Activation of CMP-N-acetylneuraminic Acid:Lactosylceramide Sialyltransferase during the Differentiation of HL-60 Cells Induced by 12-O-Tetradecanoylphorbol-13-acetate*

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We previously reported that the synthesis of NeuAc(2→3)Gal(β1→4)GlcCer (G₃₃) ganglioside was preferentially enhanced during the differentiation of HL-60 cells into a monocyte/macrophage lineage induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). Since exogously added G₃₃ ganglioside was shown to be able to induce the differentiation of HL-60 cells into the monocyte/macrophage lineage in a synthetic medium, the functional role of the G₃₃ ganglioside increase during the differentiation of HL-60 cells has become the subject of much interest. In the present study, we investigated the activity of CMP-NeuAc:lactosylceramide sialyltransferase, which catalyzes the synthesis of G₃₃ ganglioside from lactosylceramide, in cells undergoing differentiation induced by two different reagents, TPA and 1α,25-dihydroxyvitamin D₃, which induce the differentiation of HL-60 cells into the monocyte/macrophage lineage through different modes of action. We showed that the activation of CMP-NeuAc:lactosylceramide sialyltransferase and the increase in G₃₃ ganglioside were not related to the differentiated lineage but to the specific action of TPA, i.e. activation of protein kinase C.

A human promyelocytic leukemia cell line, HL-60, bipotentially differentiates into a granulocyte or monocyte/macrophage lineage on induction by various chemical reagents (1–8). Dimethyl sulfoxide and retinoic acid induce the differentiation of HL-60 cells into the granulocyte lineage (1–4), while 12-O-tetradecanoylphorbol-13-acetate (TPA)¹ and 1α,25-dihydroxyvitamin D₃, which induce the differentiation of HL-60 cells into the monocyte/macrophage lineage through different modes of action. As for the alterations of surface markers on HL-60 cells during the differentiation, a remarkable change in ganglioside expression has been demonstrated (9, 10), i.e. HL-60 cells which express both lacto- and ganglio-type gangliosides predominantly express G₃₃ ganglioside, a ganglio-type ganglioside, during differentiation induced by TPA (9).

Recently, further attention has been focused on the biological roles of gangliosides, because some gangliosides were reported to regulate cell growth and to induce the differentiation of neuroblastoma cells and leukemia cells (11–14). In particular, G₃₃ ganglioside regulates the phosphorylation of the growth factor receptor in fibroblasts (15) and induces the differentiation of HL-60 cells into a monocyte/macrophage lineage (12–14). Thus, there is a close relationship between the alteration of gangliosides and the differentiation of HL-60 cells induced by TPA. For a more detailed understanding of the biological role of G₃₃ ganglioside during the differentiation of HL-60 cells, it is necessary to determine whether the increase in G₃₃ ganglioside is caused by the differentiation into the monocytes/macrophage lineage or by the specific action of TPA, which is unrelated to the differentiation.

With regard to this, we examined the expression of G₃₃ ganglioside and measured the activity of CMP-N-acetylneuraminic acid:lactosylceramide sialyltransferase (CMP-NeuAc:LacCer sialyltransferase), the synthetase for G₃₃ ganglioside, in HL-60 cells treated with various chemical reagents which induce the differentiation of the cells into the monocyte/macrophage lineage.

EXPERIMENTAL PROCEDURES

Materials—TPA, PDBu, PDBz, 4α-PDD, and phorbol were purchased from Funakoshi Co. (Tokyo). Teleocidin and 1α,25-dihydroxyvitamin D₃ were generously donated by Dr. H. Fujiki (National Cancer Research Institute, Tokyo) and Dr. T. Suda (Showa University, Tokyo), respectively. Retinoic acid, Triton CF54 and CMP-NeuAc were obtained from Sigma, Sep-Pak C18 cartridges from Waters Associates, and precoated silica gel 60 thin layer plates from E. G. Merek (Darmstadt, Federal Republic of Germany). [9-¹⁴C]CMP-NeuAc and [4,5,6,7,8,9-¹³C]CMP-NeuAc were purchased from New England Nuclear.

Cell Culture—HL-60 cells, originally isolated by Dr. R. C. Gallo, were provided by Dr. M. Terada (National Cancer Research Institute, Tokyo). The cells were cultured in RPMI 1640 medium (Nissui Co., Tokyo) supplemented with 10% heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO₂ in air at 37 °C. In all experiments, logarithmically growing cells were inoculated into fresh medium at a concentration of 1 × 10⁵ cells/ml. For the differentiation of HL-60 cells, the cells were incubated with various reagents for the periods indicated.

