the swine anti-rabbit peroxidase conjugated second antibody. The GFAP stained sections were counterstained with hematoxylin and eosin.

The staining pattern was graded as negative (-), when no stained cells were present, weak (+), when less than 30% of the cells were stained, moderate (++) when 30%-75% of the cells were stained and intense (+++) when 75%-100% of the cells were stained.

Statistical analysis of the results was performed using the Chi-Square tests. The results were considered as statistically significant when $p < 0.05$. The program SPSS from Windows Release 10 was used for statistical analysis.

Results

Positively stained cells revealed cytoplasmic cell body and process staining with mAbH.

A summary of the results is displayed in Table 1.

All nineteen cases of astrocytomas appeared positive with mAbH though showing different degrees of staining intensity. Seven out of nineteen cases (37%) of astrocytomas revealed weak staining (Fig. 1a), ten cases (53%) moderate staining (Fig. 1b) and the remaining two cases (10%) intense staining (Fig. 1c).

Two out of eight cases (25%) of anaplastic astrocytomas appeared negative for mAbH staining, three cases (37.5%) showed weak staining (Fig. 1d) and three cases (37.5%) showed moderate staining (Fig. 1e).

Four out of fourteen cases (28.5%) of glioblastomas appeared negative for mAbH staining (Fig. 1f), seven cases (50%) showed weak staining, two cases (14%) showed moderate staining (Fig. 1g) and only one case (7.5%) showed intense staining.

Reactive astrocytes at the vicinity of the infiltrating tumor fronts showed intense cytoplasmic staining for the epitope H (Fig. 1j, k, l).

The elevation of expression of the epitope H in astrocytomas compared to anaplastic astrocytomas and glioblastomas was statistically significant (Pearson Chi-Square $p = 0.047$) (Table 1).

Discussion

The results of the present study show that neoplastic astrocytes in astrocytic tumors contain a molecule(s) bearing the epitope H that is (are) recognized by mAbH. The finding that all cases of astrocytomas appeared positive for mAbH whereas 25% of anaplastic astrocytomas and 28.5% of glioblastomas appeared negative for mAbH indicate that the expression of the epitope H decreases in parallel with the increase of the grade of astrocytic tumors from low grade to higher grade neoplasms. This could be of interest for predicting and monitoring the progression of an astrocytic tumor since it is documented that astrocytomas progress to tumors of higher grade of malignancy (Kleihues and Cavenee, 2000; Collins, 2004). The decreasing expression of the epitope H from low grade to higher grade astrocytic tumors could be attributed to downregulation of expression which is observed in many cell products during neoplastic transformation or tumor progression (Scott and Vandenberg, 1992; Sanchez-Beato et al., 2003; Collins, 2004).

Taken together, our present and previous results (Arvanitis et al., 2001), indicate that the epitope H is absent from the overwhelming majority of normal astrocytes and becomes upregulated in reactive astrocytes, whereas the expression of the epitope H is significantly higher in astrocytomas than in anaplastic astrocytomas and glioblastomas. We have recently observed such fluctuations of the expression of the epitope H in infiltrating ductal breast carcinomas and fibroadenomas by using ultrastructural immunostaining with the mAbH (Havaki et al., 2003). Indeed, the intensity of mAbH over the mitochondria, the nucleoli and the cytoplasmic vesicles appeared to be decreased in infiltrating ductal breast carcinomas when compared to fibroadenomas (Havaki et al., 2003). Since the epitope H contains an O-linked N-acetylglucosamine residue (Arvanitis et al., 2001), it is possible that the fluctuations of the expression of the epitope H reflect differences in O-GlcNAc glycosylation of various cellular proteins. These fluctuations may be of interest for gaining insight into the pathogenesis of astrocytic tumors since O-GlcNAc glycosylation may modify proteins involved in oncogenesis such as tumor suppressor proteins and oncoproteins as well as proteins with important

Table 1. Results of the immunostaining of astrocytic tumors with mAbH.

TUMOR TYPE	No. OF CASES	STAINING INTENSITY	
		Antibody H	% percentage
Astrocytomas	19	7 (+)	37%
		10 (++)	53%
		2 (***)	10%
Anaplastic astrocytomas	8	2 (-)	25%
		3 (+)	37.5%
		3 (++)	37.5%
Glioblastomas	14	4 (-)	28.5%
		7 (+)	50%
		2 (++)	14%
TOTAL	41	1 (+++)	7.5%

Staining intensity: (-), negative; (+) weak, (++) moderate; (+++) intense. Statistical evaluation: Pearson chi-square $p = 0.047$.

