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Expression of CD44 splice variants in human primary brain tumors

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Summary

Expression of CD44, particularly of certain splice variants, has been linked to tumor progression and metastatic potential in a number of different animal and human cancers. Although differential expression of CD44 standard epitopes (CD44s) in human brain tumors has been reported, the expression of CD44 variant exon encoded sequences (CD44v) in primary brain tumors *in situ* has not been studied in detail. In the present study, the expression of CD44s and CD44v epitopes was analyzed immunohistochemically on frozen sections of primary brain tumors. In addition, the expression of CD44 on cultured glioma cells was investigated by immunofluorescence flow cytometry. The results demonstrate the presence of CD44s epitopes and of CD44 splice variants containing CD44v4, v5 and v10 sequences in various types of brain tumors. A subgroup of highly malignant gliomas showed a strong (focal) expression of CD44v5. CD44v6 was absent in all brain tumors examined. CD44s appeared to be the dominant form of CD44 expressed in primary brain tumors, its expression was not correlated with tumor grade. We envisage that CD44 isoforms, in particular CD44s, may contribute to the invasive character of primary tumors by interacting with hyaluronate, one of the most abundant molecules in the extracellular matrix of the brain.

Introduction

CD44 is a major cell surface receptor for hyaluronate [1, 2] and exists as multiple isoforms [3–7]. These isoforms are generated by alternative splicing of at least 10 exons (exon v1–v10), encoding parts of the extracellular domain [8]. A 85–95 kD isoform of the CD44 molecule, termed standard CD44 (CD44s), is widely expressed on cells of hematopoietic and mesodermal origin and lacks all 10 variable exons [9, 10]. In contrast, CD44 isoforms containing multiple alternative spliced exons (CD44v) are predominantly expressed on normal epithelia and tumors of epithelial origin [9]. CD44 has been implicated in a variety of processes, including lymphocyte homing, hematopoiesis, lymphocyte activation and extracellular matrix adhesion [1, 2, 7, 11–13]. However, the exact physiological functions of the diverse CD44v are as yet unclear.

Interestingly, CD44 isoforms containing exon v6 have been shown to play a causal role in tumor metastasis in the rat [5, 14]. Expression of homologous isoforms in human tumors is associated with tumor progression and adverse prognosis [9, 10, 15]. Furthermore, expression of CD44 exon v5 has been related to tumor progression in colorectal tumors [15] and melanomas [16]. In astroglial brain tumors, CD44s expression has been reported [17–20]. In addition, it has been shown that CD44s mediates glioma cell invasion *in vitro* [21]. Furthermore, in a recent study it was proposed that variants of CD44 might be involved in the metastatic spread of sys-

temic tumors into the brain [22]. In order to gain more insight in the role of CD44 in the biology of brain tumors, we have studied the distribution of different CD44 isoforms in primary neoplasms of the brain.

Materials and methods

Tumor tissue

Primary brain tumors were collected from our files. A wide range of glial and non-glial neoplasms were included. The tumors studied are listed in Table 1. The tumor specimens were snap-frozen in liquid nitrogen immediately after removal. For routine histology part of the specimens were fixed in 10% buffered formalin and embedded in paraffin. All tumors were diagnosed according to the criteria of the WHO classification [23].

Cultured glioma cells

Glioma cell cultures were established as follows: specimens of glioblastomas were collected at surgery and processed within 1 hr. The tissue was minced gently and thereafter incubated with 0.1% collagenase/dispase (Boehringer Mannheim) in serum-free Dulbecco's modified Eagle's medium (DMEM) for 30 min. The processed tissue was filtered through a 70 μm nylon cell strainer (Becton & Dickinson, 2350). Cells were cultured in a humified incubator at 37° C in a 5% CO_2/95% air atmosphere. Cells were grown in DMEM supplemented with 20% heat-inactivated fetal calf serum (GIBCO BRL, England), penicillin, streptomycin and L-glutamin.

