Comparison of the expression of cell surface poly-N-acetyllactosamine-type oligosaccharides in PC12 cells with those in its variant PC12D

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Introduction

PC12D cells, a new subline of PC12 pheochromocytoma cells (Greene and Tischler, 1976), extend neurites very rapidly in response to cyclic AMP as well as nerve growth factor (NGF). In the PC12D cells these neurites are detectable within 24 h of stimulation, whereas PC12 cells do not display neurites over 48 h (Katoh-Semba et al., 1987). PC12D cells also differ morphologically from PC12 cells, being flat in shape and developing extended short processes without any stimulation (Sano et al., 1988). The behavior of PC12D cells is very similar to that of primed PC12 cells, which have been subjected to prior treatment with NGF. PC12 cells show a flat-shaped morphology when they have been treated with NGF for a few days (Sano et al., 1990).

In general, it is believed that flat cells adhere more strongly to the substratum than round-shaped cells (Rosen and Culp, 1977) and also that different antigens are expressed on the cell membrane as differentiation proceeds (Boyse and Old, 1969). The oligosaccharide portions of membrane glycoproteins and glycolipids are thought to participate in a variety of specific biological interactions (reviewed by Feizi, 1981a; Hakomori et al., 1981; Fenderson et al., 1986) and ligands in saccharide-mediated cell adhesion (Sharon and Lis, 1989, 1993; Edelman and Crossin, 1991; Varki, 1994). Temporal changes in the composition of the cell membrane and extracellular matrix are thought to correlate with the morphogenesis of undifferentiated PC12 cells as they develop into neurons. Thus, there may be other functions of oligosaccharides through which they could influence the cellular morphology and sensitivity to neurotrophic agents, such as NGF and basic fibroblast growth factor.

Results

Fluorography of membrane glycoproteins

PC12 and PC12D cells were grown in medium containing [3H]-glucosamine to label the oligosaccharide moieties of the glycoproteins both in the presence and absence of 100 ng/ml of NGF. After 48 h incubation, the cells were lysed by sonication in a lysis buffer containing 1% Triton X-114, and then the solubilized cellular proteins were divided into two fractions, the membrane protein fraction (detergent phase) and the cytosol protein fraction (aqueous phase), by means of phase separation (Bordeir, 1981). The membrane glycoproteins recovered in the detergent-phase were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by either staining with Coomassie brilliant blue or detection of labeled

Key words: N-acetylglucosaminyltransferase i/neurite formation/PC12 cells/poly-N-acetyllactosamine extension enzyme/poly-N-acetyllactosamine

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Received on December 15, 2000; revised on January 22, 2001; accepted on January 23, 2001

To explore the biological role of carbohydrate chains in the process of nerve cell differentiation, we carried out a characterization of the carbohydrate structure of glycoproteins by comparing conventional PC12 cells with variant cells (PC12D). In vitro metabolic labeling of cells with either [3H] glucosamine or [3H] threonine, together with tomato lectin staining, revealed that nerve growth factor (NGF) stimulation caused a decrease in the poly-N-acetyllactosamine synthesis of high-molecular-weight glycopeptides from PC12 cells. By comparison, the amount of glycopeptides with poly-N-acetyllactosamine from PC12D cells was already significantly low and it was not changed by NGF stimulation.

By assaying the glycosyltransferases that participate in poly-N-acetyllactosamine synthesis, the decrease in the amount of the poly-N-acetyllactosamine in PC12D cells as well as NGF-stimulated PC12 cells could be accounted for by a reduction in the activity of poly-N-acetyllactosamine extension enzyme (GnT-i), because the amount of poly-N-acetyllactosamine in both cells precisely correlated with changes in GnT-i activity, whereas the activities of N-acetylglucosaminyltransferase V (GnT-V) and β1,4 galactosyltransferase remained unchanged.

These results demonstrate that the decrease in poly-N-acetyllactosamine synthesis in PC12 cells occurred prior to neurite formation, whereas PC12D cells were insensitive to this effect. Next, we showed that GnT-i but not GnT-V catalyzed a rate-limiting reaction in the expression of poly-N-acetyllactosamine chains, especially in pheochromocytoma.

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glycoproteins on a fluorogram. Although no differences in the Coomassie brilliant blue staining pattern were observed between PC12 and PC12D cells both with and without NGF treatment, the labeled glycoproteins on the fluorogram, as a whole, differed among the cells as shown in Figure 1. Although the fluorogram did not give any distinct bands, the two broad bands at around 60–70 kDa and 80–90 kDa in PC12D cells appeared not only to migrate faster but also to be somewhat fainter than those of PC12 cells. These results suggested that the membrane glycoproteins in the smear band from PC12D cells might have a smaller molecular weight than those of PC12 cells, owing to either a shorter chain length or a lower amount of carbohydrate chains. Furthermore, PC12D cells (Figure 1, lanes 3 and 4) as well as NGF-treated PC12 cells (Figure 1, lane 2) gave a slightly darker band with a molecular weight of 35 kDa.

Preparation of glycopeptides from the membrane glycoproteins in PC12 and PC12D cells

To metabolically label the cells, they were cultured for 48 h in the presence or absence of 100 ng/ml of NGF together with either [3H]-glucosamine or [3H]-threonine. The membrane proteins recovered in the detergent phase were then precipitated by adding ethyl alcohol, and to isolate them from the lipid they were subjected to chloroform:methanol (2:1) extraction. By comparing the specific activity of membrane proteins from PC12 and PC12D cells, we found that the amount of [3H]-threonine incorporated into the membrane proteins was almost the same for both cells, that is, 1.29 × 10^7 d.p.m./mg for PC12 cells and 1.27 × 10^7 d.p.m./mg for PC12D cells. However, the incorporation of [3H]-glucosamine differed significantly between the cells, that is, 3.79 × 10^7 d.p.m. for PC12 cells and 2.39 × 10^7 d.p.m. for PC12D cells. Especially in the case of [3H]-glucosamine, NGF treatment reduced the specific activity by 11% in PC12 cells (3.39 × 10^7 d.p.m./mg), but there was no change in PC12D cells (2.48 × 10^7 d.p.m./mg). These results, together with the profiles of the fluorogram, indicated that the content and structure of the carbohydrate portions in membrane glycoproteins may differ between PC12 and PC12D cells and also between PC12 cells and NGF-stimulated PC12 cells. Next, the membrane proteins were digested with pronase to obtain the glycopeptides. After removal of free amino acids by gel filtration on a column of Sephadex G-25, the glycopeptides were fractionated on a column of Sephadex G-50. As shown in Figure 2B, the amount of [3H]-glucosamine recovered in the void fraction (peak I) of PC12D cells was markedly lower than that of PC12 cells. Similarly, the amount of [3H]-threonine recovered in peak I was also reduced to half that of PC12 cells (Figure 2A). In contrast to peaks I and II, the amount of radioactivity incorporated into peak III was increased in PC12D compared to PC12 cells.