Sialyltransferase Activity Assay—Sialyltransferase activity was measured using Sep-Pak C18 cartridges (16). Briefly, HL-60 cells were washed with phosphate-buffered saline three times and then suspended in 500 μl of 30 mM Tris/Cl buffer (pH 7.3), 0.25 mM sucrose, 1 mM EDTA. The suspended cells were then sonicated twice for 15 s each time. After centrifugation at 700 × g for 10 min, the resulting supernatant was used as the enzyme source. Unless other-
wise stated, the incubation mixtures contained, in a total volume of 500 μl of 100 mM sodium cacodylate buffer (pH 6.0), 0.3% Triton CF54, 2 μg of LacCer, 250 nmol of [3H]CMP-NeuAc (4 μCi/mmol), 50–100 μg of enzyme protein, and 500 nmol of MnCl₂. Each mixture was incubated at 37 °C for 30 min. The reaction products and [3H]CMP-NeuAc were separated with a Sep-Pak C18 cartridge as described previously (16). Each assay was performed in triplicate, and the value obtained without the added lipid acceptor was subtracted from all data as a control, except for that in Fig. 3. The protein content of the membrane fraction was determined by the method of Lowry et al. (17).

Analysis of Gangliosides by Thin Layer Chromatography—Gangliosides were prepared from untreated cells and cells treated with 10 ng/ml of TPA, teleocidin, mezerein, PDBu, PDBz, 4a-PDD, phorbol, or 50 ng/ml of 1α,25-dihydroxyvitamin D₃ for the periods indicated (9). The gangliosides from 10⁷ cells were applied to a thin layer plate, which was developed with chloroform/methanol/water (60:40:9, v/v/v). Gangliosides on the plate were detected with resorcinol reagent.

Identification of the Reaction Products—[14C]CMP-NeuAc was used instead of [3H]CMP-NeuAc to detect the reaction products. 500 μM [14C]CMP-NeuAc (54 μCi/mmol) was incubated with 2 μg of LacCer and 1 mg of homogenate protein from TPA-treated HL-60 cells in a reaction mixture as described above at 37 °C for 120 min. The reaction was stopped by adding 100 μl of 0.25 M EDTA (pH 7.4) and 1 ml of 0.1 M KCl. The reaction mixture was then applied to a Sep-Pak C18 cartridge to separate [14C]NeuAc-labeled gangliosides from [14C]CMP-NeuAc. After the cartridge had been washed with distilled water, the [14C]NeuAc-labeled gangliosides were eluted with 2 ml of methanol and 4 ml of chloroform/methanol (2:1, v/v). The elate was evaporated under a N₂ stream, dissolved in 20 μl of chloroform/methanol (2:1, v/v), and then applied on a silica gel thin layer plate, which was developed with chloroform/methanol/water (60:40:9, v/v/v). The plate was exposed to Kodak X-Omat AR film at −80 °C for 1 week.

Assay for Nonspecific Esterase Activity and Phagocytic Activity—Nonspecific esterase activity of the cells was determined using α-naphthylbutyrate as a substrate (18). Phagocytic activity was determined as described by Rabelino et al. (19) using latex particles.

RESULTS

Characterization of CMP-NeuAc:LacCer Sialyltransferase—In order to determine the optimal conditions for measuring the activity of CMP-NeuAc:LacCer sialyltransferase, which catalyzes the synthesis of Ga₃ ganglioside from LacCer, the homogenate prepared from HL-60 cells treated with TPA for 2 days was used as the enzyme source. The optimum pH of the enzyme activity was 6.0. The enzyme activity increased linearly with the protein concentration from 0.1 to 0.25 mg/ml and with time from 15 to 60 min. The reaction product at pH 6.0 was identified by thin layer chromatography and autoradiography as shown in Fig. 1. Ga₃ was the only ganglioside detected in the reaction with the homogenate from TPA-treated HL-60 cells. The apparent Kₘ values for LacCer and CMP-NeuAc were 6.3 μM and 0.3 mM, respectively.

