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Melanoma-Associated Adhesion Molecule MUC18/MCAM (CD146) and Transcriptional Regulator Mader in Normal Human CNS

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Abstract

The proteins MUC18 and Mader have been identified as markers of tumor progression in melanoma cells. MUC18, also known as MCAM (melanoma cell adhesion molecule) and as CD146 (endothelial antigen), is a cell adhesion molecule belonging to the immunoglobulin superfamily. Mader is a transcriptional regulator shown to negatively regulate EGR-1. As it is known that neoplastic cells of neuroectodermal origin frequently express neuron-specific molecules, we studied whether these melanoma-associated antigens are found in normal CNS tissue. We investigated the expression of MUC18/MCAM and Mader in adult human post mortem CNS tissue by immunohistochemistry, immunoblot and two-dimensional gel electrophoresis. Our results show that Mader is preferentially expressed on neurons and glial cells and that the adhesion protein MUC18/MCAM is mainly expressed on vasculature within the CNS. These observations may have important implications for further studies investigating their possible roles in cell adhesion and proliferation control within the CNS.

Key Words

Adhesion molecules
Transcription factor
Central nervous system
MUC18
MCAM
CD146
Mader
Egr-1

Introduction

Tumors of neuroectodermal origin are known to express neuron-related proteins that are undetectable in the tissue prior to malignant transformation. A typical example of this phenomenon is the expression of neuron-specific enolase (NSE) and neural cell adhesion molecule (N-CAM) in small cell lung carcinoma [1]. Detection of these proteins is important for diagnosis and prognosis of small cell lung carcinoma. At the same time, these molecules are known to be involved in the normal functions of nervous tissue. This raises the question of whether additional proteins that are identified as markers in neoplastic processes on neuroectodermal cells might also be expressed on normal CNS tissue. In a preliminary study, we had analyzed the expression of the melanoma-associated molecule MUC18 using two monoclonal antibodies (MAbs) MUCBA18.3 and MUCBA18.4 by immunoblot [2]. Since MUCBA18.3 cross-reacts with a second protein, called Mader, we now focused on MUC18 and Mader expression in central nervous tissue.

The MUC18 glycoprotein was first described by Lehmann et al. [3] as an integral membrane protein of 113 kD belonging to the immunoglobulin superfamily [4]. In benign melanocytic nevi and thin primary melanomas, MUC18 could be detected only sporadically. However, with increasing tumor thickness, MUC18 expression becomes more frequent and is found in 80% of advanced

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primary tumors and metastases, so that its expression correlates with a poor prognosis and the occurrence of metastasis. Occurrence of MUC18 in non-malignant tissues has been observed in smooth muscle and endothelia of several peripheral organs [5, 6]. An immunoreactivity of human cerebellar tissue has also been described, but the identification of MUC18-expressing cells was not possible [6]. Recent studies have shown that MUC18 functions as a cell adhesion molecule (melanoma cell adhesion molecule, MCAM), interacting with an as yet unidentified ligand [6, 7]. Pickl et al. [8] demonstrated that MUC18/MCAM can be induced on all T cells after stimulation.

Mader was first detected by the antibody MUCBA-18.3, which cross-reacts with both Mader and MUC18 [9]. Mader is a highly conserved cytoplasmic and nuclear protein with a molecular weight of approximately 65 kD that belongs to the set of growth-factor-induced delayed early response genes. Recent findings have shown that Mader is identical to a negative regulator of Egr-1 (NAB-2) which is a transcription factor that directs expression of several genes involved in cell proliferation [10]. Mader is strongly expressed in malignant melanoma, but has not been detected in other tissues, including epidermis and gastrointestinal tract.

In addition to these two novel proteins, the intercellular adhesion molecule ICAM-1 was found to be associated with the development of metastasis in malignant melanoma [11, 12]. ICAM-1 can be expressed in a great number of cell types. ICAM-1 expressed by endothelia is involved in the migration and extravasation of T cells [13, 14], and its expression on neurons and astroctyes enables T cells and monocytes to bind to these cells in the brain parenchyma [15, 16]. Increased expression of ICAM-1 has been found after ischemia in the cortex [17] as well as in Alzheimer's disease, where expression of ICAM-1 on a subset of astroctyes was found to be increased [18].