The decrease in the amount of radioactivity in peaks I and II from PC12D as well as NGF-treated PC12 cells (as described below) could be accounted for by less sugar, either due to a decrease in the amount of the glycopeptides, and/or shortened carbohydrate chains.
NGF stimulation of PC12 cells also induces secretion of the metalloprotease transin, and its release is coincidental with the neuronal differentiation (Machida et al., 1989). Therefore, to rule out the possibility that the decrease in content of high molecular weight glycopeptides (peak I) in PC12D cells might be caused by shedding from the cell surface during the metabolic labeling, the glycoproteins were separated from the spent media in which they had been grown with [3H]-glucosamine. The glycoproteins secreted into the medium were precipitated with ethanol, and the glycopeptides prepared from them were then fractionated by gel filtration on a column of Sephadex G-50 as for the membrane fraction. Although the amount of radioactivity recovered in the glycopeptides was almost the same as that of the membrane fraction, as shown in Figure 3, the radioactivity recovered in the void fraction which corresponded to peak I as shown in Figure 2, was much less than that of peak I from the membrane fraction. Furthermore, PC12D cells, the amount of radioactivity recovered in the void volume fraction was markedly reduced as compared with that of PC12 cells, even although there was less endo-β-N-acetylgalactosaminidase-susceptible material in these void fractions (data not shown). Therefore, the reduction in the amount of high molecular weight glycopeptides (peak I) in PC12D cells as well as NGF-stimulated PC12 cells (as described below) was not due to shedding of the membrane glycoproteins from cell surface into the medium over the 2 days of culture. Thus, the decrease in the radioactivity of peak I from PC12D cells should be attributed to a decrease in the synthesis of high molecular weight oligosaccharides.

**Effect of NGF treatment on [3H]-glucosamine incorporation**

To examine the effect of NGF stimulation on the synthesis of high molecular weight glycopeptides, PC12 and PC12D cells were cultured in the presence of [3H]-glucosamine for 48 h either with or without 100 ng/ml of 7S NGF. The NGF treatment caused a major decrease (about 10–15%) in the radioactivity of peak I from PC12 cells, whereas PC12D cells showed no change (shown in Figure 4).

**Characterization of carbohydrate chains that were present in peak I**

To rule out the possibility that the prominent difference in amount of [3H]-glucosamine radioactivity incorporated into peak I was merely due to differences in the content of sialic acid and glycosaminoglycans, the glycopeptides in peak I were subjected sequentially to neuraminidase and chondroitinase ABC digestion, followed by nitrous acid degradation to remove sialic acid, hyaluronic acid, chondroitin sulfate, and heparan sulfate. The percentages of radioactivity ascribed to sialic acid in peak I from PC12 and PC12D cells were 11% and 14%, respectively. The digestion with chondroitinase ABC followed by nitrous acid degradation produced some degraded material (less than 0.5%), which was found in retard fractions on Sephadex G-25 (data not shown). Thus, we speculated that

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**Fig. 3.** Gel filtration of labeled glycopeptides from the spent media of PC12 and PC12D cells on Sephadex G-50. PC12 and PC12D cells were labeled with [3H]-glucosamine for 48 h. The glycoproteins secreted into the medium were precipitated with ethanol, and the glycopeptides prepared from them were fractionated by gel filtration on Sephadex G-50. The amount of radioactivity recovered in the void volume fraction was much lower than that (peak I in Figure 2) for the membrane glycoproteins, although the total amount of radioactivity incorporated into the glycopeptides obtained from the secreted proteins was almost the same as that of the membrane glycoproteins.

**Fig. 4.** Effect of NGF treatment on the incorporation of [3H]-glucosamine into membrane glycoproteins. PC12 and PC12D cells were cultured in the presence of [3H]-glucosamine for 48 h either with or without 100 ng/ml of NGF. The glycopeptides obtained from the membrane fraction of PC12 (A) and PC12D (B) cells were fractionated on a column of Sephadex G-50 as described for membrane glycoproteins in Figure 2. The NGF treatment caused a prominent decrease in Peak I of PC12 cells, while PC12D cells showed no change. (filled circles) untreated cells; (open circles) NGF-treated cells.
the difference in the amount of [3H]-glucosamine recovered in peak I between PC12 and PC12D cells as well as between PC12 and NGF-treated-PC12 cells comes from the variations in the amount of carbohydrate chains in the glycopeptides. After treatments to remove sialic acid and glycosaminoglycans, alkaline reductive β-elimination was carried out to release O-linked oligosaccharides from the peptide portion. As shown in Figure 5, the glycopeptides were converted into at least three fractions (peaks Ia, Ib, and Ic). The amount of radioactivity in peak Ia derived from PC12D cells was markedly reduced compared with that of PC12 cells, whereas those of peaks Ib and Ic was almost the same in both the cells. Therefore, the marked decrease in the amount of radioactivity in peak I found in PC12D cells could be accounted for by the decrease in the incorporation of [3H]-glucosamine into peak Ia.

The carbohydrate chains in peaks Ib and Ic were supposed to be O-linked oligosaccharides by the fact of their elution profiles shifted on Sephadex G-50. Peak Ib was confirmed to be O-linked oligosaccharides, as GalNAc-ol was found in the acid hydrolyzates on paper chromatography, however, we could not confirm whether peak Ic was free GalNAc-ol (data not shown).

