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Expressions of polypeptide: *N*-acetylgalactosaminyltransferase in leukemia cell lines during 1,25-dihydroxyvitamin D3 induced differentiation

Hao Qiu · Xiang-Hong Guo · Jian-Hua Mo · Mei-Fang Jin · Shi-Liang Wu · Hui-Li Chen

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Abstract The effect of 1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3] on two leukemia cell lines, K562 and SHI-1, and its relation to the expression of different subtypes of polypeptide: *N*-acetylgalactosaminyltransferase (pp-GalNAc-T) was studied. With morphological and cell flow-cytometric method, it was found that 1,25$(OH)_2$D3 induced the differentiation of both leukemia cell lines toward monocytic lineage, but not affected the cell growth and apoptosis. The expressions of different subtypes of pp-GalNAc-T, the initial glycosyltransferase in *O*-glycan synthesis, were studied with RT-PCR before and after the treatment of different concentrations of 1,25$(OH)_2$D3. Among fourteen subtypes of pp-GalNAc-T (T1∼T14), K562 cells obviously expressed pp-GalNAc-T2, T4, T5, T7 (T2 was the highest) and SHI-1 cells apparently expressed pp-GalNAcT1, T2, T3 and T4 (T4 was the highest) only. After K562 cells were treated 1, 25$(OH)_2$D3 for 72 h, pp-GalNAc-T2, T4, T5, T7 were increased in a dose dependent manner. In contrast, pp-GalNAc-T1 and T2, especially T1, were up-regulated in SHI-1 cells by 1,25$(OH)_2$D3, but T3 was unchanged and T4 was down-regulated. The different alterations of pp-GalNAc-Ts in these two cell lines were probably related to the different structural changes of *O*-glycans during 1,25$(OH)_2$D3 induced differentiation.

H. Qiu · X.-H. Guo · J.-H. Mo · M.-F. Jin · S.-L. Wu (✉)
Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Institute of Bioengineering, Suzhou University, Suzhou, China
e-mail: shiliangwu@hotmail.com

H.-L. Chen (✉)
Key Laboratory of Glycoconjugate Research, Ministry of Health, Department of Biochemistry, Shanghai Medical College, Fudan University, Shanghai, 200032, China
e-mail: hlchen@shmu.edu.cn

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Abbreviations

1, 25$(OH)_2$ D3	1, 25-dihydroxyvitamin D3
pp-GalNAc-T	polypeptide: *N*-acetylgalactosaminyl transferase
GalNAc	*N*-acetylgalactosamine
O-glycan	Thr/Ser-linked glycan
N-glycan	Asparagine-linked glycan
RT-PCR	reverse transcriptase-polymerase chain reaction
VDR	vitamin D receptor
FCS	fetal calf serum

Introduction

Polypeptide: *N*-acetylgalactosaminyltransferase (pp-GalNAc-T, EC 2. 4. 1. 41) is a glycosyl- transferase involved in the first step of *O*-glycan synthesis [1]. It catalyses the transference of GalNAc group from UDP-GalNAc to the threonine (Thr) or serine (Ser) residue within a definite sequence on protein polypeptide scaffold to form a GalNAc α-O glycosidic linkage. This enzyme was firstly discovered in sheep submaxillary mucin by Mc Guire and Roseman in 1967 [2]. Now it has been well known that pp-GalNAc-T is a big family. At least fifteen subtypes or isoforms (pp-GalNAc-T1∼T15) of this family have been identified and characterized [3–15]. Some of them are widely distributed in various human tissues, but others show specific tissue distribution. The difference in tissue distribution and substrate specificity toward peptides are related to the diverse functions of these different subtypes of pp-GalNAc-T.

It has been well documented that the structures of sugar chains (glycans) on glycoproteins and glycolipids are altered during differentiation of leukemia cells, such as HL60, and these alterations result from the activity changes of the glycan processing enzymes [16–19]. However, these studies were mainly focused on the structural changes of asparagine-linked (*N*-linked) glycans and their processing enzymes. The expression of glycosyltransferases involved in the synthesis of Thr/Ser-linked (*O*-linked) glycan during the induced differentiation of leukemia cells was rarely studied. Up to date, the alteration of pp-GalNAc-T in leukemia cell differentiation had not been reported owing to the late discovery of this enzyme family.

1,25-dihydroxyvitamin D3 [1,25$(OH)_2$D3], also called 1, 25-dihydroxycholecalciferol or calcitriol, is the active form of vitamin D3, which is synthesized from the physiologically inactive prohormone vitamin D3 through 25-hydroxylation in the liver and subsequent 1α-hydroxylation in the kidney. In addition to its hormonal action on calcium metabolism, 1, 25$(OH)_2$ D3 has been known as an inducer of leukocyte differentiation, stimulating the differentiation of bone marrow progenitor cells or HL60 cells toward monocyte/macrophage lineage [20, 21]. It was reported that 1,25$(OH)_2$ D3 also participated in the growth arrest, apoptosis or differentiation of other cancer cells, such as prostate and thyroid cancers [22, 23]. The action of 1, 25$(OH)_2$ D3 is mediated by vitamin D receptor (VDR) in the cells. The 1,25$(OH)_2$ D3/VDR complex translocates to nuclei, and the VDR binds to RXR (retiniods X receptor) to form a hetero-dimer. Then the VDR- RXR dimer binds to vitamin D response element (VDRE) on DNA to initiate the transcription of vitamin D target genes [24]. Recently, it was reported that the 1, 25$(OH)_2$ D3 induced differentiation of HL60 cells was caused by the inhibition of 1,25$(OH)_2$ D3 on the combination of VDR with some Ser/Thr phosphatases, leading to the decrease of their phosphatase activities. Consequently, the phosphorylation of p70S6 was increased which was correlated with HL60 differentiation [25].

