Differential Spectrum of Expression of Neural Cell Adhesion Molecule Isoforms and L1 Adhesion Molecules on Human Neuroectodermal Tumors

Dominique F. Figarella-Branger, Pascale L. Durbec, and Geneviève N. Rougon

ABSTRACT

A series of four medulloblastomas, seven neuroblastomas (Nb), two ependymomas, and three gliomas, human neuroectodermal tumors, were screened for their expression of adhesion molecules L1, carcinoembryonic antigen, neural cell adhesion molecule isoforms (N-CAM) and HNK1 epitope by Western blotting and double immunofluorescence labeling.

All seven neuroblastomas, whatever their differentiated state, expressed L1, a neural cell surface developmental antigen, whereas all other tumors tested were negative. All tumors expressed N-CAM; however, a large diversity was observed among the isoforms. Low sialylated N-CAM 140 was present, with different intensity, in ependymomas and astrocytomas. High sialylated isoforms were detected by a monoclonal antibody (anti-MenB) specifically recognizing high polymers of α2-8 linked neuraminic acid. They were expressed in all medulloblastomas studied (4 of 4), and in 4 of 7 Nbs examined. Negative cases corresponded to tumors having undergone chemotherapeutic treatment or to ganglioneuroma. The interconversion from high to low sialylated forms might reflect changes which are critical for the conversion of Nbs into benign ganglioneuromas. HNK1 epitope was expressed on a large diversity of molecules by nearly all tumors except two Nbs which were also negative with anti-MenB antibody. This simultaneous loss of carbohydrate epitopes could correlate with higher maturation states of the tumors. None of the tumors expressed carcinoembryonic antigen. Therefore, anti-L1 and anti-MenB antibodies define differentiation-related antigens that could differentiate between Nbs and other tumors and may prove helpful in diagnosis and understanding of the biological nature of neuroectodermal tumors.

INTRODUCTION

Adhesiveness is clearly one central property for tumor behavior and in particular for metastatic process (for review see Refs. 1–3). This has been particularly well investigated for groups of tumors such as melanomas (4). However, little is known concerning other neuroectodermal tumors. In this report we describe the expression of adhesion molecules of the Immunoglobulin superfamily on a tumor panel including Nb, Mb, astrocytomas, and ependymomas. This family of cell surface molecules is involved in basic cell surface recognition events and many of its members have been reported to be specifically expressed in normal brain tissues (for review see Ref. 5). Their molecular diversity is due in part to differences in glycosylation which are differently expressed depending on the cell type and differentiation stage of the tissue considered (9, 10). For example, N-CAM has been found to be expressed in embryonic tissues under a polysialylated form (11, 12). Moreover a variable percentage of these immunoglobulin superfamily adhesive molecules shared the carbohydrate epitope HNK1. (13). Carbohydrate moieties had been shown to play an active role in modulating adhesion (14, 15).

A considerable body of data shows that neuroectodermal tumors can be distinguished from each other and from other types of malignancies according to their antigenic profiles (16, 17); however, the biochemical structure and the function of molecules considered as immunological markers is rarely known. We postulated that the description of a pattern of expression of developmentally regulated adhesive molecules by tumors could represent an alternative approach in defining markers; in particular, because tumors frequently express antigens found at a precise stage of development of the tissue from which they are derived (18). Such markers should be informative for phenotypic typing and definition of differentiation stage of a given neuroectodermal tumor. This could be particularly interesting for Nbs which are known to differentiate toward mature neuronal phenotype (19) either spontaneously or following chemotherapeutic treatment and for Mbs which could evolve toward either a glial or a neuronal phenotype (20). Mbs frequently become metastatic via CSF, consequently monitoring the presence of metastasis in this fluid should help both diagnosis and evaluation of chemotherapeutic treatment.

MATERIALS AND METHODS

Human Neuroepithelial Tumors

Eighteen surgically removed neuroepithelial tumors were diagnosed and studied.

