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Retinoic acid induction of sialyltransferase activity in neuroblastoma cells of differing sialylation potentials

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In order to determine how glycosylation changes associated with cellular differentiation may be influenced by the basal cellular sialylation potential, the effect of retinoic acid (RA)-induced differentiation was investigated in neuroblastoma cells expressing differing levels (and activities) of the α2,6(N) sialyltransferase (ST6N) enzyme. The increase in ST activity was proportional to the basal cellular sialylation potentials with the high activity clones showing the greatest increase. This was paralleled by an up-regulation of the level of overall sialoglycoprotein glycosylation level. An increase in the levels of the polysialic acid (PSA) epitope was associated with a parallel increase in the levels of the neural cell adhesion molecule (NCAM) protein backbone although there was no overall change in the PSA:NCAM ratio following RA treatment.

Keywords: **sialyltransferase, retinoic acid, polysialic acid, neural cell adhesion molecule, sialoglycoproteins**

Introduction

Glycosylation in general, and sialylation in particular, is a key event in the posttranslational processing of membrane-bound and secreted glycoproteins [1]. The protein glycosylation state is controlled by the activities of specific glycosyltransferase enzymes at the level of the endoplasmic reticulum and Golgi apparatus within the cell [2]. The negatively charged sialic acid residue occupies a terminal position in both N- and O-linked oligosaccharides and is a key determinant in the structure and function of the protein backbone. The transfer of sialic acid is controlled by a family of sialyltransferase (ST) enzymes, each of which catalyses the transfer of sialic acid to a particular acceptor in a specific linkage [3]. The catalytic activity of the enzyme is reflected in the general glycosylation state of the cellular sialoglycoproteins. As the terminal sialic acid residue in N-linked oligosaccharides can be linked to the penultimate galactose residue in either an α2,3 or α2,6 linkage, there is a local competition between the two ST enzymes (ST3N and ST6N) for access to the acceptor sugar. The ultimate linkage of sialic acid is therefore determined by the isozyme with highest specific activity [4].

Because of its key role, it is important to gain a clear understanding of the factors that control the expression and activity of the ST family. These include corticosteroids, which exert a tissue-specific regulatory effect [5,6], heavy metals [7], divalent cations [8], enzyme glycosylation [9] and second messenger system activation [10,11]. ST activity in the CNS is developmentally regulated [12,13] and the activities of individual ST enzymes are modified following neuronal cellular differentiation *in vitro*. In particular, induction of cellular differentiation with retinoic acid (RA) results in an increase in both ganglioside [14,15] and protein [16–18] ST activities. This effect was specific to RA-induced differentiation and was not observed, for example, following treatment with dimethylsulfoxide [15]. The effect of RA is not limited, however, to sialylation and there are reports of an up-regulation in the general incorporation of sugar residues into cellular glycoproteins with a parallel increase in cellular lectin binding, for example, in the squamous carcinoma cell line MDA886In [19].

The neural cell adhesion molecule (NCAM) plays a pivotal role in the processes of neural cell development and also in synaptic plasticity in the adult brain. A key posttranslational

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modification is the attachment of the α2,8-linked polysialic acid (PSA) oligosaccharide epitope that serves to modify the adhesivity of the protein backbone. PSA expression is of particular importance in early brain development in order to ensure the correct positioning of the differentiating neurons. It is also reexpressed at a time coincident with key plastic events in the CNS [20]. Previous studies using neuronal and non-neuronal cells have reported that RA-induced differentiation results in a parallel increase in both PSA and NCAM so there is no change in the PSA:NCAM ratio [21–23].

While RA-induced differentiation increases basal ST activity, there have been no studies to determine the fold increase in cells with altered basal sialylation potentials. The aim of the present study was to determine whether an increase in basal ST activity in ST6N-transfected cells influences the rate of induction of enzyme activity following the stimulation of cellular differentiation by RA.

Materials and methods

The B104 rat neuroblastoma cell line [24] was a kind gift from Prof. Konrad Sandhoff, University of Bonn. The anti-NCAM antiserum was purchased from Affiniti Ltd., U.K. and the anti-PSA antibody was generated from the 5A5 hybridoma cell line (NICHD).

