Differentiation elicits negative regulation of human β-galactoside α2,6-sialyltransferase at the mRNA level in the HL-60 cell line

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Abstract We studied the regulation of the β-galactoside α2,6-sialyltransferase (hST6Gal I) gene during HL-60 cell differentiation induced with dimethyl sulfoxide (DMSO), all *trans*-retinoic acid (ATRA), and phorbol myristate acetate (PMA). During HL-60 cell line differentiation, cell surface levels of α2,6-sialic acids expression decreased, as measured by flow cytometric analysis using *Sambucus nigra* agglutinin (SNA). Activities of hST6Gal I and levels of hST6Gal I mRNA dramatically decreased after 1 day of stimulation. Using reverse transcription polymerase chain reaction (PT-PCR), we found the major hST6Gal I mRNA isoform in HL-60 cells contains 5′-untranslated exons Y and Z. These results suggest that the expression of cell surface α2,6-sialic acids is controlled at the mRNA level, which is regulated by a promoter located 5′-upstream of exon Y.

Key words: Sialyltransferase; HL-60 cell; Cell differentiation; Gene expression; *Sambucus nigra* agglutinin; Alternative splicing

1. Introduction

Systematic alteration of cell functions during cell differentiation and maturation is induced by cell-to-cell adhesion and recognition. Many of these interactions are mediated by cell surface sugar chains on glycoproteins and glycolipids. In particular, sialic acids on the reducing ends of sugar chains are key determinants for cell-to-cell interaction and cell differentiation [1–4]. Expression of sialic acids differs in various tissues; within the same tissue, remarkable changes have been observed during cell differentiation [5–12]. Differentiation-dependent regulation of α2,3- and α2,6-linked sialic acids on sugar chains is associated with mRNA regulation of α2,3- and α2,6-sialyltransferase genes [11–13]. Moreover, β-galactoside α2,6-sialyltransferase (hST6Gal I, also called SIAT 1 and hST6N, EC 2.5.99.1) mRNA levels are altered by treatment with glucocorticoids, cytokines, secondary bile acids and phorbol myristate acetate (PMA) [10,16,17]. These results suggest that the expression levels of cell surface α2,3- and α2,6-sialic acids is mainly regulated at mRNA level.

Among sialyltransferases, hST6Gal I is the most thoroughly studied. Multiple hST6Gal I mRNA forms, differing only in the 5′-untranslated region, have been described [18–21]. A short mRNA form (Form 1) has been isolated from liver. A large transcript (Form 3), containing two 5′-untranslated exons (exons Y+Z), has been isolated from several human cell types. A distinct transcript (Form 2) containing exon X, but not exons Y+Z, has been isolated from human B cell lymphoblastoma cell lines. Recently, Lo and Lau found additional mRNA isoforms in the B lymphocyte cell line, Loucks [19]. In α2,3-sialyltransferase, multiple mRNA forms, which differ only in the 5′-untranslated regions, have been also described for the hST3Gal IV gene (also called hST3O/N, hST4 and hSTZ) [13,22]. These transcripts are produced by a combination of alternative splicing and promoter utilization, suggesting that the cell type-specific transcriptional regulation of these genes will depend on utilizable alternative promoters.

The leukemia cell line HL-60 consists predominantly of promyelocytes which can be induced to differentiate into myelocytes, metamyelocytes, and granulocytes by the addition of dimethyl sulfoxide (DMSO) or all *trans*-retinoic acid (ATRA) to the growth medium. PMA also induces HL-60 cells to differentiate, but through the monocyte/macrophage pathway. Cell surface glycoprotein and glycolipid profiles are specifically altered during differentiation [23–26]. Therefore, this cell line is utilized as a useful model for studying the regulation of carbohydrate expression. Previous studies demonstrated that DMSO-induced differentiation of HL-60 cells resulted in an increase in cell surface expression of NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAc (sialyl-Le^x), and have been performed on glycosyltransferase activities in this model [27]. These results suggest that the increased expression of sialyl-Le^x is related to α2,6-sialyltransferase activity. Therefore, the regulation of hST6Gal I may be important for the expression of sialyl-Le^x in HL-60 cells.