Of 4 Mbs (Mb 1–4), histological examination showed one medulloblastoma (Mb 1), a poorly differentiated Mb with no Homer-Wright rosette (Mb 2), and two classic Mbs (Mb 3 and 4). Immunocytochemical studies were performed with NSE, neurofilament, vimentin, desmin, and GFAP antibodies. Results are summarized in Table 1.

Of 7 Nbs (Nb 1–7), histological examination showed a mature ganglioneuroblastoma (Nb 1), four poorly differentiated Nbs (Nb 2–5), and two nercotic ganglioneuroblastomas (Nbs 6 and 7). In 3 cases (Nb 2–4) surgery preceded chemotherapy and radiotherapy. Staging was as follows: Nb 3, stage I; Nb 1, 2, and 4, stage III; Nbs 5–7, stage IV.

Two ependymomas originated from the fourth ventricle of young children.

Four gliomas were malignant astrocytomas at grade III or IV. Benign infiltrating gliomas exhibiting mature neurons were excluded from our samples.

A normal frontal area from human normal adult brain was taken as control.

Antibodies

Rabbit polyclonal anti-NSE and anti-GFAP antibodies were from Ortho Diagnostic Systems (Roissy, France), mouse mAb anti-NF 70, M, 160,000 and 210,000 isoforms (clone DP5-43-12; mouse mAb anti-desmin (clone D300) and mouse mAb anti-CEA (clone 35B) were from...
Table 1: Expression of NSE, neurofilament, vimentin, desmin, and GFAP in Mbs

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<th>Antigen</th>
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* Very few positive cells were observed.

Immunoblot Detection of L1 Molecules on Brain Tumors. In normal tissues, L1 cell surface glycoprotein has been detected on developing postmitotic and mature neurons. It plays a role in neurite fascilitation and is believed to be absent from astrocytes (6, 7). L1, in vertebrate brain tissue, showed either a single band or a doublet band at M, 200,000 and, on some occasions, additional bands at M, 140,000 and 80,000 (26). We investigated its expression on tissue extracts from adult normal human brain and from neuroectodermal tumors. Results are shown in Fig. 1. Strikingly, the expression of this molecule seemed to be specific to Nbs. Mbs as well as ependymomas and gliomas were consistently negative. The molecular weights observed were similar to the ones of L1 molecules in normal brain.

Immunoblot Detection of N-CAM Isoforms on Brain Tumors. N-CAM is the name given to a class of plasma membrane sialoglycoproteins that mediate cell-cell contacts or cell-substrate interactions. The adhesive properties of N-CAM are modulated by the differential expression of isoforms in highly regulated pattern (27). N-CAM isoform diversity is generated by both alternative splicing (28) and a variety of posttranslational modifications including glycosylation (9, 10, 13). N-CAM from embryonic tissues migrate in sodium dodecyl sulfate gels as a broad band above M, 180,000 whereas N-CAM from adult tissues shows three prominent proteins with apparent molecular weights of 180,000, 140,000, and 120,000 (hereafter called N-CAM 180, 140, and 120). The two largest polypeptides span the membrane. We took advantage of the availability of antibodies recognizing various isoforms to monitor their expression in our panel of neuroectodermal tumors. The same extracts as above were reacted with a site directed rabbit polyclonal antibody recognizing N-CAM NH2 terminal domains which was able to reveal all N-CAM isoforms inasmuch as they share identical NH2 sequences (21). N-CAM was expressed by every tumor tested with great heterogeneity in the variety of isoforms expressed (Fig. 2). Ependymomas and gliomas expressed only N-CAM 140; it is noteworthy that N-CAM 120 was never detected in such tumors. Normal brain displayed N-CAM 180, N-CAM 140, and N-CAM 120.

A mAb P61, recognizing an intracytoplasmic epitope shared by all transmembrane isoforms, was also used (22). Results obtained for gliomas and ependymomas, confirmed that the M, 140,000 band revealed by anti-N-CAM polyclonal is a transmembrane isoform (not shown).

