The expression of the O-linked N-acetylglucosamine containing epitope H in the gemistocytic, pilocytic and subependymal giant cell astrocytomas

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Abstract. The epitope H contains an O-linked N-acetylglucosamine residue in a specific conformation and/or environment recognized by the monoclonal antibody H. It has previously been shown that the epitope H is present in several types of normal and pathological cells and in several polypeptides. In normal human brains the epitope H is present mostly to a minority of fibrous astrocytes, whereas it is greatly up-regulated in reactive astrocytes and is increased in well differentiated fibrillary astrocytomas compared to anaplastic astrocytomas and glioblastomas. In this study the expression of the epitope H was investigated in thirty cases of gemistocytic (WHO grade II), pilocytic (WHO grade I), and subependymal giant cell (WHO grade I) astrocytomas using the mAbH with the indirect immunoperoxidase method. The ten cases of gemistocytic astrocytomas revealed an overall high expression pattern. The ten cases of pilocytic astrocytomas revealed a biphasic pattern of epitope H expression. The dense tumor areas composed of elongated pilocytic cells revealed high expression of the epitope H. The loose cystic tumor areas composed of stellate cells revealed low expression of the epitope H. The ten cases of subependynal giant cell astrocytomas occurring in tuberous sclerosis revealed an overall high expression pattern. This study shows that there is high expression of the epitope H in gemistocytic, pilocytic and subependymal giant cell astrocytomas. Collectively considering, the present and our previous data, it appears that there is a spectrum of the expression levels of the epitope H ranging from the high expression in the reactive astrocytes and low grade astrocytomas to the low/null expression in the normal astrocytes and glioblastomas.

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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. The most common brain tumors in adults are the diffuse astrocytic tumors which include the astrocytomas, the anaplastic astrocytomas and the glioblastomas. 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. The astrocytomas and the anaplastic astrocytomas have been documented to progress to tumors of higher grade (1,2).

Astrocytic tumors are biologically heterogeneous neoplasias (2-4) 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 (2). 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 (3,5). 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 (3,5-7). 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 on their cell surface (3).

In the context of the brain glycobiology, the monoclonal antibody H (mAbH) has recently been generated, which stains two bands with $M_r \times 10^{-3}$ 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 (8). 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 (9-11). O-GlcNAcylated proteins include cytoskeletal proteins, transcription factors, heat-shock proteins, tumor suppressor proteins, oncoproteins and chromatin proteins (9-20). The widespread distribution of O-linked N-acetylglucosamine on proteins and its regulatory