**Endo-β-galactosidase treatment of peaks Ia, Ib, and Ic**

Because the glycopeptides recovered in peak Ia were resistant to alkaline reductive β-elimination, they appeared to be N-linked carbohydrate chains with relatively high molecular weights. This result led us assume the carbohydrate chains in peak Ia contained poly-N-acetyllactosamine. To confirm this possibility, peaks Ia, Ib, and Ic were treated with endo-β-galactosidase and the digests fractionated on a column of Sephadex G-50. As shown in Figure 6A, peak Ia was converted into two fractions of lower molecular weights, one (the “disaccharide” fraction) was found close to where the reference disaccharide, N-acetyllactosamine, had migrated, and the other (the “core” fraction) migrated to a position between the fractions of the undigested peak Ia and the “disaccharide” fraction.

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**Fig. 5.** Gel filtration of oligosaccharides obtained from peak I after alkaline reductive β-elimination on Sephadex G-50. After sequential treatments with neuraminidase, chondroitinase ABC, and nitrous acid to remove sialic acid and glycosaminoglycans included in peak I, alkaline reductive β-elimination was carried out to release the O-glycosidic type oligosaccharides. The glycopeptides were converted into three fractions (peaks Ia, Ib, and Ic). The decrease in peak I of PC12D cells (as shown in Figure 2) could be accounted for by the low content of peak Ia, whereas the content of peaks Ib and Ic was almost the same. (A) PC12 cells; (B) PC12D cells; (filled circles) NGF-untreated cells; (open circles) NGF-treated cells.

**Fig. 6.** Gel filtration of endo-β-galactosidase treated peak Ia and peak Ib on Sephadex G-50. By treatment with endo-β-galactosidase, peak Ia was converted into two fractions, that is, a “disaccharide” fraction and “core” fraction (A), but peak Ib was unchanged (B). It was found that poly-N-acetyllactosamine was present exclusively in peak Ia. The bold solid bars indicate the position of untreated peak Ia (A) and peak Ib (B), respectively.
On the other hand, peaks Ib (Figure 6B) and Ic did not change their elution profile on gel filtration even after endo-β-galactosidase treatment (data not shown). From these results, we concluded that poly-N-acetyllactosamine was present in peak Ia, but not in peaks Ib and Ic. Based on the assumption that the radioactivity found in the “core” fraction represents the amount of poly-N-acetyllactosamine-bearing carbohydrates and also that the radioactivity in the “disaccharide” fraction represents the length of poly-N-acetyllactosamine chains (although the “disaccharide” fraction does also contain Galβ1-4GlcNAcβ1-3Gal as well as GlcNAcβ1-3Gal; Fukuda et al., 1979), we speculated that the number of oligosaccharides in peak Ia derived from PC12D cells were fewer than those of PC12 cells and that the poly-N-acetyllactosamine chains were shorter in length.

Identification of radioactive sugars in peaks Ia and Ib

To confirm that the “core” fraction was in the core portion of the N-linked oligosaccharide, and also that the oligosaccharides of the “disaccharide” fraction had the structure of GlcNAc-Gal, the radioactive sugars in these two fractions were analyzed by paper chromatography after acid hydrolysis with 2.5 M TFA and re-acetylation with acetic anhydride (Figure 7). If the oligosaccharides in peak Ia were composed of N-glycosidic type oligosaccharides, only GlcNAc and/or GalNAc should be detected in the “core” fraction as radioactive sugar. If those of peak Ib were O-glycosidic-type oligosaccharides, then GalNAc-ol should also be found as a sugar from the reducing end. Again, if oligosaccharides in the “disaccharides” fraction were undoubtedly the disaccharides derived from poly-N-acetyllactosamine chains, then only GlcNAc should be detected. Because the “core” and “disaccharides” fractions gave only GlcNAc as the radioactive sugar, while peak Ib gave GalNAc-ol in addition to GlcNAc, we confirmed that the glycopeptides of peak Ia were indeed N-linked oligosaccharides with poly-N-acetyllactosamine chains, and also that those of peak Ib were O-linked oligosaccharides. Table I shows the distribution of the radioactivity recovered in these two fractions (“core” and “disaccharide”). Based on the assumption that N-linked oligosaccharides with poly-N-acetyllactosamine are of a conventional structure with at least five GlcNAc residues as shown in Figure 8, we estimated the chain length of the poly-N-acetyllactosamine in peak Ia. Peak Ia from PC12 cells seems to be characterized by the presence of up to nine or more disaccharide repeating units with the structure (Galβ1-4 GlcNAcβ1-3)n linked to a conventional complex oligosaccharide core. Similarly, the decrease in the amount of [3H]-glucosamine incorporated in peak Ia from PC12D cells was accounted for by a lower content of carbohydrate in the core and with short poly-N-acetyllactosamine chain.

Histological determination of poly-N-acetyllactosamine chains on the cell surface

To prove that the amount of [3H]-glucosamine incorporated into the oligosaccharides of the glycoproteins from PC12 and PC12D cells correctly reflects the chemical amounts, we carried out lectin staining to show that there is a distinct difference in the content of poly-N-acetyllactosamine bearing oligosaccharides between PC12 and PC12D cells. It has been suggested that tomato (Leucopersicon esculentum) lectin has an affinity for N-acetyllactosamine core chains besides N-acetyllacto-chitobiose (Nachbar et al., 1980). So, we next compared the lectin binding to the cell surface histochemically. In this experiment, the cells that have more poly-N-acetyllactosamine on their cell surface exhibit a greater difference in their staining profiles taken before and after endo-β-galactosidase treatment. As shown in Figure 9, PC12 cells were found to have more poly-N-acetyllactosamine on their cell surface than PC12D cells had. However, NGF-stimulated cells showed the same staining intensity as PC12 cells (not shown). However, a

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**Table I.** Incorporation of [3H]-glucosamine into “core” and “disaccharide” fractions

<table>
<thead>
<tr>
<th>Cells</th>
<th>Disaccharide</th>
<th>Core</th>
<th>Repeating units/oligosaccharide corea</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12</td>
<td>7.09 × 10⁴</td>
<td>3.70 × 10⁴</td>
<td>9.58</td>
</tr>
<tr>
<td>PC12D</td>
<td>0.78 × 10⁴</td>
<td>0.59 × 10⁴</td>
<td>6.62</td>
</tr>
</tbody>
</table>

*Based on the assumption that N-linked oligosaccharides with poly-N-acetyllactosamine were of a conventional structure with at least five GlcNAc residues, the number of repeating units per oligosaccharide core was estimated.
small reduction in the amount of poly-N-acetyllactosamine on the cell surface might not result in a significant decrease in the fluorescent intensity.