N-CAM plays an important role in CNS development, directing cell migration and axon outgrowth, as has been shown in vitro and in vivo developmental studies [19, 20]. In healthy adult humans, N-CAM isoforms are selectively found on neurons, glial cells, skeletal muscle and natural killer cells. N-CAM, like ICAM-1 and MUC18, belongs to the immunoglobulin superfamily. Although NSE is, as the name indicates, normally expressed exclusively on neurons and APUD (amin precursor uptake and decarboxylation) cells, it appears on neuroectodermal cells after malignant transformation [21, 22].

In the present study, we investigated the presence of MUC18/MCAM and Mader in mature human CNS frontal lobe tissue from postmortem brains using immunohistochemistry, immunoblotting and two-dimensional electrophoresis and compared the protein expression patterns with those of the adhesion molecules N-CAM and ICAM-1 as well as NSE.

Table 1. Characterization of post mortem brains used in this study

Patient No.	Sex	Age years	Cause of death	Post mortem interval
1	male	22	accident	8
2	female	56	suicide	5
3	female	25	intoxication	5
4	male	60	heart attack	3
5	male	30	homicide	9
6	female	27	suicide	3
7	male	35	suicide	4
8	male	47	suicide	2
9	female	37	suicide	4
10	female	54	suicide	2

Materials and Methods

Cells, Tissues and Preparation of Brain Homogenates

After exclusion of pathological findings, 10 brains examined by a pathologist during autopsy were used in this study. The characteristics of the donors of frontal lobe tissues are given in table 1. At autopsy, the samples were prepared, frozen in liquid nitrogen and stored at –196 °C within 2–9 h after death.

The human melanoma cell line Mel Juso was maintained as described [23]. The dissected CNS tissues (1-cm^3 pieces) were homogenized in ice in 10% sucrose in 0.05 *M* Tris HCl buffer, pH 7.8, containing 1 m*M* PMSF and 1.5% Triton X-100.

SDS-PAGE

Brain homogenate was centrifuged at 20,000 *g* for 60 min. Aliquots of the supernatant containing 5 µg of protein were heated in equal volumes of SDS sample buffer containing 0.5% dithiothreitol. After cooling, 0.5 µg dithiothreitol was added to a sample volume of 100 µl to avoid reaggregation of the polypeptides. To detect possible postmortem degradation products or alterations in electrophoretic migration of the detected proteins following autopsy and tissue incubation and preparation, we conducted preliminary experiments. Human brain tissue was investigated at different preparation times after death by SDS-PAGE.

Two-Dimensional Gel Electrophoresis

Solid urea was added to aliquots of brain homogenate containing 45 µg of protein to give 9.5 *M* solution. Samples were then solubilized by addition of an equal volume of lysis buffer [24]. Centrifugation was carried out at 20,000 *g* for 60 min and the supernatants used for two-dimensional PAGE.

Western Blot Analysis and Immunodetection

Semidry blotting of the separated proteins onto polyvinyldifluoride membranes (Biorad, Munich) was performed in a discontinuous buffer system at a constant voltage of 60 V and 1.7 mA/cm^2 for 150 min without additional cooling. After blocking with 5% nonfat dried milk in TTBS the membranes were incubated overnight with the different primary antibodies (diluted 1:50 in TTBS). Membranes were then incubated with biotinylated goat anti-rabbit immunoglobulin as secondary antibody (1:3,000 in TTBS) for 90 min. The membranes were washed and then incubated for 90 min with streptavidin-alkaline phosphatase (1:3,000 in TTBS). The color was developed by immersing the membranes in a 1% solution of BCIP and NBT as chromogen substrates in 0.1 *M* Tris, 0.05 m*M* $MgCl_2$, pH 9.5.