The control B104 rat neuroblastoma cells and the ST6N transfectants were cultured in Dulbecco's modification of Eagle's medium (DMEM) containing 10% foetal calf serum, penicillin (2 U/ml) and streptomycin (0.25 mg/ml) and maintained at 37°C in a humidified atmosphere containing 9% CO_2 as previously described [4]. The cells were routinely passaged every 3–4 days upon reaching confluency and seeded at a density of 2.2×10^6 cells per 75 cm^2 tissue culture flask. The retinoic acid (final concentration 10 μM) was added to the cells 24 h after plating and the cells were allowed to differentiate for 4 days at 37°C, prior to being harvested.

Total cellular ST activity was monitored throughout the study using cytidine-5-monophosphate-4,5,6,7,8,9-^{14}C-N-acetylneuraminic acid (CMP-^{14}C-Neu5Ac, Radiochemical Centre, Amersham; specific activity 293 mCi/mmol) as the sialic acid donor and asialofetuin (ASF; Sigma) as an exogenous acceptor [25].

The protein content of the harvested cells was determined using the Folin phenol reagent [26]. The individual cellular polypeptide components were separated by discontinuous SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore) by electroblotting. Sialoglycoprotein expression was analysed by lectin affinity blot analysis (DIG glycan differentiation kit, Boehringer Mannheim) and the NCAM and PSA epitopes were visualised by Western blot analysis. The blots were scanned using a Hewlett Packard 6100C flatbed scanner and densitometric analysis was carried out using the NIH image software [4].

Results

RA treatment resulted in the differentiation of both the control and ST6N-transfected cells with a parallel decrease in the rate of cell division as determined by the quantification of cell number (Figure 1). This was observed in both the control (low activity) and 50B3 (high activity) clones, indicating that the effect was not dependent on ST activity levels. This was accompanied by an induction of total ST activity in all of the clones examined with a trend towards a proportionally greater increase in enzyme activity in the clones with a higher basal ST activity (Figure 2). This increase in activity was paralleled by an up-regulation of the expression levels of the ST6N protein (Figure 3). Two protein bands (of 46 and 44 kDa) were observed. The existence of this dimeric form of the enzyme has been reported previously and may be due to alternative splicing of the ST6N gene [4]. The change in enzyme catalytic

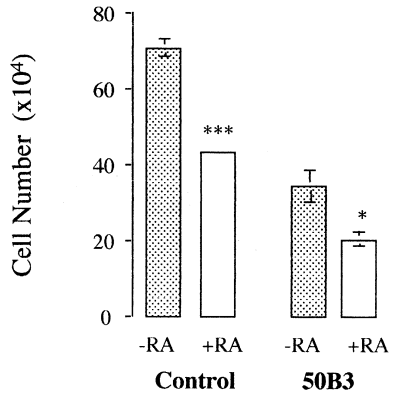

Figure 1. The effect of 10 μM RA treatment on cell number of control and the high ST activity clone, 50B3. The cell numbers were determined by nuclear counting. Figures represent mean ± SEM (n = 3). $^{*}p < 0.05$, $^{***}p < 0.01$ (student's t-test).

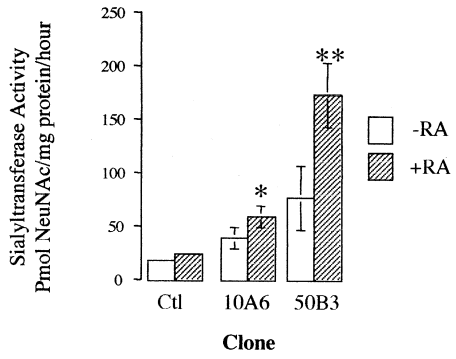

Figure 2. (A) ST activity in clones following treatment with 10 μM RA. *indicates $p < 0.05$, $^{**}p < 0.01$ RA vs control (ANOVA followed by Tukey-Kramer multiple comparisons test). Figures represent mean ± SEM.