CMP-NeuAc:LacCer Sialyltransferase Activity in HL-60 Cells after Differentiation Induced by Various Inducers—The enzyme activity increased in HL-60 cells treated for 2 days with reagents that induce their differentiation into a monocyte/macrophage lineage, such as TPA, teleocidin, and mezerein (10 ng/ml). However, 1α,25-dihydroxyvitamin D₃ (50 ng/ml), which has the same type of effect on HL-60 differentiation, did not increase the enzyme activity. Retinoic acid, an inducer of the differentiation into a granulocyte lineage, did not increase the enzyme activity either (Fig. 2). CMP-NeuAc:LacCer Sialyltransferase Activity and Ga₃ Ganglioside Content in HL-60 Cells Treated with TPA or 1α,25-Dihydroxyvitamin D₃—A 5-fold increase in the activity of CMP-NeuAc:LacCer sialyltransferase was observed in HL-60 cells treated with TPA at the concentration of 10 ng/ml for 12 to 24 h (Fig. 3). During 24 to 48 h, the content of Ga₃ ganglioside also increased (Fig. 4). Unlike TPA, 1α,25-dihydroxyvitamin D₃ did not significantly increase either the enzyme activity or the Ga₃ ganglioside content, even after 5 days (Figs. 3 and 4).

Characterization of the Differentiated Cells Induced by TPA and 1α,25-Dihydroxyvitamin D₃—Number of nonspecific esterase activity positive cells increased during 12 to 24 h and 2 to 3 days after the cells had been treated with TPA and 1α,25-dihydroxyvitamin D₃, respectively (Fig. 5). More than 80% of the cells treated with TPA for 2 days and 1α,25-dihydroxyvitamin D₃ for 5 days were positive for both nonspecific esterase activity and phagocytic function, which are markers for monocytes/macrophage cells. They showed a significant decrease in the nuclear cytoplasmic ratio, and their nuclei became reniform in shape. Their morphologies were

FIG. 1. Identification of the reaction products. The homogenate (100 μg of protein) obtained from TPA-treated HL-60 cells was incubated in a reaction mixture (pH 6.0) containing 500 μM [14C]CMP-NeuAc (0.1 μCi) and 2 μg of LacCer for 2 h at 37 °C. The reaction products were identified by TLC and autoradiography as described under "Experimental Procedures."

FIG. 2. Activity of CMP-NeuAc:LacCer sialyltransferase in differentiated HL-60 cells. The enzyme activities of cells which had differentiated into the monocyte/macrophage lineage on induction by TPA, teleocidin, mezerein, and 1α,25-dihydroxyvitamin D₃, and into the granulocyte lineage on induction by retinoic acid (RA) were measured at pH 6.0 and 37 °C for 1 h. Columns and bars show the mean values and range for triplicate values, respectively.
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**FIG. 3.** Activity of CMP-NeuAc:LacCer sialyltransferase in HL-60 cells treated with TPA or 1α,25-dihydroxyvitamin D$_3$ for the periods indicated. The enzyme activity of cells treated with (●) or without (O—O) TPA (10 ng/ml) for 24 h and the enzyme activity of cells treated with (■—■) or without (□—□) 1α,25-dihydroxyvitamin D$_3$ for several days is shown.

**FIG. 4.** Gangliosides in HL-60 cells treated with TPA (10 ng/ml) or 1α,25-dihydroxyvitamin D$_3$ (50 ng/ml) for the periods indicated. Gangliosides obtained from 10$^7$ cells were applied on the thin layer plates, which were developed with chloroform/methanol/water (60:40:9, v/v/v). Spots indicated by arrow are neuraminic acid-negative (resorcinol reagent-negative) substances. St. indicates the authentic ganglioside standards, G$_{M3}$, G$_{M2}$, and NeuAc-nLc$_4$Cer.

**FIG. 5.** Nonspecific esterase activity (NEA) of cells treated with TPA and 1α,25-dihydroxyvitamin D$_3$. Cells were treated with 10 ng/ml of TPA or 50 ng/ml of 1α,25-dihydroxyvitamin D$_3$ for the periods indicated. Then the nonspecific esterase activity-positive cells were counted.

irregular and fine ruffles were observed in their peripheries.

**Relationship between Phorbolester Derivatives and the Activation of CMP-NeuAc:LacCer Sialyltransferase**—As shown in Fig. 6, PDBu and PDBz, which show moderate tumor promotion activities, were less potent in increasing the enzyme activity than was TPA. 4α-PDD and phorbol, which are inactive as tumor promoters, did not increase the enzyme activity at all. As to the gangliosides in these cells, the G$_{M3}$ ganglioside content increased in the cells treated with PDBu and PDBz as in the cells treated with TPA, teleocin, and mezerein (Fig. 7). However, in the cells treated with 4α-PDD and phorbol, the G$_{M3}$ ganglioside content did not increase.

