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Differentiation and Prognosis of Neuroblastoma in Correlation to the Expression of CD44s

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Cell–cell and cell–extracellular matrix interactions mediated by cell adhesion molecules (for example CD44) play an important role in the cascade of metastasis and the progression of human malignant tumours. The most important aim of this review was, on the basis of our results and the literature, to show the correlation between the expression of CD44s and differentiation and prognosis of neuroblastoma. Surprisingly and in contrast to most other malignant tumours, neuroblastomas exhibited an inverse correlation between CD44s expression and tumour progression. It can be stated that CD44s is a prognostic marker in neuroblastoma which correlates significantly with the grade of tumour cell differentiation, but not with clinical stage. Moreover, there exists a statistically significant correlation between *MYCN* oncogene amplification and the lack of CD44s expression.

Key words: neuroblastoma, CD44, *MYCN*
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INTRODUCTION

CELL–CELL and cell–extracellular matrix interactions mediated by cell adhesion molecules play an important role in the cascade of metastasis and the progression of human tumours [1–4]. A qualitative and/or quantitative effect on the expression of these adhesion molecules, of which CD44 is a member, is exerted by genomic DNA alterations, such as amplifications, translocations, insertions, deletions and point mutations, but also by alterations in mRNA composition, such as alternative splicing or post-translational changes. The changed expression of certain adhesion molecules is apparently brought about by a selective process and in such a way that only a particular subpopulation of tumour cells acquires the ability to separate itself from the tumour cell cluster by cell–cell and cell–extracellular matrix interactions, invade through the basement membrane, migrate in the extracellular matrix (active locomotion), or disseminate into blood or lymphatic vessels.

For CD44, such interactions have been established *in vitro* for the first time in the vascular dissemination of melanoma and lymphoma cells, and in the migration of rat pancreas carcinoma cells in the extracellular matrix [5–7].

STRUCTURE OF CD44 AND EXPRESSION IN VARIOUS TUMOURS

CD44 is a multifunctional transmembranous cell surface molecule, which comprises a group of glycosylated proteins (glycoproteins of 85–300 kDa). It was first described by Dalchau and associates [8] on T-cells, granulocytes and cortical thymocytes, and also occurs on numerous other human cells [9]. The gene locus is on the short arm of chromosome 11 (11p13) [10]. The genomic structure of the CD44 gene comprises 20 exons over a length of 60 kb 10 exons (exons 1–5 and 16–20) code for the standard molecule (CD44s), whereas 10 exons (exons 6–15), which are formed by alternative splicing, code for different isoforms (CD44v) of CD44 [11]. The cDNA sequence codes for approximately 360 amino acids, which form a 37–38 kDa core protein of the CD44 molecule [12]. Via N- and O-glycosidic bindings, this protein links with sugars, thus giving rise to the 85–90 kDa form of the standard molecule. Further linkage with chondroitin sulphate results in a molecule of 180–220 kDa [13].

The CD44 standard molecule is composed of a long extracellular, a transmembranous, and a cytoplasmic domain. The extracellular domain consists of 250 amino acids and contains disulphide bridges. The N-terminal portion shows approximately 30% homology with the link protein of cartilaginous tissue [14]. Further amino acids can be incorporated into the extracellular domain by alternative splicing processes. This enlarges the extracellular domain, which then corresponds to an isoform of CD44. The transmembranous domain of the CD44 molecule consists of 21 amino acids, the COOH-terminal cytoplasmic domain of 73 amino acids. Ligands of the CD44 molecule in the extracellular matrix are hyaluronic acid, the collagens I and IV, fibronectin and laminin, as well as a recently described proteoglycan sulphate (gp 600) [3,15–17]. Binding to hyaluronic acid is effected via regions in the standard molecule, which are encoded by the exons 2s and 5s [18]. In the process of lymphocyte homing, a 58–66 kDa protein occurring on the endothelial cells (vessel addressin-Mad) plays an important role in the interaction with CD44 [14]. Moreover, activated T-lymphocytes expressing CD44s are apparently able to establish direct cell–cell contact with CD44s expressing smooth muscle cells via so-called "hyaluronic acid bridges" [19]. Specific ligands for CD44 isoforms have not been identified so far.

