Neural Cell Adhesion Molecule Distribution in Primary and Metastatic Uveal Melanoma

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Tumor cell adhesion, detachment, and aggregation play an important part in tumor invasion and metastasis, and a variety of cell adhesion molecules have been found on tumor cells. Cell adhesion molecules, including those of the immunoglobulin superfamily, are associated with the development of metastatic behavior in cutaneous melanomas. The neural cell adhesion molecule (NCAM) belongs to this family. To investigate its possible role in the development metastatic behavior of uveal melanomas, the authors studied immunohistochemically the expression of NCAM by using an antibody that recognizes all three major isoforms of NCAM and an antibody that recognizes the HNK-1 epitope present on some isoforms of NCAM. The authors studied 32 primary uveal melanomas from 32 patients (among these, 12 were rapidly metastasizing and 16 slowly metastasizing) and 29 metastases from 19 patients. From 13 patients the primary, as well as the metastatic, tumors were available. With one exception, all HNK-1 positive primary and metastatic tumors were also

positive for NCAM. NCAM was significantly more expressed in aggressive, rapidly metastasizing primary tumors ($P = .02$ and $.04$, respectively) and in metastases. HNK-1 was significantly ($P = .04$) more expressed in larger tumors. In liver metastases HNK-1 immunoreactivity was significantly ($P = .005$) less frequently expressed than NCAM. Therefore, NCAM isoforms that lack the HNK-1 epitope might play a role in the organ specific metastatic behavior of uveal melanomas. HUM PATHOL 26:1185-1190. Copyright © 1995 by W.B. Saunders Company

Key words: uveal melanoma, metastasis, neural cell adhesion molecule, HNK-1.

Abbreviations: NCAM, neural cell adhesion molecule; Ig, immunoglobulin; RT, room temperature; ICAM, intercellular adhesion molecule; PBS, phosphate-buffered saline; LTD, largest tumor diameter; GPI, glycosyl phosphatidyl inositol; CAM, cellular adhesion molecule.

There is increasing evidence that changes in adhesiveness and motility are of considerable importance in tumor progression and may be the prime features determining aggressiveness and metastatic potential.¹ Adhesive properties of malignant cells must change repeatedly to allow them to detach from their primary location, attach to the extracellular matrix, enter a blood vessel, and eventually lodge at a metastatic site.² Adhesion molecules that comprise several complex families, mediate intercellular interactions, and interactions between cells and the extracellular matrix.³ They are grouped into four main classes on the basis of their molecular structure: the integrin family, cadherin family, immunoglobulin superfamily, and selectin superfamily.⁴ The neural cell adhesion molecule (NCAM) and the intercellular adhesion molecule (ICAM) belong to the immunoglobulin (Ig) superfamily.^{3,4} Although the members of the Ig superfamily are functionally diverse, most are cell surface molecules involved in recognition of other surface molecules. Several neural cell adhesion molecules have been shown to mediate intercellular adhesion during the development of the nervous system including the neural cell adhesion molecule (NCAM), myelin-associated glycoprotein, L1, amalgam, contactin, and fascicilin II.⁵ The HNK-1 carbohydrate epitope is associated with several of these adhesion molecules including NCAM.⁶ Although the

exact role the HNK-1 epitope plays is unknown, it seems to serve as a ligand in cell adhesion.⁷

For cutaneous melanoma it has been shown that the development of metastatic potential is associated with de novo expression of ICAM-1^{8,9} and MUC18,¹⁰ an antigen that shows sequence similarity to NCAM.^{10,11} In contrast, others showed expression of MUC18 on a full range of benign and malignant melanocytic lesions.¹² In uveal melanoma, ICAM-1 could not be detected in one study,¹³ but using a different anti-ICAM-1 monoclonal antibody (MAb) most of the uveal melanomas stained,^{14,15} with a preferential reactivity of the mixed and epithelioid cell type.¹⁵ In several human malignancies tumor progression was paralleled by changes in NCAM expression.^{1,16-18} A role for NCAM in the development of malignant potential of uveal melanomas has, however, not been reported.