We report here that DMSO-, ATRA- and PMA-induced differentiation elicits negative regulation of hST6Gal I at the mRNA level in the HL-60 cell line. Decreased expression levels of hST6Gal I mRNA during differentiation cause decreased enzyme activity of hST6Gal I and decreased levels of cell surface α2,6-sialic acid moieties. Furthermore, we provide evidence that hST6Gal I mRNA expression is regulated by a promoter located 5′-upstream of exon Y.

2. Materials and methods

2.1. Cell culture

The myeloid cell lines HL-60, U937 and THP-1 were obtained from the American Type Culture Collection (USA). The Burkitt's lymphoma cell lines Daudi and Ramos and the hepatoma cell lines HepG2 and HuH-7 were obtained from the RIKEN Cell Bank (Japan). HL-60, U937, THP-1, Daudi and Ramos cell lines were grown in RPMI 1640 (Nissui, Japan), and HepG2 and HuH-7 were cultured in Dul-

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Abbreviations: hST6Gal I, β-galactoside α2,6-sialyltransferase; DMSO, dimethyl sulfoxide; ATRA, all *trans*-retinoic acid; PMA, phorbol myristate acetate; SNA, *Sambucus nigra* agglutinin; RT-PCR, reverse transcription-polymerase chain reaction; Dig, digoxigenin; FITC, fluorescein isothiocyanate; Le^x, Galβ1,4[Fucα1,3]GlcNAc; sialyl-Le^x, NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAc

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becco's modified Eagle's medium (Nissui, Japan) at 37°C in a humidified atmosphere with 5% CO_2. Each medium was supplemented with 100 U of penicillin, 100 μg of streptomycin and 10% heat-inactivated fetal bovine serum (JRH Biosciences, USA).

2.2. Flow cytometry analysis

Sambucus nigra agglutinin (SNA) binding was assessed by indirect lectin-fluorescence on freshly harvested HL-60 cells or on HL-60 cells after inducing differentiation by treatment with 1.35% of DMSO or 1×10^{-7} M ATRA or 10 ng/ml PMA. Cell suspensions (100 μl containing 2×10^5 cells) were incubated for 60 min at 4°C with SNA-digoxigenin (Dig) (10 μg/ml, Boehringer Mannheim, Germany). Cells were washed three times with PBS containing 2% BSA and then incubated with anti-Dig-fluorescein isothiocyanate (FITC) (10 μg/ml, Boehringer Mannheim, Germany) for 40 min at 4°C, and then washed three times with PBS containing 2% BSA. Stained cells were analyzed by flow cytometry (cytoACE-300 flow cytometer, JASCO, Japan).

2.3. Sialyltransferase assay

Sialyltransferase activities were determined according to the method of Dall'Olio et al. [28], using CMP-[^{3}H]sialic acid (75 Ci/mol, American Radiolabeled Chemicals, USA) and *N*-acetyllactosamine as a donor and an acceptor, respectively. Briefly described, incubation mixtures were composed of 12 μl of cell homogenate (100 μg of protein), 100 mm HEPES, pH 6.5, 10 mM $MnCl_2$, 6 μM CMP-[^{3}H]sialic acid and 60 μM cold CMP-sialic acid, and 250 μg of *N*-acetyllactosamine in a total volume of 20 μl. After incubation at 37°C for 6 h, the radioactive products, α2,3- and α2,6-sialyl *N*-acetyllactosamine, were separated from nucleotide sugar donor and free sugar by normal phase HPLC and detected by a flow scintillation analyzer (Radiomic-500 TR, Packard, USA).