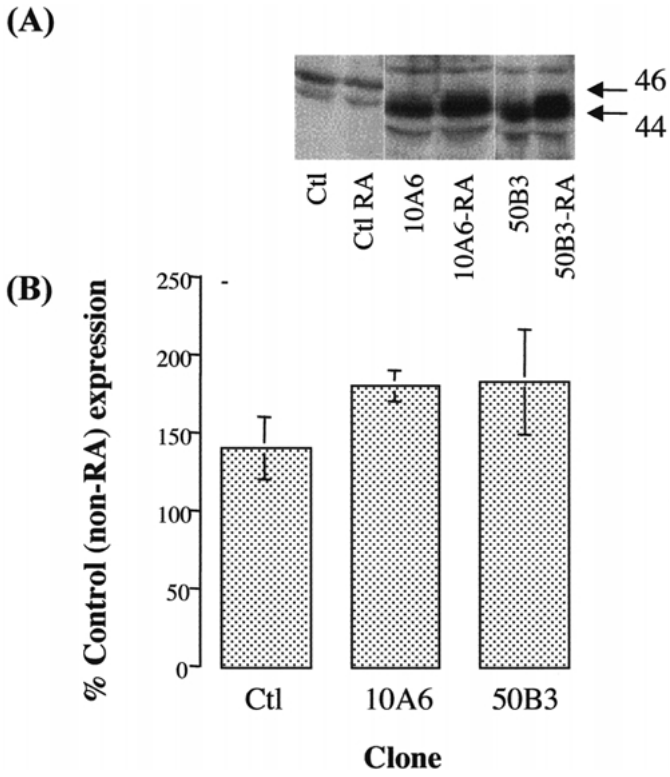

Figure 3. The expression of the ST6N proteins in B104 clones following RA treatment as determined by Western blot analysis (A). The band density was estimated by semi-quantitative densitometric analysis (B). Figures represent mean ± SEM (n = 3).

function was confirmed by lectin blot analysis using the *sambucus nigra* (SNA) lectin, which specifically identifies the expression patterns of cellular sialoglycoproteins (SGP) containing α2,6-linked sialic acid residues. A general increase in the expression levels of α2,6-SGP levels correlated well with the up-regulation of enzyme activity, although this did not reach levels of statistical significance (ANOVA followed by Tukey-Kramer multiple comparisons test). These results confirm that the increased enzyme protein levels expressed within the cell following RA treatment were functionally active (Figure 4). In particular, there was an increase in the glycosylation of the 58, 105 and 160 kDa sialoglycoprotein bands, although the identities of these proteins are unknown. They are, however, similar in molecular size to those previously observed in B104 cells whose sialylation pattern was altered following ST6N transfection [4].

Previous studies in this lab have shown that an up-regulation in ST6N expression levels is associated with an increase in the expression of PSA epitope, although there was no change in the expression levels of the NCAM protein backbone [4]. It was of interest, therefore, to determine if an RA-induced increase in ST6N activity would have a similar effect on PSA expression and if such a change may be influenced by the basal cellular ST activity. The two NCAM isoforms expressed primarily in the B104 cells were the 120 and 140 kDa protein bands and the expression of both was increased equally in all of the clones following RA-induced differentiation. There were no isoform-specific effects and the fold increase was independent of the basal ST activity (Figure 5a). The expression levels of the PSA epitope were proportional to the level of ST expression, as previously described. However, the RA-induced increase in PSA expression also paralleled that of the NCAM backbone (Figure 5b). Therefore, the ratio of PSA to the core NCAM protein was similar in all of the cell lines and was independent of the basal cellular ST activity (data not shown). This suggests that the increase in PSA expression observed following RA-induced differentiation was due primarily to an increase in the levels of the protein backbone rather than to an increase in the overall level of PSA expression per unit of NCAM protein.

Discussion

The activity of the ST family of enzymes, and the consequent expression of sialoglycoconjugates, has been demonstrated to be influenced by the state of cellular differentiation and this modulation plays a key role in neuronal cell development [1]. Previous studies have reported an RA-associated increase in ST enzymes acting on both glycoproteins [27] and glycolipids [15,22]. The level of increase in enzyme activity associated with RA treatment in cells of differing basal sialylation potentials has not, however, been determined.

We have previously documented the generation of B104 rat neuroblastoma cell lines that display increased levels of ST6N expression and activity. The enzyme was fully functional as illustrated by an increase in the sialylation of cell surface glycoproteins [4,28]. The present study aimed to determine if an RA-induced increase in ST catalytic activity may be independent of the basal enzyme activity or if, for example, there is a ceiling above which no further increase in enzyme activity can occur. The level of increase in ST catalytic activity was directly proportional to the basal cellular enzyme activity. In the low activity clones, there was an average 1.3 fold increase following RA treatment while the clones with higher basal activities showed a 2.3 fold increase suggesting that these cells may be more sensitive to the actions of agents that stimulate enzyme generation. It must be considered that certain ST enzymes are also capable of autoglycosylation and this may also be a determining factor in the change in enzyme activity following RA treatment [29,30]. This may influence certain key events associated with brain development where neuronal cell differentiation (as induced *in vitro* by RA treatment) requires the expression of PSA that is catalysed by the ST enzyme family. It is therefore critical that the protein sialylation state is strictly controlled during this developmental period. However, the increase in enzyme activity was paralleled by changes in the expression levels of the ST6N protein, suggesting that RA-associated enzyme induction was

