

Expression of the neural cell adhesion molecule and polysialic acid in human neuroblastoma cell lines

URSULA VALENTINER¹, MARTINA MÜHLENHOFF², ULRICH LEHMANN³,
HERBERT HILDEBRANDT² and UDO SCHUMACHER¹

¹Institute of Anatomy II: Experimental Morphology, University Hospital Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg; ²Institute of Cellular Chemistry and ³Institute of Pathology, Medical School Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

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Abstract. The neural cell adhesion molecule NCAM is a cell surface glycoprotein of the immunoglobulin superfamily and is widely expressed in tumours of neuroectodermal origin such as neuroblastoma. NCAM can be decorated by the carbohydrate polymer polysialic acid (polySia), which attenuates NCAM-mediated cell adhesion and increases cellular motility. The key enzymes in the biosynthesis of polySia are the two polysialyltransferases ST8SiaII and ST8SiaIV. In the present study, expression of NCAM, polySia-NCAM, ST8SiaII and ST8SiaIV was investigated in five human neuroblastoma cell lines before and after xenografting into SCID mice by immunohistochemistry, Western blot analysis and real-time PCR. Results were correlated with the metastatic potential. *In vitro*, three cell lines (LAN-1, LAN-5 and SH-SY5Y) were positive for polySia attached to the transmembrane isoforms NCAM-140 and NCAM-180, whereas Kelly and SK-N-SH cells were negative for NCAM and polySia. In the presence of NCAM, the level of polySia correlated with the amount of polysialyltransferase transcripts, which were highest in LAN-1, LAN-5 and SH-SY5Y cells. In the respective primary tumours grown in SCID mice, the expression patterns of NCAM, polySia and polysialyltransferases were similar to those observed *in vitro*. After subcutaneous engraftment, polySia-NCAM-positive neuroblastoma developed disseminated micrometastases, a metastatic pattern that was not observed for tumours derived from NCAM-negative cell lines. Together, this indicates that the presence of polySia reduces the adhesiveness of tumour cells and promotes dissemination.

Introduction

Neuroblastoma is the most common extracranial solid tumour in children. It is derived from embryonic neural crest cells of the peripheral sympathetic nervous system and can occur anywhere along the strands of the sympathetic nervous system (1). The tumour is noted for its variable clinical and biological behaviour, but most children over the age of one year present with high-risk disease with long-term survival still <40% (1,2). Therefore reliable markers with prognostic potential for the different clinical stages and the search for new therapeutic concepts are of great clinical importance.

Like other malignant tumours of neuroectodermal origin, nearly all neuroblastomas express the neural cell adhesion molecule (NCAM) (3,4). NCAM is a cell surface glycoprotein of the immunoglobulin (Ig) superfamily and by extensive alternative mRNA splicing a variety of NCAM isoforms can be generated from a single-copy gene (5). All isoforms can be grouped into three classes: NCAM-120, a glycosylphosphatidylinositol (GPI) anchored form predominantly found in normal and differentiated tissue (6), as well as NCAM-140 and NCAM-180 which are both transmembrane proteins that differ in the length of their cytoplasmic domain. These two isoforms are mostly expressed in less differentiated or malignant cell types (4,6,7).

NCAM mediates cell-cell adhesion by a calcium-independent homophilic interaction between NCAM molecules on opposite cells (8); on the other hand it can act as a plasticity-promoting molecule by decreasing the overall cell-adhesion (9). The key regulator between these two opposite functions of NCAM is polysialic acid (polySia), a dynamically regulated posttranslational modification found in mammals exclusively on NCAM. Polysialic acid is a unique linear homopolymer with up to 90 sialic acid residues in α 2,8-glycosidic linkage (10). The presence of this large polyanionic carbohydrate moiety attenuates NCAM-mediated cell adhesion and increases cellular motility (10).

Polysialic acid represents an oncodevelopmental antigen that is re-expressed on several malignant neuroectodermal and neuroendocrine tumours, including neuroblastoma (10). Several studies indicate that downregulation of NCAM or polysialylation of NCAM reduces the adhesiveness of tumour

Correspondence to: Dr Ursula Valentiner, Institute of Anatomy II: Experimental Morphology, University Hospital Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany
E-mail: valentin@uke.uni-hamburg.de

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cells and facilitates the detachment of cells during the first steps of the metastatic cascade (11,12). Thus, expression of polysialic acid has been shown to correlate with tumour progression, metastasis and invasion in several tumours (13,14). In neuroblastoma expression of polySia-NCAM was highest in patients with advanced stage of disease and correlated with other prognostic markers, such as MYCN amplification (15). Furthermore serum levels of polySia-NCAM were shown to correlate with tumour content and were found to decrease during successful therapy (16).