This study investigates whether NCAM expression is correlated with the development of metastatic potential in uveal melanoma. The authors studied primary tumors with known clinical outcome (including rapidly metastasizing and clinically nonmetastasizing or slowly metastasizing tumors) and all available metastases. The authors report here on NCAM, which was stained by a polyclonal antibody that recognizes all three major NCAM isoforms, and by MAb Leu-7, which recognizes the HNK-1 epitope¹⁹ of the cell-binding domain of some NCAM isoforms.^{20,21}

MATERIALS AND METHODS

Patient Selection

From the files of patients with uveal melanoma related death, the authors were able to collect metastatic tissue from 20 patients (nine liver biopsies, one skin biopsy, one autopsy, and nine fine needle aspirations). From 14 of these patients

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TABLE 1. Immunohistochemical Data of Melanomas Metastasizing Within 3 Years

Patient No.*	Cell Type	LTD	Primary Uveal Melanoma		Corresponding Metastasis	
			NCAM	HNK-1	NCAM	HNK-1
1	S	2	1	0	NA	NA
2	E	2	2	0	NA	NA
3	M	2	2	0	0	0
4	M	2	2	2	NA	NA
5	E	2	2	0	NA	NA
6	M	2	NA	0	NA	NA
7	E	3	2	2	NA	NA
8	M	2	0	3	3	0
9	M	2	3	0	NA	NA
10	M	2	2	0	NA	2
11	M	3	2	2	0	0
12	M	2	2	NA	0	NA

NOTE. Score 0 = negative; score 1 = 0% to 5% tumor cells positive; score 2 = 5% to 50% tumor cells positive; score 3 = 50% to 100% tumor cells positive.

Abbreviations: E, epithelioid cell type; M, mixed cell type; S, spindle cell type; LTD, largest tumor diameter: 1: <10 mm, 2: 10 to 15 mm, 3: >15 mm; NA, paraffin block not available; NCAM, neural cell adhesion molecule.

* All patients died of tumor-related death.

the paraffin blocks from the primary ciliary body or choroidal melanoma were available; their follow-up varied between 6 and 147 months. From five of these patients, paraffin blocks from the primary, as well as the metastatic tumor were available. Uveal melanomas create a peak incidence of mortality during the second and third years after enucleation, irrespective of the largest tumor diameter.²² Tumor related death within 3 years was, therefore, considered to be caused by rapidly metastasizing melanoma ($n = 8$; mean follow-up, 21.3 months) (Table 1). Furthermore, the authors selected nine patients with a follow-up of at least 10 years after enucleation without clinical evidence of metastatic disease, of whom paraffin blocks of the primary tumor were available. These melanomas were considered to be of low metastatic potential (Table 2). The total mean follow-up of this group ($n = 11$ including two cases of tumor related death after >10 years) was 169.5 months.

From nine patients, the paraffin blocks of the primary uveal melanomas and eight corresponding metastases (four autopsies and four biopsies) were obtained from the Eye Pathology Institute in Copenhagen: the follow-up varied between 2 and 32 years. Four were rapidly metastasizing melanomas (Table 1), and five were of low metastatic potential (Table 2). All these patients died of tumor-related death.

The total material consisted of 32 primary tumors. Among these, 12 were rapidly metastasizing (Table 1) and 16 slowly metastasizing (Table 2); the remaining four died because of uveal melanoma-related death between 3 and 10 years. We investigated 29 metastases (Table 3) from 19 patients. Of 13 patients, tissue of the corresponding primary and metastatic tumors were available.

Histology

Sections were cut at 5 to 6 μ m and stained with hematoxylin-eosin. Of the primary tumor, the predominant cell type (spindle, mixed, or epithelioid) and largest tumor diameter (LTD) (≤ 10 , 10 to 15, and >15 mm) were recorded.