K562 is a chronic myeloid leukemia cell line established by Lozzio et al in 1977 [26] with rapid growth rate and poor differentiation. It can be induced to erythroid differentiation by various compound, but 1,25$(OH)_2$ D3 may inhibit the erythroid differentiation and shift the pathway of differentiation from the erythoid to the monocytic lineage [27]. SHI-1 is an acute monocyte leukemia cell line established from the bone marrow of a patient with acute monocytic leukemia (AML-M5b) at relapse, which was characterized by t(6;11)(q27;q23) translocation and the alteration of p53 allele as well as the high tumorigenicity in nude mice [28]. 1, 25$(OH)_2$ D3 was reported to induce the differentiation of monocytic leukemia cell lines *in vitro* without inducing cytotoxicity of the cells [29].

In the present investigation, in order to verify that the effect of 1,25$(OH)_2$ D3 on K562 and SHI-1 is differentiation to the monocytic lineage, but does not affect cell growth and apoptosis, the morphological changes, cell cycle and apoptosis after treatment with different concentrations of 1,25$(OH)_2$ D3 were studied. On the basis of these results, the expressions of pp-GalNAc-T1 to T-14 were observed in these two leukemia cell lines by using RT-PCR. Then some subtypes of pp-GalNAc-T with higher expressions were selected to study their expression changes during 1,25$(OH)_2$ D3 induced differentiation for elucidating the relation between pp-GalNAc-T and leukemia cell differentiation.

Materials and Methods

Materials

K562 and SHI-1 leukemia cell line were obtained from Shanghai Institute of Cell Biology and the First Affiliated Hospital of Suzhou University respectively. Fetal calf serum (FCS), RPMI-1640 and IMEM mediums were purchased from GIBCO/BRL. 1, 25$(OH)_2$ D3 was the product of Roche Company. Random primer (6 bases) was purchased from TaKaRa Company. Taq E polymerase, dNTP mixture and PCR buffer were purchased from MBI Company. TRIzol, RNase inhibitor, Superscript II RNase H reverse transcriptase and pUC Mix DNA marker were from Invitrogen Company. The other reagents were commercially available in China.

Cell culture and treatment of 1, 25$(OH)_2$ D3

Cells were cultured at 37°C, 5% CO_2 in RPMI-1640 medium containing 10% FCS (for K562) or IMDM medium containing 15% FCS (for SHI-1). 1, 25$(OH)_2$ D3 was added to the culture medium at a concentration of 10^{-8}, $10^{-7}$and 10^{-6} mol/L for 72 h before the cells were harvested.

Determination of VDR in K562 and SHI-1 cells

Total RNA was extracted from cells using TRIzol/ Chloroform/isopropanol method according to the protocol provided by Promega. Complementary DNAs (cDNAs) were synthesized from 2μg of the total RNA with 150 ng random primer, 1μl dNTP Mix, 40U RNase inhibitor, and 200U Superscript II RNase H reverse transcriptase in 20 μl reaction mixture. The mixture was incubated at 30°C for 10 min, 42°C for 30 min and 99°C for 5 min. After the addition of 2U RNase H, the PCR was performed in a volume of 50μl containing 2μl cDNA, 10pM primer pair of VDR or β-microglobulin (loading control), 0.2 μM of each dNTP, 2 IU Taq E polymerase and PCR buffer. The cDNA was

subjected to denaturation at 94°C for 3 min, followed by 30 cycles of PCR. Each cycle included denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 1 min. Finally, the sample was further incubated for elongation at 72°C for 5 min. After completion of the RT-PCR, 10 μl products or pUC Mix DNA marker were applied to 1.5% agarose gel for electrophoresis and stained by ethidium bromide. The intensities of the amplified DNA bands were scanned by ImageMaster System (UVP), including the analysis of the screened photos with NIH Image software. The semi-quantitative data were obtained by the intensity ratios of VDR bands to the β- microglobulin band. The primer of VDR was designed from the cDNA sequence of VDR as follow [30], F: 5′-TCAGGAGATCTCATTGCCAAAC-3′, R; 5′-CAGACCAGAGTTCTTTTGGTTG-3′ and was synthesized by Boya Biotech. Company in Shanghai.

Morphological observation of K562 and SHI-1 cells during 1,25$(OH)_2$ D3 induced differentiation

The cells treated with different concentrations of 1,25$(OH)_2$ D3 were smeared on microscope glass plates and stained with Wright's solution for 5 minutes. After naturally drying, the morphological changes of the cells were observed under the light microscope.