Enhanced expression or upregulation of CD44s or CD44v has been found to be related to tumour progression in malignant non-Hodgkin's lymphomas [20–22], gastric carcinomas [23], colorectal carcinomas [24, 25], brain tumours [26, 27], carcinomas of the uterine cervix [28], and malignant epithelial tumours of the kidney [29]. In contrast, decreased expression or downregulation of CD44v has been shown to be co-responsible for the progression of squamous cell carcinomas [30] and endometrial carcinomas [31].

IS CD44s INVOLVED IN NEUROBLASTOMA TUMOUR PROGRESSION?

Neuroblastoma is a malignant childhood tumour of the autonomic nervous system. It originates histogenetically from primitive neuroectodermal cells, with an incidence of 9 per one million children under 15 years of age [32]. In contrast to most other malignant tumours, neuroblastoma has the ability to differentiate or spontaneously regress. Its biological behaviour, clinical pattern and prognosis depend on the age of the patient [33–34]. In addition to its clinical staging (stages 1–4 and 4s) and histological classification, other prognostic markers have been described, such as partial monosomy of chromosome 1 [35], amplification of the *MYCN* oncogene [36, 37] and tumour cell ploidy [38].

CD44s is expressed in many fetal and adult tissues, and in fetal sympathoblasts (10th week of gestation) of the adrenal medulla [9]. A first study of CD44s and CD44v expression in 20 primary neuroblastomas showed that a subgroup of these tumours failed to express CD44s, and that none of the tumours expressed CD44v [39].

Surprisingly and in contrast to most other malignant tumours, primary neuroblastomas (52 diffuse stroma-rich (DSR) tumours, 82 stroma-poor differentiated (SP-D) tumours and 71 stroma-poor undifferentiated (SP-U) tumours) [33] exhibited an inverse correlation between CD44s expression and tumour progression. All investigated tumours were CD44v negative. The results showed that CD44s negative tumours (approximately 14%) are associated with a poorer event-free survival probability in comparison with CD44s positive tumours (approximately 86%) ($P<0.05$) [40]. The CD44s negative neuroblastomas were mainly found in the group of SP-U tumours (32.4% of the SP-U tumours were CD44s negative). The frequency of CD44s non-expression in the SP-U tumours differs significantly from that observed in the CD44s negative tumours (4.5%) of the DSR and SP-D cases ($P<0.0001$) [40]. The event-free survival probability of patients with CD44s negative SP-U tumours was also significantly poorer ($P = 0.0305$) than that of patients with CD44s positive SP-U tumours [40]. It can, therefore, be stated that this morphologically defined tumour group obviously consists of highly heterogenous tumour cell populations and possesses different biological properties. This might occasion a new assessment of the morphological classification of SP-U neuroblastomas.

In this context, it is interesting that all of the investigated SP-U stage 4s neuroblastomas ($n = 8$) showed CD44s at a high antigen density. In infants, these 4s tumours have a particular clinical pattern with a generally good prognosis, even though metastasis to the liver, lymph nodes and bone marrow occurs very early. In view of the fact that these tumours strongly express CD44s the immunohistological determination of CD44s in neuroblastomas will be meaningful in the future in order to provide the clinical oncologist with prognostic information at an early date. A statistically significant difference of CD44s expression in correlation to the tumour stages 1–4 has not been demonstrated ($P>0.09$) [40].

In conclusion, it can be stated that CD44s, but not CD44v, is a prognostic marker in neuroblastoma which correlates significantly with the grade of tumour cell differentiation ($P<0.0001$), but not with clinical stage ($P>0.09$), as recently reported by Favrot and associates [41].