Immunohistochemistry

Paraffin sections, 6 to 7 μ m thick were cut and mounted on aminopropyltriethoxysilane (Sigma, St. Louis, MO)-coated glass slides and dried overnight at 37°C. After deparaffinizing and rehydrating, endogenous peroxidase activity was blocked by incubation for 20 minutes in methanol containing 3% hydrogen peroxide. After rinsing the slides in water, antigen retrieval was performed by microwave irradiation (Bio-Rad 37°C; 750 W; 2 $\times$ 5 minutes in 0.1% pronase). The slides were incubated with phosphate-buffered saline (PBS) at 4°C for 10 minutes and subsequently at room temperature (RT) for 5 minutes. To detect all three major NCAM isoforms, irrespective of the presence or absence of polysialylation,²³ a polyclonal antibody was used²⁴ in a 30-minute incubation in a dilution of 1:100 at RT. This antibody was a generous gift from E. Bock (Research Center for Medical Biotechnology, University of Copenhagen). Visualization of antibody binding was performed as described subsequently. HNK-1, a sulfated glucuronic carbohydrate epitope that is present on the 145- and 180-kD isoforms of NCAM and in related proteins^{21,25} was detected using the anti-Leu-7 monoclonal antibody (Becton Dickinson, Sunnyvale, CA). This antibody was originally generated against a human T-cell line and is present on a subpopulation of natural killer cells,¹⁹ but is also associated with several adhesion molecules.^{20,21} This antibody was used in a dilution of 1:10. The slides were incubated for 30 minutes at RT with biotinylated goat-anti-mouse-rabbit-rat-guinea-pig Ig (Biogenex, San Ramon, CA) in a dilution of 1:50, in PBS with 5% BSA. After washing in PBS/Tween 0.05%, the slides were incubated with the streptavidin-biotin-peroxidase complex (Biogenex) in a dilution of 1:50. The peroxidase was visualized using hydrogen peroxide in N-N-dimethylformamide with 3-amino-9 ethylcarbazole as chromogenic sub-

TABLE 2. Immunohistochemical Data of Melanomas With a Follow-Up of More Than 10 Years

Patient No.	Cell Type	LTD	Primary Uveal Melanoma		Corresponding Metastasis	
			NCAM	HNK-1	NCAM	HNK-1
1	M	1	2	0	*	*
2	S	2	0	0	*	*
3	S	2	0	0	*	*
4	S	2	0	0	*	*
5	S	2	0	0	*	*
6	S	1	0	0	*	*
7	S	3	2	0	*	*
8	M	2	0	0	*	*
9	S	2	1	0	*	*
10†	S	2	2	3	NA	NA
11†	M	1	1	0	3	0
12†	S	3	3	3	2	2
13‡	S	2	0	0	2, 2	3, 2
14‡	S	1	3	0	2	0
15‡	S	2	0	0	0, 2, 3, 3	0, 0, 0, 0
16‡	M	3	2	0	3	0

NOTE. Score 0 = negative; score 1 = 0% to 5% tumor cells positive; score 2 = 5% to 50% tumor cells positive; score 3 = 50% to 100% tumor cells positive.

Abbreviations: S, spindle cell type; M, mixed cell type; E, epithelioid cell type; LTD, largest tumor dimension: 1: <10 mm, 2: 10 to 15 mm, 3: >15 mm; NA, paraffin block not available; NCAM, neural cell adhesion molecule.

* Patients alive and free of metastatic disease after >10 years.

† Tumor-related death after >10 years.

‡ Multiple metastatic sites obtained at autopsy.

TABLE 3. Staining Pattern in the Metastases

	NCAM					HNK-1				
	Negative	Score			Ratio Positive	Negative	Score			Ratio Positive
		1	2	3			1	2	3	
Liver	3	1	6	5	12/15	14	—	1	—	1/15
Lung	1	—	6	2	8/9	5	—	3	1	4/9
Skin*	1	—	2	—	2/4	1	1	2	—	3/4
Abdomen*	—	—	—	—	0/1	—	—	—	1	1/1
Total*	5	1	14	7	—	20	1	6	2	—

NOTE. Score 1 = 0% to 5% positive tumor cells, score 2 = 5% to 50% positive tumor cells, score 3 = 50% to 100% positive tumor cells.

* One metastasis from the skin and one metastasis from the abdomen were unclassifiable for NCAM.

† Ratio of positive lesions/total lesions in the metastatic site.

Abbreviation: NCAM, neural cell adhesion molecule.

strate. The red stain allowed easy detection of immunoreactivity in pigmented lesions. The sections were counterstained with Mayer's hematoxylin and mounted in glycerin gelatin.