2.4. Northern blot analysis

Isolation of total RNA, gel electrophoresis, Northern blotting and hybridization with radiolabeled cDNA probes were performed as described previously [13]. hST6Gal I cDNA fragments, generated by PCR amplification of HuH-7 cDNA using specific primers, were used as the probes in Northern blot analysis. The specific primers were 5′-ATGATTCACACCAACCTGAAG-3′ and 5′-TTAGCAGTGAATGGTCCGGAA-3′. The amplification products were cloned into a TA cloning vector (Invitrogen, USA) and were verified by sequence analysis.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of hST6Gal I mRNA

Four μg of total RNA was heated at 70°C for 10 min and placed on ice for 2 min. Reverse transcription into cDNA was achieved using the Super Script preamplification system (Gibco-BRL, USA) according to the manufacturer's protocol with random hexamers as initiation primers in a final reaction volume of 22 μl. Four μl of the retrotranscription reaction were subjected to PCR amplification using exons Y+Z, exon X and β-actin. Specific primers were as follows: pY, 5′-GCCCCGGCGTTAACAAAGGGAGCCG-3′; pX, 5′-ACAACCAGGGAGGGCGTGGAAGCT-3′; pII, 5′-TTCTTTTCCTTCCACACACAGATG-3′; β-actin, 5′-ATCATGTTTGAGACCTTCAA-3′ and 5′-CATCTCTTGCTCGAAGTCCA-3′. PCR was performed as described previously [13].

3. Results

3.1. Decreased expression of cell surface α2,6-sialic acids during HL-60 cell differentiation

In the beginning of the study, we estimated the degree to which HL-60 cells will differentiate after induction with DMSO, ATRA, and PMA. We found, based on morphological findings, that the maturation population compared to 80–90% of the total cell count after a 4-day treatment with inducer. DMSO-, ATRA- and PMA-stimulated superoxide production was detected by nitroblue tetrazolium reduction in more than 80% of the cells by 7 days (data not shown). To evaluate the expression of cell surface α2,6-sialic acids during in vitro HL-60 cell line differentiation, we performed flow cytometric analysis using SNA lectin with undifferentiated HL-60 cells and with HL-60 cells after DMSO-, ATRA- and PMA-induced differentiation. As shown in Fig. 1, SNA binding to the cells treated with DMSO, ATRA or PMA continued to decrease gradually, falling to levels about 15% of those in control HL-60 cells at 7 days. The results indicated that cell surface α2,6-sialic acid expression is down-regulated during HL-60 cell differentiation.

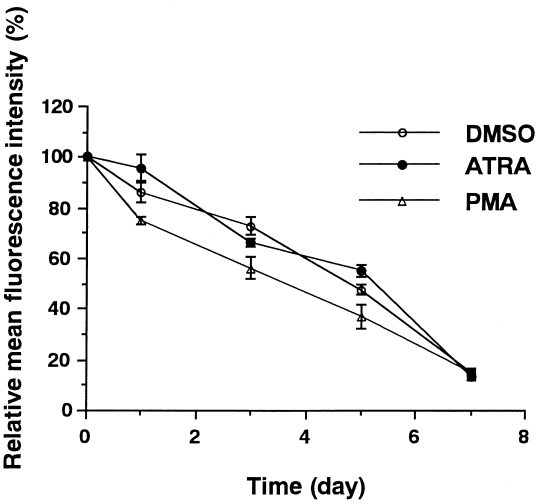

Fig. 1. Flow cytometric analysis of SNA binding in HL-60 cells before and after exposure to DMSO, ATRA and PMA. HL-60 cells were grown in the absence (control) or presence of DMSO or ATRA or PMA for periods up to 7 days and stained with SNA-lectin. Each value includes data from 3 independent experiments. The error bars define the standard error for each data set.