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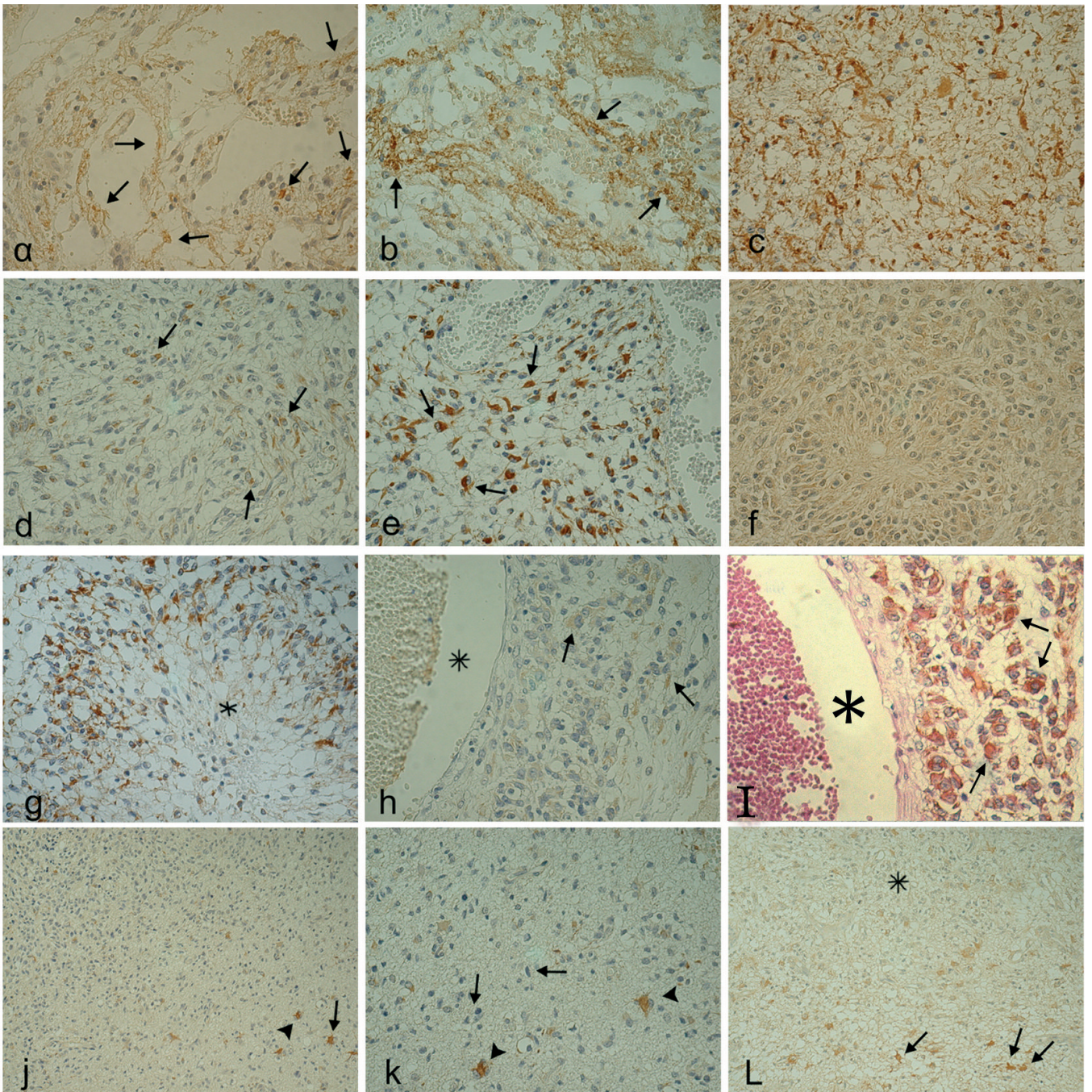


