Glycolipid Glycosyltransferases in Human Embryonal Carcinoma Cells during Retinoic Acid Induced Differentiation†

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ABSTRACT: Retinoic acid induced differentiation of TERA-2-derived human embryonal carcinoma cells is accompanied by a dramatic reduction of extended globo-series glycolipids, including galactosyl globoside, sialylgalactosyl globoside, and globo-A antigen (each recognized by specific MoAbs). Associated with these glycolipid changes, the activities of two key enzymes, α1→4galactosyltransferase (for synthesis of globotriaosyl core structure) and β1→3galactosyltransferase (for synthesis of galactosyl globoside), were found to be reduced 3- to 4-fold. The latter enzyme plays a key role in the synthesis of extended globo-series structures, and its characterization has not been reported previously. Therefore, its catalytic activity was studied in detail, including substrate specificity, detergent and phospholipid effects, pH and cation requirements, and apparent K_m. During retinoic acid induced differentiation, a series of Le^x glycolipid antigens (recognized by anti-SSEA-1 antibody) and their core structures (lacto-series type 2 chains) increase dramatically. In parallel with these changes in glycolipid expression, the activities of two key enzymes, β1→3*N*-acetylglucosaminyltransferase (for extension of lacto-series type 2 chain) and α1→3fucosyltransferase (for synthesis of Le^x structure), were found to increase by 4- and 2-fold, respectively. Similarly, an increase in the expression of several gangliosides (e.g., GD_3 and GT_3) during retinoic acid induced differentiation was mirrored by a 4-fold increase in the activity of α2→3sialyltransferase (for synthesis of ganglio core structure, GM_3). The results suggest a coordinate regulation of key glycosyltransferases involved in core structure assembly and terminal chain modification. Changes in the expression of these glycosyltransferases may mediate the changes in glycolipid antigen expression observed during embryonal carcinoma cell differentiation and may provide an important basis for changes in cell surface phenotype during embryogenesis.

The rapid, dramatic changes of glycoconjugates observed during embryogenesis and during the differentiation of cultured embryonal carcinoma (EC)[1] cells suggest that cell surface glycoconjugates play a vital role in embryonic development (Solter & Knowles, 1979; Feizi, 1981; Hakomori et al., 1982). A glycoconjugate having an Le^x determinant (recognized by anti-SSEA-1 MoAb) was found to be maximally expressed at the morula stage and to decline greatly after compaction (Solter & Knowles, 1978; Fenderson et al., 1986). Since compaction was inhibited by multivalent Le^x oligosaccharide, this structure may play a role in this process, the very first overt morphogenic change during embryogenesis (Fenderson et al., 1984). Dramatic alteration of many other carbohydrate antigens, including SSEA-3/4 (Kannagi et al., 1983c), I/i (Kapadia et al., 1981), and Le^y (Fenderson et al., 1986), also occurs and is a focus of current research in molecular embryology. These studies may provide a clue for understanding the biological significance of cell surface glycoconjugates in general.

Recently, we presented a detailed study of changes in the expression of glycolipid antigens during retinoic acid induced differentiation of the pluripotent human EC cell NTERA-2 clone D1 (Fenderson et al., 1987). Initially, the EC cells, which we believe may resemble early embryonic cells (Andrews, 1988), express predominantly globo-series glycolipids. However, once differentiation is initiated, there is a marked shift to expression of lacto- and ganglio-series glycolipids and a corresponding decrease in globo-series structures. We now

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[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagles's medium; EC, embryonal carcinoma; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MoAb, monoclonal antibody; NT2/D1, NTERA-2 clone D1; PBS, 10 mM phosphate buffered 0.15 M NaCl, pH 7.2; SSEA, stage-specific embryonic antigen; TLC, thin-layer chromatography. Glycolipids are designated according to the recommendation of the IUPAC Nomenclature Committeee (1978). Gangliosides are designated according to the nomenclature of Svennerholm (1964), and structures corresponding to each designation are as shown below. Structures of other glycolipids mentioned in this study are shown in Table I.

GM_3	NeuAcα2→3Galβ1→4Glcβ1→Cer
GD_3	NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→Cer
GT_3	NeuAcα2→8NeuAcα2→8NeuAcα2→3Galβ1→4Glcβ1→Cer
GM_1	Galβ1→3GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→Cer
Le^x	Galβ1→4[Fucα1→3]GlcNAcβ1→R
Le^y	Fucα1→2Galβ1→4[Fucα1→3]GlcNAcβ1→R
Gb_3 (CTH)	Galα1→4Galβ1→4Glcβ1→Cer
Lc_3	GlcNAcβ1→3Galβ1→4Glcβ1→Cer
Gg_3 (asialo GM_2)	GalNAcβ1→4Galβ1→4Glcβ1→Cer
Gb_4 (globoside)	GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer
Forssman	GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer
I	Galβ1→4GlcNAcβ1→3[Galβ1→4GlcNAcβ1→6]Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer
i	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer
A^b	GalNAcα1→3[Fucα1→2]Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer

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report that this shift in expression of different glycolipids is accompanied by corresponding changes in the activities of the glycosyltransferases responsible for their synthesis.

Materials and Methods

Cells. NTERA-2 cl.D1 (abbreviated NT2/D1) is a cloned EC subline of the human teratocarcinoma cell line TERA-2 (Andrews et al., 1984, 1985). Cells were maintained by growth at high cell density (>5 × 10^6 per 75-cm^2 flask) in the high-glucose formulation of DMEM supplemented with 10% FCS under a humidified atmosphere of 5% CO_2 in air, at 37 °C. Differentiation was induced by seeding cells at 10^6 cells per 75-cm^2 flask in DMEM/10% FCS containing 10^{-5} M *all-trans*-retinoic acid (Eastman Kodak Chemical Co., Rochester, NY) as described previously (Andrews, 1984). Retionic acid treated cultures were fed with fresh medium containing retinoic acid at weekly intervals.

Antibodies. The following MoAbs were obtained as hybridoma supernatants: HH5 (Clausen et al., 1986), 1B2 (Young et al., 1981), A2B5 (Eisenbarth et al., 1979), and ME311 (Thurin et al., 1985). The A2B5 hydriboma cell line was purchased from the American Type Culture Collection. Additional MoAbs were obtained as ascites fluids: anti-SSEA-1 (Solter & Knowles, 1978), MC630 (anti-SSEA-3) (Shevinsky et al., 1982), MC813-70 (anti-SSEA-4) (Kannagi et al., 1983c), and R24 (Pukel et al., 1982).

Immunofluorescence Analysis of Cell Surface Carbohydrate Expression. Indirect immunofluorescence analysis of cell surface antigen expression was carried out by flow cytofluorometry using an Ortho Cytofluorograf 50H as described previously (Andrews et al., 1980, 1984; Andrews, 1982). P3X63Ag8 ascites (Kohler & Milstein, 1975), diluted 1:100, was used as a negative control. Antibodies were pretitered on appropriate target cells and used as dilutions giving maximum binding. The second (detecting) antibody was either FITC-conjugated goat anti-mouse IgM or IgC (Cooper Biomedical, Malvern, PA), as appropriate.

Immunostaining of Glycolipids. Immunostaining of glycolipids separated on HPTLC plates was carried out according to the procedures of Magnani et al. (1980) as modified by Kannagi et al. (1982). In the case where product identification was sought, unlabeled UDP-Gal was used for the transfer of Gal to glycolipid acceptors.