SIGNIFICANCE OF CD44s EXPRESSION VERSUS OTHER PROGNOSTIC MARKERS

In accordance with a study of Gross and associates [42], we found a statistically significant correlation between *MYCN* oncogene amplification and the non-expression of CD44s ($P<0.0001$). *MYCN* expression in *MYCN* amplified neuroblastomas is significantly higher than in non-amplified tumours [43]. Moreover, enhanced *MYCN* expression has been demonstrated

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in the early stage of embryonic development [44]. It can be assumed that the non-expression of CD44s in neuroblastoma cells is associated with an undifferentiated phenotype, and that CD44s is thus a marker of differentiation. Moreover, the connection between *MYCN* and CD44s expression depends apparently on transcriptional control mechanisms. It is conceivable that CD44s expression is prevented in neuroblasts of the early developmental phase which have a high *MYCN* expression, and that a decreasing transcriptional actvity of the *MYCN* oncogene upregulates CD44s expression. In this context, it is interesting that the regulation of CD44 expression by other oncogene products, for instance *RAS* and *SRC* oncogenes, has been demonstrated in fibroblasts and intestinal epithelial cells of the rat [45, 46]. It is also known that similar to CD44s expression, enhanced expression of the oncogene products of *HRAS* (*HA-RAS* p21 and *SRC* ($pp60^{c\text{-}src}$)) as well as *C-HA-RAS* and *CSRC* amplification in neuroblastomas correlate with a better prognosis, whereas non-expression or reduced expression of *HA-RAS* p21 and $pp60^{c\text{-}src}$ are correlated with a poorer prognosis [47–50].

Studies of Shtivelman and Bishop [51] on neuroblastoma cell lines have shown that CD44 repression in these cell lines is associated with reduced activity of the CD44 promoter. The authors identified different *cis*-acting elements that are thought to be responsible for the negative regulation of the CD44 promoter in neuroblastoma cells. Since *MYCN* oncogene products apparently play a role as transcriptional factors [52], they might similarly be involved in the regulation of CD44 promoter activity. In the case of N-CAM, another transmembrane adhesion molecule, such involvement has already been demonstrated in rat neuroblastomas [53].

EXPRESSION OF OTHER ADHESION MOLECULES IN CD44s NEGATIVE NEUROBLASTOMAS

As already mentioned, approximately 14% of the investigated neuroblastomas were CD44s negative [40]. The patients with CD44s negative tumours had a significantly poorer event-free survival probability than those with CD44s positive neuroblastomas. Our studies on the expression of other adhesion molecules, such as integrins and N-CAM (neural cell adhesion molecule), revealed that nearly all CD44 positive and negative tumours expressed N-CAM (140 kDa form) and $\alpha 3/\beta 1$ integrin at high antigen densities. $\alpha 3/\beta 1$ integrin is a heterodimer involved in cell–cell and cell–matrix interactions in normal and malignant cells [54, 55]. Ligands of the molecule are laminin, epilegrin, entactin, fibronectin, and collagen I and VI. N-CAM plays an important role in embryogenesis [56] and is downregulated in the migration of embryonal stem cells of the neural crest [57]. *In vitro* studies have shown that $\alpha 3/\beta 1$ integrin and N-CAM are of importance in the adhesion of tumour cells to vascular endothelial cells [58, 59]. Moreover, enhanced expression of $\alpha 3/\beta 1$ integrin correlates with tumour progression in the case of another neuroectodermal tumour, namely malignant melanoma [60]. Whether the constant expression of $\alpha 3/\beta 1$ integrin and N-CAM in neuroblastomas is important for the biology of the tumour, for example, with regard to dissemination, must remain an open question. Future *in vitro* studies will have to show whether these two adhesion molecules also play a functional role in the dissemination events of neuroblastoma, a role that has been described for other malignant tumours [58, 59].

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