Measurement of cell percentages at different phases of cell cycle and cell apoptosis with FCM before and after 1,25$(OH)_2$ D3 treatment

The cells were synchronized with serum hunger method (2% FCS in RPMI-1640 medium) for 48 h, then transferred to 10% FCS/RPMI-1640 (for K562) or 15% FCS/IMDM medium (for SHI-1) and treated with different concentrations of 1,25$(OH)_2$ D3 for further 72 h. Then the cells were washed with phosphate-buffered saline (PBS), and suspended in PBS containing 20μg/ml RNase A, 0.2% Triton X-100, 0.2 mM EDTA and 20μg/ml propidium iodide. After incubation at 37°C for 20 min, the cells were subjected to FACScan for determination of DNA contents. The cell percentage at each phase of cell cycle and the appearance of apoptosis (sub-G1 hypoploid cell fraction) were analyzed, and the flow-cytometric histogram was drawn automatically.

RNA extraction and determination of the mRNA of pp-GalNAc-T with RT-PCR

The method for extraction of total RNA and the reaction mixture of RT were the same as described above, and then the mixture was incubated at 42°C for 50 min and 70°C for 20 min. The PCR was also performed in a volume of 50μl containing 2μl cDNA, 10pM primer pair of different subtypes of pp-GalNAc-T or β-microglobulin, 0.2μM of each dNTP, 2 IU Taq E polymerase and PCR buffer. The PCR reaction was initiated at 95°C for 3 min, followed by 35 cycles. Each cycle included the incubation at 94°C for 45 sec, 56°C for 1 min and 72°C for 1 min. Subsequently, the sample was further incubated at 72°C for 7 min. Then 10μl of RT-PCR products or pUC Mix DNA marker were applied to electrophoresis and staining with the method as mentioned above. The quantification of the DNA bands was also performed with ImageMaster System. The primers of pp-GalNAc-T1∼T14 were designed by using Genetyx software as well as GenBank Blast, and synthesized by Sheng-gong Bioengineering Company in Shanghai. Their sequences were listed at Table 1.

Results

Expression of nuclear VDR in K562 and SHI-1 cells

By using RT-PCR it was discovered that the product of VDR mRNA was 1301 bp, which was in accordance with the predicted length. Both K562 and SHI-1 cells expressed VDR, but the expression in K562 cells was lower than that in SHI-1 cells (Figure 1).

Morphological changes of K562 and SHI-1 cells after 1,25$(OH)_2$ D3 treatment

After the treatment of 1,25$(OH)_2$D3, both K562 and SHI-1 cells were differentiated toward monocytic lineage. This was evidenced by the increased vacuoles in the cytoplasm and the condensed nuclear chromatin. The morphological change showed roughly dose dependent and was more obvious in SHI-1 cells than that in K562 cells (Figure 2A, B).

Cell percentages at different phases of cell cycle and cell apoptosis after 1,25$(OH)_2$ D3 treatment

As shown in Table 2, there was no significant alteration in cell percentages at different phases of cell cycle after K562 and SHI-1 cells were treated with different concentrations of 1,25$(OH)_2$D3. At the high concentration of 1,25$(OH)_2$D3 (10^{-6}mol/L), the percentage of K562 cells in G1 phase and that of SHI-1 cells in G2 phase were somewhat increased as compared with the untreated controls. However, at low concentration of 1,25$(OH)_2$D3, the changes of the cell percentages at G1, S, and G2 phases were not significant, or not proportional with the concentration of 1,25$(OH)_2$D3. These results suggested that 1,25$(OH)_2$D3 showed little effect on the cell cycle of K562 and SHI-1 cells.

With morphological study, apoptosis was not appeared in 1,25$(OH)_2$D3 treated cells (Fig. 2). In addition, the sub-G1 (apoptotic cell) fraction was not found in the flow

Table 1 The primer sequences of pp-GalNAc-Ts and β-microglobulin

pp-GalNAc-T	Sequence	Sequence no.	DNA length (bp)
T1	F: 5′- GCTGAACGTGCTGTCATAATGAAG-3′ R: 5′-TGCCTGCAGTGAAAGTCCTTGAT-3′	NM_020474	1177
T2	F: 5′-AAGAAAGACCTTCATCACAGCAATGGAGAA-3′ R: 5′-ATCAAAACCGCCCTTCAAGTCAGCA-3′	NM_004481	669
T3	F: 5′-GCGTTGGTCAGCCTCTATGTCTG-3′ R: 5′-GGAAGACTTAAGTGCTTCAAGTG-3′	NM_004482	1028
T4	F: 5′-GACGTCCTCACTTTCCTGTATTGT-3′ R: 5′-TACATCAGGTCGGCCCTCCGGTGT-3′	NM_003774	1020
T5	F: 5′-TCATCAAGGAGATTCTGCTGGTAG-3′ R: 5′-TATCACAAGGGTGCAGCCTTACGG-3′	XM_050509	1044
T6	F: 5′-CAGGATCTGGTACCTGCCTGACAT-3′ R: 5′-GGTGCTGTCTTGGCTCACTGCAAC-3′	NM_007210	1046
T7	F: 5′-TGACCAGTGTTTGACAAAGGGAG-3′ R: 5′-GACAGAGGGCACTGGGAATGCCAG-3′	NM_017423	1033
T8	F: 5′-TCTGAGGATCACTGGCTGTTGGTC-3′ R: 5′-CTGTGATTGTGTCTCCTGTGTTTG-3′	NM_017417	1024
T9	F: 5′-TCTTTGGACATCTCCACCTCCAGG-3′ R: 5′-GTCGGCTTCTTTGATGCCCACGTC-3′	NM_024572	1004
T10	F:5′-GAAGCGGCAAAGGGGACCAT-3′ R:5′-TGTGCAACAGAGGCTGGAGC-3′	NM_022087	676
T11	F:5′-ACTGGGGCTGTGGCGGAAAT-3′ R:5′-TGAAGCAGGTGTGCTGGCGT-3′	NM_022087	604
T12	F:5′-TTGGTGCTCCTGGCGCTACT-3′ R:5′-AGGTCAGAACATCGCCCCTC-3′	NM_024642	613
T13	F:5′-TGAGGAGATCTGTCTACTGC-3′ R:5′-AAGTTATGACCTGCCCTTTT-3′	AB078142	616
T14	F:5′-CTGACTCGTCGGCTGGTTCT-3′ R:5′-ACCTCACAGTGGCTGTCGAG-3′	AB078114	602
β-Microglobulin	F: 5′-CTCGTGCTACTCTCTCTTTC-3′ R: 5′-CATGTCTCGATCCCACTTAAC-3′	X00357	330