Glycosyltransferase Assays. (*A*) *General Procedure.* Analyses of glycosyltransferase activities of NT2/D1 cells treated or untreated with retinoic acid were performed by using cell crude homogenate. The cell crude homogenate was prepared by homogenization of cell pellet in 2 volumes of homogenization buffer, consisting of 20 mM HEPES (pH 7), 1 mM EDTA, and 0.5 M sucrose, with a Polytron homogenizer (Beckman Instruments). Protein concentrations of cell crude homogenate were determined by fluorescamine protein analysis (Udenfriend et al., 1972), using BSA as standard. General methods for preparation of substrate glycolipid in enzymatic reaction, determination of enzymatic products, and other common procedures are described below. A mixture of chloroform/methanol (2:1) solutions of glycolipid substrate and detergent in a small glass test tube was dried under N_2. Appropriate buffer was added subequently, and the mixture was then sonicated. If necessary, bivalent cations and inhibitors for both phosphodiesterase (e.g., CDP-choline) and β-galactosidase were then added, followed by the addition of UDP-[^{14}C]galactose and cellular protein extract. The reaction mixture was incubated for various durations at 37 °C, and the reaction was terminated by the addition of 6 μmol of EDTA and 100 μL of chloroform/methanol (2:1). The entire reaction mixture was streaked quantitatively onto a 4-cm-wide strip of Whatman No. 3 filter paper and developed overnight with distilled water. The paper was then air-dried. The origin was cut out and extracted twice with 5 mL of chloroform/methanol/water (10:5:1). Recovery of labeled glycolipids after extraction from the paper was greater than 95%. The combined eluate was concentrated to dryness under N_2 and dissolved in 20 μL of chloroform/methanol (2:1). An aliquot (10 μL) of this was chromatographed on HPTLC plates (Merck, Darmstadt, West Germany) along with glycolipid standards in a solvent system composed of chloroform/methanol/water (50:40:10) and 0.05% $CaCl_2$. Radioactive glycolipids were located by autoradiography, scraped from the plates, and quantitated by liquid scintillation counting. Standard glycolipids were visualized by orcinol/sulfuric acid reaction. Counting efficiency of ^{14}C-products in the presence of silica gel was about 70%. Methods for determination of activity of each glycosyltransferase were as described in the following subsections.

(*B*) *UDP-galactose: Lactosylceramide α1→4Galactosyltransferase (α1→4Gal Transferase).* The α1→4Gal transferase activity was determined by procedures similar to those of Kijimoto and Hakomori (1971) as modified by Wiels et al. (1984). The reaction mixtures contained 50 μg of lactosylceramide, 2.5 μmol of sodium cacodylate buffer, pH 5.9, 0.3 mg of Triton X-100, 1 μmol of $MnCl_2$, 100 μg of phosphoglycerol, 0.5 μmol of galactonolactone, 0.5 μmol of CDP-choline, 42 nmol of UDP-[^{14}C]galactose (17600 cpm/nmol), and 150 μg of cellular protein in a total volume of 50 μL. The reaction mixture was incubated for 2 h at 37 °C, and the reaction product was determined as described above.

(*C*) *UDP-galactose: Globoside β1→3Galactosyltransferase (β1→3Gal Transferase).* The activity of β1→3Gal transferase was determined in reaction mixtures that contained 30 μg of globoside, 0.5 μmol of CDP-choline, 0.5 μmol of galactonolactone, 2.5 μmol of HEPES buffer, pH 7.0, 0.5 μmol of $MnCl_2$, 0.1 mg of Triton X-100, 25–50 nmol of UDP-[^{14}C]-galactose (15 000–19 000 cpm/nmol), and 250 μg of cellular protein in a final volume of 50 μL. The reaction mixture was incubated at 37 °C for 2 h. The reaction was terminated and the reaction product was examined as described above. Reaction mixtures that did not contain exogenously added globoside served as negative controls. Neutral glycolipids of Folch upper phase isolated from erythrocytes of type A individuals were used as glycolipid standards. The identity of component glycolipids in these standards had been ascertained previously by radioimmunostaining with MoAbs and by chemical analysis. In addition to comigration on HPTLC with Gal-globoside of Al upper neutral glycolipids, the reaction product was also identified as Gal-globoside by TLC immunostaining using reaction mixtures after incorporation of unlabeled sugars into acceptor globoside. The enzyme activity is susceptible to repeated freezing and thawing. This is reflected in the reduced activity seen in "complete" activity in Tables IV and V as compared to values in Table III and Figure 3.

(*D*) *UDP-N-acetylglucosamine: Lactosylceramide β1→3N-Acetylglucosaminyltransferase (β1→3GlcNAc Transferase).* β1→3GlcNAc transferase activity was determined according to the method of Holmes et al. (1987), in reaction mixtures that contained 50 μg of lactosylceramide, 0.5 μmol of $MnCl_2$, 0.025 mg of Triton CF-54, 2.5 μmol of sodium cacodylate buffer, pH 7.0, 0.5 μmol of CDP-choline, 0.025 μmol of UPD-[^{14}C]GlcNAc (26 300 cpm/nmol), and 150 μg of cellular protein in a final volume of 50 μL. The reaction mixtures were incubated at 37 °C for 1 h, and the reaction

product was determined as above.

(*E*) *CMP-sialic Acid: Lactosylceramide α2→3Sialyltransferase.* The activity of α2→3sialyltransferase was determined under optimal conditions for α2→3sialyltransferase of rat liver and human placenta. The reaction mixtures contained 50 μg of lactosylceramide, 2.5 μmol of sodium cacodylate, pH 5.5, 0.5 μmol of $MgCl_2$, 0.25 mg of Triton X-100, 54 nmol of CMP-[^{14}C]sialic acid (7060 cpm/nmol), and 150 μg of cellular protein in a final volume of 50 μL. The reaction mixture was incubated at 37 °C for 2 h, and the reaction product was determined as described above.

(*F*) *GDP-fucose: Paragloboside α1→3Fucosyltransferase (α1→3Fuc Transferase).* The activity of α1→3Fuc transferase was determined according to the procedures of Holmes et al. (1985) in a reaction mixture that contained 30 μg of paragloboside, 0.5 μmol of CDP-choline, 2.5 μmol of HEPES buffer, pH 7.2, 1 μmol of $MnCl_2$, 100 μg of detergent G3634A, 17 nmol of GDP-[^{14}C]fucose (15 000 cpm/nmol), and 650 μg of cellular protein in a final volume of 100 μL. The reaction mixture was incubated at 37 °C for 2 h, and the reaction product was quantitatively determined as described above.

Glycosylhydrolase Assays. To evaluate glycolipid-hydrolytic activity in retinoic acid treated and nontreated NT2/D1 EC cells, an aliquot of biosynthetically labeled glycolipid (see method below) was dried in a glass test tube under N_2 with 0.3 mg of Triton X-100. The dried mixture was then incubated either alone in a buffer containing 5 μmol of sodium citrate, pH 4.5, in a final volume of 100 μL or with 500 μg of cell homogenate prepared from cells treated with retinoic acid for 0, 7, 14, or 21 days in the same buffer. The incubation was carried out for 1 h at 37 °C. The reaction was terminated with chloroform/methanol (2:1). The entire incubation mixture was streaked onto a Whatman No. 3 filter paper, air-dried, and subjected to descending paper chromatography in distilled water overnight at room temperature. The paper was air-dried the next day, and the origins where the samples were applied were cut out and counted in 5 mL of scintillant, Formula 963 (New England Nuclear, Piscataway, NJ), in a scintillation counter. The amount of hydrolytic activity was expressed as percent radioactivity recovered relative to the blank, with lower values reflecting higher hydrolytic activity.