3.2. Decreased α2,6-sialyltransferase activities during HL-60 cell differentiation

The synthesis of α2,6-sialic acid is mediated by the action of α2,6-sialyltransferase. In order to investigate whether decreased levels of hST6Gal I are responsible for decreased expression of α2,6-sialic acids during HL-60 differentiation, α-2,6-sialyltransferase activities were measured with *N*-acetyllactosamine. As shown in Fig. 2, α2,6-sialyltransferase activities in the cells treated with DMSO, ATRA and PMA decreased rapidly during the first day of stimulation and continued to decrease more gradually thereafter, falling to levels 20–50% of those in control HL-60 cells at 7 days. Levels of α2,3-sialyltransferase activity were unaltered by DMSO, ATRA and PMA for 7 days (data not shown). DMSO and PMA exerted a stronger influence on α2,6-sialyltransferase activities than ATRA. The results suggest that a reduced activity level of α2,6-sialyltransferase is responsible for the reduction in levels of α2,6-sialic acids on the cell surface during HL-60 differentiation.

3.3. Down-regulation of hST6Gal I mRNA expression during HL-60 cell differentiation

In order to evaluate hST6Gal I gene expression during HL-60 differentiation, hST6Gal I mRNAs in HL-60 cells stimulated with DMSO, ATRA and PMA were subjected to Northern blot analysis using hST6Gal I cDNA as a probe. As shown in Fig. 3, levels of hST6Gal I mRNA dramatically decreased one day after stimulation. DMSO and PMA exerted a stronger influence on hST6Gal I mRNA than ATRA. The results suggest that the down-regulated hST6Gal I transcript

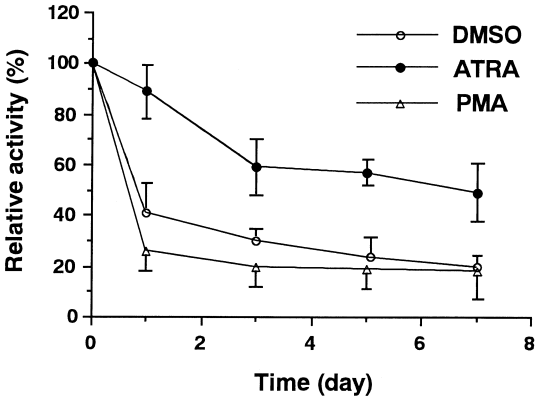

Fig. 2. α2,6-Sialyltransferase activities in HL-60 cells before and after exposure to DMSO, ATRA and PMA. HL-60 cells were grown in the absence (control) or presence of DMSO or ATRA or PMA for periods up to 7 days and cell lysates were prepared and assayed for hST6Gal I activity with *N*-acetyllactosamine. Each time point includes data from 3 independent experiments. The error bars define the standard error of each data set.

expression is an essential event leading to decreased levels of cell surface α2,6-sialic acids observed during HL-60 differentiation.

3.4. hST6Gal I mRNA isoforms in HL-60 cell line using RT-PCR

Expression of hST6Gal I in various cell types has recently been investigated by Wang et al. [21]. They propose, based upon their findings, that tissue-specific expression of hST6Gal I depends on the use of alternative promoters. Fig. 4A is a schematic diagram of the 5′-untranslated region of various hST6Gal I cDNAs. The liver predominantly expresses Form 1 mRNA, a form that lacks sequences from either exon Y, Z or X [18,29]. Form 2, containing exon X, has been isolated from various human lymphoblastoma cell lines of B-lineage [20]. Form 3 (exons Y+Z) has been isolated from several human cell types. In order to determine the major hST6Gal I transcript in the HL-60 cell line, we performed RT-PCR analysis using exon Y+Z and X specific primers. As shown in Fig. 4B, exon Y+Z specific primers gave fragments with the expected size after amplification of cDNA from all cells tested. Exon X sequence is found only in Daudi and Ramos cell lines, but not in HL-60, THP1 and U-937 cell lines. The results suggest that the isoform which contains exon Y+Z (i.e. Form 3) is the major transcript in the HL-60 cell line, and that transcriptional regulation of hST6Gal I in this cell line depends on a promoter that exists 5′-upstream of exon Y.