**GnT-V, GT, and GnT-i activities in PC12 and PC12D cells**
It has been shown that N-acetylgalactosaminyltransferase (GnT-V), β1-4 galactosyltransferase (GT), and poly-N-acetyllactosamine extension enzyme (GnT-i) are specific enzymes for the synthesis of poly-N-acetyllactosamine chains on N-linked oligosaccharides (Yousefi et al., 1991; Heffernan et al., 1993). To determine the precise activities of GnT-V and GnT-i, we used two fluorescence-labeled sugar chains, Gn, Gn-bi-PA and Gal-Gn-Gn-PA, as the substrates for each of these two transferases, respectively. The reaction products were then...
determined by reverse-phase high-performance liquid chromatography (HPLC) according to the method of Nishikawa et al. (1990). The GnT-V activity in PC12 and PC12D cells was 156 pmol/mg·h and 192 pmol/mg·h, respectively. These values were almost the same as those of malignant cells, such as rat ascites hepatoma cells (Nishikawa et al., 1990), metatarsus murine tumor cell lines and a spontaneous mammary carcinoma cell line SP1 (Yousefi et al., 1991), and a mouse F9 teratocarcinoma (Hefferman et al., 1993). NGF treatment slightly raised the GnT-V activity rather than lowering it.

The GT activity of PC12D cells was also the same as that of PC12 cells, and it did not vary with NGF-stimulated cells.

Figure 10 showed the reaction profile and a typical elution pattern of the reaction products from the GnT-i assay on HPLC. The substrate (Gal-Gn-Gn-PA), by-product (Gn-Gn-PA, which was produced by endogenous β-galactosidase as the enzyme source), and the newly formed product were eluted at the retention times of 10, 8, and 13 min, respectively. The newly formed product was confirmed to be GlcNAcβ1-3/4 Galβ1-4 GlcNAcβ1-4 GlcNAc-2-aminoxyridine (Gn-Gal-Gn-Gn-PA), because β-hexosaminidase (jack bean) converted it into Galβ1-4 GlcNAcβ1-4 GlcNAc-2-aminoxyridine (Gn-Gn-Gn-PA), whereas endo-β-galactosidase converted it to GlcNAc-2-aminoxyridine as measured by HPLC. To determine the terminal GlcNAc linkage of this reaction product, the standard material was prepared by a large-scale enzyme reaction using a PC12 cell lysate as the source of GnT-i. The Gn-Gal-Gn-Gn-PA was collected on a ODS-80TM column (7.8 mm × 300 mm). The 1H nuclear magnetic resonance measurement with a Varian Unity spectrometer at 500 MHz revealed that the terminal GlcNAc was linked to galactose through a β1-3 linkage (data not shown).

The increase in the amount of the reaction product (Gn-Gal-Gn-Gn-PA) in this experiment was linear with incubation time up to at least 4 h. With the standard deviation of three to four experiments, the calculated values for the GnT-i activity in PC12D cells (43.1 ± 14.5 pmol/mg·h) were significantly lower than those of PC12 cells (13.1 ± 17.1 pmol/mg·h). The activity of GnT-i was reduced by NGF treatment in the case of PC12 cells (97.0 ± 12.6 pmol/mg·h), but not in PC12D cells.

The GnT-i activity found in PC12 and PC12D cells was much lower than that of other cells, which are known to synthesize or contain a large amount of poly-N-acetyllactosamine in N- and/or O-linked oligosaccharides. Malignant cell lines, such as murine lymphoma (Yousefi et al., 1991) and human B lymphoma (Higgins et al., 1991), show activity of 880 and 1600 pmol/mg·h, respectively.

The activities of these three transferases are shown in Figure 11. The levels of GnT-V and GT activity were almost the same in PC12 and PC12D cells regardless of incubation with or without NGF. In contrast, the GnT-i activity of PC12D cells was markedly lower than that of PC12 cells, and NGF treatment reduced the activity in PC12 cells while PC12D cells showed no effect. The changes in GnT-i activity between PC12 and PC12D cells and between PC12 and NGF-stimulated PC12D cells correlated well with the changes in the poly-N-acetyllactosamine content of each cell.

To elucidate whether the decrease in the GnT-i activity of PC12D cells was due to a difference in the nature of GnT-i itself or not, cell homogenates without any purification were used for this kinetic study. The results shown in Figure 12 suggest that the reduced GnT-i activity could be attributed to an increase in the Km value for the acceptor oligosaccharide (Gal-Gn-Gn-PA) but not the donor nucleotide sugar (uridine-5′-diphosphogalactose [UDP]-GlcNAc). A more biochemical approach will be necessary to elucidate how the reduced activity in PC12D cells occurs. The NGF treatment also down-regulated the activity of GnT-i in PC12 cells. Therefore, we presume that the reduction in the amount of poly-N-acetyllactosamine due to the decreased activity of GnT-i has led PC12D cells to acquire the same abilities as primed cells.

Discussion

In the present study, we provide evidence that the poly-N-acetyllactosamine content of pheochromocytoma cells is down-regulated during neurite formation by NGF treatment, and that the decrease in the content is led by a reduction in the activity of β1,3 N-acetylglucosaminyltransferase I (GnT-i), one of the enzymes that participates in the synthesis of the poly-N-acetyllactosamine chain.