Immunohistochemistry

Small blocks of the frozen samples were sectioned into 7-Ìm-thick serial sections, air dried overnight at 4°C and then fixed in acetone for 10 min at 4°C. Endogenous peroxidase activity was quenched by treatment with 3% H_2O_2 for 10 min at 4°C and nonspecific binding blocked with 1% bovine serum albumin (Sigma, St. Louis, Mo., USA) for 20 min at room temperature. The first investigations were done by the avidin-biotin complex (ABC) method with a counterstaining of the nuclei by hematoxylin. A monoclonal IgG directed against an *Aspergillus niger* enzyme (DAKO, Denmark) was used for negative control. To allow a better identification of location of the stained structures, a double-staining technique using two MAbs was established. The ABC method was combined with the alkaline phosphatase-antialkaline phosphatase (APAAP) method and followed by a hematoxylin counterstaining. Although the two primary antibodies were of the same species, the system could be optimized to eliminate any cross-reactivity between the two staining systems. Slides showing localization of different epitopes in different cells indicate a good specificity of this double-staining technique. The two staining procedures were done after each other, so that the first primary antibody was no longer detectable by the secondary antibody of the second staining. This specificity was only achievable when the ABC staining was done first. The following steps were carried out at room temperature, and the sections were washed 3 times for 3 min in 0.05 *M* TBS (pH 7.6) after each incubation step. The sections were incubated in the first primary antibody, diluted 1:250 in TBS with 1% BSA for 30 min. The first secondary antibody [biotinylated rabbit anti-mouse-F(ab′)$_2$; DAKO, Hamburg, Germany] was diluted 1:250 in TBS containing 1% rabbit serum (DAKO), and the sections incubated for 30 min. Horseradish-peroxidase-coupled streptavidin (1:750 in TBS, DAKO) was added for 30 min followed by diaminobenzidine substrate for 5 min (Sigma; 10 mg in 15 ml TBS). Counterstaining as described below or incubating with antibody directed against a second antigen was done afterwards. Following 45 min of incubation with the second primary antibody, the rabbit anti-mouse bridge antibody (1:30 in TBS rabbit serum, DAKO) was added for 30 min followed by the APAAP complex (1:25, 30 min, DAKO). The reaction was developed using new fuchsin (5 min, DAKO). The nuclei were counterstained using hematoxylin (3 min), and the sections were embedded in Kaiser's glycerinated gelatin and evaluated with a Zeiss-Optiplan microscope. Cells which had bound the first primary antibody stained brown, while those which bound the second primary antibody stained red. Staining with the anti-Mader antibodies was performed using a carbazole substrate as described [9].

Antibodies

MAbs MUCBA18.3 (IgG1) and MUCBA18.4 (IgG1) were produced against purified, denatured MUC18 antigen as described [4]. MAb MUCBA18.4 recognizes an extracellular epitope of the MUC18 glycoprotein. MAb MUCBA18.3 recognizes both the protein Mader and an intracellular epitope of MUC18. These hybridoma culture supernatants were diluted 1:10. The anti-MUC18 antibody A32 (IgG1; [6]) was kindly provided by M. Herlyn, Wistar Institute, Philadelphia, Pa., USA, as ascites and was used at a dilution of 1:500. MAb A32 reacts with an extracellular epitope distinct from that recognized by MUC18BA4. The anti-Mader antibodies 5H1 and 1C4 (both IgG1) were produced against recombinant Mader protein as described [9] and were used as undiluted culture supernatants.

Additional primary MAbs included: anti-human NSE (IgG1, clone DAKO-NSE H14, DAKO) diluted 1:50; anti-human N-CAM (DAKO) diluted 1:500; anti-human I-CAM-1 (IgG1, clone BBIG-I1, R & D Systems, Minneapolis, Minn., USA) diluted 1:200; anti-human CD31 (IgG1, clone JC/70A, DAKO) diluted 1:25; anti-*A. niger* glucose oxidase (IgG1, clone DAK-GO1, DAKO) as a negative control diluted as the compared anti-human monoclonals.

As secondary antibodies we used: biotinylated rabbit anti-mouse immunoglobulin diluted 1:200; biotinylated rabbit anti-mouse immunoglobulin [F(ab′)$_2$ fragment], diluted 1:250; rabbit anti-mouse immunoglobulin (for APAAP) diluted 1:30. All secondary antibodies were obtained from DAKO. Peroxidase-conjugated streptavidin (diluted 1:750) and murine APAAP complex (diluted 1:30) were also obtained from DAKO.

Results

Immunohistochemical Analysis of MUC18 and Mader Expression in Brain Tissue

Frontal lobe brain tissue was examined for the expression of the cell adhesion molecule MUC18/MCAM and of the N-CAM and the inducible leukocyte binding adhesion molecule ICAM-1. Neuronal cells were identified by staining for NSE, and vascular endothelia were identified using an antibody to CD31. CD31 was found to be expressed on endothelial cells of all vessels but was not observed on any other cells in the brain tissue. ICAM-1 expression was noted on endothelial cells of the vasculature except in capillaries and also on cells surrounding those blood vessels. Additionally, some cells in the brain parenchyma apart from the blood vessels also showed positive reaction (fig. 1). N-CAM expression was observed throughout the examined brains.