The key enzymes in the biosynthesis of polySia are the two closely related polysialyltransferases ST8SiaII and ST8SiaIV, formerly named STX and PST, respectively (17). ST8SiaII is described as the predominant form in embryonic and early postnatal brain, whereas ST8SiaIV was found to be the major form in adult brain (18-20). Expression of ST8SiaII mRNA was recently shown to be a potential molecular marker of metastatic neuroblastoma (21).

Human tumour cell xenograft models using immunodeficient mice have provided an *in vivo* model system for the study of prognostic markers (22). Based on our previous study demonstrating that the SCID mouse model is ideally suited to study metastasis of human neuroblastoma cell lines (23), we now used this xenograft model to investigate the role of polySia-NCAM and the two polysialyltransferases for tumour growth and metastasis. Therefore, the expression level of ST8SiaII and ST8SiaIV as well as polySia-NCAM and the NCAM isoform pattern were investigated in five human neuroblastoma cell lines before and after xenografting into SCID mice and results were correlated with the metastatic potential.

Materials and methods

Cell lines. Five human neuroblastoma cell lines, namely Kelly, LAN-1, LAN-5, SH-SY5Y and SK-N-SH were investigated in this study. Kelly, LAN-1, LAN-5 and SK-N-SH were a kind gift from Dr R. Erttmann (Department of Paediatric Haematology and Oncology, University Medical Center Hamburg-Eppendorf, Germany); SH-SY5Y cells were obtained from Dr H. Hildebrandt (Institute of Cellular Chemistry, Medical School Hannover, Germany). The cell lines were cultured *in vitro* under standard cell culture conditions (37°C, 100% relative humidity, 5% CO₂) in RPMI medium (Gibco/Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco). Before reaching confluence, cells were routinely harvested for passaging using 0.05% trypsin-0.02% EDTA (Gibco). Prior to all experiments, all cells were checked for the absence of mycoplasma infections using the VenorGeM[®]-Mycoplasma detection kit (Minerva Biolabs GmbH, Berlin, Germany).

For the production of cytopins, cells were harvested using 0.05% trypsin-0.02% EDTA (Gibco). A 200 µl cell suspension (~10⁶ cells/ml) was centrifuged onto HistoBond[®] microscope slides (Marienfeld, Lauda-Königshofen, Germany) at 1500 x g and 4 min at 20°C using 1-cm diameter cytocentrifuge funnel and Hettich centrifuge (Hettich, Tuttlingen, Germany). The supernatant was withdrawn and the cells were air dried overnight. Cytopins were fixed in acetone for 15 min at room temperature before immunohistochemistry.

For injection into pathogen-free BALB/c severe combined immunodeficient (SCID) SCID/SCID mice, neuroblastoma cells were harvested by trypsination and viable cells (5x10⁶) were suspended in 1 ml cell culture medium. An aliquot of 200 µl of this suspension was injected subcutaneously between the scapulae of each SCID mouse. The mice bearing neuroblastomas were sacrificed when the tumour had reached maximal growth (up to 10% of the body weight of the animal at the beginning of the experiment) or started to ulcerate. Primary tumours were removed and divided into several parts. One part of the tumour was frozen for cryosectioning. The animal experiment was approved by the local animal experiment approval committee (Behörde für Soziales, Familie, Gesundheit, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, Hamburg, Germany, project no. 56/03) and was performed in compliance with local animal welfare laws, guidelines and policies.

Antibodies and enzymes. NCAM-specific mouse monoclonal antibody (mAb) 123C3 (24) and polySia-specific mAb 735 (25) were used after affinity purification on Protein G- and Protein A-sepharose (GE Healthcare), respectively. Endosialidase (endoN) of bacteriophage K1F was purified as described previously (26).