Fig. 1. **a.** Astrocytoma with weak staining. Stained astrocytes (arrows). x 400. **b.** Astrocytoma with moderate staining. Stained astrocytes (arrows). x 400. **c.** Astrocytoma with intense staining. All neoplastic astrocytes are stained. x 400. **d.** Anaplastic astrocytoma with weak staining. Stained astrocytes (arrows). x 400. **e.** Anaplastic astrocytoma with moderate staining. Stained astrocytes (arrows). x 400. **f.** Glioblastoma with negative staining. No stained cells are seen. x 400. **g.** Glioblastoma with moderate staining. necrotic center (asterisk). x 400. **h.** Anaplastic astrocytoma with weak staining. occasional astrocytes (arrows) show faint cytoplasmic staining. Blood vessel lumen (asterisk). x 400. **i.** Anaplastic astrocytoma consecutive section of h stained for GFAP. All astrocytes show strong cytoplasmic stain for GFAP (arrows). Same blood vessel (asterisk) seen in picture h. Section is counterstained with hematoxylin and eosin. x 400. **j.** Infiltrating front of a glioblastoma. Glioblasts mainly at upper left corner are negative. Reactive astrocytes at the periphery show strong cytoplasmic staining (arrow head and arrow). x 200. **k.** Higher magnification of J. Glioblasts are negative (arrows). Reactive astrocytes are strongly stained (arrow heads). Same astrocyte indicated in picture J with arrow head is indicated with the low arrow head in picture K. x 400. **l.** Infiltrating front of an anaplastic astrocytoma. Neoplastic astrocytes are largely negative (asterisk). Several reactive astrocytes at the periphery of the tumor are strongly stained (arrows). x 200

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biological functions such as cytoskeletal proteins, transcription factors, heat-shock proteins and chromatin proteins (Jackson and Tijian, 1989; Privalsky, 1990; Chou et al., 1995; Shaw et al., 1996; Haltiwagner et al., 1997; Hart, 1998; Kreppel et al., 1998; Van den Steen et al., 1998; Miller et al., 1999; Chou and Hart, 2001; Wells et al., 2003; Zachara and Hart, 2002, 2004). It could be hypothesized that O-GlcNAc modification of tumor-related proteins such as the tumor suppressor protein p53 and the oncoprotein c-myc, which play important roles in the pathogenesis of various malignancies (Chou and Hart, 2001; Sanchez-Beato et al., 2003), may also be involved in gliomagenesis. Indeed, the oncoprotein c-myc is modified by O-GlcNAc at threonine 58, a known phosphorylation site and a mutational hot-spot in human lymphomas (Chou et al., 1995). In addition, there is evidence that O-GlcNAc modification at the carboxy-terminus of the tumor suppressor protein p53, may have a role in the regulation of specific DNA binding by p53 (Shaw et al., 1996). This could be of particular interest for gliomagenesis since alterations of the p53 pathway are involved in the pathogenesis of diffuse astrocytic tumors, mainly of glioblastomas (Collins, 2004). On the other hand, it has been suggested that the putative involvement of O-GlcNAc modifications in oncogenesis may be mediated by addition/removal of O-GlcNAc in oncoprotein and tumor suppressor proteins (Chou and Hart, 2001).

Our present findings that the expression of the epitope H decreases in parallel with the increase of the grade of human astrocytic tumors from low to higher grade neoplasms can be parallel to previous glycopathological findings in relation to tumor progression in human astrocytic tumors (Camby et al., 2001a,b; Rorive et al., 2001). Indeed, Camby et al. (2001a) showed that the expression of galectins 1 and 3 significantly change during the progression of malignancy in these tumors, while that of galectin 8 remains unchanged. These three galectins are involved in tumor astrocyte invasion since their levels of expression were higher in the invasive parts of xenografted glioblastomas than in their less invasive parts (Camby et al., 2001a). Galectins 1 and 3, and to a lesser extent galectin 8, markedly stimulate glioblastoma cell migration *in vitro* (Camby et al., 2001a). Rorive et al. (2001) reported that the level of galectin-1 expression correlated with tumor grade in astrocytic tumors and that immunopositivity of high-grade astrocytic tumors from patients with short-term survival. In another study, Camby et al. (2001b) showed by glycohistochemistry that malignant progression in diffuse astrocytic tumors was paralleled by a decrease in cells' ability to bind distinct sugar epitopes, especially the D-GalNAc (alpha 1-3)-D-GalNAc-beta1-R determinant of the Forssman pentasaccharide in tumors, the alpha-L-fucose in perivascular tumor areas, the beta-D-glucose in tumor vessel walls and the alpha-D-mannose in tumors, perivascular tumor areas and tumor vessel walls. In addition, the expression of β -1,4-galactosyltransferase V