Detection of CD44 variants

Frozen sections, 5 μm thick, were tested for expression of standard CD44 (CD44s) and CD44 splice variants (CD44v) by immunohistochemistry as described previously [9, 10], using a polyclonal antibody (pAb) and a panel of monoclonal antibodies (mAbs) raised against a bacterially expressed fusion protein encoded by exon v3–v10 of the human HPKII-type CD44 [9, 10]. Exons v3–v10 are corresponding to exons 7–14 of the human genomic CD44 [8]. The structure of CD44 and the location of the epitopes that are recognized by the pAb, anti-CD44v, and by the mAbs, NKI-P1, VFF11, VFF8, VFF4, VFF7, VFF18, VFF14 and VFF16 are shown in Fig. 1. A detailed description of most of these antibodies and their specificity has been published elsewhere [9, 10]. When more than 10% of the tu-

Table 1. Expression of CD44 isoforms on brain tumors as analyzed by immunohistochemistry

Tumor type	Antibodies						
	CD44s (NKI-P1)	CD44v4 (VFF11)	CD44v5 (VFF8)	CD44v6 (VFF7)	CD44v6 (VFF18)	CD44v10 (VFF14)	CD44v10 (VFF16)
Gliomas							
Pilocytic astrocytoma	3/3	1/3	2/3	0/3	0/3	2/3	1/3
Astrocytoma I or II	3/3	1/3	2/3	0/3	0/3	2/3	1/3
Anaplastic astrocytoma	4/4	3/4	3/4	0/4	0/4	3/4	2/4
Glioblastoma multiforme	7/7	1/7	6/7	0/7	0/7	5/7	5/7
Oligoastrocytoma	3/3	0/3	1/3	0/3	0/3	1/3	1/3
Oligodendroglioma	3/3	0/3	3/3	0/3	0/3	1/3	0/3
Anaplastic oligodendroglioma	2/2	1/2	2/2	0/2	0/2	2/2	2/2
Ependymoma	4/4	0/4	1/4	0/4	0/4	3/4	3/4
Others							
Schwannoma	4/4	0/4	3/4	0/4	0/4	2/4	2/4
Meningioma	3/3	0/3	2/3	0/3	0/3	1/3	2/3
Medulloblastoma	3/3	0/3	0/3	0/3	0/3	0/3	0/3