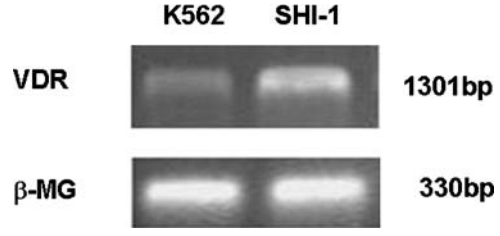

Fig. 1 Expression of VDR in K562 and SHI-1 cells $\beta-$MG: β-microglobulin; The RT-PCR method for the detection of VDR was described in the "Methods". Three independent and reproducible experiments were performed.

cytometric histogram even in the presence of high concentration (10^{-6}mol/L) 1,25$(OH)_2$D3 (Figure 3), revealing that 1,25$(OH)_2$D3 also did not affect the cell apoptosis of K562 and SHI-1 cells.

Expressions of pp-GalNAc-T1∼T14 in K562 cells and SHI-1 cells

Only pp-GalNAcT-2, -T4, -T5 and -T7 were obviously expressed in K562 cell. The others were expressed very slightly or undetectable. In Figure 4A it was shown that the expression order of these four ppGalNAcTs was $2 = 4 > 7 > 5$. However, if the smaller size of pp-GalNAcT-2 PCR product was taken into account (Table 1), it would be reasonable to assume that pp-GalNAcT-2 was more abundantly expressed than pp-GalNAcT-4. Therefore, the actual expression order was $2 > 4 > 7 > 5$. This assumption will be evidenced in the results of Figure 5. On the contrast, SHI-1 cells expressed pp-GalNAcT-1, -T2, -T3 and -T4 apparently. Among them, GalNAcT-4 was the highest, and the other three were almost equally expressed (Figure 4B). Therefore, only pp-GalNAcT-2 and -T4 were commonly expressed in these two leukemia cell lines, but the expression patterns of pp-GalNAc-T1∼T14 in K562 and SHI-1 were quite different to each other.

Alterations of the expressions of pp-GalNAc-T2, -T4, -T5, -T7 in K562 cells after treatment of 1,25$(OH)_2$D3

After K562 cells were treated with 10^{-8}, 10^{-7}, 10^{-6} mol/L of 1,25$(OH)_2$D3 for 72 h, all the expressions of pp-GalNAc-T2,