(β -1,4-Gal T V), which along the β 4-Gal T I and II are the enzymes responsible for the biosynthesis of N-acetylglucosamine or N-glycans, was increased in astrocytomas II, III and IV, successively (Xu et al., 2001). Furthermore, in the tumor-cell associated ECM of diffuse astrocytic tumors the expression pattern of proteoglycans is altered (Gladston, 1999). Indeed, the proteoglycans phosphacan and neurocan are expressed in normal brain but not in astrocytic gliomas (Gladston, 1999). In addition, BEHAB (brain-enriched hyaluronan-binding protein) expression is upregulated and versicna expression is downregulated in neoplastic glial cells, as compared with normal brain (Gladston, 1999). On the other hand, the CD15 epitope (fucosyl-N-acetyl-lactosamine) is present on normal human astrocytes but human glioma cells express little or no CD15 on their surface (Martin et al., 1995; Gocht et al., 1996). However, while human glioma cells express little or no CD15, other fucosyl-related oligosaccharide moieties exert very important roles in glioma cell biology. Indeed, fucose-containing glycoproteins have been suspected as candidates for the tumor suppressor function in the glioblastoma cell line SNB 19 using the fucose specific lectin *Ulex Europaeus* I (van der Meulen et al., 1994). Fucose-containing glycans with potential clinical applications, might inhibit the development of malignant gliomas. Indeed, Nieto-Sampedro et al. (1996) identified the mitogen inhibitors for the proliferation of the C6 glioma cell line as immunologically related to blood group oligosaccharides (i.e. Lewis antigen-related structures) and to glycan epitopes of the epidermal growth factor receptor. On the basis of this data Aguilera et al. (1998) synthesized several disaccharides with a common Lewis-X-type structure (i.e. fucosyl LacNAc structures) and these disaccharide derivatives were found to be inhibitors of cell division of the astrocytoma cell lines U-373 and U-118.

Previous biochemical data (Arvanitis et al., 2001) showed that the epitope H is not located on glial fibrillary acidic protein (GFAP) of the rat astrocytes and that epitope H is present on two polypeptides with Mr $\times 10^{-3}$ of 209 and 62. Since the immunohistochemical staining patterns of the normal rat brains and normal human brains are similar for the epitope H, it may be suggested that the same polypeptides bear the epitope H in the normal human astrocytes. Taking into consideration that the staining pattern of normal human brains for the epitope H is different from the staining pattern for glial fibrillary acidic protein (GFAP), which constitutes the major protein of the intermediate cytoskeletal filaments of the astrocytes (Eng, 1985), it is implied that normal human astrocytes do not bear the epitope H on GFAP (Arvanitis et al., 2001). The results of the present study indicate that the neoplastic astrocytes also do not bear the epitope H on their GFAP, because in tumor cases areas of tumors of high grade astrocytomas which are positive for GFAP are negative for the epitope H (Fig 1h, i). The antigen(s) which bear(s) the epitope H in the human neoplastic astrocytes

might be one or both rat astrocytic polypeptides or in addition, other antigen(s) which are expressed by the neoplastic astrocytic cells due to dysregulation of the genes.

In conclusion, the present study shows a statistically significant elevation of expression of the epitope H in astrocytomas compared to anaplastic astrocytomas and glioblastomas. These results indicate that the expression of the epitope H decreases in parallel with the increase of the grade of astrocytic tumors from low grade to higher grade neoplasms. This could be of interest for predicting and monitoring the progression of an astrocytic tumor since it is documented that astrocytomas progress to tumors of higher grade of malignancy. Further investigation of the antigens bearing the epitope H might help to gain further insight into the mechanisms which regulate the progression of astrocytic tumors and to examine the relevance of the mAbH staining with respect to the prognosis of these neoplasms. Future studies, including a large number of cases with a clinical follow-up, are necessary to confirm that the expression of the epitope H is a predicting factor of prognosis of astrocytic tumors.

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