Preparation of Biosynthetically Radiolabeled Glycolipids. In order to study the enzyme activity of specific glycosylhydrolases cleaving specific residues of glycolipids, various glycolipid substrates radiolabeled by biosynthesis were prepared.

(*A*) *GM_3.* The sialic acid moiety of GM_3 was labeled with CMP-[^{14}C]sialic acid by the use of GM_3 synthetase (CMP-sialic acid:lactosylceramide α2→3sialyltransferase) from lung squamous cell carcinoma QG56. Choice of this cell line was made after an initial screening of a number of cell lines for high GM_3 synthetase activity. The packed cell pellet (0.3 mL) was suspended in 2 volumes of 50 mM sodium cacodylate buffer, pH 5.5, 40% glycerol, and 1 mM EDTA and homogenized by two strokes of a Potter–Elvehjem homogenizer. A mixture of 500 μg of lactosylceramide, 5 μmol of $MgCl_2$, and 5 mg of Triton X-100 was incubated with an aliquot of 480 μL of QG56 cell homogenate and 20 μL of CMP-[^{14}C]sialic acid (7000 cpm/nmol, 10.8 mM). The reaction mixture was incubated at 37 °C for 4 h, and the product was isolated as described above.

(*B*) *Globotriaosylceramide (Gb_3).* The terminal αGal residue of Gb_3 was labeled with [^{14}C]galactose by incubating lactosylceramide with UDP-[^{14}C]galactose and α1→4-galactosyltransferase of Burkitt lymphoma Ramos cells as previously described (Wiels et al., 1984). The reaction mixture contained 500 μg of lactosylceramide, 25 mmol of sodium cacodylate buffer, pH 5.9, 3 mg of Triton X-100, 10 μmol of $MnCl_2$, 1 mg of phosphoglycerol, 5 mmol of galactonolactone, 5 mmol of CDP-choline, 480 μL of Ramos homogenate (prepared as described for QG56 homogenate), and 20 μL of UDP-[^{14}C]galactose (19 000 cpm/nmol, 5.26 mM) and was incubated at 37 °C for 4 h.

(*C*) *Le^x Pentasaccharide Ceramide ($III^3FucnLc_4$).* The fucose moiety of this glycolipid was radiolabeled by incubation of nLc_4 with GDP-[^{14}C]fucose and α1→3fucosyltransferase of H69 cell extract. The method was essentially the same as that previously described (Holmes et al., 1985). The reaction mixture contained 300 μg of nLc_4, 1 mg of G3634A, 35 mmol of HEPES buffer, pH 7.2, 5 μmol of CDP-choline, 10 μmol of $MnCl_2$, 42.5 nmol of GDP-[^{14}C]fucose (15 000 cpm/nmol), and H69 homogenate, to total volume 1 mL. The reaction mixture was incubated at 37 °C for 4 h.

(*D*) *Gal-globoside.* The terminal galactose moiety of Gal-globoside was radiolabeled through incubation of globoside with UDP-[^{14}C]galactose with the crude homogenate of NTERA-2 cells grown at high density in the high-glucose formulation of DMEM supplemented with 10% FCS. Conditions of the reaction were as described under Glycosyltransferase Assays, except that quantities of substrate and reagent were scaled up five times.

Results

Cell Surface Expression and Chemical Changes of Carbohydrates during Retinoic Acid Induced Differentiation. Table I lists the major glycolipid antigens of NT2/D1 human EC cells and their differentiated derivatives. As we have shown previously (Fenderson et al., 1987), undifferentiated stem cells express predominantly globo-series structures, including galactosyl globoside, sialylgalactosyl globoside, and the globo-A antigen. In contrast, differentiated derivatives express predominantly lacto-series glycolipoids, including Le^x, and ganglio-series glycolipids, including GD_3, 9-*O*-acetyl-GD_3, and GT_3. In order to investigate the enzymatic basis of this dramatic, differentiation-dependent switch in glycolipid expression, NT2/D1 cells were cultured in the presence of 10^{-5} M retinoic acid for 0–28 days and then assayed in parallel experiments for either surface glycolipid expression or glycosyltransferase enzyme activity. The results of indirect immunofluorescence assays are presented in Figure 1 (see Table I for MoAb specificity). The reactivity of anti-SSEA-3 (recognizing Gal-globoside and sialyl-Gal-globoside) and anti-SSEA-4 (recognizing sialyl-Gal-globoside) with NT2/D1 cells decreased dramatically by 8 days following retinoic acid induction. By contrast, the reactivity of MoAbs R24 and A2B5 (recognizing polysialogangliosides GD_3 and GT_3, respectively) increased to a peak on days 8–12 of retinoic acid induction and then declined. Reactivity with MoAb ME311 (recognizing 9-*O*-acetyl-GD_3) also increased. A small increase in the reactivity of anti-SSEA-1 antibody (recognizing Le^x structures) was also observed on day 4 of retinoic acid treatment. These results confirm our previous immunological and biochemical evidence for glycolipid core structure switching from globo- to lacto- and ganglio-series pathways during the differentiation of NT2/D1 human EC cells (Fenderson et al., 1987).

Glycolipid Glycosyltransferase Activity during Retinoic Acid Induced Differentiation. Glycolipid core structure switching and associated terminal chain modification may depend fundamentally on the ability of cells to regulate the synthesis of a few key structures, i.e., switch from Gb_3 to Lc_3 for induction of lacto-series synthesis and switch from Gb_3 to

Table I: Glycolipid Antigens of NT2/D1 EC Cells and Their Differentiated Derivatives[a]

series	glycolipid structure	glycolipid designation	defining MoAb	expression: stem[b]	expression: dif[c]
globo	Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer	Gb_5	SSEA-3	++	-
	NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer	Gl_7	SSEA-4	++	-
	GalNAcα1→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→Cer (2 ↑ Fucα1)	globo A	HH5	++	-
lacto	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→Cer (3 ↑ Fucα1)	Le^x	SSEA-1	-	+
ganglio	NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→Cer	GD_3	R24	-	++
	(9-O-acetyl)NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→Cer	9-*O*-AcGD_3	ME311	-	++
	NeuNAcα2→8NeuNAcα2→8NeuNAcα2→3Galβ1→4Glcβ1→Cer	GT_3	A2B5	-	++

[a] Data are summarized from Fenderson et al. (1987). Asterisks refer to key enzymes whose activities were analyzed during retinoic acid induced differentiation of NT2/D1 EC cells: (*) enzymes involved in core structure assembly; (**) enzymes involved in terminal chain modification. [b] Development expression of undifferentiated stem cells. [c] Developmental expression of differentiated derivatives (various somatic cell types including neurons).