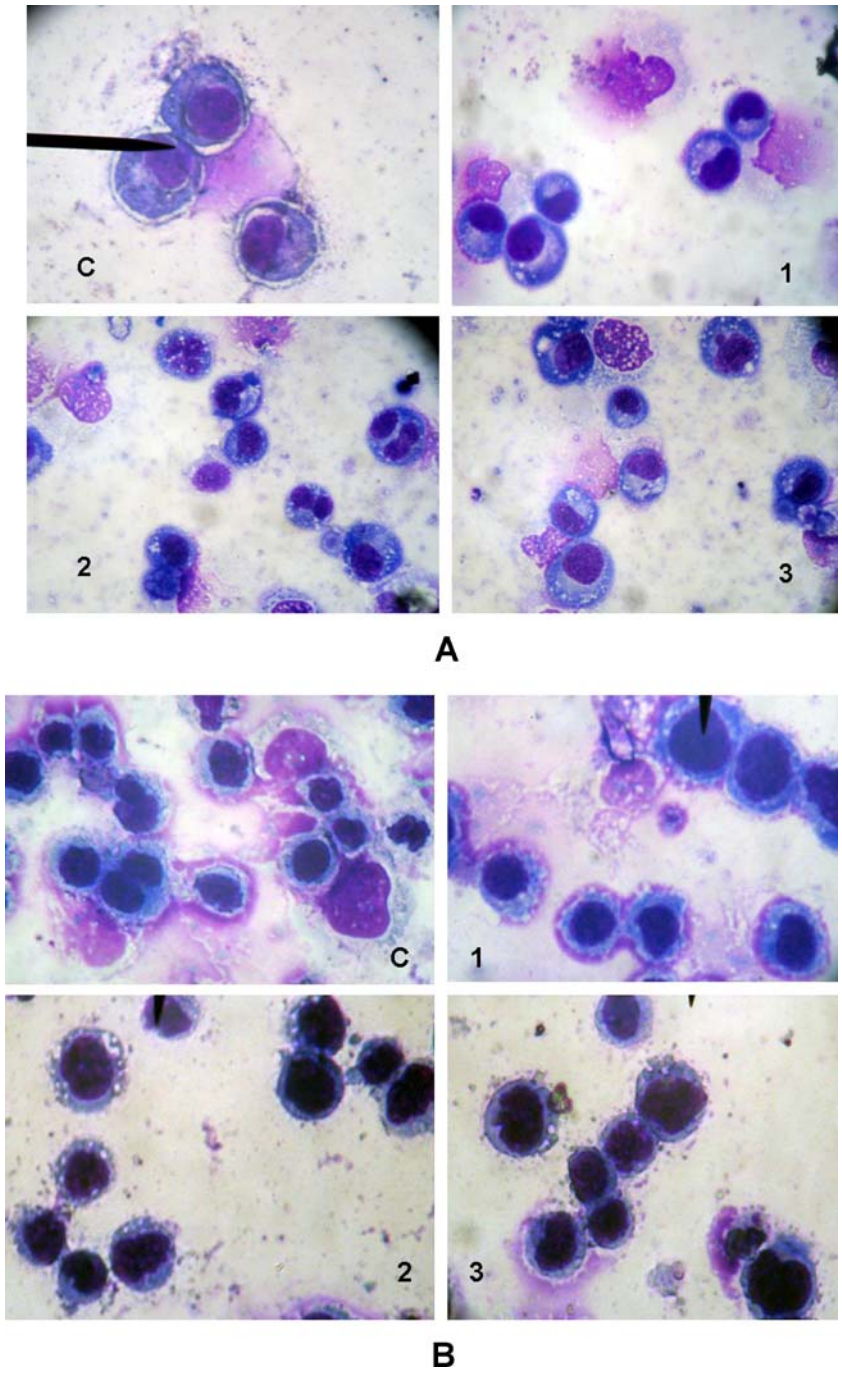

Fig. 2 Morphological changes of K562 and SHI-1 cells after treated with different concentrations of $1,25(OH)_2D3$ A. K562 cells × 400 B. SHI-1 cells × 400 C: Control cells without $1,25(OH)_2D3$ treatment; 1: Cells treated with 10^{-8} mol/L $1,25(OH)_2$ D3; 2: Cells treated with 10^{-7} mol/L $1,25(OH)_2$ D3; 3: Cells treated with 10^{-6} mol/L $1,25(OH)_2$ D3; β-MG: β-microglobulin.

-T4, -T5, -T7 were increased in a dose dependent manner, except that pp-GalNAc-T2 was not increased at low concentration (10^{-8}mol/L) of $1,25(OH)_2D3$. Among them, pp-GalNAc-T4 was up-regulated most obviously ($p < 0.01$ at 10^{-8}, 10^{-7} and 10^{-6} mol/L of $1,25(OH)_2D3$ vs untreated control), T5 was also elevated significantly ($p < 0.01$ at 10^{-7} and 10^{-6}mol/L of $1,25(OH)_2D3$ vs untreated control), but pp-GalNAc-T2 and -T7 were increased medially ($p < 0.05$ and $p < 0.01$ at 10^{-7} and 10^{-6}mol/L of $1,25(OH)_2D3$ respectively vs untreated control) (Figure 5 A, B)

Alterations of the expressions of pp-GalNAc-T1, -T2, -T3, -T4 in SHI-1 cells after treatment of $1,25(OH)_2D3$

The changes of pp-GalNAc-T expressions in SHI-1 cells were quite different from those in K562 cells after treated with different concentrations of $1,25(OH)_2D3$. Pp-GalNAc-T1 was elevated one time at 10^{-7} mol/L of $1,25(OH)_2D3$ as compared with that at 10^{-8} mol/L of the drug ($p<0.01$), and further increased to 10 times at 10^{-6} mol/L of $1,25(OH)_2D3$

Table 2 Cell percentages in different phases of cell cycle during $1,25(OH)_2D3$ induced differentiation

$1,25(OH)_2D3$ concentration	Cell percentages		
	G1	S	G2/M
K562 cells			
Control	25.4	57.3	17.3
10^{-8}M	27.0	54.4	10.6
10^{-7}M	29.4	54.5	16.1
10^{-6}M	32.9	55.2	11.9
SHI-1 cells			
Control	57.0	33.5	9.5
10^{-8}M	60.1	29.4	10.5
10^{-7}M	59.0	31.2	9.8
10^{-6}M	55.1	31.8	13.1

Data were the mean value of two independent experiments.