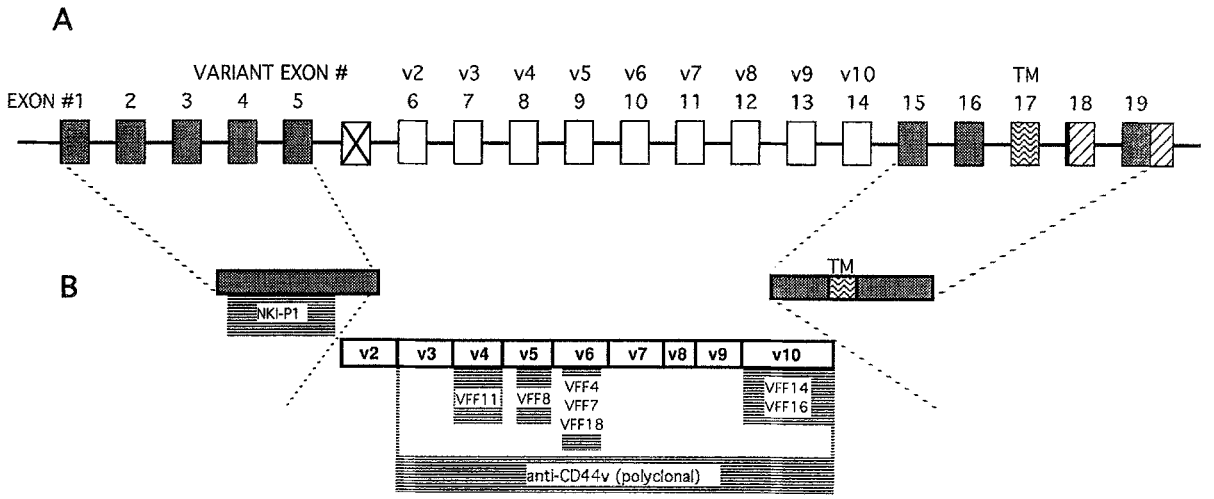

Fig. 1. Schematic representation of the CD44 gene. Open boxes indicate exons that are spliced out of the standard form of CD44. TM, transmembrane region. B: Schematic representation of the CD44 protein with location of the epitopes which are recognised by the monoclonal antibodies: NKI-P1, VFF4, VFF7, VFF8, VFF11, VV14 and VFF16, and by the polyclonal antibody anti-CD44v. Dark area, standard form of CD44; v1–v10, domains encoded by the variant exons.

mor cells were estimated to show staining, tumors were scored as 'positive'.

The expression of CD44s and CD44v epitopes on glioma cell cultures was studied by FACS analysis. Three primary glioma cell cultures of early (2 to 5) passages were used: Gli15, Gli16, Gli25. Cells were harvested mechanically. Next, cells were sequentially incubated with the appropriate dilutions of the different anti-CD44 antibodies diluted in phosphate-buffered saline containing 1 mg/ml bovine serum albumin. Thereafter, cells were incubated with biotin-conjugated Rabbit anti-Mouse Ig, for the mAbs, (Dakopatts, Glostrup, Denmark) or biotin-conjugated Swine and anti-Rabbit Ig (Dakopatts) for the pAb, for 30 min at 0° C. Subsequently, cells were incubated for another 30 min at 0° C with streptavidin-phycoerythrin (Dakopatts). Flow cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA).

Results and discussion

Table 1 shows the CD44 expression of the various primary brain tumors. All tumors showed expression of the standard CD44 (CD44s) epitopes, but the various tumor types differed in their expression pattern of CD44s. Astrocytomas (Fig. 2a), oligoastrocytomas, oligodendrogliomas and schwannomas were strongly positive for CD44s epitopes in most of the tumor cells. Ependymomas also showed uniform positivity, but stained less intensively. Focal immunoreactivity for CD44s was observed in meningiomas and medulloblastomas (Fig. 2b). Compared to the cells of origin in the normal brain [Kaaijk *et al.*, manuscript in preparation], CD44 appears to be overexpressed in primary brain tumors, but we did not find a correlation between CD44s expression and tumor grade. For example, low-grade astrocytomas showed a similarly strong expression of CD44s as the highly malignant glioblastomas. It has to be taken into consideration, however, that the number of the tumors investigated is rather small.

CD44 splice variants (CD44v) were also expressed in the primary brain tumors studied. With the exception of medulloblastomas, CD44v5 and CD44v10 were present in all tumor types. Expression of CD44v4 was only found in astroglial tumors, although not in those of mixed oligo-astroglial type. CD44v6 was absent in all tumors examined. It should be noted that, with the exception of strong