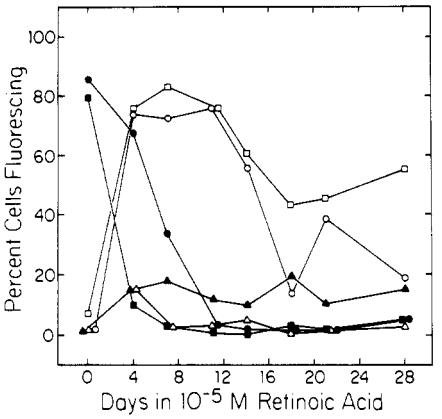

FIGURE 1: Surface carbohydrate changes associated with the retinoic acid induced differentiation of NT2/D1 EC cells, as analyzed by flow cytoflurometry. Cells were cultured in the presence of 10^{-5} M retinoic acid for 0, 4, 8, 12, 16, 20, 24, or 28 days. Trypsin-dissociated cells were labeled in suspension with either anti-SSEA-3 (anti-Gb_5 and anti-GL7, ■), anti-SSEA-4 (anti-GL7, ●), A2B5 (anti-GT_3, ○), R24 (anti-GD_3, □), ME311 (anti-9-*O*-acetyl-GD_3, ▲), or anti-SSEA-1 (anti-Le^x, △). A fluorescence threshold was set to exclude >95% of cells in samples treated with P3x63Ag8 hybridoma control supernatant.

GM_3 for induction of ganglio-series synthesis. The glycosyltransferases involved in synthesizing these key core structures include the α1→4Gal transferase (globo core), the β1→3GlcNAc transferase (lacto core), and the α2→3sialyltransferase (ganglio core). Retionic acid induced changes in the levels of these enzymes or their activities would provide a mechanism for core switching during EC differentiation. Alternatively, glycosyltransferases involved in core structure assembly, chain elongation, or terminal chain modification may all be coordinately regulated during this differentiation process. In order to address this question, the activities of enzymes involved in the core synthesis or terminal modification of globo-, lacto-, or ganglio-series glycolipids were analyzed. Similar results were obtained in replicate assays using replicate cell cultures. A 3- to 4-fold reduction of α1→4Gal transferase activity (Figure 2A for synthesis of Gb_3) and of β1→3Gal transferase (Figure 2B for synthesis of Gal-Gb_4) was clearly observed by 8 days following retinoic acid induced differentiation. These changes in enzyme activity parallel the differentiation-dependent decline in globo-series glycolipid antigens SSEA-3 and SSEA-4 observed at the cell surface (Figure 1). By contrast, the activities of β1→3GlcNAc transferase (Figure 2C for synthesis of Lc_3) and α1→3Fuc transferase (Figure 2D for synthesis of Le^x structure) were enhanced 2- to 4-fold during differentiation. Similarly, the activity of α2→3sialyltransferase (Figure 2E for synthesis of GM_3) was also enhanced approximately 4-fold during retinoic acid induction. Although these differentiation-dependent changes in enzyme activity broadly parallel increases in both lacto- and ganglio-series glycolipid antigens Le^x, GD_3, 9-*O*-acetyl-GD_3, and GT_3 observed in cell surface labeling assays (Figure 1), the enzyme activity does not necessarily reflect the net level of glycolipid antigen on the cell surface throughout the entire period of differentiation. This is evident in comparison of data in Figures 1 and 2. The enzymes involved in lacto-series synthesis (Figure 2C,D) have increased activity throughout the entire culture period in retinoic acid, although Le^x glycolipids on the cell surface decline after 4 days in culture. Similarly, gangliosides are at maximal levels after 4–12 days of culture and then decline, while the enzyme activity continues to rise about 4-fold throughout the rest of the culture period (Figure 2E). One possible explanation for this might be that the antigen expression follows glycolipid core chain synthesis.

Glycolipid Glycosylhydrolase Activity during Retinoic Acid Induced Differentiation. The results described above suggested that coordinate changes in glycosyltransferase activities may indeed regulate glycolipid core structure switching during the differentiation of human NT2/D1 EC cells. However, dramatic changes in the synthesis and expression of membrane glycolipids can also be induced by the enhancement or reduction of specific hydrolytic enzymes. Therefore, radiolabled glycolipids were used as substrates to assay relevant hydrolytic enzymes. The results of these assays are presented in Table II. As shown in the first two columns, α1→4galactosidase (which hydrolyzes the terminal αGal residue of Gb3) and β1→3galactosidase (which hydrolyzes the terminal β1→3Gal residue of Gal-globoside) were unchanged during retinoic acid induced differentiation. Similarly, α2→3sialidase (which hydrolyzes the terminal sialic acid of GM_3), α1→3fucosidase (which hydrolyzes the fucosyl residue of Le^x structures), and β1→3*N*-acetylglucosaminidase (which hydrolyzes the terminal GlcNAc of Lc_3) were unchanged during the retinoic acid

Table II: Glycolipid Hydrolase Activity in NT2/D1 EC Cells during Retinoic Acid Induced Differentiation[a]

^{14}C-labeled biosynthetic substrate	hydrolytic enzyme tested	conditions	rel recovery[b] of radioact. (%)
CTH ([^{14}C]Gal)	α1→4galactosidase	no enzyme	100
		0 days in RA	123
		7 days in RA	108
		14 days in RA	110
		21 days in RA	115
Gal-Gb_4 ([^{14}C]Gal)	β1→3galactosidase	no enzyme	100
		0 days in RA	102
		7 days in RA	115
		14 days in RA	97
		21 days in RA	100
GM_3 ([^{14}C]sialic acid)	α2→3sialidase	no enzyme	100
		0 days in RA	150
		7 days in RA	151
		14 days in RA	160
		21 days in RA	160
Le^x pentasaccharide ([^{14}C]Fuc)	α1→3fucosidase	no enzyme	100
		0 days in RA	118
		7 days in RA	115
		14 days in RA	110
		21 days in RA	130
GlcNAc-Gal-Glc-Cer ([^{14}C]GlcNAc)	β1→3*N*-acetylglucosaminidase	none	100
		0 days in RA	94
		7 days in RA	84
		14 days in RA	92
		21 days in RA	95

[a] Procedures for the biosynthesis of radiolabeled substrates and assay conditions for the endogenous hydrolytic enzymes are described under Materials and Methods. In brief, endogenous hydrolytic activity in NT2/D1 cells that were cultured in the presence of retinoic acid (RA) for 0, 7, 14, or 21 days was determined by incubating each of the biosynthetically labeled glycolipid substrates with or without cell homogenate under the optimal conditions for the hydrolytic enzymes. [b] The data represent the amount of substrate recovered from samples that contained cell homogenate compared with samples that did not. Similar results were obtained in replicate assays.

induced differentiation of NT2/D1 cells. Thus, glycosylhydrolases do not appear to play a significant role in controlling changes in glycolipid antigen expression in differentiating NT2/D1 EC cells.

Characterization of UDP-galactose: Globoside β1→3-Galactosyltransferase. The β1→3Gal transferase (for synthesis of Galβ1→3Gb_4) is a previously uncharacterized enzyme that plays a key role in the synthesis of extended globo-series structures. These structures include the oncodevelopmental antigens SSEA-3 and SSEA-4, as well as the globo-series ABH blood group antigens. Therefore, the catalytic properties of this enzyme were explored in detail.

Effects of Detergent. Among various detergents tested, Triton CF-54 showed the strongest enhancing activity: 25-fold (Table III). Empigen BB, G3634A, hexadecyltrimethylammonium bromide, and Brij 58, some of which have been shown to markedly enhance the activity of other enzymes, were found to be rather inhibitory for this enzyme. Since Triton CF-54 showed the strongest enhancement of β1→3Gal transferase activity, its concentration dependence was determined (Figure 3). The concnetration range of Triton CF-54 yielding maximum enhancement was relatively narrow, with an optimum effect at 0.2% (v/v).