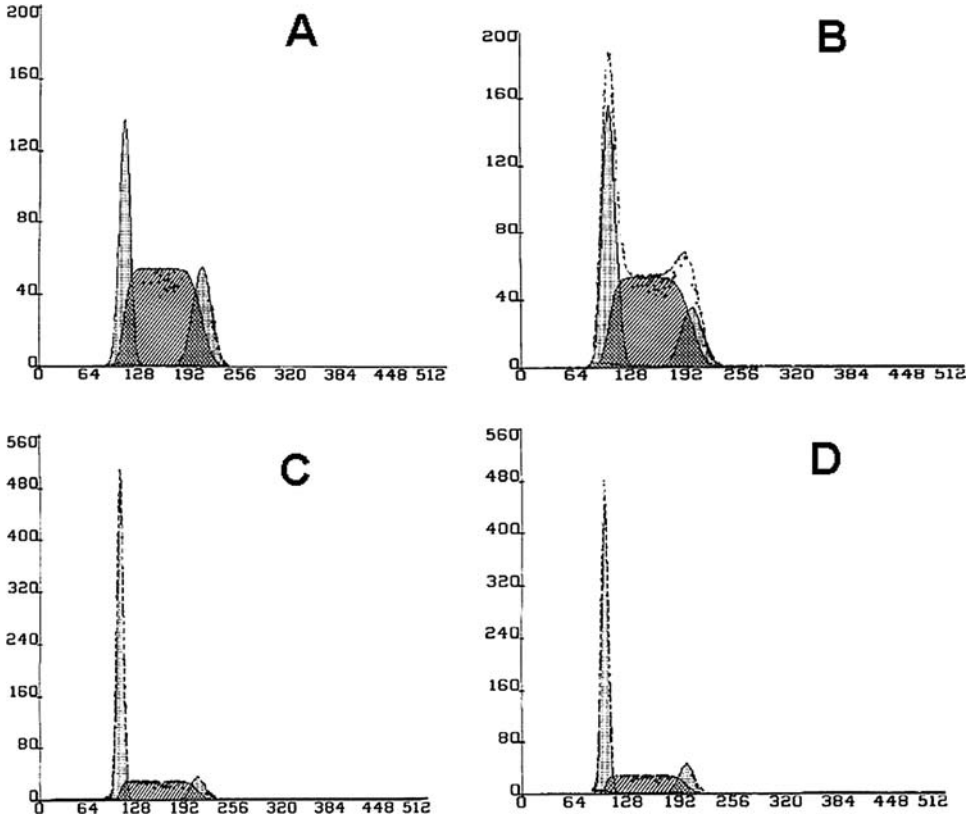

Fig. 3 Flow cytometric analysis of cell apoptosis. A. K562 control cells without $1,25(OH)_2D3$ treatment. B. K562 cells treated with 10^{-6} mol/L $1,25(OH)_2D3$. C. SHI-1 control cells without $1,25(OH)_2D3$ treatment. D. SHI-1 cells treated with 10^{-6} mol/L $1,25(OH)_2D3$. X axis: Relative content of cell DNA; Y axis: Cell number.

when compared with that at 10^{-7} mol/L concentration ($p<0.001$). T2 was up-regulated to 178% of the control value at 10^{-8} mol/L of $1,25(OH)_2D3$ ($p < 0.05$) and increased to the highest level, approximately 230% of the control at 10^{-7} mol/L of $1,25(OH)_2D3$ ($p < 0.01$), but went down when the concentration of $1,25(OH)_2D3$ was increased to 10^{-6} mol/L ($p < 0.05$). In contrast, T3 expression was almost not altered after $1,25(OH)_2D3$ treatment. The expression of T4 was just opposite to those of T1 and T2; it was down-regulated with a dose dependent manner ($p < 0.01$ at 10^{-7} and 10^{-6} mol/L of $1,25(OH)_2D3$ vs untreated control) (Figure 6 A, B).

Discussion

The presence of VDR in both K562 and SHI-1 cells indicates that these two cell lines are the target cells of $1,25(OH)_2D3$. In a morphological study, it was observed that K562 cells were differentiated toward monocyte after the treatment of $1,25(OH)_2D3$. This was further evidenced by the increased expression of CD11b epitope, an adhesion marker on monocyte surface [28], detected by antibody and flow-cytometry (to be published).

The results shown in Figure 2, Table 2 and Figure 3 indicated that the effect of $1,25(OH)_2D3$ on pp-GalNAc-Ts was not related to cell growth or apoptosis, but associated with cell differentiation.

We have used real time RT-PCR to check the data shown in Figure 5 and 6 using RT-PCR, the results from two methods were similar to each other. These findings indicate that the same subtype of pp-GalNAc-T in different cell lines responses to $1,25(OH)_2D3$ differently, which may be related to the different expressions or nuclear localization of VDR in K562 and SHI-1 cells [29]. On the other hand, the different