PC12D cells, which have been established from rat pheochromocytoma cells (PC12), extend neurites within 24 h of treatment with NGF (Katoh-Semba et al., 1987). This is quite different from the behavior of conventional PC12 cells, which extend their neurites gradually in response to NGF after a lag period of a few days. This finding, together with morphological observations (Sano et al., 1988), means that PC12D cells can be thought of as primed cells, for they are flat in shape, as are PC12 cells that have been treated with NGF for a few days (Greene and Shooter, 1980). A comparative study of the carbohydrate chains from PC12 and PC12D cells will give insight into the functional properties of specific oligosaccharides that may be involved in nerve cell differentiation.
Metabolic labeling and separation of cell surface glycoproteins

We characterized the carbohydrate structure of membrane glycoproteins isolated from PC12 and PC12D cells. For the purpose of this comparative study, the cells were incubated for 48 h in media containing either \[^3H\]-glucosamine to label the carbohydrate chains, or \[^3H\]-threonine to label the peptide chains. As a means of isolating the membrane glycoproteins quantitatively and separating them easily from other cellular proteins, we used the method of Triton X-114 phase separation. Whole cells or membranes isolated by differential centrifugation are commonly used as a starting material to characterize oligosaccharides, but the former is not always suitable for detecting minute changes in composition that may occur when only a small quantity of cell surface glycoproteins is present, because the membrane glycoproteins are removed from sight. When we tried to isolate the glycopeptides from whole cells, we could not detect any defects in carbohydrate synthesis that would lead to a decrease in the high molecular weight glycopeptides from PC12D cells, in spite of the fact that a remarkable difference in the high molecular weight glycopeptides was found on Sephadex G-50. The latter method to isolate plasma membranes also made it difficult to separate the membrane quantitatively among the different cells.

High molecular weight glycopeptides containing poly-N-acetyllactosamine

The membrane glycoproteins recovered from the detergent phase were digested with pronase after delipidation, and the digests were then fractionated on Sephadex G-50. In PC12D cells, the amount of \[^3H\]-glucosamine as well as \[^3H\]-threonine recovered into the void volume fraction (peak I) was significantly lower than that of PC12 cells. Furthermore, in PC12D cells the radioactivity recovered in the \[^3H\]-threonine-labeled high molecular mass glycopeptides was 45% of that of PC12 cells, whereas that of \[^3H\]-glucosamine-labeled glycopeptides was about 25%. These results indicated that the high molecular weight glycopeptides in PC12D cells were about half as abundant as in PC12 cells and also that they had, as a whole, relatively little sugar. Furthermore, the 48-h NGF stimulation caused a decrease in these oligosaccharides from PC12 cells but not from PC12D cells. By the sequential digestion and chemical treatment of the high molecular weight glycopeptides, we found that the carbohydrate chains that we attributed to the difference between PC12 and PC12D cells were N-linked oligosaccharide with poly-N-acetyllactosamine. The oligosaccharides of interest were characterized by the presence of up to six or more disaccharide repeating units with the structure \((\text{Gal}\beta 1-4\text{GlcNAc})\_n\) linked to a conventional...
complex oligosaccharide core. However, their precise structure remains unknown.

**Tomato lectin staining of cells before and after endo-β-galactosidase treatment**

As metabolic labeling experiments are often influenced by the turnover rate of metabolites, the amount of radioactivity does not always correctly reveal the chemical mass, even if the specific activity of [3H]-threonine-labeled glycoproteins is almost the same in PC12 and PC12D cells. To ensure that the decrease in radioactivity of high molecular weight glycopeptides in PC12D cells implies a lowered expression of poly-N-acetyllactosamine chains at the cell surface, the cells were stained with FITC-conjugated tomato lectin before and after treatment with endo-β-galactosidase. Tomato lectin has been shown to agglutinate all human A, B, and O phenotypes equally, as well as mouse and sheep erythrocytes, and the agglutination is inhibited by oligosaccharides of GlcNAc but not the monomer itself or other monosaccharides (Nachbar et al., 1980). Therefore, the lectin should bind poly-N-acetyllactosamine chains in which the blood-type antigens are formed.

From the difference in staining intensity between nontreated cells and endo-β-galactosidase-treated cells, we could show that the results of metabolic labeling were well reflected in the chemical mass of poly-N-acetyllactosamine bearing glycopeptides. Although we could not detect any difference in the staining intensity between PC12 and NGF-treated PC12 cells, NGF stimulation might still cause the decreased synthesis of these oligosaccharides in PC12 cells.

**Enzymes that participate in the synthesis of the poly-N-acetyllactosamine chain**

The comparative study of three glycosyltransferases, which have been shown to participate in the synthesis of the poly-N-acetyllactosamine chain on N-linked oligosaccharides, that is, GnT-V, GT, and GnT-i, demonstrated that in PC12D cells as well as NGF-stimulated PC12 cells the activity of GnT-i was reduced and that the changes in the activity reflected well the amount of poly-N-acetyllactosamine.

From the observation of neuritic behavior and morphology, PC12D cells are reported to resemble the "primed" PC12 cells (Katoh-Semba et al., 1987). Our results support this idea because NGF induces a decrease in the synthesis of poly-N-acetyllactosamine in PC12 cells only, and PC12D cells have already lost most of their poly-N-acetyllactosamine chains. Figure 13 shows a schematic model accounting for the relationship between the loss of poly-N-acetyllactosamine on the cell surface glycoproteins in PC12 cells and the ability to extend neurites in response to NGF. In the beginning of the process for neurite formation, cell flattening and a decrease in poly-N-acetyllactosamine are commonly observed, which are the characteristics found in PC12D cells.

Recent studies have concluded that GnT-V and core 2 GlcNAc transferase were the only enzymes to regulate poly-N-acetyllactosamine levels in the N- and/or O-linked oligosaccharides, respectively, such as in the retinoic acid induced F9 teratocarcinoma (Heffernan et al., 1993), immortalized rat2 fibroblasts and their malignant T24H-ras-transfected counterpart (Yousefi et al., 1991), benign SP1 mammary carcinoma and the metastatic subline of SP1 (Yousefi et al., 1991), malignant transformation of BHK cells by polyoma virus (Yamashita et al., 1985), and human B lymphocytes (Higgins et al., 1991). As mentioned above, the levels of GnT-V as well as GT did not differ between PC12 and PC12D cells, irrespective of NGF treatment. The specific activity of GnT-V calculated from PC12 and PC12D cells was nearly as high as that of the various transformed cells.