Immunohistochemistry. Immunoreaction of the neuroblastoma cells was examined on cytopins of *in vitro* grown cells and cryosections of neuroblastomas grown in SCID mice. The following monoclonal antibodies were used: polySia-specific mouse mAb 735 (anti-polySia) and NCAM-specific mouse mAb 123C3 [5 µg/ml in 3% BSA (bovine serum albumin) in PBS, respectively]. Briefly, sections incubated with mAb 735 and mAb 123C3 were treated twice with lysine (18.3 mg/ml) in PBS (phosphate-buffered saline, pH 7.6) for 15 min and rinsed in PBS. Non-specific binding was blocked by incubating the sections with 3% BSA (bovine serum albumin) and 0.3% Triton X-100 in PBS for 30 min at room temperature. Sections were incubated overnight at 4°C with primary antibody, rinsed, and incubated for 30 min at room temperature with a respective biotinylated secondary antibody (Dako) diluted 1:200 in PBS and 3% BSA. Subsequently, sections were rinsed and incubated for 30 min with a streptavidin-alkaline phosphatase kit (Vector Laboratories). Enzyme reactivity of the alkaline phosphatase complex was visualized using Naphtol-AS-biphosphate as a substrate and hexatozised New Fuchsin for simultaneous coupling. Sections were counterstained with Mayer's hemalum diluted 1:1 in distilled water for 5-10 sec, rinsed in tap water and covered with Crystal Mount and Clarion (Biomedica, Foster City, CA). They were examined and photographed under a Zeiss Axioplan photomicroscope.

Specificity of mAb 735 was controlled on samples pretreated with 1 µg/ml endosialidase in 3% BSA and 0.3% Triton X-100 in PBS for 2 h at 37°C, an enzyme that specifically degrades polySia. These controls showed an insignificant background staining. Negative controls were treated the same way, omitting the incubation step with the primary antibody, and showed no immunoreactivity.

Protein extraction and Western blot analysis. Neuroblastoma cells (5x10⁶) were washed twice with phosphate-buffered

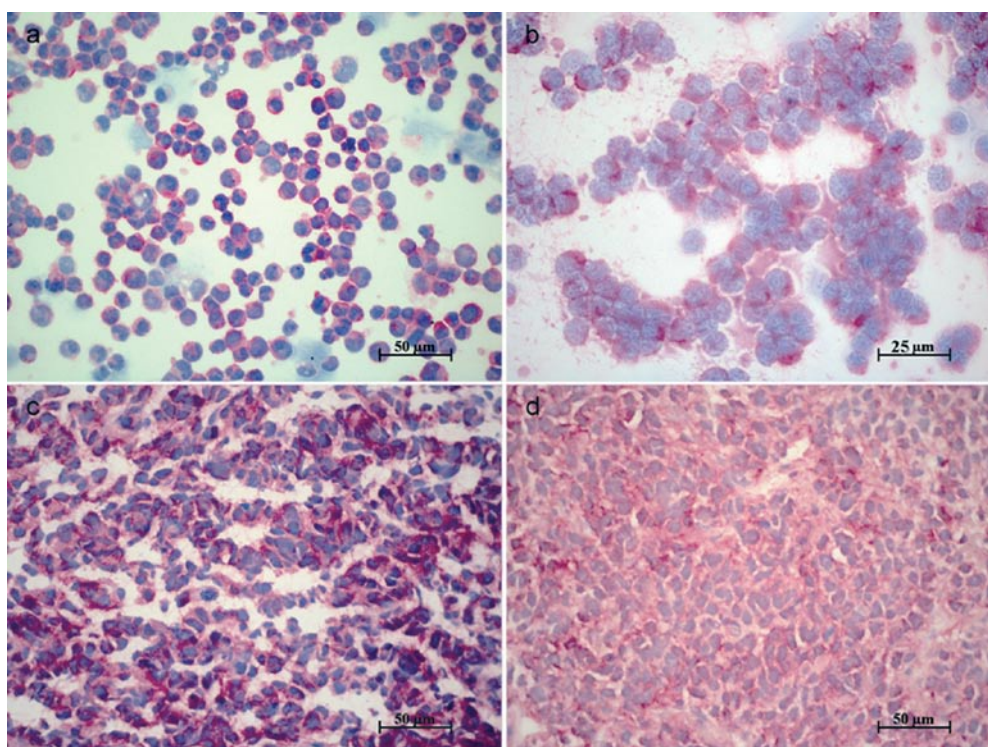


Figure 1. NCAM (a and c) and polySia (b and d) immunohistochemistry of SH-SY5Y neuroblastoma cells grown *in vitro* and LAN-5 cells grown in SCID mice. SH-SY5Y cells showed a moderate immunostaining with anti-NCAM antibody mAb 123C3 (a) and anti-polySia antibody mAb 735 (b). LAN-5 primary tumours showed a moderate to strong NCAM expression (c) and a moderate polySia expression (d).