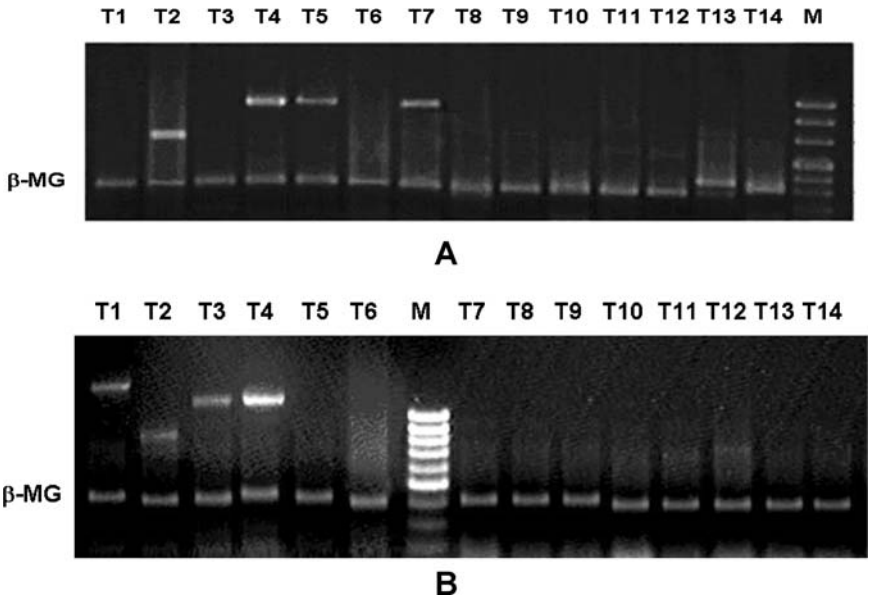

Fig. 4 Expressions of pp-GalNAcT1∼T14 in K562 and SHI-1 cells A. K562 cells. B. SHI-1 cells. T1∼T14: pp-GalNAcT1∼T14; β-MG: β-microglobulin; M: pUMix DNA marker. The RT-PCR method for the detection of pp-GalNAcT1∼T14 was described in the "Methods". Three independent and reproducible experiments were performed.

subtypes of pp-GalNAc-T in the same cell line also response to $1,25(OH)_2D3$ diversely, suggesting that the regulatory mechanisms of the expressions of different subtypes of pp-GalNAc-T are not the same. The different changes of pp-GalNAc-T expressions after $1,25(OH)_2D3$ treatment indicate the diverse functions of different pp-GalNAc-Ts in the $1,25(OH)_2D3$ induced differentiation of SHI-1 cells.

We have not determined the activity changes of pp-GalNAc-Ts, the reasons are as following. First, different subtypes of pp-GalNAc-T share the same substrate frequently. If a definite substrate is used, the detected activity is the total activity of pp-GalNAc-Ts in the enzyme preparation that can act on the same substrate. Secondly, it is almost impossible for us to purify each pp-GalNAc-T from the cell lysates for using in the assay of activity.

From our findings, it is reasonable to suggest that pp-GalNAc-T2, -T4, -T5 and -T7 are involved in the differentiation of K562 cells, while only pp-GalNAc-T1 and -T2 are participating in the differentiation of SHI-1 cells. However, pp-GalNAc-T3 is supposed to be not related to, and T4 may negatively associate with the differentiation of SHI-1 cells.

Pp-GalNAc-T1 and -T2 are widely distributed in most human tissues, while T3 is found predominantly in human testis, kidney, digestive and reproductive tracts [3,4]. Pp-GalNAc-T4 is highly expressed in sublinguar gland, stomach and colon, but less in small intestine, urogenital tract and lung [5]. Pp-GalNAc-T7 expression is similar to that of T4 [31]. The distribution of pp-GalNAc-T5 was reported in rodent tissues, but not in human [32]. High expression was found in rat sublingual gland, with lesser amounts in the stomach, small intestine and colon. In this study, we have discovered that pp-GalNAc-T1∼-T5, and -T7 were expressed in K562 and/or SHI-1 leukemia cells. However, only T1 and T2, but not T3, T4, T5 and T7 have been reported to be present in human normal leukocytes [3∼5,7].

Pp-GalNAc-T1, -T2 and -T3 show broad substrate specificity toward Thr/Ser-containing peptides. They have distinct but partially overlapping specificities with peptide acceptor substrates. Glycosylation of tandem repeat peptides MUC1 and MUC2 revealed that multiple potential sites in the tandem repeat were GalNAc-glycosylated by all three enzymes, but with different rates on individual sites and different numbers of incorporated GalNAc residues. Only pp-GalNAc-T3 can *O*-GalNAc-glycosylate the clustered Thr residues in the synthesis of clustered carbohydrate antigens [33,34]. Pp-GalNAc-T4 can further glycosylate the remaining Thr/Ser residues in MUC1 after the catalysis of T1, T2 and T3, suggesting that T4 can complement other pp-GalNAc-Ts in the complete *O*-glycosylation of the MUC1 tandem repeat. In other words, pp-GalNAc-T4, unlike T1∼T3, may use the already GalNAc-glycosylated peptides as its substrate, but T4 also catalyses non-glycosylated peptides [5]. In contrast, the substrate specificity of pp-GalNAc-T7 is strict, it shows apparent exclusive specificity for partially GalNAc-glycosylated acceptor substrates, and has no activity with non-glycosylated peptides [7]. On the base of the above discussion, we can imagine that *O*-GalNAc-glycosylation in K562 cells is increased after the treatment with $1,25(OH)_2D3$, indicating that more peptides and more sites on a single peptide are glycosylated by the up-regulated T2, T4, and T7. Similarly, GalNAc-glycosylation is also promoted in SHI-1 cells, which results from the increased expressions of T1 and T2. The decrease of T4 in SHI-1 cells was not significant, so its effect would not exceed that of the increased T1 and T2, especially in the high concentration (10^{-6} mol/L) of $1,25(OH)_2D3$. In K562 cells, the clustered