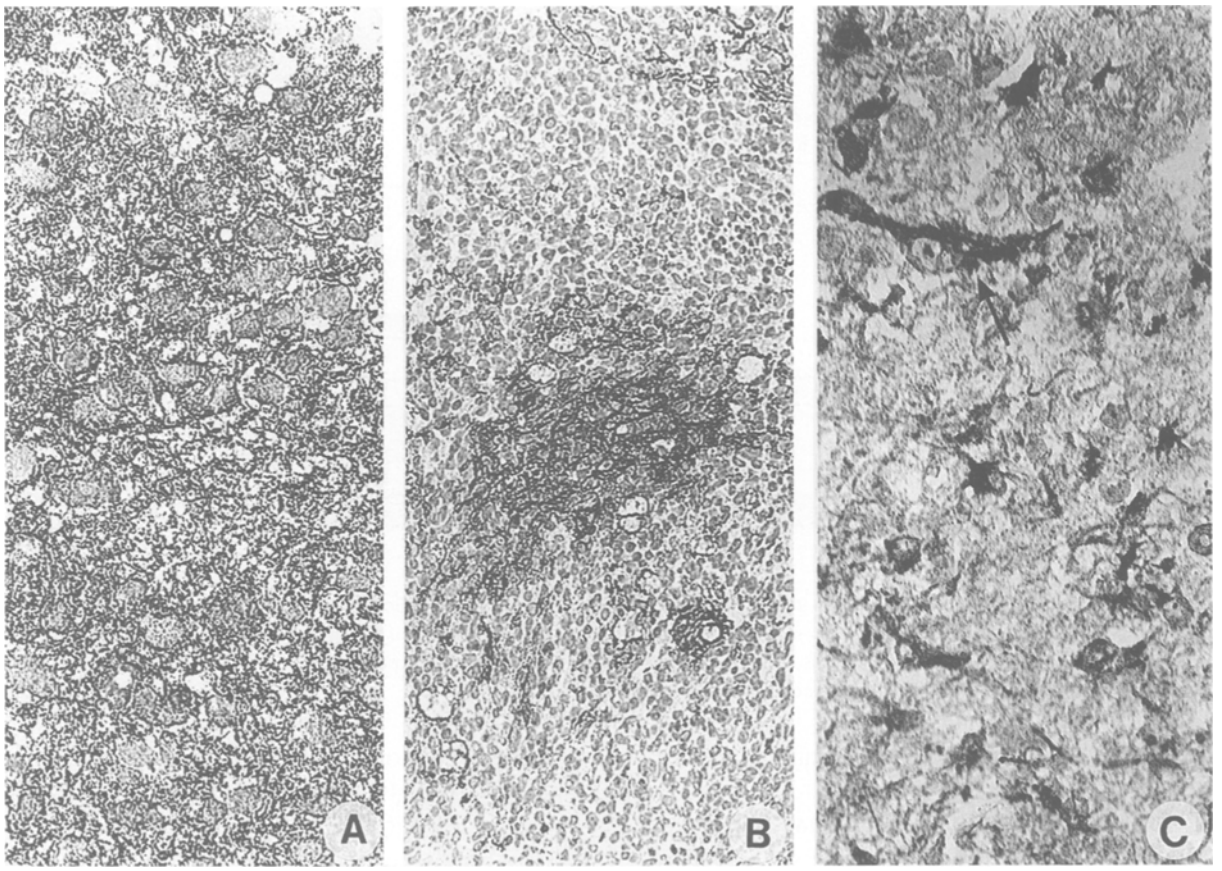

Fig. 2. Expression of CD44 isoforms on primary brain tumors. A: CD44s was strongly expressed on glioblastomas × 400. B: CD44s showed to be focally expressed on medulloblastomas × 200. C: CD44v5 was strongly expressed on some tumor cells of a glioblastoma × 400.

staining for CD44v5 in three glioblastomas (Fig. 2c) and in one anaplastic oligodendroglioma, the staining for the CD44v epitopes was far less intense than for CD44s. Furthermore, CD44v epitopes were generally focally expressed, presumably reflecting the cellular heterogeneity usually found in these tumors. The strong expression of CD44s compared to CD44v epitopes strongly suggests that CD44s is the dominant form of CD44 expressed in primary brain tumors. However, the observation of strong (focal) expression of CD44v5 seen in a subgroup of highly malignant tumors (three glioblastomas and one anaplastic oligodendroglioma) suggests a possible role in the biological behavior of these tumors.

The expression pattern of CD44s and CD44v epitopes in primary brain tumors is comparable with the expression pattern of CD44 in malignant melanomas: strong expression of CD44s, (weak) expression of CD44v5 and CD44v10, but no expression of CD44v6 as detected by immunohistochemistry [16]. These tumors have their neuro-ectodermal origin in common. Hence, the CD44 expression pattern as observed in primary brain tumors and melanomas, might be a reflection of neuro-ectodermal differentiation.