By contrast, the specific activity of GnT-i in PC12 and PC12D cells (131, 43 pmol/h·mg, respectively) was remarkably lower than that of transformed cells (i.e., 770 pmol/h·mg from immortalized rat2 fibroblasts, and 880 pmol/h·mg from benign SP1 mammary carcinoma; Yousefi et al., 1991). Although van den Eijnden et al. (1988) have shown that linear sugar chains with N-acetyllactosamine residues in their nonreducing termini, such as Galβ1-4 GlcNAcβ1-6 Man, and Galβ1-4 GlcNAc, as well as lactose, were three or four times less effective as acceptors than those containing the Galβ1-4 GlcNAcβ1-2(Galβ1-4 GlcNAcβ1-6) Man pentasaccharide sequence as a structural element, the low specific activities found in PC12 and PC12D cells were not merely due to differences in the substrate used (Galβ-4 GlcNAcβ1-4 GlcNAc-2-aminopyridine) for our assay. The GnT-i activities between PC12 and PC12D cells with or without treatment with NGF correlated well with the amount of high molecular weight glycopeptides containing poly-N-acetyllactosamine; for this reason, we suggest that GnT-i catalyzes a rate-limiting reaction in the expression of poly-N-acetyllactosamine in PC12 and PC12D cells.
Possible role of poly-N-acetyllactosamine

Poly-N-acetyllactosamine chains have been shown to exist on the surface of a variety of animal cells, including rat (Matsumoto et al., 1982), rabbit (Egge et al., 1985), and human erythrocytes (Koscielak et al., 1976; Feizi, 1981b; Fukuda et al., 1984); human leukocytes (Childs et al., 1983; Maemura and Fukuda, 1992); normal and malignant granulocytes (Fukuda et al., 1985a); malignant human colon (Ito et al., 1996); PC12 cells; and rat sympathetic neurons (Margolis et al., 1986). They are present as i-antigens, which are associated with N- and/or O-linked oligosaccharides. They are also carried on ABH and Lewis blood group determinant X (stage-specific embryonic antigen) (Watkins, 1980; Spooncer et al., 1984; Feizi, 1985), and Le^x and sialyl Le^x haptenic structures at their nonreducing termini (Fukuda et al., 1985b). It was also shown that the transition of linear (i-active) to branching (I-active) poly-N-acetyllactosamine chains takes place during the development of erythrocytes (Koscielak et al., 1979) and the differentiation of murine leukemia cells (Kannagi et al., 1983). The carbohydrate structures constructed from poly-N-acetyllactosamine chains are well known to serve as ligands for cell adhesion molecules, such as selectins in endothelial cells and platelets (Sharon and Lis, 1989, 1993; Edelman and Crossin, 1991). The common feature of these ligands originates from the structure of the N-acetyllactosamine backbone, being either type 1 (Galβ1-3 GlcNAc) or type 2 (Galβ1-4 GlcNAc) chains. Most of the cells that express poly-N-acetyllactosamine on their cell surface appear to undergo dispersion to prevent them from adhering to each other or to other cells. The poly-N-acetyllactosamine chains obtain the ability to serve as recognition and cell adhesion molecules once they are modified once by sugars or some unknown factor.

As far as the development of sympathetic nerve cells is concerned, it has been shown that the cells disperse or migrate from the neural crest and travel along the supporting cell layers to where they differentiate into neurons. Therefore, the changes in poly-N-acetyllactosamine content could exert an influence on these phenomena in the process of nerve cell differentiation. In the case of growing olfactory axons, an endogenous lactosamine-binding lectin in non-neuronal cells may mediate their fasciculation by cross-linking adjacent cell membrane via the lactosamine-containing glycolipids (Mahanthappa et al., 1994). Recently, Puche and Key (1996) have shown that solidified N-acetyllactosamine on substratum strongly promoted neural outgrowth of primary olfactory neurons.

Poly-N-acetyllactosamine bearing glycoproteins in nerve cells

There are three well-characterized receptor molecules (e.g., neuronal cell adhesion molecule, N-cadherin, and NILE glycoprotein or L1) that exist at the surface of neural cells. These are known recognized to function as adhesion molecules to where they differentiate into neurons. It should be noted that most of the glycopeptides with a high molecular weight (peak I) both in PC12 and PC12D cells were susceptible to treatment with endo-β-galactosidase, and converted into only two fractions, that is, core oligosaccharide and disaccharide. Because highly branched or highly sulfated poly-N-acetyllactosamine, and those that contained only a limit number of N-acetyllactosamine repeats, are relatively resistant to endo-β-galactosidase digestion (Fukuda et al., 1978; Scudder et al., 1984), the majority of poly-N-acetyllactosamine chains found in PC12 and PC12D cells may have chicken, which contain several poly-N-acetyllactosamine chains (Stallcup et al., 1985).

Neuronal differentiation of PC12 cells begins with the binding of growth factors to their receptors. The process of differentiation to fully differentiated neural cells is very complex and requires a few days. Poly-N-acetyllactosamine may be involved in the early stages of nerve cell differentiation, such as cell-migration and cell–cell interaction, rather than tropic interaction and signal transduction.

The results of fluography, together with a previous report (Margolis et al., 1986), have revealed that most of the membrane glycoproteins from PC12 and PC12D cells were present as a smear band ranging from 60 kDa to 160 kDa on SDS–PAGE. When the membrane glycoproteins were treated with endo-β-galactosidase, they were converted into several distinct bands with molecular weight of 82, 60, 32, and 29 kDa (unpublished data), suggesting that there were several poly-N-acetyllactosamine-bearing glycoproteins in addition to NILE glycoprotein in PC12 cells.