saline and lysed in 300 μ l of ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 70 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2% Triton X-100). Detergent extracts were clarified by centrifugation and one aliquot was treated with 20 ng/ μ l endoN for 45 min on ice. For xenograft tumours 200 μ l of lysis buffer was used per 100 mg wet weight and homogenization was performed in a Precellys 24 homogenizer using 1.4-mm beads (PepLab). Proteins were separated by SDS-PAGE (30 μ g total protein per lane) and electroblotted on nitrocellulose membranes. Equal loading and protein transfer were controlled by Ponceau S staining. After removal of the dye, immunostaining was carried out using anti-polySia mAb 735 or anti-NCAM mAb 123C3 in a concentration of 2.5 μ g/ml. Bound antibodies were detected with peroxidase-coupled anti-mouse IgG and developed by enhanced chemiluminescence.

RNA isolation, cDNA synthesis and quantitative real-time PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Karlsruhe, Germany) and treated with RQ1 DNase I (Promega, Mannheim, Germany), according to the manufacturer's recommendations. The cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) and 1 μ g of DNase I-treated total RNA as template in a 20 μ l reaction volume.

For quantitative gene expression analyses 1 μ l cDNA was subjected to PCR amplification using SYBR-Green (Invitrogen) and the ABI Prism 7700 system (Applied Biosystems, Darmstadt, Germany). Each PCR reaction of 25 μ l contained 2.5 μ l 10X concentrated universal PCR buffer (Invitrogen), 1.5 or 2.5 μ l MgCl₂ (25 mM), 0.5 μ l dNTP's (10 mM), 0.25 μ l

ROX reference dye (Invitrogen), 2 μ l SYBR-Green (Invitrogen), 0.6 μ l mixed forward and reverse primer 10 pmol/l each, 0.125 μ l Platinum Taq DNA polymerase (5 U/ μ l, Invitrogen) and 1 μ l cDNA. The following primer pairs were used: STII_f (5'-TGACGCCACAGCTTCG-3') and STII_r (5'-CATCCCGGGCATACTCCTG-3') for *ST8SIA2*, STIV_f (5'-GACAAAGAAATAGCAAGAAGACTGAGGA-3') and ST-IV_r (5'-CCGACTCAAAGACAATTCACCA-3') for *ST8SIA4*, hNCAM_f (5'-GAGATCAGCGTTGGAGAGTCC-3') hNCAM_r (5'-GGAGAACCAGGAGATGTCTTTATCTT-3') for *NCAM1*, β GUS_f (5'-CTCATTTGGAATTTTGCCGATT-3') and β GUS_r (5'-CCGAGTGAAGATCCCCTTTT-3') for the β -glucuronidase gene β GUS, and TBP_f (5'-GCCCGAAACGCCG AATAT-3') and TBP_r (5'-CCGTGGTTCGTGGCTCTCT-3') for the TATA-box binding protein gene TBP. Relative mRNA levels were calculated by normalization of target gene mRNA level to the mean of the expression level of two endogenous reference genes (β GUS and TBP). For the calculation of delogarithmized relative expression values the mean relative expression of every target gene (the mean ΔC_T -value) across all samples was set equal to one. All individual values were normalized to this value.

Results

Immunohistochemistry. The results of the immunohistochemical analysis are summarized in Table I. Three of the five neuroblastoma cell lines grown *in vitro* reacted with anti-human NCAM mAb (LAN-1, LAN-5, SH-SY5Y), whereas Kelly and SK-N-SH cells were negative for NCAM (Fig. 1a). The NCAM-positive cells also showed a moderate staining