4. Discussion

In this study, alterations in the expression of cell surface α2,6-sialic acids, α2,6-sialyltransferase activities and hST6Gal I mRNA levels were investigated in the HL-60 cell in the process of differentiation induced by treatment with DMSO, ATRA and PMA. Our findings of decreased α2,6-sialyltransferase activities in the HL-60 cell during induction of differentiation coincide well with previous results [27,30]. Moreover, our results indicated that down-regulation of hST6Gal I mRNA is essential for the differentiation-induced reduction of cell surface α2,6-sialic acids. A good correlation between the hST6Gal I mRNA level and cell surface α2,6-sialic acids level had previously been reported in various cell lines [14,15,17]. These results suggests that the expression of cell surface α2,6-sialic acids is mainly controlled at the mRNA level. While all types of induction led down-regulation of cell surface α2,6-sialic acids as measured by SNA staining with very similar kinetics, DMSO and PMA exerted a stronger influence on α2,6-sialyltransferase activities and hST6Gal I mRNA than ATRA. These results suggest that the expression of cell surface α2,6-sialic acids in HL-60 cells may be partially affected by sialidase.

Although DMSO- and ATRA-induced HL-60 differentiation to granulocyte-like cells will be mediated through different intracellular pathways, decreased expression of hST6Gal I mRNA was commonly detected during the differentiation; therefore, down-regulation of hST6Gal I expression will be essential for differentiation or a sequence of differentiation. Our results indicate that PMA also induces down-regulation of hST6Gal I expression. Others have found that hST6Gal I is altered by PMA in colon adenocarcinoma cell lines [17]. However, Form 3 mRNA is not found in this type of cell line [14]. We found Form 3 mRNA in HL-60 cells, suggesting that regulation of hST6Gal I in this cell line depends upon a different promoter from that for colon adenocarcinoma cell lines. It is not clear whether the PMA-mediated down-regulation of hST6Gal I expression is differentiation-dependent or not. PMA-mediated induction of protein kinase C in HL-60 cells has been demonstrated to activate the mitogen-activated kinase (MAP kinase) pathway [31]. It is possible, therefore, that PMA might directly mediate down-regulation of hST6Gal I mRNA via the MAP kinase signal transduction pathway.

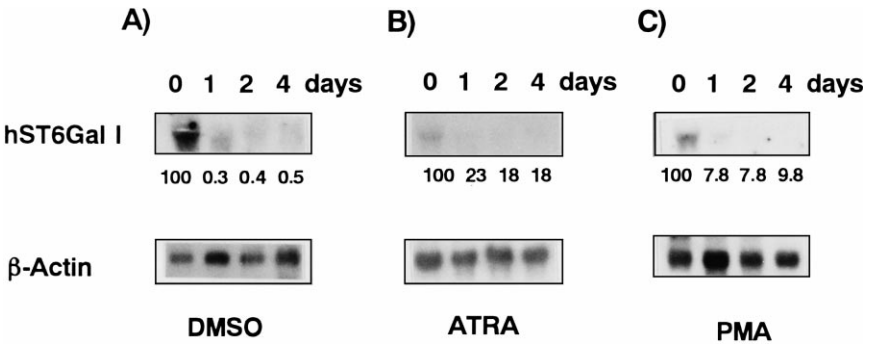

Fig. 3. Expression of hST6Gal I mRNA from HL-60 cells before and after exposure to DMSO, ATRA and PMA. Total RNA (5 μg) prepared from HL-60 cells before (day 0) or after growing in DMSO (panel A), ATRA (panel B) or PMA (panel C) for periods up to 4 days were electrophoresed, transferred to a membrane, and hybridized with ^{32}P-labeled hST6Gal I (upper panel) and β-actin (lower panel) cDNA. Values at the bottom of the Northern blot indicate the % of control (day 0) levels of hST6Gal I mRNA indicated by densitometric measurement.

