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Basic Investigations

IMMUNOCYTOCHEMICAL CHARACTERIZATION OF CD44 MOLECULES EXPRESSED IN HUMAN BRAIN METASTASES

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On the basis of analyses of differential CD44 expression patterns between primary brain tumours and brain metastases and the high incidence of CD44v detection in brain metastases formed by various types of systemic tumours, we further characterised CD44v proteins expressed in brain metastases studied previously with the antibodies specific for variant CD44v isoforms. The results demonstrate that CD44v expression in brain metastases is extremely heterogeneous. Generally, v5/v6 is homogeneously expressed in the cell populations, while up-regulated v7/v8 or v7-v10 could be usually found in part of tumor cells, especially the ones close to necrosis, at tumour borders and with strong tendency of invasiveness. v7/v10 could also be detected in microvascular endothelial cells of the tumoours. Our data thus describe multi-spectrum CD44v expression in the same tumours and even in individual tumour cells and suggest the necessity to use more than one antibodies in the study of CD44 expression in human malignancies.

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We have recently shown that CD44v (variant CD44) expression is a very common feature of human brain metastases (Bms) irrespective to their origins and morphologies,[1,2] indicating either a general distribution of CD44v in metastatic tumours or the potential importance of these molecules in the formation of brain metastases.

The generation of CD44v molecules is due to the insertion of up to ten alternative spliced variant exons and/or due to posttranslational modification, resulting in tremendous heterogeneity of CD44v expression.[3] As well as the findings in gastric and colorectal carcinomas and their metastatic tumours in regional lymph nodes and liver,[4-7] the heterogeneous CD44v expression can also be detected in different types of Bms, e.g. squamous cell carcinomas express mainly CD44v containing variant exons v3-v10, adenocarcinomas mainly have v4-v7 or v7-v10 or v4-v10.[2] So far, it is not clear whether these individual CD44v isoforms have an identical function or somewhat different ones. Since more than one type of CD44v molecules were found to be expressed in the same tumours or a groups of tumour cells,[3] it might be possible that different CD44v molecules may confer

different properties to the cells or that their expression may reflect the cells under different physiological or pathological conditions. In order to assess these speculation, we characterized the types of CD44v molecules expressed in 25 brain metastases with monoclonal antibodies (mAbs) recognizing epitopes on "variant" exon encoded portions[3] and mAb specific for the common portion that is shared by all isoforms of CD44. [9] The results showed that more than 90% of the tumours was stained positively with a polyclonal antisera against the whole variant region of CD44, but the composition of individual exons varies dependent upon the location of the tumour cells within the tumour masses: particularly at the regions where the cell turnover occurs. These findings suggest that except heterogeneity of CD44v expression during stepwise carcinogenesis, CD44v expression is also highly variable even in the same cell populations. Therefore, the influence of certain micro environmental factors and cell-cell interaction in CD44v splicing should be taken into account.

MATERIALS AND METHODS

Sample Collection

25 brain metastases originated from different organs were collected immediately after removal from patients, part of the tumours was placed in liquid nitrogen and stored at -70 ℃ until use. The remaining parts were fixed in 10% bufferred formalin, then embedded into paraffin. The paraffin sections were prepared as usual and subjected to histopathological examination.

Anti-CD44 Antibodies

The staining for standard and variant CD44 was performed using mAb Ab-2 detecting all CD44 isoforms (Oncogene Science, Inc., Uniondale, NY) with 1:50 dilution and a rabbit polyclonal antibody directed against human CD44v exons v3-v10 (a generous gift from Drs. Karl-Heinz Heider and Petra Skroch-Angel, Institute of Genetics, Karnfors-chungszentrum, Karlsruhe, Germany). For immunocytochemical characterization of CD44v isoforms, the monoclonal antibodies specific for the variant exons v5 (VFF-8), v6(VFF-7), v7(VFF-9) and v7/v8-10 (VFF-17) and v10(VFF-16)(the generous gifts from Dr. Erik Patzelt, Berder+ Co Ges mbH, Vienna, Austria) were used.