There is some evidence that NGF may stimulate glyco-protein synthesis immediately after treatment in PC12 cells. For example, McGuire et al. (1978) and Margolis et al. (1986) showed that a large increase (three- to fivefold) was observed in the synthesis of cell surface glycoproteins with an apparent molecular weight of 230 kDa (NILE glycoprotein). McGuire and Greene (1979) also found that NGF selectively increased the capacity of PC12 cells to synthesize a minor cell protein with a molecular weight of 80 kDa. However, we could not detect such bands on fluorogram in either the membrane glycoproteins (detergent phase) or aqueous phase of both in NGF-treated PC12 and PC12D cells, although the radioactivity detected close to the top of the gel appeared to be somewhat intensified in case of NGF-treated PC12 cells as shown in Figure 1. This discrepancy may be due to the different conditions for metabolic labeling. McGuire et al. (1978) and Margolis et al. (1986) used PC12 cells that had been cultured for 10 or 12 days in the presence of NGF at a concentration of 50 ng/ml, and this was followed by a 72- or 48-h incubation with ^3H-glucosamine to label the glycoprotein, respectively. In contrast to theirs, the PC12 and PC12D cells in our experiment were metabolically labeled for 48 h with ^3H-glucosamine immediately after the addition of 100 ng/ml of NGF, and thus PC12D cells would have finished their neurite formation within the incubation time. These results indicate that the majority of membrane glycoproteins for which NGF treatment could influence the structure of their carbohydrate chains would be constitutive glycoproteins rather than induced, and also that the decrease in synthesis of the poly-N-acetyllactosamine chains in these glycoproteins occurs prior to the expression of NILE glycoprotein and the 80-kDa molecular weight protein.

It should be noted that most of the glycopeptides with a high molecular weight (peak I) both in PC12 and PC12D cells were susceptible to treatment with endo-β-galactosidase, and converted into only two fractions, that is, core oligosaccharide and disaccharide. Because highly branched or highly sulfated poly-N-acetyllactosamine, and those that contained only a limit number of N-acetyllactosamine repeats, are relatively resistant to endo-β-galactosidase digestion (Fukuda et al., 1978; Scudder et al., 1984), the majority of poly-N-acetyllactosamine chains found in PC12 and PC12D cells may have
no such structural features. A recent paper has shown that the oligosaccharides with poly-N-acetyllactosamine chains in PC12 cells had considerable amounts of fucose residues in their chains (Kojima et al., 1993).

It is well known that the formation of the nervous system during development depends on cell lineage, inductive and trophic interactions between cells, target-derived and target-independent navigational cues, specific cell–cell recognition, and ongoing activity-dependent refinement of connections (Nicholls et al., 1992). A number of questions arise about morphogenesis during the formation of the nerve system. How do the neuroblasts migrate to their correct destinations where they are destined to differentiate? How do the cells stay there? How do the cells extend their axons? How do the neurites find their target? Answers to these questions may come from studies on the function of poly-N-acetyllactosamine chains and their glycoproteins.

Materials and methods

Materials

\[1^{-3}H\]-glucosamine-HCl, 2.7 mCi/mmol and \[^3H\]-threonine, 14.7 mCi/mmol were obtained from Amersham, UK. Dulbecco’s modified Eagle’s medium (high glucose) was from Grand Island Biochemical Co. (Grand Island, NY). Fetal calf serum was purchased from Hyclone, (Utah) and horse serum was purchased from M.A. Bioproduct (Walkerville, MD). Nerve growth factor (Eagle’s medium (high glucose) containing with 10% horse serum was purchased from Hyclone, (Utah) and horse serum was purchased from M.A. Bioproduct (Walkerville, MD). Nerve growth factor was added to remove the lipid. For the pronase digestion, the residue was suspended in 0.1 M borate buffer, pH 5.0, containing 10 mM calcium acetate. The pronase P (actinase E) was then added to the suspension at the ratio of 1 part enzyme to 75 parts of membrane protein, and the mixture incubated for 3 days at 37°C (Yamauchi et al., 1968). The glycopeptides derived from the membrane glycoproteins were sequential fractionated on a column of Sephadex G-25 and G-50 as described previously (Fukui et al., 1981, 1991).

Characterization of oligosaccharide chains

To hydrolyze the glycosidic linkage with terminal sialic acid, the glycopeptides were digested with neuraminidase (A. ureafaciens). After the free sialic acid had been removed by gel filtration on a column of Sephadex G-25 (1 cm × 44 cm) equilibrated with 0.05 M pyridine-acetic acid buffer, pH 5.0, the glycopeptides were then treated with 0.4 units of chondroitin ABC in 50 mM Tris–HCl buffer, pH 8.0, at 37°C for 2 h to degrade proteoglycans that might have contaminated the detergent phase. The digests were subjected to gel filtration on Sephadex G-25 to remove the unsaturated disaccharides derived from chondroitinsulfate and hyaluronic acid (Fukui et al., 1981). The residual glycopeptides were then treated with 2.5% nitrous acid to degrade heparansulfate (Lagunoff and Warren, 1962), and the degraded carbohydrate chains were removed by gel filtration in the manner described above. To release O-linked oligosaccharides, the glycopeptides were subjected to reductive alkaline β-elimination (0.05 M NaOH and 1 M sodium borohydride at 45°C) according to the method of Carlson (1968). After a 16-h incubation, 1 M acetic acid solution was added to terminate the reaction. The reaction mixture was then applied to a column of Sephadex G-50 (1 cm × 61 cm) to separate the O-linked oligosaccharides from the N-linked carbohydrates bearing high molecular weight glycopeptides. To confirm the existence of poly-N-acetyllactosamine chains in these glycopeptides, they were treated with endo-β-galactosidase (E. freundii) in 0.2 M sodium acetate buffer, pH 5.8, for 16 h at 37°C in the presence of one drop of toluene. The reaction mixtures were then applied to a column of Sephadex G-50. To identify the radioactive sugars in the separated oligosaccharides, each oligosaccharide was treated with 2.5 M trifluoroacetic acid at 100°C for 6 h. After re-N-acetylation according to the method of Suzuki et al. (1991), the monosaccharides were separated by paper chromatography using borate-imregnated paper according to the method of Rajilo and Renkonen (1981).