As a negative control, specimens were stained following the same incubation protocol without use of the primary antibodies. A human neuroblastoma served as a positive control for NCAM. For HNK-1, nerve tissue and tumor infiltrating lymphocytes present in the tissue section served as an internal positive control.

NCAM and HNK-1 immunoreactivity were scored semi-quantitatively as a percentage of positive cells: score 0: 0%; score 1: 0% to 5%; score 2: 5% to 50%; score 3: 50% to 100%. The immunohistochemical staining was scored without knowledge of the clinical data and was repeated after 2 months to assess reproducibility. Major (more than two classes) discrepancies did not occur.

Statistical Analysis

Spearman's rank correlation was used to test the relation between the variables cell type, LTD and rapidly/slowly metastasizing tumors, and NCAM and HNK-1 staining, respectively. Fisher's exact test was used to test correlations of NCAM and HNK-1 immunoreactivity in the metastatic sites.

RESULTS

Primary Tumors

Thirteen tumors were of the spindle cell type, 15 of the mixed-cell type and four of the epithelioid-cell type. Four tumors were small (≤ 10 mm), 22 were intermediate size (10 to 15 mm), and six were large (> 15 mm).

NCAM-positive tumors showed strong cytoplasmic staining; in three, cytoplasmic (Fig 1A) and membrane-bound staining was noted (Fig 1B). The results for NCAM expression in the different cell types are illustrated in Fig 2. NCAM was significantly more expressed in mixed-cell type and epithelioid-cell type melanomas ($P = .02$) and in rapidly metastasizing tumors ($P = .04$). Rapidly and slowly metastasizing tumors are summarized in Tables 1 and 2.

In the HNK-1-positive tumors, strong cytoplasmic staining was noted (Fig 3). HNK-1 was significantly more expressed in larger tumors ($P = .04$).

Metastases

The staining pattern of all metastases for NCAM is illustrated in Fig 2; the staining pattern in the different organs is specified in Table 3. For NCAM, 72% of the metastases stained (score 2 + 3). For HNK-1, 27% of the metastases stained (score 2 + 3). HNK-1 was significantly ($P = .005$) less expressed in liver metastases compared with other metastatic site. In five autopsies, different metastases from the same subject had a varying score (from negative to score 3) both for NCAM and HNK-1.

Corresponding Primary and Metastatic Tumors

Of 13 tumor pairs (partly reflected in Tables 1 and 2), nine primary tumors and their metastases showed

FIGURE 1. Field of uveal melanoma of the epithelioid cell type immunostained for NCAM. (A) Cytoplasmic staining. (Original magnification $\times 361$.) (B) membrane-bound staining. (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with 3-amino-9 ethylcarbazole dimethylformamide substrate; original magnification $\times 880$.)

FIGURE 2. Results for NCAM immunostaining in primary and metastatic uveal melanoma. Percentage of positive cells: ■ 50% to 100%; ▨ 5% to 50%; □ <5%; □ 0%.

positive (score 2 + 3) staining for NCAM; in four major (negative vs positive) discrepancies between the primary tumor and the metastases were noted. For HNK-1 major discrepancies were noted in three tumor pairs.

Relationship Between HNK-1 and NCAM Immunostaining

Of the primary tumors, 71% was NCAM positive, but only 20% was HNK-1 positive. Similarly, of the metastases 72% were NCAM positive, but only 27% was HNK-1 positive. However, with one exception, all HNK-1 positive primary and metastatic tumors were also positive for NCAM.

DISCUSSION

Three major isoforms of NCAM have been identified, which are generated by alternative splicing of a single gene. Two of these isoforms are transmembrane proteins, differing in only their cytoplasmic domain. NCAM-140 kD has a short cytoplasmic tail, whereas NCAM-180 kD has a large cytoplasmic domain thought to interact with the cytoskeleton. NCAM-180 expression is restricted to neural tissues; the 120- and 140-kD isoforms also occur on other cell types.²⁶ One role of the different isoforms may be to target them to different cellular destinations: It has been found that glycosyl phosphatidyl inositol (GPI) membrane anchoring (small cytoplasmic domain NCAM-120) acts as an apical targeting signal in epithelia.²⁷ The antibody against