Requirements for Cation, Hydrolase Inhibitors, and Other Reagents. Optimum conditions for enzyme activity are shown in Table IV. These include Gb_4 substrate, Triton CF-54 detergent, and the presence of Mn^{2+}. However, CDP-choline (which inhibits phosphodiesterase) and galactonolactone (which inhibits β-galactosidase) were not required for maximal expression of the enzyme, as shown in Table IV. Addition of *N*-ethylmaleimide did not reduce the enzyme activity, suggesting that the Gal transferase is not a sulfhydryl enzyme.

Optimal pH. The optimum pH for enzyme activity was determined by various buffer systems. As shown in Figure 4, maximal activity was obtained with Tris buffer, pH 7.0.

Effects of Phospholipids. The activity of glycosyltransferases is often affected by addition of phospholipid (Basu et al., 1968; Holmes & Hakomori, 1987). Therefore, the activity of this enzyme, with and without addition of various phospholipids, was compared (Table V). With the exception of an enhancement caused by phosphatidic acid (PA), all

Table III: Effects of Detergent on β1→3Gal:Globoside Galactosyltransferase Activity[a]

detergent	sp act. [pmol/(mg of protein·h)]
none	75
Triton X-100	430
Triton CF-54	1944
Triton CF-54/Tween 80 (2:1)	229
Brij 58	13
sodium deoxycholate	657
Empigen BB	2
G3634 A	8
hexadecyltrimethylammonium bromide	3

[a] The reaction mixtures contained 250 μg of crude homogenate of cell protein, 50 mM of sodium cacodylate, pH 7.0, 10 mM of $MnCl_2$, 50 μg of globoside, and 25 nmol of UDP-[^{14}C]galactose (13754 cpm/nmol) in a final volume of 50 μL with or without detergent supplement at a final concentration of 0.1%. Incubation was conducted at 37 °C for 2 h. Similar results were obtained in four separate experiments.

Table IV: Cation and Hydrolase Inhibitor Requirements for β1→3Gal:Globoside Galactosyltransferase Activity[a]

conditions	pmol/(h·mg of protein)	conditions	pmol/(h·mg of protein)
complete	352	$-Mn^{2+}$, $+Ca^{2+}$	21
−Gb4	25	$-Mn^{2+}$, +EDTA	12
−CF-54	54	−CDP-choline	283
$-Mn^{2+}$	15	−galactonolactone	310
$-Mn^{2+}$, $+Mg^{2+}$	15	+*N*-ethylmaleimide	462

[a] The standard reaction mixture was modified as shown. Divalent cations, EDTA, CDP-choline, galactonolactone, or *N*-ethylmaleidmide was present at 10 mM final concentration where indicated. Similar results were obtained in four separate experiments.

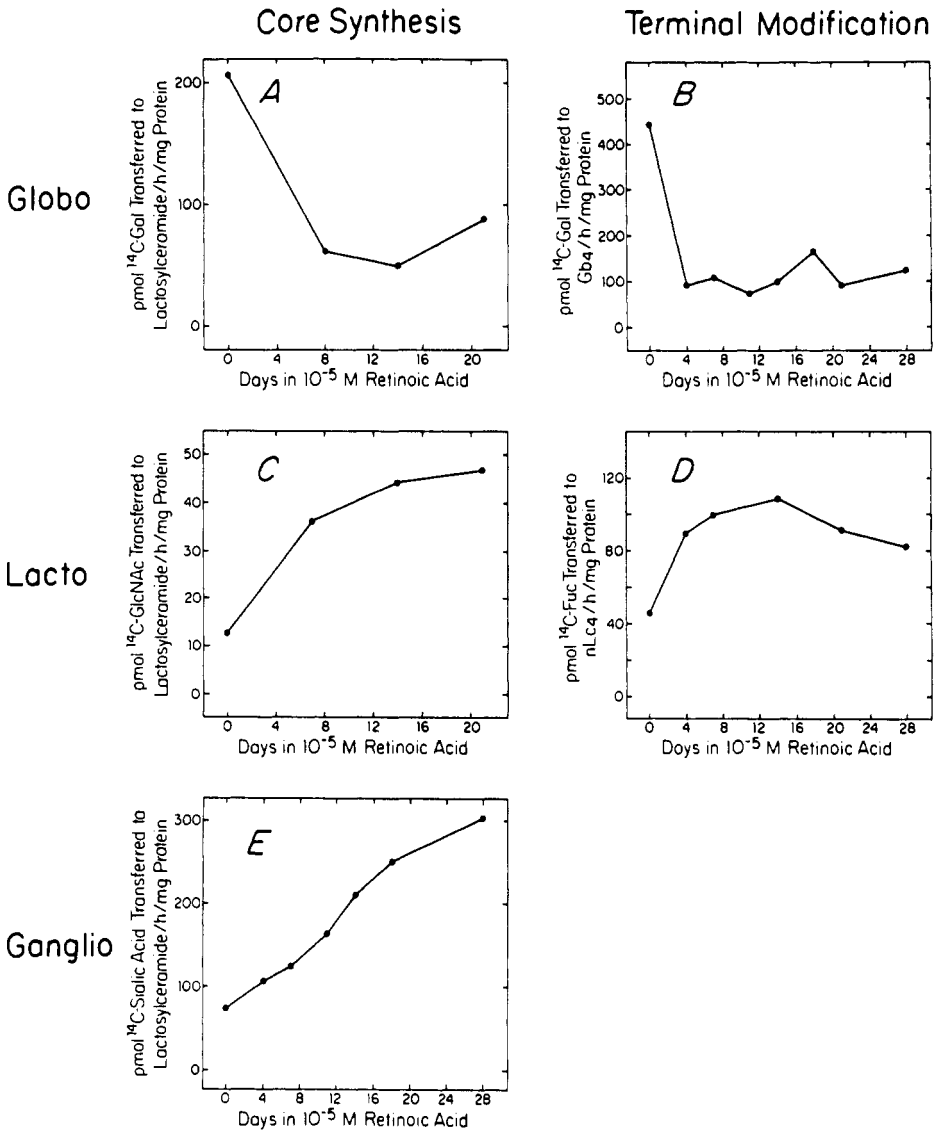

FIGURE 2: Changes in NT2/D1 glycolipid glycosyltransferase activity during retinoic acid induced differentiation. NT2/D1 EC cells were cultured in the presence of 10^{-5} M retinoic acid for 0, 4, 8, 12, 16, 20, 24, or 28 days. The activities of enzymes involved in either *core synthesis* or *terminal modification* of globo-, lacto-, or ganglio-series glycolipids were analyzed: (A) α1→4Gal transferase; (B) β1→3Gal transferase; (C) β1→3GlcNAc transferase; (D) α1→3Fuc transferase; (E) α2→3sialyltransferase. Enzyme assays were performed under optimal conditions as described under Materials and Methods. During differentiation, the activities of glycosyltransferases involved in globo-series biosynthesis (A, B) declined, while the activities of glycosyltransferases involved in lacto-series (C, D) and ganglio-series (E) glycolipid biosynthesis increased. Similar results were obtained in replicate assays, using replicate cell cultures.