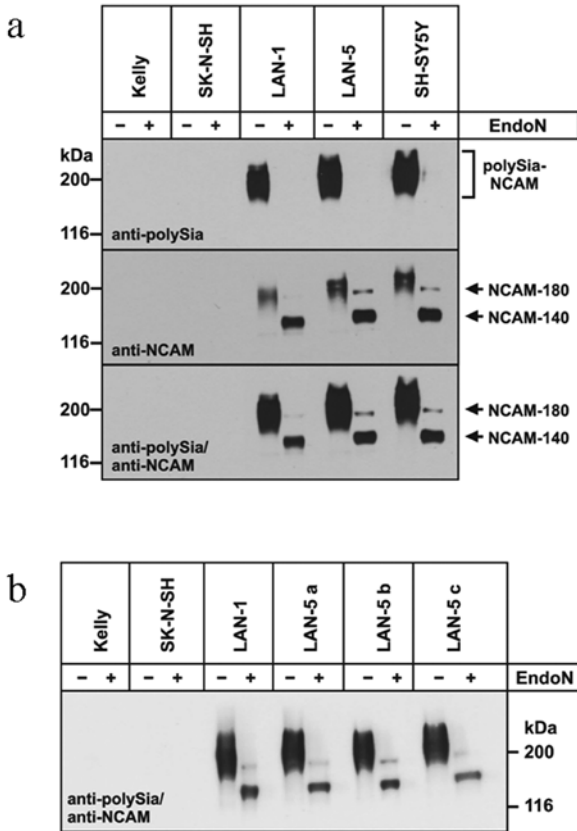


Figure 2. Western blot analysis of NCAM and polySia levels in human neuroblastoma cells grown *in vitro* (a) and in SCID mice (b). Lysates were separated before (-) and after (+) endoN treatment by 7% SDS-PAGE. (a) Western blot analysis of cell grown *in vitro*. Blots were stained with anti-polySia mAb 735 (upper panel), anti-NCAM mAb 123C3 (middle panel) or a combination of both antibodies (lower panel). In the case of Kelly and SK-N-SH 10-fold higher amounts of total protein (50 μ g) was loaded per lane compared to LAN-1, LAN-5 and SH-SY5Y. Bands corresponding to the major NCAM isoforms NCAM-140 and NCAM-180 are indicated by arrows. Kelly and SK-N-SH were negative for NCAM and polySia, whereas LAN-1, LAN-5 and SH-SY5Y showed strong expression of polySia-NCAM. EndoN treatment revealed the expression of NCAM-140 and NCAM-180. (b) Western blot analysis of cells grown in SCID mice. Equal amounts of total protein were separated by SDS-PAGE and Western blot analysis was performed with anti-polySia mAb 735 and anti-NCAM mAb 123C3. For LAN-5, three independent samples were analyzed. Kelly and SK-N-SH were negative for both polySia and NCAM, whereas LAN-1 and LAN-5 showed strong expression of polySia-NCAM of the isoforms NCAM-140 and NCAM-180.

with anti-polySia antibody and the NCAM negative neuroblastoma cells were also negative for polySia (Fig. 1b). SK-N-SH and Kelly primary tumours grown in SCID mice were also negative for NCAM and polySia. LAN-5 tumours reacted moderately with anti-NCAM antibody as well as with anti-polySia antibody (Fig. 1c and d). LAN-1 tumours showed inter-individual variations with weak or moderate NCAM and moderate polySia expression. SH-SY5Y tumours could not be examined because these cells did not adequately grow in SCID mice.

Western blot analyses. The NCAM isoform pattern and polysialylation status of all neuroblastoma cell lines used in this study were analysed by Western blotting using mAb 735 specific for α 2,8-linked polySia with ≥ 8 residues (25,27) and

Table I. Results of immunohistochemistry on human neuroblastoma cells grown *in vitro* and in SCID mice.

Cell line	Cells grown <i>in vitro</i>		Primary tumours	
	NCAM	PolySia	NCAM	PolySia
Kelly	-	-	-	-
SK-N-SH	-	-	-	-
LAN-1	++	++	+ or ++	++
LAN-5	++ to +++	++	++	++
SH-SY5Y	++	++	no data	no data

-, negative; +, weak staining; ++, moderate staining; +++, strong staining.

mAb 123C3 recognizing all isoforms of human NCAM (24). Antibodies were either applied separately (Fig. 4a, upper and middle panel) or combined in order to allow enhanced detection of polySia-NCAM (Fig. 2a, lower panel and Fig. 2b). In LAN-1, LAN-5 and SH-SY5Y staining with mAb 735 revealed a broad high molecular weight signal typical for polysialylated NCAM (Fig. 2a, upper panel). In the case of Kelly and SK-N-SH 10-fold higher amounts of protein were applied compared to LAN-1, LAN-5 and SH-SY5Y. However, even under these conditions no polySia was detectable in Kelly and SK-N-SH. To analyze the NCAM isoform pattern, a parallel blot was stained with anti-NCAM antibody (Fig. 2a, middle panel). Clear NCAM signals were detected in the highly polySia-positive cell lines LAN-1, LAN-5 and SH-SY5Y and removal of polySia by endoN treatment resulted in the appearance of two focused bands with an apparent molecular mass of 140 and 180 kDa, indicating the expression of the two transmembrane isoforms NCAM-180 and -140. By contrast, Kelly and SK-N-SH were completely devoid of both polySia and NCAM and even after combined application of anti-polySia and anti-NCAM mAb no signals were observed (Fig. 2a, lower panel).