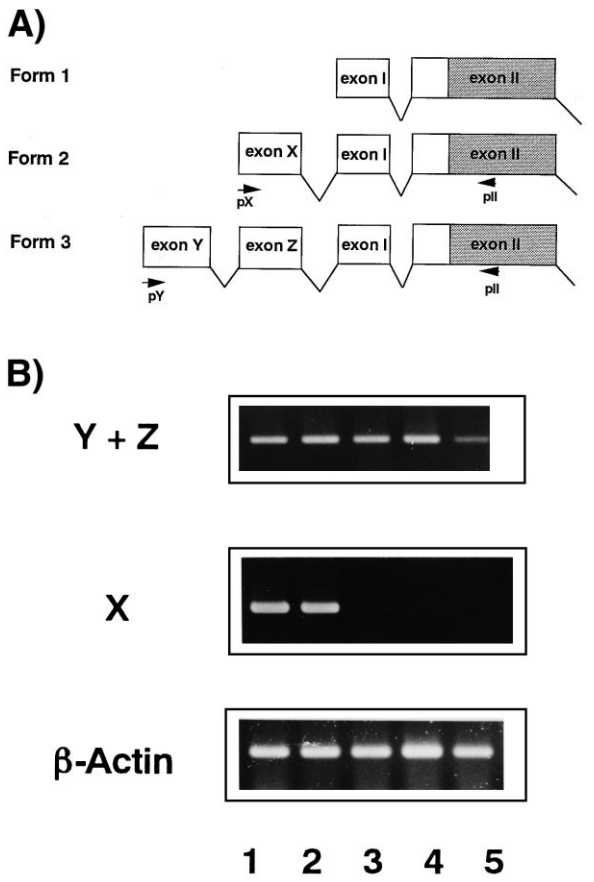

Fig. 4. RT-PCR analysis of B cell lymphocyte cell lines (Daudi and Ramos) and myelocyte cell lines (HL-60, THP-1 and U-937) hST6Gal I mRNA isoforms. A: Comparison of the structure of the 5′-untranslated region in Form 1, Form 2 and Form 3. Exon regions are denoted by boxes. Black boxes represent coding sequence and open boxes denote 5′-untranslated sequences. Arrows and pX, pY and pII indicate position and polarity of synthetic oligonucleotide primers used in PCR analysis. B: cDNAs from Daudi (lane 1), Ramos (lane 2), HL-60 (lane 3), THP-1 (lane 4) and U937 (lane 5) were used as target DNA in the PCR analysis. The primers used were as follows: upper panel, exons Y+Z; middle panel, exon X; bottom panel, β-actin. PCR products were run on 2% agarose gel and stained with ethidium bromide.

The 5′-flanking region to the hepatic (Form 1) and B-cell-specific (Form 2) transcriptional starting sites have been defined [18]. A number of *cis*-acting regulatory elements with consensus to transcriptional factors are noted in these regions. Some of them are cell type specific factors that are believed to participate in the cell type specificity of Form 1 and Form 2 mRNA expressions. The 5′-flanking region to Form 3 is not yet identified and may be important for the differentiation-dependent reduction of hST6Gal I mRNA levels in HL-60 cells.

Sialyl-Le^x is well expressed on the surface of mature granulocytes and is believed to be the major ligand recognized by E- and P-selectin [32–34]. Previous studies demonstrated that DMSO-induced differentiation of HL-60 cells resulted in an increase in cell surface expression of sialyl-Le^x, and have been performed on α2,3-, α2,6-sialyltransferase and α1,3-fucosyltransferase activities [27]. These results suggest that the reduced expression of sialyl-Le^x in undifferentiated HL-60 cells is related to the presence of the strong α2,6-sialyltransferase, which uses the precursor at the expense of α1,3-fucosyltransferase and competes the synthesis of Le^x and sialyl-Le^x. Therefore, the down-regulation of hST6Gal I during HL-60 cell differentiation may play an important role for the expression of sialyl-Le^x.

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