MAbs MUCBA18.3, MUCBA18.4, and A32 directed to the protein MUC18/MCAM reacted with endothelial cells of the vasculature. Double staining with CD31 indicated that the endothelial cells of blood vessels showing a muscular layer, but not capillaries, were MUC18/MCAM positive (fig. 2). In contrast to the uniform staining pat-

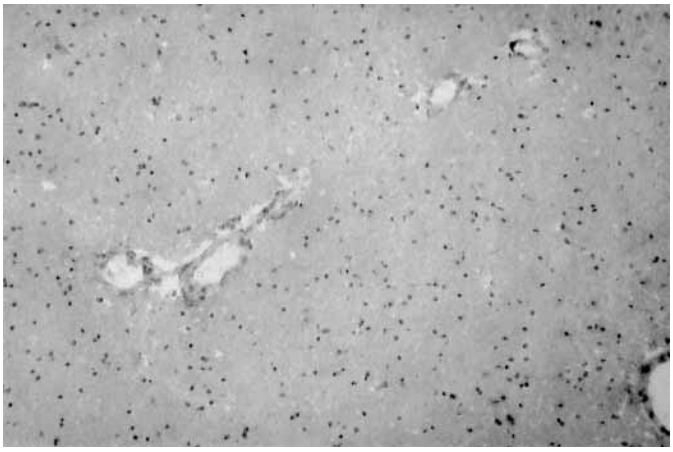

Fig. 1. Frozen section from human frontal lobe tissue (brain No. 6). Immunostaining with antibody to ICAM-1 shows reactive cells in blood vessels greater than capillaries and in the surrounding of these vessels. Additionally, there are some reactive cells stained in the brain parenchyma, possibly indicating ICAM-1-positive astrocytes or microglial cells. DAB = brown color. × 160.

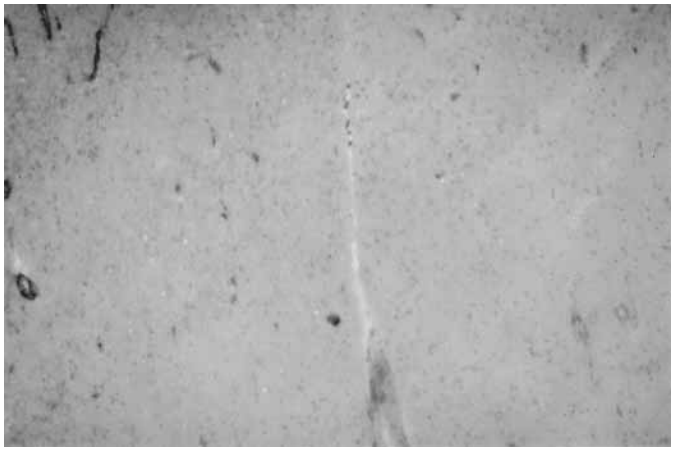

Fig. 2. Frozen section from human frontal lobe tissue (brain No. 4). The double staining with antibodies to MUCBA18.3 (DAB = brown color) and CD31 PECAM (newfuchsin = red color) shows reaction to MUCBA18.3 only in blood vessels greater than capillaries and additionally on cells in the brain parenchyma. × 100.

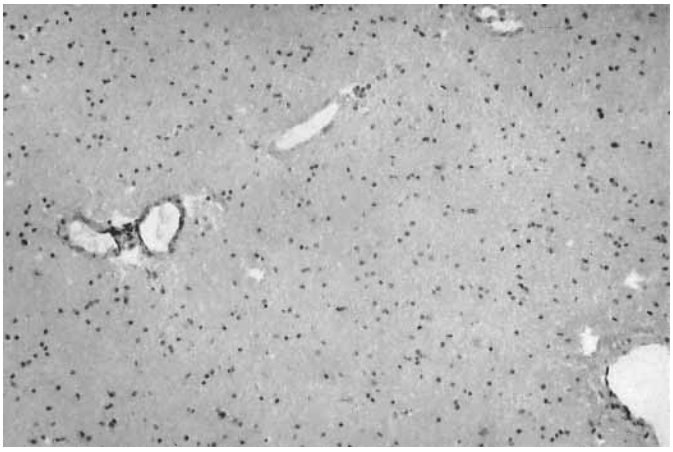

Fig. 3. Serial section of the same sample as shown in figure 1. Immunostaining with antibody to MUCBA18.4 (DAB = brown color). Mainly the pre- and postcapillary blood vessels are stained, but there are also some reactive cells in the brain parenchyma away from the vasculature. × 160.