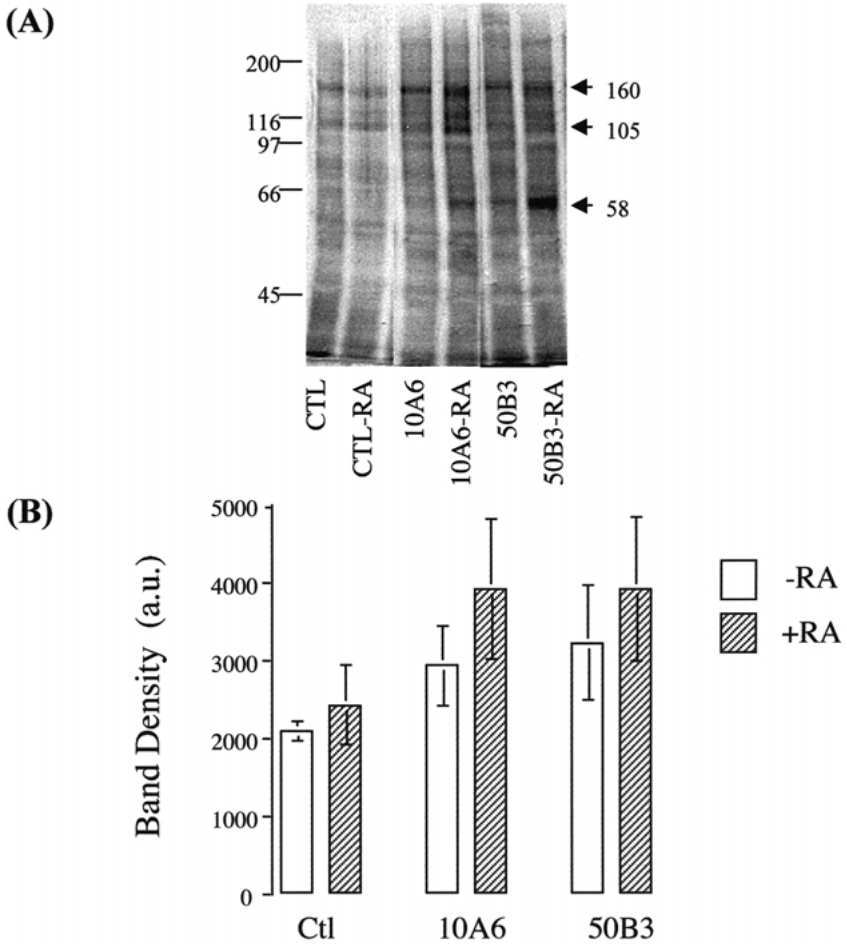

Figure 4. α2,6-linked sialoglycoprotein expression in control and RA-treated cells as determined by SNA lectin Western blot analysis (A). Band density was estimated by semi-quantitative densitometric analysis (B). Figures represent mean $\pm$ SEM. The migration of the molecular weight standards is indicated.

due primarily to an increase in the expression levels of the ST6N protein rather than altering its actual catalytic activity.

While there was a general increase in cellular levels of SNA lectin-labelled sialoglycoproteins, there was a particular increase in the sialylation of individual proteins (58, 105 and 160 kDa). Previous studies have also reported changes in the glycosylation of specific proteins associated with an increase in the expression levels of the protein backbone [7]. Although ST6N is itself a glycoprotein, none of the altered bands correspond with the molecular weight of the enzyme.

Changes in the levels of the NCAM protein backbone were observed in all of the cell lines. We have previously reported that an increase in ST6N expression (and activity) in stably transfected cells induces PSA levels without a change in the levels of the NCAM protein backbone [4]. While the α2,8-linked PSA is primarily linked to the protein backbone via an α2,3 linkage, we have shown previously that, under certain circumstances, it can also be attached with an α2,6 bond [4]. Therefore, PSA expression is controlled jointly by the ST3N/ST6N enzymes that attach the oligosaccharide chain and the polysialyltransferase that is required for chain elongation [31]. Although RA stimulates an increase in both ST6N activity and the level of PSA expression levels in the cell lines, the latter appears to be a consequence of an increase in the level of the protein backbone rather than a *de novo* increase in PSA associated with the increased ST6N activity (Figure 5). This was confirmed by determining the PSA:NCAM ratio which was similar in both the control and RA-treated cells, irrespective of their basal ST activity (data not shown). These results are in good agreement with previous studies that reported an RA-stimulated increase in NCAM expression with a parallel up-regulation of PSA [21,23]. Furthermore, while the expression of the mRNA coding for the PST polysialyltransferase is increased in cells following RA treatment, there is no increase in PSA levels [32]. Thus, the changes in PSA levels are associated with changes in the protein rather than an overall increase in the PSA:NCAM ratio. This would suggest that the expression of