Immunocytochemical Staining

Mab, Ab-2, for both CD44s (standard CD44) and CD44v and the polyclonal antibody against exons v3-v10 of CD44v were employed to determine the type of CD44 expression in brain metastases. The four variant exon specific mAbs were then used to characterise the main CD44 expression pattern and distribution of individual exons in each of the tumours. The staining procedures were done on frozen sections as described previously.[8] Briefly, 7 μm frozen sections were put onto gelatine coated slides, air dried, fixed in cold acetone for 3-5 minutes and hyrated in PBS. The slides were incubated with 1: 15 diluted primary antibodies for one hour, then with biotinylated antibodies for 30 minutes. Between each of incubations, the slides were rinsed with PBS for three times. Then, incubate the sections for 30 minutes with VECTASTAIN^{R} ABC reagents. After being washed the in PBS for 10 minutes, the slides were incubated for 7 minutes in peroxidase substrate solution. The counter staining was performed with hematoxylin. The keratinocytes in normal skin were used as positive control for CD44v,[9] the glioblastoma tumour, G-765, as positive control for CD44s. [7] Supernatant of P3X63Ag8, a γ-1- producing myeloma cell line was used as negative control.

Immunocytochemical Characterisation

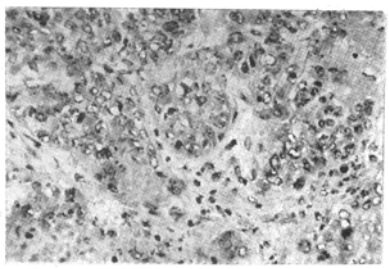
The characterisation was done on each of brain metastases according to the following parameters: the types of CD44 expression; overall expression of CD44 varian exons in more than 90% of tumour cells; the main CD44v expression pattern with one or more than one expressed variant exons and the heterogenecity of individually expressed variant exons. In the later context, more attention was paid to the regions of tumour invasion, necrosis and tumour border. Meanwhile, CD44 expression in infiltrating lymphocytes and endothelial cells of intraneoplastic vessels was also examined.

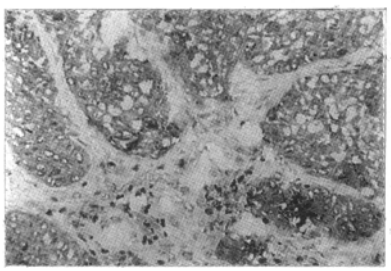
Results And Discussion

By the use of a mAb against CD44s/v (Ab-2) and a polyclonal antibody specific for variant exons v3-v10, CD44 and CD44v were detected in 24/25 and

25/25 brain metastases respectively, demonstrating a remarkable correlation of tumor cell dissemination and CD44v expression. It was found that under the same staining conditions, the positivity of mAb Ab-2 stained protein (αpan-CD44) in brain metastases and keratinocytes is much weaker than that of anti-CD44v polyclonal antibody reacting protein(s) (Figure 1a and b). Moreover, BM-748, an adenocarcinoma originated from the testis, show local CD44v expression irrespective of its negative reaction to Ab-2 antibody. These results might indicate that Ab-2 is not always able to recognize their epitopes in all CD44v isoforms. Whether this is due to an unusual type of posttranslational modification, leading to "masking" of the antibody's epitope or the presence of additional peptide sequences in CD44v molecules resulting in steric hinderens remain to be analysed. Therefore, it is recommendable to use both Ab-2 and CD44v specific antibodies in evaluation of CD44 production. A fine analysis of Cd44v isoforms expressed in brain metastases was performed using a panel of αCD44v mAbs (see Materials and Methods). As shown in Table 1, variant exons of CD44 could be detected in all of 25 cases studied. Among them, 17 cases (68%) were found to express v5-v10, 5 (20%) expressed v7-v10 and one (4%) expressed v5/v6 or v5-v7 or v7-v10, respectively. However, the expression levels of different exons are found to be heterogeneous in 18 cases in the form of one or more than one overexpressed exons, v5 in one case, v5/v6 in four, v5-v7 in one, v7/v8 in 10 and v7-v10 in four. Our data thus demonstrate a multiple spectrum of variant exon expression not only among different types of tumours or different cases of the same kind of tumours but also within individual cases.