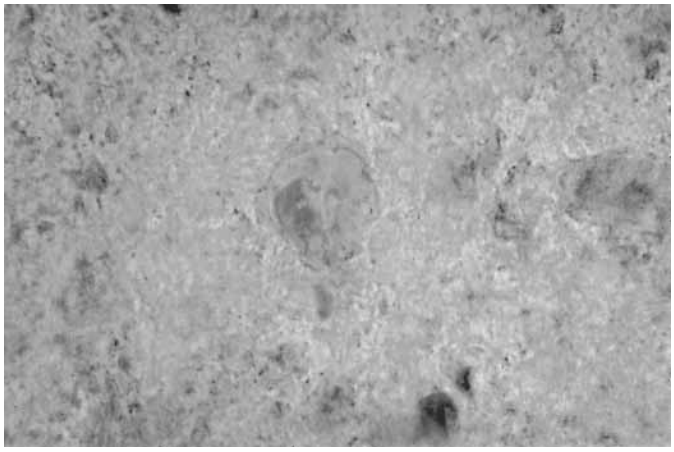

Fig. 4. Frozen section from human frontal lobe tissue (brain No. 8). Immunostaining with antibodies to NSE (DAB = brown color) and MUCBA18.3 (newfuchsin = red color). The neuron shown is positive for both NSE and MUCBA18.3. × 1,600.

tern of the three anti-MUC18 antibodies on the vasculature, distinct patterns of reactivity were observed on non-vascular elements in the brain tissue.

While reactivity with MUCBA18.4 and A32 was mainly seen on vascular elements in the brain samples and only sporadically detected on parenchyma cells away from the vasculature (fig. 3), MUCBA18.3 was observed to react with a higher proportion of parenchymal cells. Double staining with the antibody to NSE identified most of these cells as neurons. The MUCBA18.3-expressing neurons accounted for less than 25% of the NSE-positive cells in all brains examined (fig. 4). In addition some NSE-negative cells – thought to be glial cells – were also reactive. Although a few cells reacted with both MUCBA18.3 and anti-N-CAM, most cells reacted with either MUCBA18.3 or with anti-N-CAM. As shown in figure 5, the anti-Mad-

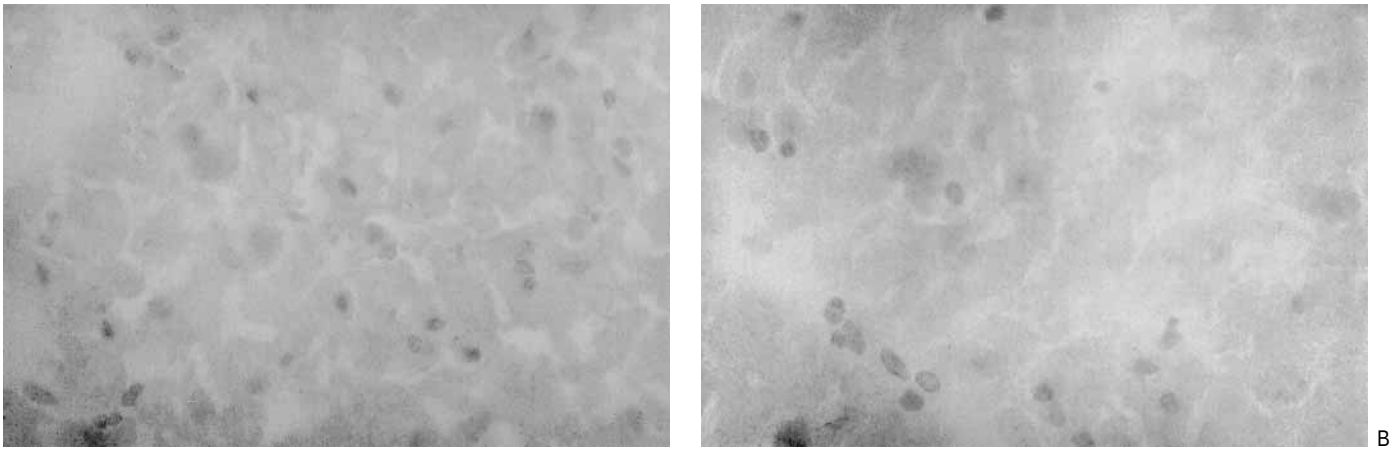

Fig. 5. Expression of Mader protein. A Frozen section from human frontal lobe tissue (brain No. 10) stained with the anti-Mader MAb 5H1 (1:400). Red staining of several cells can be seen. B Similar section stained with the MUC18-specific MAb A32 (1:400).

