Polysialic Acid on the Neural Cell Adhesion Molecule Correlates with Expression of Polysialyltransferases and Promotes Neuroblastoma Cell Growth

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ABSTRACT

Neuroblastomas and cell lines derived from these tumors bear the oncodevelopmental antigen polysialic acid (PSA) bound to the neural cell adhesion molecule. Polysialylation of neural cell adhesion molecule can be achieved by two different polysialyltransferases, STSSialI and STSSialV. This study was undertaken to investigate the pattern of polysialyltransferases expressed in the human neuroblastoma cell line SH-SY5Y. Reverse transcription-PCR showed simultaneous expression of the two enzymes, and in situ hybridization demonstrated that the polysialyltransferase mRNA expression parallels immunoreactivity with the PSA-specific monoclonal antibody 735. After retinoic acid-induced differentiation, only the PSA-positive, neuron-like cell type gave clear signals for STSSialI and STSSialV in situ hybridization; whereas both signals were drastically reduced in the weakly PSA-positive substrate adherent phenotype. Like the SH-SY5Y cells, a primary, PSA-positive neuroblastoma specimen revealed expression of the two polysialyltransferases. To investigate the role of PSA for cell growth and differentiation, SH-SY5Y cells were treated with the PSA-specific endo-N-acety neuraminidase E. Although loss of PSA was accompanied with a marked reduction of cell growth, it did not interfere with retinoic acid-induced differentiation. Together, our results suggest that PSA surface expression is regulated on the level of polysialyltransferase transcription. Moreover, the similarity to the primary neuroblastoma tissue makes SH-SY5Y cells a suitable model system to examine further the role of polysialylation in tumor cell growth and the orchestration of PSA synthesis in neuroblastoma.

INTRODUCTION

PSA is a developmentally regulated carbohydrate polymer that is mainly attached to NCAM (5–7). It modulates the adhesive functions of NCAM and interferes with other cell surface interactions. The latter, general effect appears to depend on the steric properties of the highly negative and heavily hydrated polymer (6, 8). PSA is abundant in embryonic tissues but decreases progressively during development. In the nervous system, only regions of persistent proliferation and neurogenesis retain PSA (9–11). Although PSA is diminished in the majority of tissues during development, some tumors are known to reexpress PSA (12). A comparative study carried out with isogenic cell lines of the phenotype NCAM-‡/PSA§ and NCAM-§/PSA§ identified PSA as a modulator of the malignant potential of small cell lung carcinoma (13). Thus, PSA represents an oncodevelopmental antigen that may play an important role in tumor development and metastasis (14).

Neuroblastoma, a childhood tumor of neural crest origin, belongs to the highly malignant and metastatic tumors that express polysialylated NCAM (15–19). Like other human neuroblastoma cell lines, SK-N-Sh cells express PSA-NCAM (20, 21). In addition, these cells have been reported to contain high polysialyltransferase activity (19). The best studied subclone of this cell line, SH-SY5Y, can be induced to differentiate in a well-described manner (1). Although lacking the MYCN gene amplification typical for most neuroblastoma, SH-SY5Y cells serve as a model system for neuroblastoma cells that are arrested in their differentiation but have an intact and inducible capacity to mature in vitro (1, 3, 22, 23). On addition of retinoic acid, the proliferation rate of the neuroblast-like cells decreases. Some of the cells differentiate into a flat, substrate-adherent cell type (S cells). As characterized from respective clonal sublines of SK-N-Sh cells, these cells have properties of melanocytic, Schwannian, or meningial cell types (reviewed in Ref. 3). The majority of the cells, however, develop the characteristic phenotype of differentiating neurons, with long neuritic extensions guided by growth cones (NCI cells; Refs. 1, 2, 24). Together with enhanced expression of neuron-specific enolase, strong PSA immunoreactivity for the neuronal phenotype has been reported previously (2).

Recently, two different polysialyltransferases, STSSialI and STSSialV, have been cloned and characterized (25–28). As determined in transfection studies, both enzymes are capable of inducing synthesis of PSA on NCAM (26–30). Both are developmentally regulated, and although there is only limited information on the specific expression patterns (25, 28, 31, 32), it seems obvious that at least in some tissues STSSialI and STSSialV are expressed simultaneously; therefore, both enzymes may contribute to PSA biosynthesis.