**Substrate Specificity of the Sialyltransferase**—The homogenate prepared from TPA-treated HL-60 cells was used as the enzyme source. GalCer and G$_{M2}$Cer, which are not expressed in HL-60 cells, did not act as substrates for the enzyme. nLc$_4$Cer, which is one of the major glycolipid components of HL-60 cells, was not sialylated by the homogenate of TPA-treated HL-60 cells.

**DISCUSSION**

Previous studies have shown a strong correlation between reduced levels of G$_{M3}$ ganglioside and increase in the onco-
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The activity of the differentiation into the monocyte/macrophage lineage. The other is that the increase in GM\(_3\) ganglioside is due to the specific action of TPA, which is unrelated to the differentiated lineage.

CMP-NeuAc:LacCer sialyltransferase, which catalyzes the formation of GM\(_3\) ganglioside, was at first characterized by Kaufman et al. (26). The properties of this enzyme in the TPA-treated HL-60 cells such as pH optimum, substrate specificity, and \(K_m\) values were essentially similar to those in NIL cells described by Burczak et al. (27) except for the \(K_m\) value for LacCer, i.e., the \(K_m\) value for LacCer in the TPA-treated HL-60 cells was much lower than that in NIL cells.

The activity of CMP-NeuAc:LacCer sialyltransferase and the content of GM\(_3\) ganglioside increased in cells treated with monocyte/macrophage differentiation inducers such as TPA, telocidin, and mezerein, but not with a glucocysteine differentiation inducer, retinoic acid (Fig. 2). These results appear to support the former possibility that GM\(_3\) ganglioside is a marker for the monocyte/macrophage differentiation in the differentiated HL-60 cells. However, 1a,25-dihydroxyvitamin D\(_3\), which also induces the differentiation of HL-60 cells into the monocyte/macrophage lineage (7, 8), did not increase the enzyme activity or the GM\(_3\) ganglioside content during the 5 days after the cells had been treated (Figs. 3 and 4), although the HL-60 cells differentiated into monocyte/macrophage cells which were positive for nonspecific esterase and phagocytic activities. This result supports the latter possibility.

Such a possibility was also demonstrated in a recent study on the expression of the c-fos oncogene in HL-60 cells (28). TPA induces the expression of the c-fos gene in HL-60 cells 45 to 90 min after the HL-60 cells have been treated, but 1α,25-dihydroxyvitamin D\(_3\) does not induce its expression even in 2 days. Thus, like the expression of the c-fos gene, the increase in CMP-NeuAc:LacCer sialyltransferase is considered to be caused by the specific action of TPA and not by the initiation of the differentiation into the monocyte/macrophage lineage.

TPA, telocidin, and mezerein, which are active tumor promoters for mouse skin fibroblasts, are known to activate protein kinase C, when substituted for diacylglycerol (29-31). In HL-60 cells, 1α,25-dihydroxyvitamin D\(_3\), which are unable to activate protein kinase C, did not act as a substrate for the sialyltransferase in TPA-treated HL-60 cells, but LacCer was a good substrate. Protein kinase C may specifically activate CMP-NeuAc:LacCer sialyltransferase, and the activation may result in an increase in the content of GM\(_3\) ganglioside during differentiation of HL-60 cells induced by TPA.

However, the possibility that CMP-NeuAc:LacCer sialyltransferase may not be activated through direct phosphorylation by protein kinase C still remains, because cycloheximide inhibited the activation of the enzyme in the TPA-treated HL-60 cells (data not shown). This result suggests that new protein synthesis is necessary for the activation of CMP-NeuAc:LacCer sialyltransferase. Furthermore, addition of the partially purified protein kinase from rat brain did not stimulate the enzyme activity (data not shown). Protein kinase C activated by TPA may regulate the expression of the CMP-NeuAc:LacCer sialyltransferase at the transcriptional level in HL-60 cells, as in the case of the expression of the c-fos gene, or the activity of CMP-NeuAc:LacCer sialyltransferase may be regulated by unidentified proteins, which are activated through the phosphorylation by protein kinase C.

REFERENCES