Western blot analysis of cells xenografted in SCID mice (Fig. 2b) revealed that LAN-1 and LAN-5 were still positive for polySia-NCAM and displayed the same isoform pattern as the corresponding cells grown *in vitro*. For Kelly and SK-N-SH neither polySia nor NCAM could be detected. As mentioned above, SH-SY5Y tumours could not be examined due to the fact that SH-SY5Y cells did not adequately grow in SCID mice.

NCAM and polysialyltransferase transcript levels. Parallel to the Western blot analyses, all neuroblastoma cell lines were characterized by quantitative real-time RT-PCR using gene-specific primers for NCAM and the two polysialyltransferases ST8SiaII and ST8SiaIV. Primers specific for NCAM transcripts were designed for amplification of all possible splice variants. Consistent with the protein expression profiles, high levels of NCAM mRNA were detected in LAN-1, LAN-5 and SH-SY5Y, whereas very low or no mRNA expression was observed in Kelly and SK-N-SH (Fig. 3a). Highest expression levels of ST8SiaII mRNA were detected in the highly polySia-

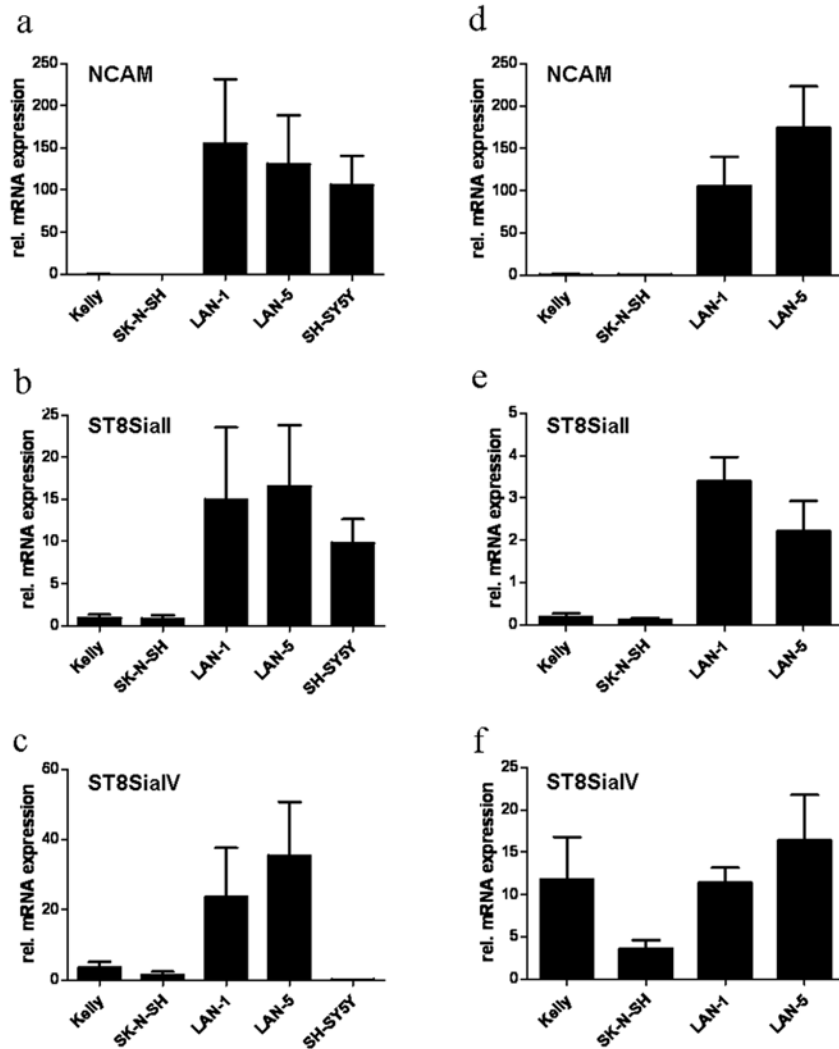


Figure 3. Quantitative RT-PCR analysis of transcript levels of NCAM (a and d), ST8SiaII (b and e) and ST8SiaIV (c and f) in neuroblastoma cells grown *in vitro* (a-c) and in SCID mice (d-f). All measurements were performed at least two times independently. Shown are the mean expression levels of all measurements \pm SD.