In this study, we investigated the role of PSA for growth and differentiation of the neuroblastoma cell line SH-SY5Y. To analyze the biosynthetic system underlying PSA surface expression in neuroblastoma, the expression of polysialyltransferases was studied by RT-PCR and in situ hybridization. The expression patterns in undifferentiated and differentiated cells were compared with PSA immunoreactivity. We provide evidence that both enzymes are expressed in SH-SY5Y cells as well as in neuroblastoma tissue, and that their expression parallels the regulation of PSA.

MATERIALS AND METHODS

Cell Culture. Human neuroblastoma cells of the cell line SH-SY5Y were seeded at a density of fewer than 2 × 10^4/cm^2 and grown with DMEM-Ham’s F-12 medium (1:1) containing 15% heat-inactivated FCS, 10 µg/ml tetracycline, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was changed every second day, and cells were passaged weekly. All experiments were conducted with cells in their exponential growth phase. To induce differentiation, cells were grown for 8 days in the presence of 10 µM retinoic acid.

Neuroblastoma Tissue. A tumor specimen rapidly frozen in liquid nitrogen and stored at –80°C was obtained from a 6-month-old girl suffering from a neuroblastoma grade 3 of the left adrenal gland (staging according to Ref. 33). Tumor tissue was obtained at surgery, following Ethics Committee approval.

PSA and NCAM-specific Reagents. Affinity-purified recombinant endo-neuraminidase Endo NE that specifically degrades PSA (34) and PSA-specific mAb 735 (IgG2a; Ref. 35) were used for sensitive and reliable identification of PSA (36). Although some other PSA antibodies may cross-react with DNA,
mAb 735 has been shown to bind exclusively to PSA (37). mAb 123C3 (IgG1), reactive with all isoforms of human NCAM (38, 39), was kindly provided by Dr. R. Michalides (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

**Western Blotting.** Cells were harvested with a cell scraper, counted, washed three times with PBS, and frozen at -80°C. Cells were homogenized by sonication in Tris-HCl (100 mM, pH 7.4) containing 1% (w/v) Igepal (substitute for NP40; Sigma Chemical Co., Deisendorf, Germany), 20 mM mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 units/ml aprotinin as protease inhibitors. To analyze NCAM isoforms, the homogenate was incubated for 20 min at 20°C in the presence or absence of Endo NE. After centrifugation (20,000 × g for 15 min at 4°C), supernatants were mixed with reducing electrophoresis buffer and heated carefully (10 min, 100°C). Proteins were resolved on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose sheets at 2.5 mA/cm² for 2 h. After blocking with 2% BSA, 1% (v/v) normal goat serum, and 150 mM NaCl in 10 mM Tris (pH 7.5), the blots were incubated for 2 h with mAb 735 (1 µg/ml) or mAb 123C3 (5 µg/ml) and for 1 h with alkaline phosphatase-labeled, anti-mouse IgG, followed by staining with phosphatase substrate BCIP-NBT.

**Immunostaining and Cellular ELISA.** Cells were grown in 35-mm Petri dishes, fixed with 3.8% paraformaldehyde-PBS for 30 min at 4°C, washed, blocked overnight with 2% (w/v) BSA-PBS at 4°C, and incubated for 2 h at room temperature with 5 µg/ml mAb 735 or 25 µg/ml mAb 123C3 in 2% (w/v) BSA-PBS. As secondary antibody, alkaline phosphatase-labeled, anti-mouse IgG was coupled for 1 h and stained with BCIP-NBT.

For quantitation of PSA or NCAM on the cell surface by cellular ELISA, cells were grown in 96-well microtiter plates and processed as for immunostaining with alkaline phosphatase-labeled, anti-mouse IgG as secondary antibody but with p-nitrophenylphosphate as substrate. Absorbencies were determined by an SLT ELISA plate reader at 405 nm against a reference wavelength (750 nm).

**Endo NE Treatment and Cell Growth Assays.** SH-SY5Y cells were cultivated in 96-well plates, and exponentially growing cultures were treated for 2 days with or without Endo NE. Efficiency of PSA removal was tested in a cellular ELISA with mAb 735 and mAb 123C3. To assess cell proliferation, an individual well was photographed at days 0 and 2 of Endo NE treatment. The medium was not changed during this period. Cells per well were counted by stereological methods in a double-blind procedure. The increase was calculated by the ratio of the number of cells at days 2 and 0. To quantify cell growth by determination of metabolic rates, a colorimetric tetrazolium-formazan assay (40) was applied at day 0, 1, or 2 to groups of cells seeded in parallel on the same 96-well plate.