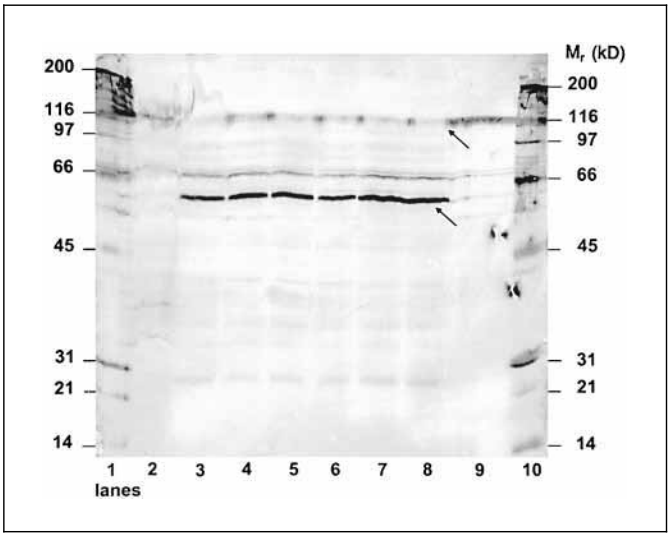

Fig. 6. Immunoblot with MAb MUCBA18.3 following SDS-PAGE. Lanes 1 and 10 = Biotinylated marker proteins; lanes 2 and 9 = melanoma cell line Mel Juso; lanes 3–8 = postmortem frontal lobe tissue. Arrows indicate molecular weights 65 and 113 kD.

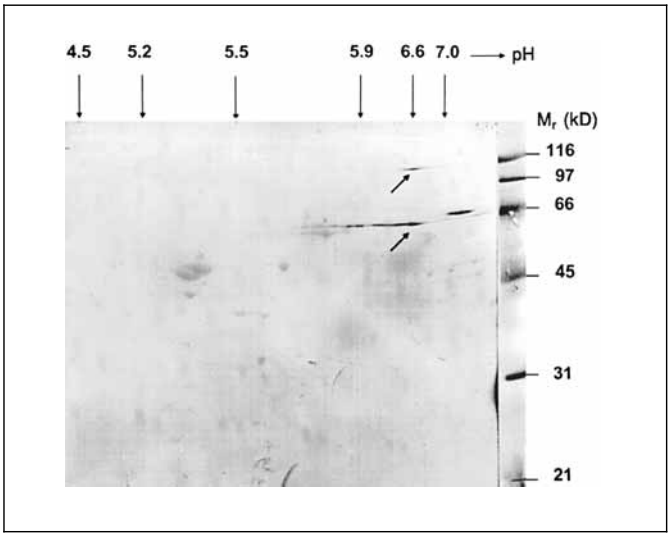

Fig. 7. Two-dimensional PAGE gel electrophoresis of a frontal lobe homogenate incubated with the MUCBA18.3 antibody showing proteins in isoelectric range of pH 5.9–8 at molecular weights of 65 and 113 kD (arrows).

er MAb 5H1 also stained many of the cells in these forebrain sections, suggesting that a portion of the MUCBA18.3 reactivity reflects Mader expression. These staining patterns were seen in all investigated brains.

Western Blot Analysis of Anti-MUC18-Reactive Proteins

Experiments at different times before tissue preparation and incubation after death revealed no alterations in electrophoretic migration patterns of the protein bands detected by the MAbs used. Therefore, postmortem degradation artifacts could be excluded.