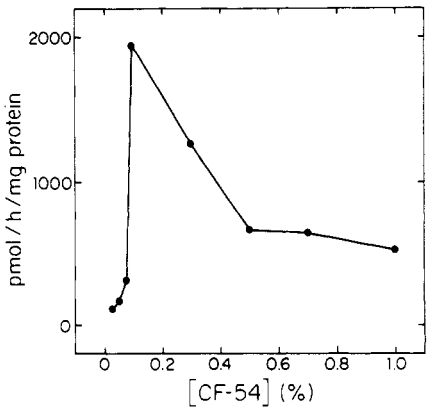

FIGURE 3: Effect of Triton CF-54 detergent concentration on the activity of β1→3Gal:globoside galactosyltransferase in NT2/D1 EC cells. Assay mixtures contained 250 μg of cell homogenate protein from undifferentiated stem cells. With the exception of varying detergent concentration, the assays were performed under optimum conditions as described under Materials and Methods.

Table V: Effects of Phospholipid on β1→3Gal:Globoside Galactosyltransferase Activity[a]

conditions	pmol/(h·mg of protein)	conditions	pmol/(h·mg of protein)
without addition	352	+PA	468
+egg yolk PC	39	+PG	150
+egg yolk PE	129	+PS	199
+bovine brain PE	263	+sphingomyelin	126

[a] The standard reaction mixture was modified as shown. The phospholipid was present at a final concentration of 2 mg/mL. Similar results were obtained in four separate experiments.

phospholipids added to the assay system decreased enzyme activity.

Substrate Specificity and Characterization of Biosynthesized Product. The β1→3Gal transferase is highly specific in its requirement of globoside as a substrate (Table VI). The identity of product Gal-globoside was ascertained by radioimmunostaining with anti-SSEA-3 MoAb. The band corresponding to galactosyl β1→3globoside increased greatly only

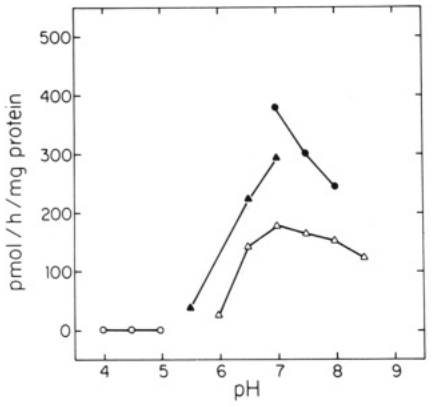

FIGURE 4: Determination of pH optimum for β1→3Gal:globoside galactosyltransferase activity in NT2/D1 EC cells. Assays were performed in either 50 mM ammonium acetate buffer (○), 50 mM sodium cacodylate buffer (▲), 50 mM Tris buffer (●), or 50 mM HEPES buffer (△), under optimal conditions as described under Materials and Methods.

Table VI: Acceptor Specificity of β1→3Gal:Globoside Galactosyltransferase

acceptor	pmol/(h·mg of protein)	acceptor	pmol/(h·mg of protein)
Gb_4	898	A^b	4
CMH	21	asialo-GM_2	15
CDH	274		

[a] The reaction mixtures were supplemented with 50 μg of the indicated glycolipid and incubated as described under Materials and Methods. Similar results were obtained in four separate experiments.

in the presence of globoside (Gb_4) as a substrate, as indicated by strong staining by anti-SSEA-3 MoAb (Figure 5, lane +Gb_4). Asialo-GM_2 (Gg_3), which has a terminal GalNAcβ1→4Gal structure, was not an acceptor for this enzyme. However, a small degree of Gal transfer to lactosylceramide was observed. This product was identified as Galα-CTH by radioimmunostaining with MoAb 1A4-E10. Thus, the crude homogenate of undifferentiated NT2/D1 cells may contain a small amount of α1→4Gal transferase in addition to the large amount of β1→3Gal transferase.

K_m Analysis. Enzyme kinetics were analyzed by varying the concentration of globoside (acceptor) from 0.01 to 0.4 mM and by varying the concentration of UDP-galactose (donor) from 0.02 to 0.5 mM. A Hanes–Woolft plot of these data is presented in Figure 6. K_m values for the β1→3Gal transferase were determined to be 0.02 mM (acceptor) and 0.18 mM (donor).

Discussion

The most remarkable information obtained by application of MoAbs has been the identification of dramatic changes in chemically defined glycoconjugate structures during ontogeny, as well as oncogenesis. In the present study, we have use a model system, i.e., retinoic acid induced differentiation of the human embryonal carcinoma cell line NT2/D1, to study the enzymatic basis of these changes. When cultured in the presence of retinoic acid, NT2/D1 EC cells differentiate into a variety of somatic cell types, including neurons (Andrews, 1984). Associated with these changes in cellular phenotype, NT2/D1 EC cells display dramatic switching of glycolipid structure from globo series to lacto and ganglio series (Fenderson et al., 1987).

In the present study, clear changes in three glycosyltransferase systems were observed. The first system includes two Gal transferases involved in globo-series glycolipid bio-

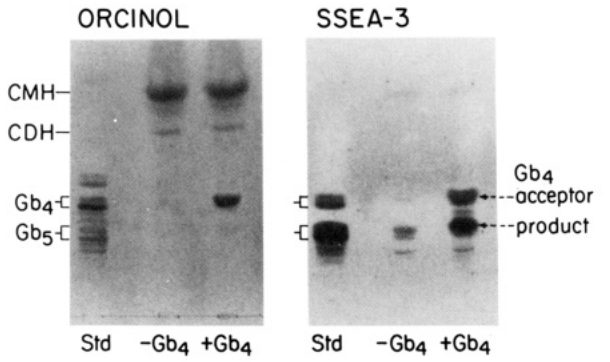

FIGURE 5: Identification of product Galβ1→3 globoside by TLC immunostaining. Glycosyltransferase assays were performed as described under Materials and Methods with unlabeled UDP-Gal and with Gb_4 as an exogenous acceptor. Glycolipids extracted from samples, with or without Gb_4 acceptor (±Gb_4), are included on the right side of each panel. Upper-phase neutral glycolipids obtained from NT2/D1 EC cells are also included as standards (Std). Plates were developed using chloroform/methanol/water (50:40:10) containing 0.05% $CaCl_2$ and either stained for carbohydrate by the orcinol/H_2SO_4 reaction (orcinol) or labeled with MoAb MC630, defining Gb_4 and Galβ1→3Gb_4 (SSEA-3).

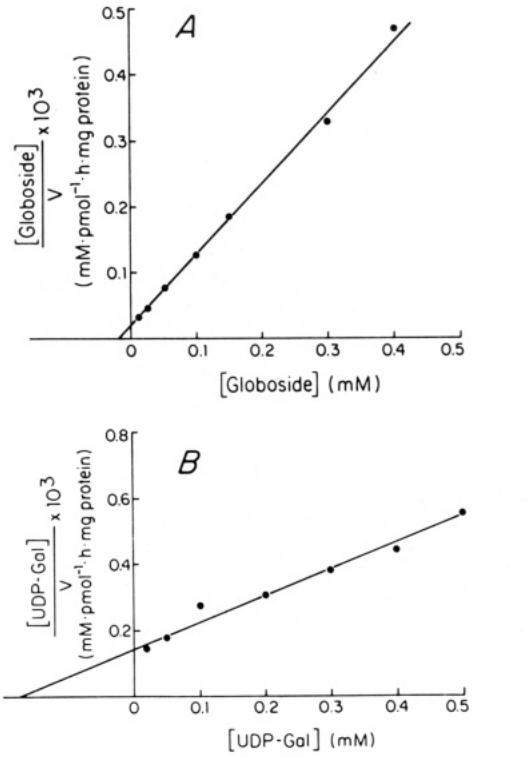

FIGURE 6: Hanes–Woolf plot of enzyme saturation kinetics. Data for β1→3Gal:globoside galactosyltransferase activity were obtained by varying the concentration of globoside from 0.01 to 0.4 mM in the presence of 250 μg of protein and 35 nmol of UDP-[^{14}C]galactose per 50-μL reaction mixture (panel A), and by varying the concentration of UDP-[^{14}C]galactose from 0.02 to 0.5 mM in the presence of 250 μg of protein and 765 μM of Gb_4 (panel B).