The expression pattern of CD44 on cultured glioma cells as measured by immunofluorescence flow cytometry was consistent with the immunohistochemical data; CD44s as well as CD44v epitopes were expressed at the cell surface of the cultured glioma cells (Fig. 3). Clearly positive staining was observed with antibodies against: CD44s, CD44v3–v10, CD44v4, CD44v5 and CD44v10. In addition, CD44v6 appeared to be present at low levels. Hence, CD44v6 expression was detected by flow cytometry and not by immunohistochemistry. This may either indicate that the relatively low levels of CD44v6 are not detectable by immunohistochemis-

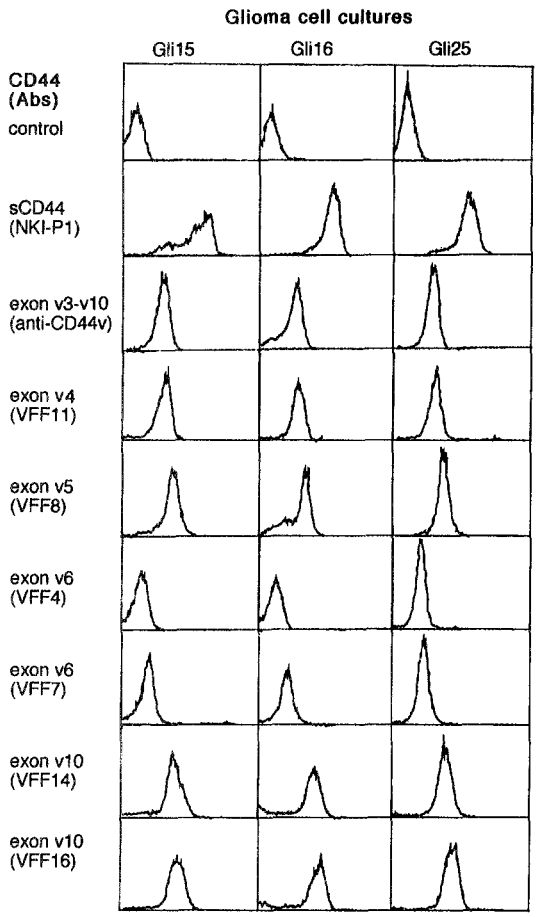

Fig. 3. Flowcytometric analysis of CD44 expression on glioma cell cultures. Abs, antibodies.

try or that CD44v6 is upregulated as a result of activation due to culture conditions. Our findings that different CD44v epitopes are expressed in glioblastomas both with immunohistochemistry on frozen sections and with flow cytometric analysis on cultured glioblastoma cells is in contrast with observations of Li *et al.*, who did not detect CD44v expression in glioblastomas [22]. However, Radotra found a 120 kDa and a 95 kDa isoform of CD44 expressed on astrocytic tumour cultures as detected by immunoblotting [20].

Diffuse local invasion precludes effectiveness of therapy in the majority of primary brain tumors. Even low-grade tumors are poorly demarcated. Invasiveness is greatly influenced by interactions of the tumor cells with other cells and extracellular matrix components [24, 25]. In this process, adhesion molecules play a crucial role. It is noteworthy that glioblastomas preferentially invade the white matter of the brain, in which high levels of hyaluronate, for which CD44 is the principal receptor, have been found [17]. Furthermore, CD44 has been reported to be involved in human glioma cell invasion *in vitro* [21]. We conceive that CD44 molecules, in particular CD44s, which appears to be the dominant form of CD44 in primary brain tumors, may contribute to the invasive character of primary brain tumors by interacting with hyaluronate, one of the most abundant molecules in the extracellular matrix of the brain. Alternative splicing of CD44, which can greatly influence hyaluronate binding [1, 2] may regulate this interaction.

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