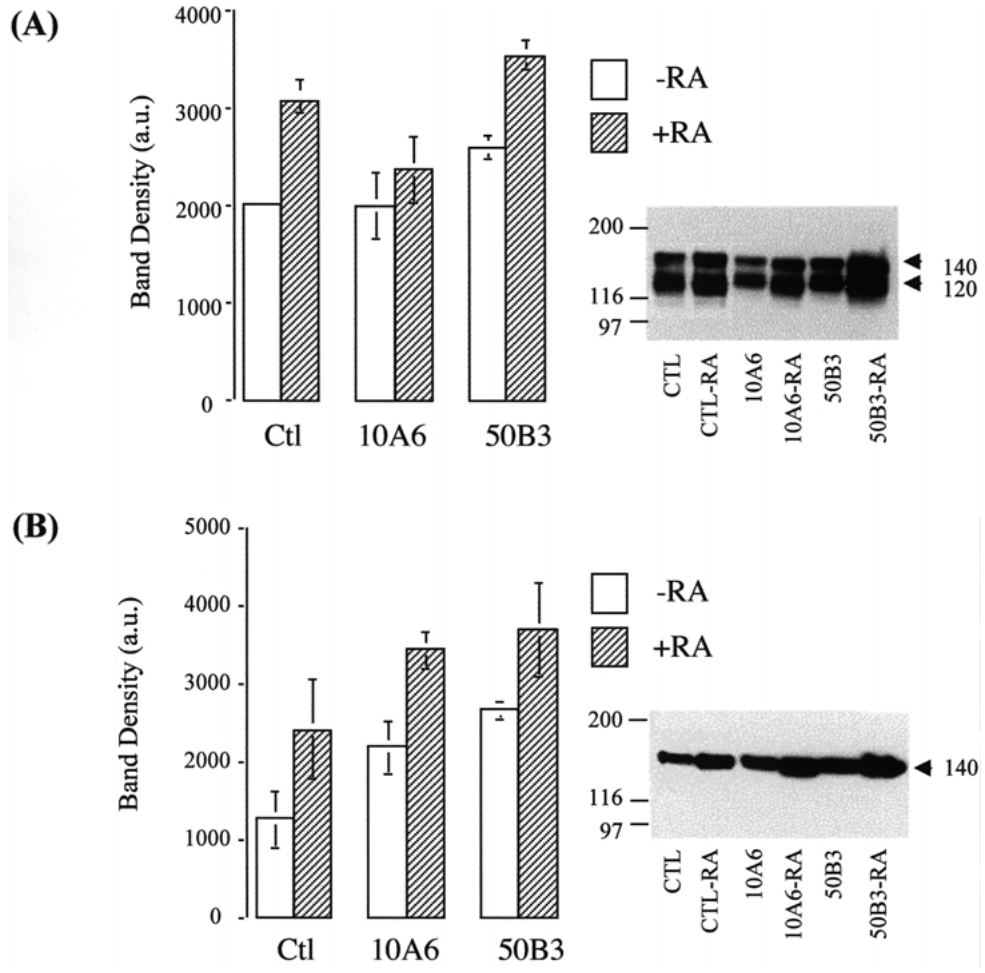

Figure 5. Semi-quantitative densitometric analysis of (a) NCAM and (b) PSA expression in control and RA-treated cells. *indicates $p < 0.05$, vs control (ANOVA followed by Tukey-Kramer multiple comparisons test). Figures represent mean ± SEM.

NCAM-associated PSA is a complex process with input from both ST activity in undifferentiated cells and the expression of the protein backbone following cellular differentiation.

Without doubt, the regulation of glycosyltransferase activity and the subsequent post-translational modification of glycoprotein substrates is a complex one. We have previously demonstrated, for example, that a change in the expression of the ST3N enzyme results in a general upset in cellular SGP levels [33] and that there is a competition between ST3N and ST6N for a limited available substrate.

Further studies will be required to gain a greater insight into the glycosylation process within the nervous system and particularly the physiological consequences on subtle changes that may occur.

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