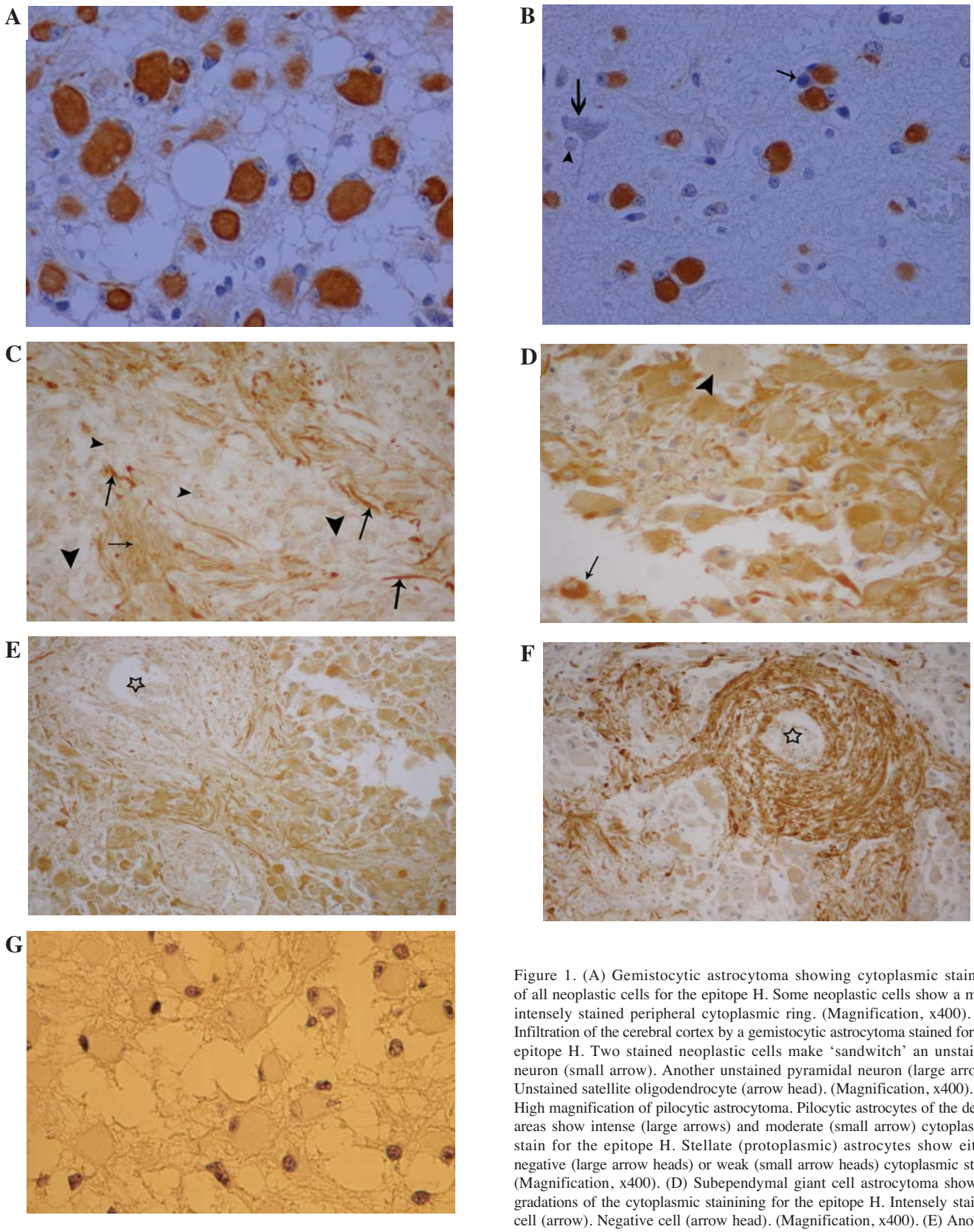

Figure 1. (A) Gemistocytic astrocytoma showing cytoplasmic staining of all neoplastic cells for the epitope H. Some neoplastic cells show a more intensely stained peripheral cytoplasmic ring. (Magnification, x400). (B) Infiltration of the cerebral cortex by a gemistocytic astrocytoma stained for the epitope H. Two stained neoplastic cells make 'sandwitch' an unstained neuron (small arrow). Another unstained pyramidal neuron (large arrow). Unstained satellite oligodendrocyte (arrow head). (Magnification, x400). (C) High magnification of pilocytic astrocytoma. Pilocytic astrocytes of the dense areas show intense (large arrows) and moderate (small arrow) cytoplasmic stain for the epitope H. Stellate (protoplasmic) astrocytes show either negative (large arrow heads) or weak (small arrow heads) cytoplasmic stain. (Magnification, x400). (D) Subependymal giant cell astrocytoma showing gradations of the cytoplasmic stainining for the epitope H. Intensely stained cell (arrow). Negative cell (arrow head). (Magnification, x400). (E) Another subependymal giant cell astrocytoma showing many cells with cytoplasmic staining and relatively few stained cell processes. Many unstained processes are present around a blood vessel. Lumen of the vessel is marked with an asterisk. (Magnification, x250). (F) Consecutive section of the region of the picture (E) stained for GFAP. Around the same blood vessel marked with an asterisk in (E) multiple processes are stained whereas the majority of them remain unstained for the epitope H. Lumen of blood vessel (asterisk). Note that the majority of neoplastic cells show either weak or negative stain of the cell body cytoplasm. (Magnification, x250). (G) Negative control. Gemistocytic astrocytoma. All cells are unstained. (Magnification, x400).

function on basic biological processes, suggests that it may play a role in the pathology of many diseases, including cancer (11,20). In this respect, the mAbH was used to investigate by immunostaining the expression of the epitope H in infiltrating ductal breast carcinomas, in fibroadenomas, in mitochondria-rich normal, metaplastic and neoplastic cells,

in pre-decidualized, decidualized and non-decidualized normal endometrial stromal cells, in normal myometrium and uterine smooth muscle cell tumors, in normal human brains, in human brains with a variety of lesions and in astrocytic tumors (8,21-26).

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 (8). 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 (8). Furthermore, we investigated the expression of the epitope H in 41 cases of astrocytic tumors including 19 cases of fibrillary astrocytomas, 8 cases of anaplastic astrocytomas and 14 cases of glioblastomas and we found a statistically significant elevation of the expression of the epitope H in fibrillary astrocytomas compared to anaplastic astrocytomas and glioblastomas (24). 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. These latter findings prompted us to investigate the expression pattern of the epitope H in other subtypes of the human astrocytic tumors. Therefore, in the present study the expression of the epitope H was investigated in thirty cases of gemistocytic, pilocytic, and subependymal giant cell astrocytomas using the mAbH with the indirect immunoperoxidase method.