Heterogenecity with respect to expression of variant exons could be observed in some special regions. In 21 out of 25 brain metastases, highly expressed v7/v8 or v7-v10 was usually found in the cells close to tumour boder (Figure 2a) or in cell clusters that invaded into surrounding tissues (Figure 2b). In these cases, 13 (62%) showed overexpression of v7/v8 and 8 (38%) showed v7-v10. A similar phenomenon could be observed in cells close to the regions of necrosis. These results suggest that the expression of v7/v8 and v7-v10 are highly variable in comparison with that of v5 and v6. The real role(s) of v7/v8 and v7-v10 in the cells remains conjectural at this time. However, their up-regulated expression might reflect an interaction between tumour cell-matrix and/or a response of tumour cells to their environments, because of the frequent detection of them in poorly nourished cells near necrosis and in the cells toward expansion and invasion.





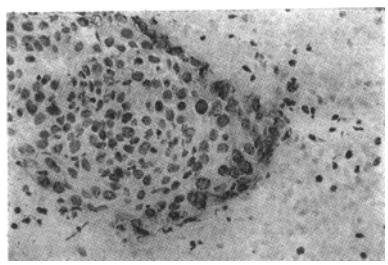


Fig 1. Immunocytochemical stainings performed on frozen sections of a brain metastasis spread from squamous cell carcinoma of the lung with mAb Ab-2 detecting all CD44 isolforms and a polyclonal antibody. against human CD44v vexons v3-v10.

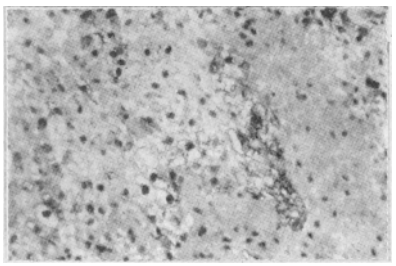


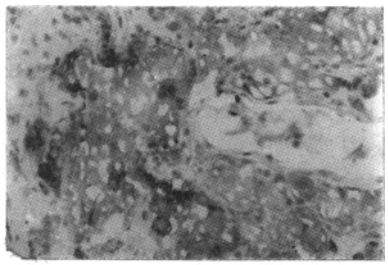
Fig 2. Representative figures of the immunocytochemical stainings showing the upregulated expression of exon v7/v8 in the cells close to tumor border (a) and with strong tendency of invasiveness(b)

Normally, endothelial cells are negtive for CD44. [12] However, a high incidence (20/24 or 83%) of v7/v8 (Figure 3a) and a small fraction (2/24 or 8%) of v7-v10 expression could be found in microviscular endothelial cells inside the tumours; while v5/v6 remain negative in those cells (Figure 3b). In a separate study, we also found v7/v8 positive endothelia within glioblastomas and medulloblastomas in which the tumour cells are totally negative for CD44v expression. [2] Additionally, with the same mAbs used in our study, Wielenga, et al. found focal expression of v7-v10 encoding epitopes in normal colon epithelium and an increased expression level and extent during stepwise colorectal carcinogenesis. [4] All these data indicate that v7-v10 expression can be easily induced not only in cancer cells but also in the preneoplastic cells and even in non-malignant cells where cell proliferation and tunover occurs.