synthesis: α-Gal transferase for synthesis of Gb_3 and β-Gal transferase for synthesis of β1→3Gal-globoside (IV$^3\beta$-GalGb_4), the "stage-specific antigen 3". These enzyme activities declined dramatically in parallel with the decline in cell surface globo-series structures during retinoic acid induced differentiation. The second enzyme system, for the synthesis of lacto-series glycolipids, includes β1→3GlcNAc transferase and α1→3Fuc transferase. These enzymes are responsible for the synthesis of Le^x structures, and the activity of these enzymes increased during differentiation. The third enzyme system is responsible for the first step in the synthesis of ganglio-series glycolipids;

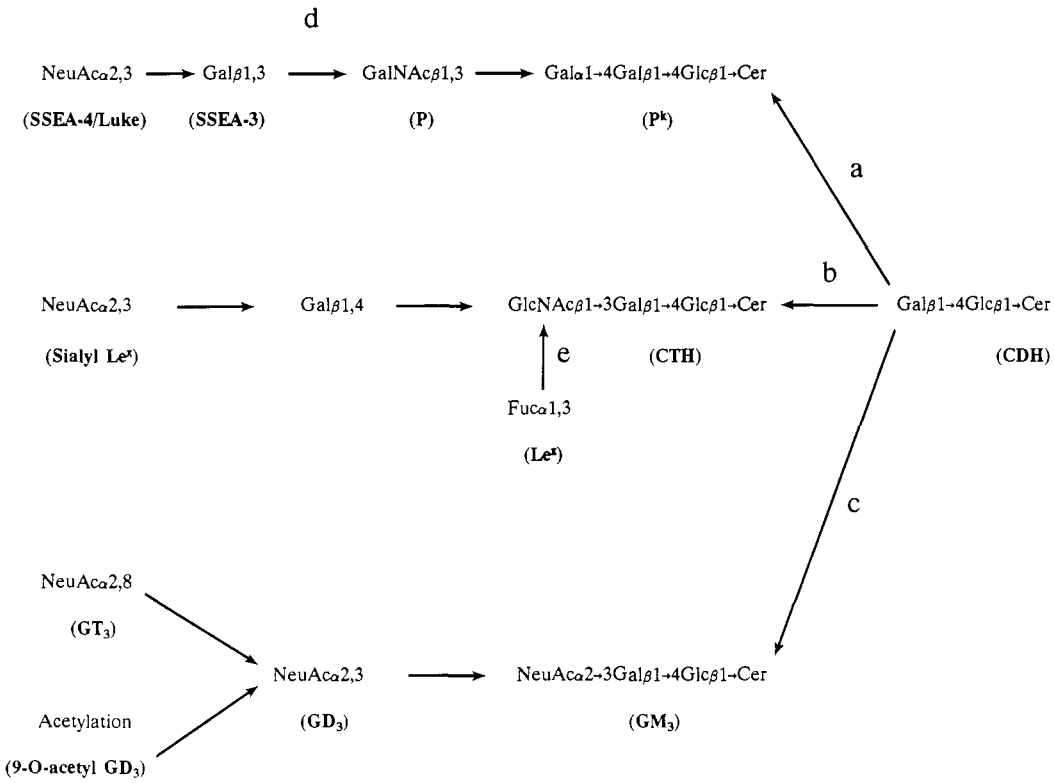

FIGURE 7: Scheme summarizing the major glycolipid antigens of human EC cells, their changes during retinoic acid induced differentiation, and the enzymatic basis of these glycolipid changes. Undifferentiated NT2/D1 stem cells are characterized by the predominance of globo-series glycolipids, including histo-blood group antigens P^k, P, SSEA-3, and SSEA-4 (LUKE). When differentiation of these cells is induced, the synthesis of globo-series glycolipids declines, while the synthesis of lacto-series glycolipids (including sialyl type 2 chain and Le^x) and that of ganglio-series glycolipids (including GM_3, GD_3, GT_3, and 9-*O*-acetyl-GD_3) increase dramatically. The results presented here indicate that this process of glycolipid core structure switching is regulated by appropriate changes in the activities of key glycosyltransferases. Undifferentiated NT2/D1 stem cells express high levels of α1→4Gal transferase activity (pathway a) controlling globo-series biosynthesis. During differentiation, this enzyme activity is greatly reduced, while α1→3GlcNAc transferase (pathway b) and α2→3sialyltransferase (pathway c) are greatly enhanced. Glycosyltransferases involved in terminal chain modification are also developmentally regulated: the activity of the β1→3Gal transferase for globoside extension (pathway d) is reduced, whereas the activity of the α1→3Fuc transferase for Le^x synthesis (pathway e) is enhanced during differentiation.

i.e., CMP-sialic acid:LacCer-α2→3sialyltransferase, also increased greatly during retinoic acid induced differentiation. These changes in glycosyltransferase activity, and the proposed effect of these enzyme changes on glycolipid antigen expression, are summarized in Figure 7. It should be noted that because NT2/D1 EC cells differentiate into a variety of somatic cell types, the changes observed represent an average change of enzymatic activity for a heterogeneous cell population.

Retinoic acid induced differentiation of NT2/D1 human EC cells appears to be accompanied by coordinate changes in the expression, levels, or activities of multiple glycosyltransferases involved in core structure assembly and terminal chain modification. Although the specific sequence of molecular events leading to altered enzyme activity in differentiating EC cells remains totally unknown, the effect of retinoic acid on the glycosyltransferases seems to be an indirect one. Preliminary observations indicate that the incubation of crude cell homogenate of NT2/D1 (which had not been exposed to retionic acid) with 10^{-8}–10^{-2} M retionic acid under standard assay conditions does not affect β1→3Gal:globoside Gal transferase activity to any appreciable extent. Retinoic acid is known to be natural morphogen (Thaller & Eichele, 1987), and an intracellular receptor with DNA-binding activity has been identified recently (Giguere et al., 1987). Thus, retinoic acid treatment may result in specific changes in the transcription of multiple glycosyltransferase genes. Alternatively, epigenetic mechanisms of enzyme regulation can also be envisioned. The activities of glycosyltransferases may be controlled posttranslationally by phosphorylation (Burczak et al., 1984) or glycosylation (Ivatt, 1981). It may be possible to discriminate among these alternative mechanisms in the near future as cDNA probes for the glycosyltransferases become available.

Glycolipid core structure switching from one series to another has been observed previously. The first report described globo- to lacto-series changes associated with the carcinogenic transformation of hamster NIL cells induced by polyoma virus. Nontransformed NIL cells were characterized by the presence of globo-series structures (Gb_3, Gb_4, and Forssman) and the absence of lacto-series structures. Upon transformation, synthesis of globo series (e.g., Gb_3) was inhibited, and synthesis of lacto series (e.g., paragloboside) was initiated (Gahmberg & Hakomori, 1975). Another example is the differentiation of murine myelogenous leukemia M1 cells along a macrophage lineage. Undifferentiated cells were characterized by the predominance of lacto-series structures, which were converted to ganglio- and finally to globo-series structures, at the terminal stage of macrophage differentiation (Kannagi et al., 1983b). However, the enzymatic basis of these changes has not been explored.