Materials and methods

Tissues from thirty cases of human astrocytic tumors including ten cases of gemistocytic (WHO grade II), ten cases of pilocytic (WHO grade I) and ten cases of subependymal giant cell astrocytomas (WHO grade I) were procured as previously (24) and investigated by immunohistochemistry. For the immunodetection of the epitope H, the indirect immunoperoxidase procedure was applied as described in details previously (8). Briefly, tissue sections about 4 μ were cut from formalin fixed paraffin-embedded tumor blocks. After deparaffinization and blocking of endogenous peroxidase activity by immersing the sections in 3% H_2O_2 in Tris-Saline buffer pH 7.6, the sections were incubated in 10% normal rabbit serum in buffer for 30 min in order to inhibit the non-specific binding of antibodies. Then the sections were incubated in undiluted supernatant containing the mouse monoclonal antibody H (8) for 2 h at room temperature. After washing 3x10 min in buffer the sections were incubated with peroxidase conjugated rabbit anti-mouse antibody diluted 1:50 in buffer for 1 h. After washing 3x10 min the color was developed by incubating the sections in DAB-H_2O_2 in buffer for 8 min, then after washing, counterstaining 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. The immunohistochemical expression pattern was graded as negative (-), when no stained cells were present, low (+), when <30% of the cells were stained, moderate (++) when 30-75% of the cells were stained and high (+++) when 75-100% of the cells were stained.

Results

In the ten cases of gemistocytic astrocytomas almost all the cells revealed intense cytoplasmic staining either homogeneously diffuse or with a strong peripheral cytoplasmic ring, rendering an overall high expression pattern (Fig. lA and B).

The ten cases of pilocytic astrocytomas revealed a biphasic pattern of epitope H expression. The dense tumor areas composed of elongated pilocytic cells revealed high expression of the epitope H with the overwhelming majority of cells showing intense and, a minority of cells, moderate staining. The loose cystic tumor areas composed of stellate cells revealed low expression of the epitope H with the overwhelming majority of cells showing either negative or weak cytoplasmic staining (Fig. lC).

The ten cases of subependynal giant cell astrocytomas occurring in tuberous sclerosis revealed an overall high expression pattern, although gradations of cytoplasmic staining were noted within each case. The overwhelming majority of cells revealed intense cytoplasmic staining, a small percentage moderate, and a very small percentage negative cytoplasmic staining (Fig. 1D). The staining was present mostly in the cell body cytoplasm, whereas very few of the many fine cell processes in the background were positively stained (Fig. 1E). All these fine processes were intensely stained for Glial Fibrillary Acid Protein (GFAP) in consecutive tissue sections (Fig. 1F). In the negative control the primary antibody H was omitted (Fig. 1G).

Discussion

This study shows that there is high expression of the epitope H in gemistocytic, pilocytic and subependymal giant cell astrocytomas. In previous studies (8,24) it was found that: i) the epitope H is absent from the overwhelming majority of normal astrocytes and becomes upregulated in reactive astrocytes; and ii) that all cases of low grade fibrillary astrocytomas appeared positive for mAbH whereas 25% of anaplastic astrocytomas and 28.5% of glioblastomas appeared negative for mAbH indicating 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. Taking together into consideration, the present and the previous data (8,24), it appears that there is a spectrum of the expression levels of the epitope H ranging from the high expression in the reactive astrocytes and low grade astrocytomas to the low/null expression in the normal astrocytes and glioblastomas.

Our present and previous data (8,24) document the occurence of fluctuations of the expression of the epitope H in normal and neoplastic astrocytes. It is possible that the fluctuations of the expression of the epitope H reflect differences in O-GlcNAc glycosylation of various cellular

proteins, since the epitope H contains an O-linked N-acetylglucosamine residue (8). These fluctuations may be of interest for gaining insight in 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 biological functions such as cytoskeletal proteins, transcription factors, heat-shock proteins and chromatin proteins (9-20,27).

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 (20,28), 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 (14). 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 (15). 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 (2). 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 on oncoproteins and tumor suppressor proteins (20).

In conclusion, this study shows that there is high expression of the epitope H in gemistocytic, pilocytic and subependymal giant cell astrocytomas. Considering collectively, our present and previous data, it appears that there is a spectrum of the expression levels of the epitope H ranging from the high expression in the reactive astrocytes and low grade astrocytomas to the low/null expression in the normal astrocytes and glioblastomas.

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