The most striking patterns observed with polyclonal anti-N-CAM on Mbs and Nbs were fuzzy large bands, migrating above M, 200,000; this migration is usually associated with highly sialylated forms of N-CAM or with lipids tightly associated with the molecule and perturbating gel separation. In addition, in some cases (Mbs 2 and 4 for instance), bands at M, 140,000 and 120,000 were also easily discernible. In some instances delipidation of tissues prior to membrane preparation removed the fuzziness of the band. This is shown for Nb 1 (Fig. 2, Lane ‘’I‘’ for which bands at M, 180,000, 140,000, and 120,000 become easily discernible. By contrast, Nb 2 exhibited the same profile after this treatment (Fig. 2, Lane ‘’2‘’).

Immunoblot Detection of Polysialylated N-CAM on Brain Tumors. We used anti-MenB, a mAb directed against N-acetyl-neuraminic acid (α2–8) polymers consisting of at least eight residues and known to react only with highly sialylated isoforms (12), to probe the glycosylation state of N-CAM expressed by tumors.

As expected from results obtained with the site directed
ADHESION MOLECULES ON BRAIN TUMORS

Fig. 1. Western blotting analysis of L1 molecules. Membrane extracts from various tumors and normal brain tissue (N) were applied (250 μg/lane), under reducing conditions, to a 7% polyacrylamide gel and then electroblotted and incubated with anti-L1 polyclonal antiserum at 1:1000 dilution. The bound antibody was visualized by incubation with 125I-labeled protein A. No bands were detected by incubation with protein A alone (not shown).

Fig. 2. Western blotting analysis of overall N-CAM isoforms. Same extracts as in Fig. 1 were analyzed for their expression of N-CAM. Lanes 1' and 2' were, respectively, Nb 1 and Nb 2 after the delipidation procedure; a rabbit polyclonal recognizing the NH2 terminal domain common to all the N-CAM was used at 1:1000 dilution. Procedure was the same as in Fig. 1. Arrows, position of N-CAM 180, 140, and 120 in normal adult brain (N). Positive bands observed in Nb 2 and Nb 1' are likely degradative products from N-CAM.
Fig. 3. Western blot analysis of polysialylated N-CAM isoforms. Membrane extracts were processed as in Fig. 1 and probed for their content in polysialic acid polymers using anti-MenB mouse IgM mAb. Note that normal adult brain (N) was negative. Nylon sheets were first incubated with immunopurified rabbit anti-mouse IgM prior to application of protein A.

Fig. 4. Western blotting analysis of HNK1 positive molecules. Membrane extracts were processed as in Fig. 1 and reacted with a mouse IgM mAb recognizing HNK1, a carbohydrate epitope shared by various adhesion molecules. For revelation the same procedure was used as in Fig. 3. Arrows, position of high molecular weight positive molecules in Mb, of M, 140,000 positive molecule in ependymomas, and of M, 90,000 molecule in normal adult brain (N).
ADHESION MOLECULES ON BRAIN TUMORS

Fig. 5. Double immunofluorescence staining of cryostat tumor sections. Mb sections labeled with anti-N-CAM rabbit polyclonal antibody (A) and mouse anti-MenB mAb (B) (final dilution, 1:1000 for each). Nb serials sections labeled with anti-L1 polyclonal antibody (C) and anti-N-CAM polyclonal antibody (D) (final dilution 1:1000 for each). Incubation was conducted for 24 h at 4°C. Anti-MenB was revealed with anti-isotype conjugated to rhodamin, anti-L1, and anti-N-CAM antibodies with anti-isotype antibody conjugated to fluorescein.

polyclonal antibody, N-CAM expressed by ependymomas and gliomas as well as by normal adult brain was not highly sialylated (Fig. 3). By contrast, all Mbs showed high sialylation state of N-CAM. Interestingly, although most Nbs also showed highly sialylated N-CAM, three of them (Nbs 1, 6, and 7) did not. This is also in agreement with the patterns observed in Fig. 2 for N-CAM after delipidation of tissues. They corresponded (Nbs 6 and 7) to Nbs whose patients had received chemotherapeutic treatment prior to surgery. Thus, anti-MenB antibody is able to demonstrate a heterogeneity of glycosylation among N-CAM expressed by Nbs.