NCAM used in this study recognizes the three major forms of NCAM, whereas MAb Leu-7 recognizes the HNK-1 epitope present in the 145- and 180-kD isoform of NCAM. Furthermore, NCAM exhibits special carbohydrate characteristics: glycosylation of NCAM seems to be regulated during development and to influence the adhesive function of the molecule.²⁸ It has been suggested that polysialylated NCAM present in early (embryonic and fetal) stages of development is involved in cellular migration, whereas the expression of unsialylated NCAM in tissues may be important for local differentiation and organization.²⁹ Polysialylation of the NCAM molecule decreases its adhesion properties and may, therefore, play a role in connection with tumor invasion and metastasis. Immunohistochemical investigation does not provide information about NCAM polysialylation.

The percentage of NCAM positive primary tumors in our study was higher than has been reported for cutaneous melanomas.^{12,17,30} This might be explained in terms of methodological differences: The authors used a different (polyclonal) antibody on formalin-fixed, paraffin-embedded tissue and also applied an antigen retrieval method. NCAM immunostaining was mostly cytoplasmic and less frequently both cytoplasmic and membrane bound, which is in keeping with previous investigations.^{17,30,31} NCAM was significantly more expressed in tumors with presence of epithelioid cells (mixed and epithelioid cell type). The presence of epithelioid cells is one of the factors associated with progression of uveal melanomas (development of metastatic potential).³² That NCAM is expressed in epithelioid cells seems surprising because epithelioid

FIGURE 3. Field of metastatic uveal melanoma immunostained for HNK-1. (Hematoxylin counterstain with streptavidin-biotin-peroxidase complex immunoperoxidase with 3-amino-9 ethylcarbazole dimethylformamide substrate; original magnification $\times 361$.)

cells are morphologically noncohesive. It is not clear how adhesion molecules on the surface of cancer cells influence metastasis. Adhesion molecules may delay the escape of tumor cells from the primary site because of an increased adhesion to other cells and to extracellular matrix proteins. However, an altered pattern of CAM expression or expression of aberrant CAM might disrupt normal adhesion and attachment of these cells to a endothelium or extracellular matrix at a metastatic site.¹⁶

Immunoreactivity for HNK-1 was found in approximately one third of the NCAM positive lesions, which can be explained by the HNK-1 epitope being present in only two isoforms of NCAM. Interestingly, HNK-1 was significantly less expressed in liver metastases compared with other metastatic sites. This discrepancy was not found for NCAM. Uveal melanomas metastasize relatively late^{33,34} and in contrast to cutaneous melanomas primarily hematogenously, preferentially to the liver. Once hepatic metastases are clinically present, the median survival is extremely poor: only 2 to 11 months.³⁵ These findings suggest that NCAM isoforms, lacking the HNK-1 epitope, might play a role in the organ-specific pattern of uveal melanoma metastasis. Isoforms with alternative modes of membrane association are targeted to different surfaces of polarized epithelial cells; the 120-kD small cytoplasmic domain is expressed on apical surface and is attached to the cell membrane via a GPI-linkage, whereas the 140-kD small surface domain form and the 180 kD large domain form are expressed on the basolateral surface.²⁷

Furthermore, the authors found a significant increase of NCAM and HNK-1 positive lesions among rapidly metastasizing and large tumors, and in metastases. This is in keeping with the increasing evidence that changes in adhesiveness and motility are of considerable importance to tumor progression.¹ However, a de novo expression in tumor progression could not be shown as has been found for related cellular adhesion molecules ICAM-1 and MUC-18 in progression of cutaneous melanomas.⁸⁻¹⁰ The pattern of NCAM and HNK-1 expression in the primary tumors differed significantly from that in the paired metastases, suggesting that NCAM and HNK-1 expression are probably modulated by the tumor cell microenvironment.

In summary, these results show that expression of NCAM is associated with the development of malignant potential of uveal melanoma. Furthermore, this study found that NCAM isoforms, lacking the HNK-1 epitope, may be associated with the organ specific metastatic behavior of uveal melanomas. The prognostic value of NCAM and HNK-1 expression in uveal melanoma remains to be established.

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