Table 1. Immunocharacterization of CD44v isoforms expressed in brain metastases

Morphology	Origin	No.	Expres Pattern		Unidentical Expres		Intraneoplastic
			General	Main	Necrosis	Border/Invas.	Endo. Cells
Sq.Cell Carc.	Lung	1	v5-v10	v5-v7	*	v7/v8	v7/v8
		2	v5-v10	v5/v6		v7/v10	v7/v8
		3	v5-v10	v7-v10	v7/v8	v7-v10	v7/v8
	Cervix	1	v5-v10	v7/v8		v7/v8	v7/v8
	Tonsil	1	v5-v10	v5/v6		v7/v8	
Adenocarc.	Lung	1	v5-v10	v7-v10		v7-v10	v7/v8
		2	v7-v10	v7/v8		v7/v8	v7/v8
		3	v5-v10	v5-v10	v7-v10	v7/v8	v7/v8
		4	v7-v10	v7-v10	v7/v8		v7/v8
	Breast	1	v7-v10	v7-v10		v7/v8	v7/v8
		2	v5-v10	v7-v10			v7/v8
		3	v5-v10	v7-v10		v7/v8	v7/v8
		4	v5-v7	v5/v6	v7/v8	v7/v8	—
		5	v5-v10	v5-v10			v7/v8
		6	v5-v10	v7-v10		v7/v8	v7/v8
	Testis	1	v7-v10	v7/v8		v7/v8	v7-v10
		2	v8-v10	v7-v10		v7/v8	v7/v8
	Colon	1	v5-v10	v5/v6		v7-v10	—
		2	v5-v10	v5/v6			v7/v8
	kidney	1	v5-v10	v5-v10	v7/v8	v7/v8	v7/v8
Others:							
SCLC	Lung	1	v5-v10	v7-v10		v7-v10	v7/v8
		2	v7-v10	v7/v8	v7/v8	v7/v8	v7/v8
LCLC	Lung	1	v5-v10	v7-v10		v7/v8	v7/v8
Histocytiocytoma		1	v5-v10	v7-v10		v7/v8	v7-v10
Melanoma		1	v5-v10	v5		v7-v10	v7/v8

* The empty spaces mean the pathological region or change studied cannot be observed.



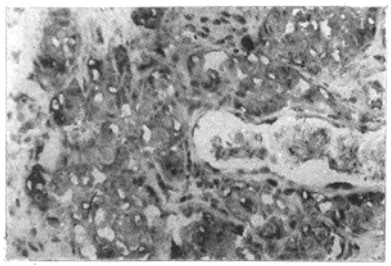


Fig 3. A case of brain metastasis formed by squanmous cell carcinoma of the lung (BM544) stained with anti-v6 mAb (a, VFF-7) and anti-v7/v8 mAb (b, VFF-14)

In addtion to the high frequency of CD44v detection in brain metastases, we also found remarkable heterogeneous spectrum of CD44v expression in those tumors. v5/v6 is usually expresssed in majority of the tumor cells and intensively distributed on the cell surface. Since v5/v6 usually appeared at relatively later stages of colorectal and gastric oncogenesis, [4,7] these two exons may be biologically essential for tumor cells during their dissemination and localization in distant organs by a not yet known mechanism. On the other hand, frequent observation of up-regulated v7/v8 or v7-v10 and their unidentical expression in peripheral tumor region and in invasive cells implicates that these exons may be required to provide cell-matrix interaction more appropriate to growth advantage and the architecture of metastasized tumor cells.

Metastasis is a very complicated process involving numerous tumor cell-host cell and tumor cell-matrix interactions. Therefore, it requires additional genetic alteration(s) beyond those related to tumorigenesis. If we consider that the general distribution of CD44v adhesion molecules in human malignancies is one of those alterations, the coexistence of multiple CD44v isoforms and, especially their appropriate combination in the same tumor/the same cell may probably allow the spreading tumor cells not only to localize but also to form metastatic foci efficiently in their target organs.

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