HNK1 Detection by Immunoblot. Polysialic acids are not the only glycosylated epitopes borne by N-CAM. It is well known that in normal tissues a variable percentage of N-CAM molecules share with other adhesion molecules such as L1 and myelin associated glycoprotein the HNK1 epitope (13).

We examined the expression of this epitope in normal as well as tumor tissues (Fig. 4). It was present in all Mbs as well as ependymomas and gliomas tested; the patterns were very heterogeneous even in a given group of tumors. Here again a heterogeneity was seen among Nbs, because two (Nbs 1 and 6) were negative and some showed weak immunoreactivity. It is very likely that a portion of the positive molecules corresponded to N-CAM based on their position of migration of gels (ependymomas for example in which a band at M, 140,000 was visible). However, other strong bands with molecular weight around 200,000–250,000 (Mbs 4, gliomas 2 and 4) and M, 90,000 (N) were also seen. According to its molecular weight, the M, 90,000 band is likely to correspond to myelin associated glycoprotein which is known to be expressed by neurons (13). On glial cells, the dispersed high molecular weight band could be cytactin, a cell substrate adhesion molecule shown to be synthesized by glial cells (29).

CEA Detection by Immunoblot. In searching for expression of potential adhesion molecules, the CEA antigen, another membrane of the immunoglobulin superfamily (30), had shown consistently negative results in all the tumors examined (not shown).

Immunohistochemical Analysis. In immunoblot experiments, in some instances, mature forms of N-CAM appeared superimposed with highly sialylated isoforms. Thus it was interesting to monitor at the histological level whether this corresponded to heterogeneous cell populations with cells expressing only the low sialylated isoforms, or whether cells always coexpressed both forms.

Double immunofluorescence labeling using anti-MenB mAb and site directed polyclonal antibodies were used. A typical result is shown in Fig. 5 (A and B) for a Mb. All the cells
was conducted as for immunoblots. With N-CAM immunoreactivity and expressed by all the cells of N-CAM positive cells in the tumor. It is most likely that the same cells could coexpress both mature and highly sialylated isoforms.

We also showed that L1 immunoreactivity was superimposed with N-CAM immunoreactivity and expressed by all the cells in the Nbs examined by immunofluorescence labeling of serial sections [Fig. 5 (C and D)].

**Immunodot Detection of Polysialylated Molecules in CSF.** As an alternative to cytological analysis of CSF to detect the presence of metastatic cells, we set up an immunodot assay. Preliminary data (Fig. 6) conducted with anti-MenB mAb (Fig. 6A) and polyclonal anti N-CAM antibody (Fig. 6B) on CSF from normal subjects and patients exhibiting metastasis are shown. The metastasis was assessed by the presence of Mb cells in CSF examined after centrifugation and staining with May-Grünwald-Giemsa reagent. Experiments have been conducted as described in “Materials and Methods” on serial dilutions of CSF. Normal subjects (Fig. 6, Lanes D, E, F) exhibited only very weak reactivity that was lost at dilution higher than 1:8 of the sample. Patients diagnosed with acute Mb metastasis (Fig. 6, Lanes A, B) showed high reactivities inasmuch as a positivity was still detectable at a dilution of 1:2048. In agreement with diagnostic data, a patient in a remission phase (Fig. 6C) shows much lower reactivity. It is noteworthy that anti-MenB and anti-N-CAM immunoreactivity correlates for every sample tested; this is in agreement with the fact that polysialylated molecules detected are indeed N-CAM.

**DISCUSSION**

Characteristic features of tumors are (a) the presence of structural elements resembling those found during embryonic development of the tissue from which they originated (18) and (b) the role played by adhesive properties in metastatic process. These observations prompted us to investigate neuroectodermally derived tumors for the expression of L1 and N-CAM that are neural cell developmentally regulated adhesion molecules. Although expression of N-CAM on such tumors has already been reported (31), information concerning the nature of isoforms expressed was missing. The availability of probes allowing discernment between the various isoforms, and in particular anti-polysialic acid polymer antibody, was instrumental in this study.