positive cell lines LAN-1, LAN-5 and SH-SY5Y (Fig. 3b). LAN-1 and LAN-5 displayed also the highest ST8SiaIV transcript levels, whereas SH-SY5Y cells were almost devoid of ST8SiaIV mRNA (Fig. 3c). Compared to cells grown *in vitro*, the corresponding xenograft tumours showed similar relative mRNA expression levels for NCAM (Fig. 3d), ST8SiaII (Fig. 3e) and ST8SiaIV (Fig. 3f). Major variations were limited to ST8SiaIV transcript level in Kelly. Compared to LAN-1, the ST8SiaIV level in Kelly was 5-fold lower when cells were grown *in vitro*, whereas similar expression levels were observed when cells were grown in SCID mice (compare Fig. 3c and f).

Correlation of polySia-NCAM expression with tumour take-rates and metastatic pattern. All NCAM-negative cell lines showed high take-rates after engraftment in SCID mice, whereas the highly polySia-NCAM-positive lines LAN-1, LAN-5 and SH-SY5Y were characterized by reduced take rates (<40%) (Table II). In the case of SH-SY5Y, which showed the highest polySia level, no tumour formation was observed. Lung metastases were found for both polySia-NCAM negative (Kelly

and SK-N-SH) and positive (LAN-1 and LAN-5) tumours. However, disseminated micrometastases in the alveolar septae were exclusively observed in animals with polySia-positive xenografts (Table II), indicating that if polySia-positive tumours grew in SCID mice, their pattern of metastases differed from NCAM-negative tumours which developed multicellular metastases located in the pulmonary artery and their tributaries (Fig. 4).

Discussion

In this study expression of polySia and NCAM in human neuroblastoma cells grown *in vitro* and in a SCID mouse model was investigated. *In vitro*, cells of three out of the five tested neuroblastoma cell lines were clearly positive for NCAM, namely LAN-1, LAN-5 and SH-SY5Y. Likewise, primary tumours that were obtained by inoculation of LAN-1 and LAN-5 in SCID mice reacted with anti-NCAM and anti-polySia antibodies.

In a lung carcinoma xenograft model, sublines positive or negative for polySia showed different tumour growth. While

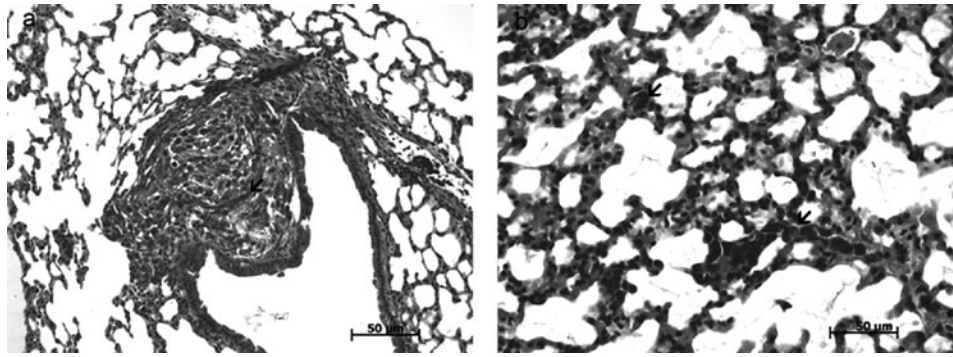


Figure 4. Pattern of metastasis formation amongst human neuroblastoma cells engrafted in SCID mice (HE staining). (a) PolySia-negative SK-N-SH tumours produced solid lung metastasis within a branch of the pulmonary artery. The arrow marks the intact smooth vascular vessel wall. (b) PolySia-positive LAN-1 tumours developed micrometastases (arrow), which are diffusely distributed in the alveolar septae of the lung.

Table II. PolySia-NCAM expression, take rates and pattern of metastasis of different human neuroblastoma cell lines xenografted in SCID mice.