In addition to characterizing changes in the glycosyltransferases responsible for switching from globo- to lacto- and ganglio-series structures during differentiation, we have also characterized the β1→3Gal transferase, responsible for the synthesis of β1→3Gal-globoside, i.e., extended globo structures. Other enzymes that are capable of transfering Gal in β linkage have been reported in the literature. These include β1→3Gal

transferases for GM_1 synthesis in chick embryonic brain (Basu et al., 1965, 1984) and rat liver (Kaplan & Hechtman, 1983), a β-Gal transferase in rabbit bone marrow for paragloboside synthesis (Basu & Basu, 1972), a soluble β-Gal transferase in bovine milk (Bushway & Keenan, 1979), a β1→4Gal transferase that transfers Gal to glucosylceramide producing CDH or to GlcNAc–CTH producing paragloboside (Yamato & Yoshida, 1982), a β1→3Gal transferase in pig trachea (Sheares & Carlson, 1983), a β1→3Gal transferase in chick embryonic liver that uses asialo ovine submaxillary mucin as acceptor (Furukawa & Roth, 1985), a β1→4Gal transferase in chick embryonic liver that transfers Gal to GlcNAc or asialo-agalacto-α_1-acid glycoprotein (Furukawa & Roth, 1985), and a β1→3Gal transferase in human kidney that transfers Gal to lactosylceramide (Bailly et al., 1988). The β1→3Gal:globoside Gal transferase in our study is presumably analogous to these other β-Gal transferases. The enzymatically synthesized product βGal-globoside was characterized only by immunostaining with anti-SSEA-3 antibody, since the Gal residue of Gal-globoside is highly resistant to various β- as well as α-galactosidases (Blomberg et al., 1982; Kannagi et al., 1983a), and its identification as β-Gal structure was based on NMR spectroscopy (Kannagi et al., 1983a). The β1→3Gal:globoside transferase characterized here is activated in the presence of Mn^{2+} and requires globoside (Gb_4) as a specific substrate. Enzyme activity is optimal at pH 7.0 and is not enhanced by addition of phospholipid. Apparent K_m values for globoside and UDP-Gal were determined as 0.02 mM and 0.18 mM, respectively. It is interesting that all of the β-Gal transferases characterized to data appear to have a similar requirement for Mn^{2+} and an optimal activity at or near neutral pH.

β1→3Gal transferase activity is necessary for the subsequent synthesis of several oncodevelopmentally regulated antigens, including SSEA-4. Since SSEA-4 has been associated with the human LUKE (or LKE) red blood cell antigen (Tippett et al., 1986), the question arises as to whether this enzyme is active in LKE (–) individuals. Aside from erythrocytes, the presence of globo-series glycolipids is highly limited in normal human adult tissues. However, globo-series glycolipids appear to be expressed predominantly during early embryogenesis (Willison et al., 1982), and their presence in large quantity in human EC cells may be seen in the light of the proposition, by analogy with murine EC cells, that human EC cells possess an early embryonic character. It is of crucial importance, therefore, to understand the mechanism of expression of this enzyme during diffentiation, since extended globo-series structures may play a role in embryonic cell recognition.

In summary, the results indicate that glycolipid core structure switching during the differentiation of human EC cells is regulated by changes in the activities of key glycosyltransferases. By implication, stage-specific changes in complex glycosyltransferase networks may provide a fundamental mechanism for controlling the ontogeny of cellular diversity during human embryonic development.

Acknowledgments

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Registry No. Gb_4, 11034-93-8; Gb_5, 82708-17-6; [^{14}C]Gb_3, 118436-86-5; [^{14}C]Gb_5, 118436-83-2; GD_3, 104443-61-0; 9-*O*-AcGD_3, 110069-37-9; Gg_3, 35960-33-9; Gl_7, 86903-81-3; [^{14}C]GM_3, 118436-84-3; GT_3, 73904-48-0; Le^x, 73201-40-8; [^{14}C]Le^x, 118436-85-4; globo A, 95032-09-0; retinoic acid, 302-79-4; glycosyltransferase, 9033-07-2; α1→4galactosyltransferase, 106769-65-7; β1→3-galactosyltransferase, 118473-70-4; β1→3*N*-acetylglucosaminyltransferase, 83682-80-8; α1→3fucosyltransferase, 118473-69-1; α2→3sialyltransferase, 55071-95-9; glycosylhydrolase, 9032-92-2; α1→4galactosidase, 58561-52-7; β1→3galactosidase, 9031-11-2; α2→3sialidase, 9001-67-6; α1→3fucosidase, 83061-50-1; β1→3*N*-acetylglucosaminidase, 9012-33-3.

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DNA Binding Properties and Replication Activity of the T Antigen Related D2 Phosphoprotein†

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ABSTRACT: According to earlier genetic experiments, a region within the N-terminal 50-100 amino acids may be important for the replication function of T antigen, the initiator protein of simian virus 40 (SV40). We have investigated this possibility using the T antigen related D2 protein in several biochemical assay systems. D2 protein, a phosphoprotein coded for by the adeno-SV40 hybrid virus Ad2$^+$D2, shares its 594 C-terminal amino acids with authentic T antigen and its 104 N-terminal amino acids with an adenovirus structural protein. We confirmed earlier studies showing that D2 protein appeared to bind well to specific binding sites in the SV40 origin of replication. We found, however, that D2 protein was rather inefficient, inducing the unwinding of the double-stranded origin region, and was much less active than authentic T antigen as an initiator of in vitro SV40 DNA replication. We interpret these findings to indicate that D2 protein molecules associate with the origin to form an aberrant complex that is quite inefficient, inducing DNA unwinding and the establishment of replication forks. The possibility that the N-terminus may be required for an optimal arrangement of T antigen at the origin was supported by results of dephosphorylation studies. Dephosphorylation of N-terminal phosphoamino acids had significant effects on the stability of D2 protein-origin complexes.

Simian virus 40 (SV40) encodes its own replication initiator, commonly referred to as the large T antigen, a product of an "early" viral gene. T antigen, a nuclear phosphoprotein of 708 amino acids, has 2 functional domains known to be associated with its replication activity [for reviews, see Bradley and Livingston (1987) and Stahl and Knippers (1987)] (Figure 1).

One domain is responsible for the specific interaction of T antigen with the viral origin of replication. An analysis of nucleotide exchange and deletion mutants has shown that the DNA binding domain begins at amino acid residue 139 and extends at least to amino residue 259 (Paucha et al., 1986; Strauss et al., 1987; Arthur et al., 1988), but T antigen regions up to amino acid residue 371 are found in close contact with DNA in DNA-T antigen complexes (Simmons, 1988). This latter region includes a Zn-finger loop (Berg, 1986), a structural element known to be involved in DNA binding and, possibly, in protein-protein interactions (Klug & Rhodes, 1987; Evans & Hollenberg, 1988).

A second domain carries an ATPase/helicase activity which is required for the opening of duplex DNA at the origin (Dean et al., 1987a,b; Wold et al., 1987; Stahl et al., 1988) as well as for the continued unwinding of double-stranded DNA ahead of the replication forks (Stahl et al., 1986; Wiekowski et al., 1987). The boundaries of the ATPase/helicase domain are

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