Of particular interest was that L1 was found expressed only by the Nbs examined. The consistent pattern of expression of this antigen, known to appear later than N-CAM in development (6), is in agreement with the fact that Nbs originate from neuronal cells. These cells are presumably neuroblasts at a unique state of normal differentiation, when cells are particularly susceptible to malignant transformation. Conceivably, the stem cell phenotype, including cell surface antigen pattern, is retained by the tumor cells. The recent demonstration that L1 and N-CAM are engaged in a close functional association within the cell surface (15, 32) might result in particular adhesive behavior of L1/N-CAM positive tumors.

We found that N-CAM was expressed by every tumor tested, in agreement with their neuroectodermal origin. Immunoblot examination with antibodies of various specificities showed that ependymomas and gliomas expressed only N-CAM 140. Survey of more cases is necessary to ascertain that this is a common feature of such tumors. Interestingly, N-CAM 120, known to occur later in development (33), was never detected. For Mbs, N-CAM patterns were more complex. Bands migrating at M, 140,000 and 120,000 were clearly discernible (Mb 2), together with higher molecular weight fuzzy bands. We cannot decide whether this is attributable to astrocytic or neuronal differentiation. However, in every case, GFAP immunoreactivity was either absent or expressed in very few cells.

Polysialylation occurred in every Mb tested; this result led us to investigate the presence of polysialylated N-CAM in the CSF from patients suffering from CSF spreading of Mbs. We set up an immunodot assay requiring only 10 μl of CSF. Preliminary data showed that CSF metastasis of Mbs was correlated with high titer of polysialylated N-CAM, whereas CSF from control subjects or from a patient in a remissive phase gave much lower titers. Larger systematic assays should determine whether this quantitation of polysialylated N-CAM could constitute a test to detect metastasis at early stages and also monitor the effect of chemotherapeutic treatment. More generally it can also be useful for assessing the malignant potential and the aggressiveness of Mb growth and help clinical staging and prognosis. MenB immunoreactivity was also frequently exhibited by Nbs. This finding corroborates with the data (31, 34) showing that human Nb cell lines IMR32 and CHP-134 express extended polysialic acid chains. However, three of the Nbs tested were negative with anti-MenB antibody and expressed mature forms of N-CAM. They corresponded to patients having received chemotherapeutic treatment. Nbs 6 and 7 showed necrotic areas and large ganglionic cells. Nb1 showed a histological pattern of ganglioneuroma. Conceivably, the embryonic toward mature N-CAM form interconversion reflects cellular changes critical for the conversion of some Nbs into benign ganglioneuromas. This was also correlated with the loss of the HNK1 epitope expression, therefore with other changes affecting adhesion molecules. Interestingly, in normal brain, this epitope was
reported to be more heavily expressed at early stages of development (13).

Expression of highly sialylated forms of N-CAM had also been reported on tumors of presumed neuroectodermal origin such as Ewing's (35) and small cell lung cancers (36). Its expression on Wilm's tumor is more enigmatic (37). It is particularly interesting that polysialic units are part of a primary cell adhesion molecule of the immunoglobulin superfamily (28), that is proposed to have a regulatory role in cell growth and differentiation (38). Changes in cell surface carbohydrates accompanying malignant transformation have been implicated in the altered growth and behavior of tumor cells. In many instances, carbohydrate moieties that were expressed during certain stages of embryonic development, but suppressed later on were found to be reexpressed in tumors. However, in very few cases could this be correlated with a specific role of the carbohydrate in cell growth and differentiation. We have shown that, in Mbs and Nbs, the highly sialylated form of N-CAM that is transiently and regionally expressed during nervous system development and nearly absent from mature neuroectodermal and other tissues is present. Further experiments must be aimed at clarifying its possible significance in tumor growth and its usefulness as a tumor marker.

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