Determination of enzyme activity for GnT-V

GnT-V activity was assayed according to the method of Nishikawa et al. (1990) as follows. To prepare the enzyme source for GnT-V, the PC12 and PC12D cells were homogenized with a sonicator in four volumes of 10 mM Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose and 1% Triton X-100.
The assay mixture was then incubated at 37°C for 2 min, the reaction tubes were centrifuged at 10,000 × g for 10 min. The supernatants were applied to a TSKgel ODS-80TM column (4.6 mm × 150 mm) that had been equilibrated with 0.1 M acetate buffer, pH 4.0, containing 0.15% n-butyl alcohol. The pyridylaminated products of this reaction were separated with a linear concentration gradient from 0.025% to 0.225% n-butyl alcohol for 30 min at 55°C with a flow rate of 1.2 ml/ min. Fluorescence of the column eluates was detected with a spectrophotometer using excitation and emission wavelengths of 320 nm and 400 nm, respectively.

**Determination of enzyme activity for GnT-i**

As a substrate compound that gives an accurate measurement of GnT-i activity, we prepared the fluorescence-labeled oligosaccharide Galβ1-4 GlcNAcβ1-4 GlcNAc-2-aminopyridine (Gal-Gn-Gn-PA for short) that has the N-acetylactosamine structure at its nonreducing terminal. To produce Gal-Gn-Gn-PA, we made GlcNAcβ1-4 GlcNAc-2-aminopyridine (Gn-Gn-PA) from N-acetylchitobiose using the method of Hase et al. (1984). To provide a N-acetylactosamine structure in the nonreducing terminal of Gn-Gn-PA, the terminal GlcNAc residue was substituted with galactose using UDP-galactose and β1-4 galactosyltransferase according to the method of Holt and Hart (1986).

To assay the enzyme activity, we used a solution that was made up of 500 mM MES buffer, pH 7.40, containing 160 mM UDP-GlcNAc, 30 mM MnCl₂, 800 mM N-acetylglucosamine, and 2% Triton X-100. To 25 μl of this solution we added 10 μl of 400 μM fluorescence-labeled sugar substrate (Gal-Gn-Gn-PA) and 15 μl of the enzyme source prepared as described above. The assay mixture was then incubated at 37°C for 3 h, before being heated at 100°C for 2 min to terminate the reaction. After centrifugation at 10,000 r.p.m. for 5 min, the supernatants were applied to a TSKgel ODS-80TM column (4.6 mm × 150 mm) equilibrated with 0.1 M acetate buffer, pH 4.0, containing 0.025% n-butyl alcohol. The pyridylaminated products of this reaction were separated with a linear concentration gradient of 0.025% to 0.225% n-butyl alcohol for 30 min at 55°C with a flow rate of 1.2 ml/ min. Detection and measurement of fluorescence-labeled products were carried out as stated for GnT-V.

**FITC-conjugated tomato lectin staining of cells**

PC12 and PC12D cells were seeded in a chamber slide that had been coated with 0.01 mg/ml of L-polysulphide. After 48 h cultivation, the cells were prefixed in 0.05 M sodium cacodylate buffer, pH 7.2, containing 1% paraformaldehyde, 0.0125% glutaraldehyde, 0.1 M sucrose, 0.05 M CaCl₂, and 1% polyvinylpyrrolidone for 1 h at 4°C, and then postfixed with 95% ethanol for 24 h at 4°C. After fixation, the cells were rinsed once with phosphate buffered saline (PBS) containing 10 mM glycine and twice with PBS alone. To block nonspecific binding, the cells were incubated in PBS containing 1% bovine serum albumin and 0.05% Tween 20 for 1 h at room temperature. The cell cultures were divided into halves, and one was treated with 0.1 units/ml of endo-β-galactosidase for 2 h before FITC-conjugated tomato lectin staining, and the other was treated with buffer alone. The difference in the staining intensity between the two shows the amount of poly-N-acetyllactosamine on the cell surface qualitatively, but not quantitatively. The cells were treated with FITC-conjugated tomato lectin at 5 μg/ml in PBS containing 0.1% bovine serum albumin. After incubation for 1 h in the dark, the cells were washed three times in PBS and mounted in a solution of PBS containing 10% glycerin before being viewed with a Nikon fluorescent microscope.

**SDS–PAGE**

The membrane glycoproteins recovered in the detergent phase fractions were precipitated by adding four volumes of cold ethyl alcohol. After the precipitates had been washed once with 80% ethyl alcohol, they were dissolved in SDS–PAGE sample buffer. The SDS–PAGE was performed in 10% polyacrylamide gels according to the procedure described by Laemmli (1970). Fluorography to detect radioactive membrane glycoproteins which had been metabolically labeled with [3H]-glucosamine was carried out according to the method of Bonner and Laskey (1974).

**Acknowledgments**

The authors thank Prof. N. Taniguchi (Department of Biology, School of Medicine, University of Osaka, Japan) for providing the substrate of GnT-V, and Prof. K. Sugahara (Department of Biochemistry, Kobe Pharmaceutical University) and Prof. T. Feizi (Glycosciences Lab., Imperial College School of Medicine) for helpful discussion. This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture of Japan (07807205 and 12672133), and Fugaku Trust for Medicinal Research in Japan, and a Grant-in-Aid from the Sankyo Foundation of Life Science, Japan.

**Abbreviations**

Gal, galactose; Gal-Gn-Gn-PA, Galβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine; GlcNAc, N-acetylgalactosamine; Gn, N-acetylglucosamine; Gn-Gn-PA, GlcNAcβ1-4GlcNAc-2-aminopyridine; Gn-βi-PA, GlcNAcβ1-2(GlcNAcβ1-4)Manβ1-3(GlcNAcβ1-2Manβ1-6)Manβ1-4GlcNAcβ1-4GlcNAc-2-aminopyridine; Gn-Gn-PA, GlcNAcβ1-2GlcNAc-2-aminopyridine; GnT-i, N-acetylgalactosaminyltransferase i; GnT-V, N-acetylgalactosaminyltransferase V; HPLC, high-performance liquid chromatography; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; UDP, uridine-5’-diphosphogalactose.
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