**RT-PCR:** RT-PCR was performed using 1 µg of poly(A)+ RNA isolated from undifferentiated SH-SY5Y cells as a template. Using Superscript II reverse transcriptase (Life Technologies, Inc., Eggenstein, Germany), the cDNA was synthesized with the primer 5'-TGGTTTCGTGTCTGTCACTCAG-3' specific for STSSiall and the primer 5'-TTGGCTCATCGACTTCCTTGGTCG-3' specific for STSSialV. Nested amplification was carried out with the primer combination 5'-CCAGCTGCAGTTCCGGAGCT-3' and 5'-CGT-GAGACTTATTCAT-3' specific for STSSiall and 5'-CACATTTAATGTTTTGAATTCT-3' specific for STSSialV and Kpnl to generate the sense probe for STSSiall. The transcription was confirmed by DNA sequencing.

**In Situ Hybridization.** After embedding of neuroblastoma tissue in Tissue-Tek (Miles Inc., Elkhart, IN), 12-µm cryostat sections were obtained at -20°C, thaw mounted on silanated slides, and air dried for 30 min. Cells in deposit 735 have been shown to bind exclusively to PSA (37). mAb 123C3 (IgG1), reactive with all isoforms of human NCAM (38, 39), was kindly provided by Dr. R. Michalides (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

**RESULTS**

**Immunoblot Detection of PSA-NCAM in SH-SY5Y Cells.** Fig. 1 shows detergent-solubilized extracts of undifferentiated SH-SY5Y cells analyzed in Western blot with the PSA-specific mAb 735 and the NCAM-specific mAb 123C3. After Endo NE digestion, the broad PSA-immunoreactive smear became substituted by focused bands of 120, 140, and 180 kDa. However, the protein of 140 kDa represents the major NCAM isofrom.

**Immunostaining of Undifferentiated and Differentiated SH-SY5Y Cells.** Immunostaining was used to compare the surface expression of NCAM and PSA before and after retinoic acid-induced differentiation of SH-SY5Y cells. As shown in Fig. 2, undifferentiated cells are highly variable for both antigens; however, mitotic stages were always intensively stained (Fig. 2, a and b). After treatment with retinoic acid, NCAM and PSA immunostaining of the flat substrate-adherent S cells was clearly reduced, whereas the concentration of NCAM and PSA was high on cell bodies and neurite-like extensions of NL cells (Fig. 2, d and e). The specificity of the PSA staining was demonstrated by Endo NE digestion (Fig. 2, c and f). Depending on the Endo NE concentration, the PSA immunostaining was strongly reduced (Fig. 2c) or completely abolished (Fig. 2f). Under the illumination chosen to reveal the shape of unstained cells (or cell processes; see Fig. 4), the few dark structures in Fig. 2f result from dense material that, with or without staining, occurs in most cells.

**Simultaneous Expression of STSSiall and STSSialV in SH-SY5Y Cells and in a Neuroblastoma Specimen.** Two different polysialyltransferases, STSSiall and STSSialV, have recently been identified to be involved in the biosynthesis of PSA. RT-PCR was used to analyze which of the two enzymes may govern PSA expression in SH-SY5Y cells. The results shown in Fig. 3 demonstrate simultaneous expression of STSSiall and STSSialV in undifferentiated cells. Therefore, in situ hybridization was performed.
Fig. 2. Immunocytochemical analysis of NCAM and PSA expression on SH-SY5Y cells before (a–c) and after (d–f) retinoic acid-induced differentiation. Cells were stained with mAb 123C3 (a and d) or 735 (b, c, e, and f). As controls, cells were treated with Endo NE for 1 h before fixation (c and f). Endo NE concentration was 1 ng/ml (c) or 10 ng/ml (f; see "Results" for details). Mitotic stages are marked with arrows, NL cells with arrowheads, and S cells with open arrows.

to determine whether the expression of both polysialyltransferases correlates with the pattern of PSA immunoreactivity. Antisense probes directed against ST8SiaII and ST8SiaIV gave signals in all undifferentiated SH-SY5Y cells, and, corresponding to the strong PSA immunostaining (see Fig. 2), mitotic stages appear to express high levels of both polysialyltransferases (Fig. 4a–c). On retinoic acid-induced differentiation, the NL cells were strongly labeled with ST8SiaII and ST8SiaIV antisense probes, whereas the hybridization signal was clearly reduced in S cells (Fig. 4d–f). Thus, expression of both polysialyltransferases parallels the pattern of PSA immunostaining.