It is known that MAb MUC18BA.4 reacts only with MUC18 while BA.3 detects both the 113-kD glycoprotein MUC18 and the 65-kD protein Mader. The broader immunoreactivity of MUC18BA.3 raised the question of whether this antibody is detecting Mader, or an additional cross-reactive protein in brain parenchyma. In order to

examine this, proteins in the brain samples were separated by SDS-PAGE, blotted onto nitrocellulose and incubated with the anti-MUC18 MAbs. Incubation with MAb MUCBA18.4 revealed a single band with an M_r of 113 kD, consistent with the M_r of the MUC18 glycoprotein isolated from human melanoma cells. However, incubation with MAb MUCBA18.3 revelaed a strong band with the 65-kD M_r of Mader in addition to the 113-kD band (fig. 6). Discrete additional protein bands of molecular weights between M_r 66–97 kD were seen in both MUCBA18.4 and MUCBA18.3 blots, presumably representing postmortem degradation products of MUC18. In lysates of the melanoma cell line Mel Juso (fig. 6, lanes 2 and 9) the predominant 113-kD band of MUC18 as well as the band in the 65-kD area can be seen. Western blotting of the two-dimensional PAGE-separated brain proteins revealed reactivity of MAb MUCBA18.3 with proteins in the isoelectric range between pH 5.9–8 at the observed molecular weights (fig. 7).

Discussion

Initially MUC18/MCAM and Mader were identified as human-melanoma-associated antigens [3, 9]. Since MUC18/MCAM and Mader expressions are common in tumors of neuroectodermal origin, e.g. melanomas, gliomas and neuroblastomas, it was feasible that these molecules might also be expressed in some areas of the brain. In fact Shih et al. [6] reported MUC18 expression in human cerebellum, detectable with MAb A32. However, the type of cells expressing MUC18 in cerebellum could not be identified. Mader expression has not yet been examined on normal human CNS tissue. In this study, we have examined MUC18/MCAM as well as Mader expression in forebrain samples. Because of its structural similarities belonging to the immunoglobulin superfamily, we compared MUC18 expression with those of ICAM-1 and N-CAM.

In our material three MAbs, which are directed at distinct MUC18 epitopes, reacted with blood vessels confirming that this molecule is strongly expressed in the endothelium of some pre- and postcapillary vessels. ICAM-1 was found to be expressed in the vasculature in the CNS in a similar way. Comparison of the staining patterns with the specific endothelial marker CD31 showed that only endothelium of blood vessels larger than capillaries express ICAM-1. N-CAM expression was examined throughout the brain parenchyma, and no relationship was found to ICAM-1 or to MUC18/MCAM. It is known, that ICAM-1 is an endothelial CAM that enables leukocytes to migrate through the vessel wall and mediates adhesion to astrocytes and neurons [15, 16]. The sporadically seen anti-ICAM-1-reactive cells in the brain parenchyma apart from the blood vessels could reflect ICAM-1-expressing glial cells. ICAM-1 expression by astrocytes and microglia has already been described on primary cells of rats [25]. The staining pattern of antibodies to ICAM-1 parallels that of MUCBA18.4, also in sporadically detectable anti-MUCBA18.4-reactive cells in the brain parenchyma. This underlines the hypothesis of similar expression of ICAM-1 and MUC18/MCAM in the CNS. Given that these molecules have similar structures [4] and expression patterns including activated lymphocytes [8, 14], and that both proteins act as adhesion molecules in vitro [6, 7], one might hypothesize similar functions in cell adhesion in vivo.

Mader expression was observed in nearly one quarter of neurons identified by anti-NSE. Additionally, there were some NSE-negative parenchymal cells, possibly glial cells, reacting with anti-Mader. Mader is a nuclear protein involved in the regulation of gene expression. It contains a nuclear localization signal and it can be found in both the cytoplasm and in the nucleus [9]. While Mader mRNA is ubiquitously expressed, the protein was not detectable in normal skin, lymph nodes and gastrointestinal tissue [9]. Our finding that Mader protein is present in normal CNS tissue represents the first example of normal tissue expressing detectable protein levels. This is consistent with our initial hypothesis that melanoma-associated proteins may also be expressed in normal brain tissue. Recently, a negative regulator of the transcription factor Egr-1 (NAB-2) has been shown to be identical to Mader [10]. Egr-1 is an immediate early response gene controlling transcription, either positively or negatively, of a wide range of genes involved in cell proliferation and differentiation [26]. Regarding the high levels of Mader in normal brain, its high expression in melanoma cells would appear paradoxical. However, abnormally high levels of another transcriptional regulator in a number of malignant tumors [27, 28], p53, are due to mutations rendering it dysfunctional [29].

Our observation that the two melanoma-associated proteins MUC18/MCAM (CD146) and Mader are also detectable in normal human brain tissue enables the description of novel markers of neoplastic processes in normal CNS prior to malignant transformation. Functional analysis of these proteins has to be done to clarify their possible roles in cell adhesion and proliferation control within the CNS.

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