Cell line	NCAM ^a	PolySia ^a	Tumour take-rate ^b (%)	Lung metastases ^b	Disseminated micrometastases ^b
Kelly	Negative	Negative	8/10 (80)	+	-
SK-N-SH	Negative	Negative	9/9 (100)	+	-
LAN-1	Positive	Positive	4/11 (36)	+	+
LAN-5	Positive	Positive	3/9 (33)	+	+
SH-SY5Y ^c	-	-	0/10 (0)	-	-

^aExpression in primary tumours determined by Western blot analysis. ^bData are from Valentiner *et al.* (23). ^cCell line with high polySia and NCAM expression *in vitro*.

expression of polySia-NCAM reduced tumour formation in mice, the absence of polySia enhanced tumour growth (28). This inhibitory effect of polySia expression on tumour formation is also confirmed by the engraftment of the neuroblastoma cells in our xenograft model. Neuroblastoma cell lines, which were strongly positive for NCAM and polySia (LAN-1, LAN-5 and SH-SY5Y) had lower tumour take-rates than the polySia-NCAM-negative cell lines (Kelly and SK-N-SH) (23).

NCAM isoform expression in neuroblastomas has been associated with differentiation status. A recent study assumed that benign tumours express the adhesive NCAM-120 isoform, while malignant neuroblastomas rather express NCAM-140 involved in neurite outgrowth and NCAM-180 involved in cell motility (4). In accordance with these findings, the NCAM-positive neuroblastoma cells analyzed in the present study showed highest expression of NCAM-140 followed by NCAM-180, two isoforms that are mainly expressed in less differentiated or malignant cell types (4,6,7). NCAM-120, which is predominantly found in normal and differentiated tissue (6), could not be detected in our samples.

NCAM can be post-translationally modified by the addition of polysialic acid (polySia), a linear α 2,8-linked sialic acid polymer, which is covalently attached to the NCAM molecule (29,30). In our study, NCAM-positive neuroblastoma cells were consistently positive for polySia and the polySia-NCAM positive cells LAN-1, LAN-5 and SH-SY5Y were characterized by the highest expression levels of ST8SiaII mRNA (Fig. 3b).

LAN-1 and LAN-5 displayed also the highest ST8SiaIV mRNA levels (Fig. 3c). ST8SiaII and ST8SiaIV are the key enzymes in the biosynthesis of polySia (17). Polysialylation of NCAM is crucial for NCAM-mediated regulation of tumour growth (31). In clinical studies high polySia serum levels were associated with poor prognosis and polySia and ST8SiaII were suggested as molecular prognostic markers (15,16,21,32). PolySia is thought to abrogate homophilic binding properties of NCAM and to reduce cell adhesion important in cell migration and invasion. Thus, downregulation of NCAM as well as polysialylation of NCAM facilitate the migration of the cells from the primary tumour (11,12).

Here all neuroblastoma cell lines that were engrafted in SCID mice developed lung metastases independent of polySia-NCAM expression. However, the pattern of metastatic disease was different for the individual cell lines. Thus, LAN-1 and LAN-5 cells developed numerous micrometastases in the alveolar septae, whereas Kelly and SK-N-SH cells produced multicellular metastases mostly located in the pulmonary artery and their tributaries (23). Comparing polySia-NCAM and ST8SiaII expression of the associated primary tumours, LAN-1 and LAN-5 tumours showed a high polySia-NCAM and ST8SiaII expression. This result supports the current model that expression of polySia-NCAM reduces the adhesiveness of tumour cells, contributes to cellular dissemination and may promote metastasis (21,33). Cells from neuroblastomas, which are positive for polySia-NCAM disseminate and

emigrate as single cells in the parenchyma of the lung, whereas cells of tumours without or with weak polySia-NCAM expression leave the primary tumour and adhere as cell clusters to the vascular vessel but do not transmigrate or do so very slowly, respectively.

Polysialic acid is the key regulator of NCAM-mediated interactions (34). By masking the underlying protein scaffold, polySia prevents not only homophilic NCAM-NCAM binding but also heterophilic interactions involved in signal transduction processes. In neuroblastoma cells, enzymatic removal of polySia from NCAM leads to the activation of extracellular signal-regulated kinase (ERK), resulting in reduced cell proliferation and induction of neuronal differentiation (31). In the present study, polySia was found exclusively on the 140- and 180-kDa isoforms of NCAM. Expression of these isoforms in their polysialylated state seems to reduce take-rates after subcutaneous engraftment of neuroblastoma cells in SCID mice and to have an influence on pattern of metastasis formation. In line with previous reports showing that neuroblastoma cell lines are either negative for NCAM or express the polysialylated form of NCAM (31), none of the cell lines used in our study was characterized by the expression of 'naked' polySia-free NCAM. Therefore, it was not possible to compare the malignant potential of polySia-NCAM-positive xenografts with those lacking polySia but expressing 'naked' NCAM. However, the developed xenograft model forms the basis for further studies addressing this question by manipulating the expression level of the polysialyltransferases.

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