In a first attempt, a primary neuroblastoma specimen, strongly positive for polysialylated NCAM, was analyzed by in situ hybridization. As shown in Fig. 5, the two enzymes are present in the tumor but not in the surrounding connective tissue.

Effect of PSA Digestion on Cell Growth and Differentiation. Asking whether PSA surface expression is required for growth and retinoic acid-induced differentiation of SH-SY5Y cells, the cells were cultured in the presence of increasing concentrations of Endo NE. After 2 days of culture, PSA immunoreactivity was checked by cellular ELISA (Fig. 6). An Endo NE concentration of 1 ng/ml was sufficient to degrade PSA completely but did not affect the cell viability during the 2 days of culture. This concentration, therefore, was used to assess cell growth in a metabolic assay and by counting the cells, as described in "Materials and Methods." Both evaluations demonstrated a similar, significant inhibition of cell growth in the presence of Endo NE (Fig. 7). However, as judged by morphological parameters (length of neurite-like structures and amounts of NL- and S-cell types), the retinoic acid-induced differentiation into NL and S cells was identical in parallel cultures treated with or without Endo NE (data not shown).

The reduction of cell growth by Endo NE treatment implies that this effect is mediated by altered cell-cell interactions attributable to the nonpolysialylated NCAM. To exclude that major changes in the NCAM concentration occur during the 2 days of Endo NE treatment,
Fig. 4. *In situ* hybridization analysis of ST8SialI and ST8SialV expression in SH-SY5Y cells before (a–c) and after (d–f) retinoic acid-induced differentiation. *In situ* hybridization was performed with antisense probes directed against ST8SialI (a and d) and ST8SialV (b and e) compared with sense controls (c, ST8SialI sense; f, ST8SialV sense). Arrows, mitotic stages; arrowheads, NL cells; open arrows, S cells.

NCAM immunoreactivity was monitored by cellular ELISA. PSA digestion leads to an improved accessibility of the 123C3 epitope, as can be seen in Fig. 8 from the difference in immunoreactivity between untreated cells and cells treated for 1 h with Endo NE. With regard to this immediate effect, the decline of NCAM immunoreactivity after the 2-day incubation period (Fig. 8) reflects the reduction of the cell number shown in Fig. 7. Thus, calculation of NCAM immunoreactivity per cell indicates that the amount of NCAM is not affected by PSA removal (data not shown).

DISCUSSION

The SH-SY5Y cells used in this study serve as a model for human neuroblastoma, one of the most common childhood solid tumors (1). The neural crest-derived tumor cells appear arrested in their differentiation but retain the capacity to mature *in vitro*. This property has been related to the high incidence of spontaneous regression in neuroblastoma (23). As in other malignant tumors of neuroectodermal origin, PSA-NCAM was detected in neuroblastoma (15). The occur-

Fig. 5. *In situ* hybridization analysis of ST8SialI and ST8SialV expression in a neuroblastoma specimen. *In situ* hybridization was performed with antisense probes directed against ST8SialI (a) and ST8SialV (b). Respective sense controls remained unstained (c; shown for ST8SialI). Arrowheads, connective tissue surrounding the tumor.
that only the caps of the bars can be distinguished). SH-SY5Y cells per well were grown for 2 days in the presence of different concentrations of five determinations each; bars, SD. Significant difference is as indicated U test).

The occurrence of this oncodevelopmental antigen appears to be linked to a high metastatic potential (5, 12-14).

As in the parental cell line SK-N-SH (20, 21), the 140-kDa isofrom is the major NCAM band in the subclone SH-SY5Y, whereas the 120- and 180-kDa isoforms appear as minor compounds. Regarding the expression of NCAM and PSA, clear differences were observed between the two cell types, which can be distinguished on retinoic acid-induced differentiation. Only cells differentiating toward the neuron-like cell type express high amounts of NCAM and PSA. In accordance with the lack of neuronal properties in S cells (reviewed in Ref. 3), NCAM and PSA immunoreactivity is low on cells that exhibit the flat substrate-adherent morphology. The differences observed in immunoreactivity correlate with the expression of the two polysialyltransferases STSSiall and STSSialV. Moreover, this correlation between PSA and polysialyltransferases was also found for cells that undergo mitosis. In line with earlier studies (26-28, 43), our results suggest that the PSA surface expression depends on the transcriptional level of the polysialyltransferases. However, the highly variable staining of NCAM and PSA on the cell surface of undifferentiated cells (see Fig. 2, a and b) did not correspond to the strong in situ hybridization signals found in all of these cells (see Fig. 4, a and b). This discrepancy possibly reflects variations in the intracellular transport of polysialylated NCAM. Kiss et al. (44) recently reported intracellular storage of polysialylated NCAM, which was rapidly transported to the cell surface after electrical stimulation. A related mechanism may be responsible for the modulation of PSA surface expression in SH-SY5Y cells.

Using Northern blot analysis and RT-PCR, Kojima et al. (43) were able to demonstrate that the two polysialyltransferases are expressed in the mouse teratocarcinoma cell line P19. However, only STSSiall expression appears to correlate with the occurrence of large quantities of PSA after retinoic acid-induced neuronal differentiation, and STSSiall has been reported to be the enzyme responsible for PSA synthesis in PSA-positive subclones of the small cell lung carcinoma cell line NCI H69 (26). On the other hand, in some human tissues immunoreactive for PSA, only STSSialV has been detected (28, 31). Here we demonstrate that STSSiall and STSSialV are simultaneously expressed in SH-SY5Y neuroblastoma cells. In contrast to P19 cells, in which retinoic acid-induced differentiation leads to specific induction of STSSiall (43), the expression of both sialyltransferases parallels the variations observed in PSA immunoreactivity between the different types of SH-SY5Y cells obtained after retinoic acid-induced differentiation. Moreover, STSSiall and STSSialV occur in PSA-positive neuroblastoma cells of a tumor biopsy. Therefore, both enzymes may be relevant for PSA synthesis in neuroblastoma tissue.

Quantitative measurements of PSA and NCAM immunoreactivity demonstrate efficient removal of PSA by recombinant Endo NE (34), whereas the amount of NCAM remains unaffected. As judged by cell morphology, retinoic acid-induced differentiation into NL and S cells and neurite outgrowth were not altered after removal of PSA. Thus, unspecific interactions such as enhanced attachment to the plastic surface, as reported for PSA removal from Fl1 cells (8), seem not to be involved in this process. Furthermore, our data show a significant reduction of cell growth after PSA removal. This result is in good agreement with previous reports demonstrating that overexpression of NCAM in the glioma cell line BT4Cn causes growth retardation (45, 46). Thus, proliferation appears impaired by unpolyssialylated NCAM or facilitated by polysialylation. Because PSA is important for the process of cell separation (47), the high expression of polysialyltransferases and PSA in mitotic stages may allow the cells to detach more easily from, e.g., a cell aggregate to conduct mitosis. The same antiadhesive mechanism is also favored to explain the coincidence of high metastatic behavior and PSA expression (13, 14). But it is important to keep in mind that NCAM does not only mediate cell adhesion per se but also acts as a signal transducer (48, 49). Perhaps PSA acts as a modulator of NCAM-mediated second messenger pathways that influence cell growth.

In conclusion, polysialylation of NCAM affects cell growth in neuroblastoma and is differentially regulated in the two cell types that become evident on retinoic acid-induced differentiation of SH-SY5Y cells. The simultaneous expression of STSSiall and STSSialV in a
primary neuroblastoma specimen as well as in the SH-SY5Y cell line indicates that both polysialyltransferases contribute to PSA synthesis in neuroblastoma. Consequently, the SH-SY5Y cell line appears to be a suitable model system to examine the orchestration of the two polysialyltransferases and to study the role of PSA synthesis in tumor growth. Moreover, the identification of both polysialyltransferases in neuroblastoma may be relevant for new diagnostic or therapeutic options in the future.

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