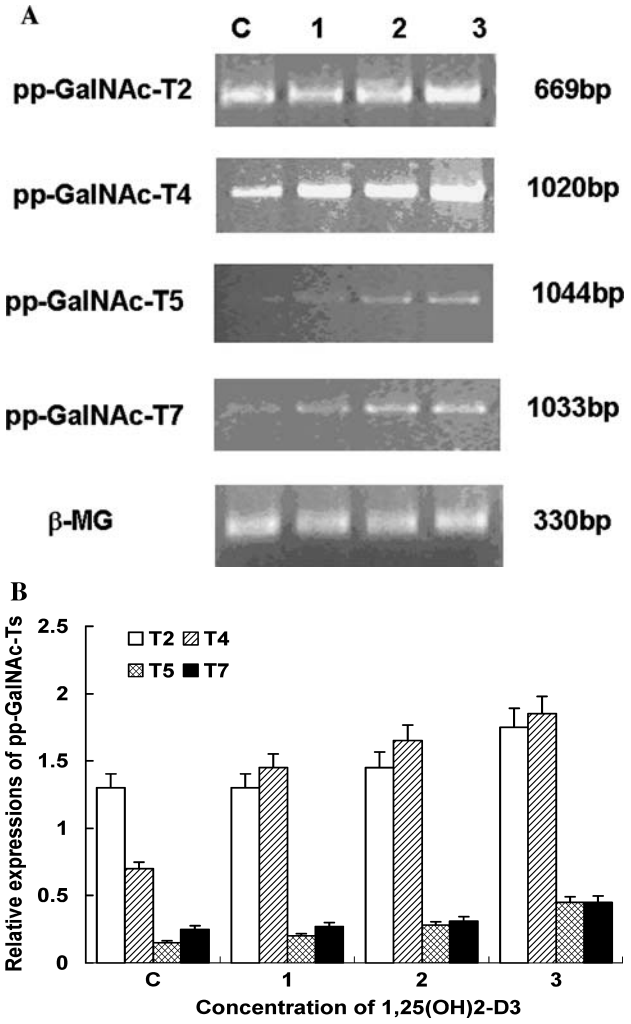

Fig. 5 Alterations of pp-GalNAcT expressions in K562 cells after the treatment with $1,25(OH)_2D3$ at various concentration. A: RT-PCR profile of pp-GalNAc-T2, T4, T5, T7. B: Semi-quantification of the results in A ($n = 3$). C, 1, 2, 3: Same as in Fig. 2, β-MG: β-microglobulin. The RT-PCR method for the detection of pp-GalNAcTs was described in the "Methods". Three independent and reproducible experiments were performed.

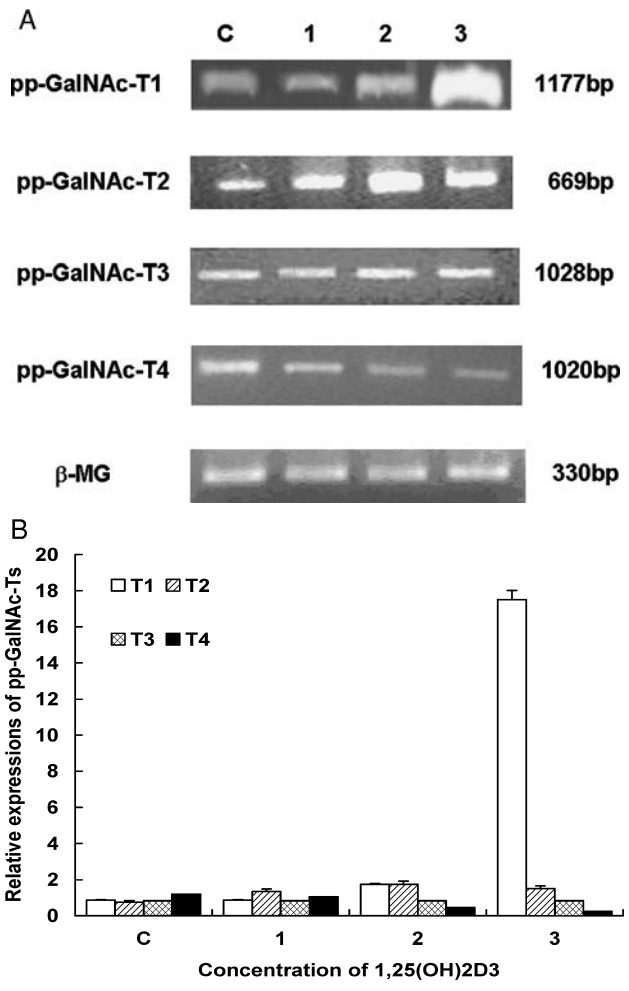

Fig. 6 Alterations of pp-GalNAcT expressions in SHI-1 cells after the treatment of $1,25(OH)_2D3$ with various concentration. A: RT-PCR profile of pp-GalNAcT1, T2, T3, T4. B: Semi-quantification of the results in A ($n = 3$). C, 1, 2, 3: Same as in Fig. 2, β-MG: β-microglobulin. The RT-PCR method for the detection of pp-GalNAcTs was described in the "Methods". Three independent and reproducible experiments were performed.

GalNAc-glycosylation might be increased owing to the up-regulated T4 and T7, while this structure was supposed to be decreased in SHI-1 cells due to the unchanged T3 and reduced T4. However, the changes of other glycosyltransferases involved in the synthesis of *O*-glycan, such as galactosyltransferases, *N*-acetylglucosaminyltransferases, sialyltransferases and fucosyltranferases after the treatment with $1,25(OH)_2D3$ have not been studied or reported, hence, we cannot know whether the whole *O*-linked sugar chains and mucin synthesis are increased by $1,25(OH)_2D3$ treatment. Therefore, the biological significance of the altered *O*-GalNAc- glycosylation in the differentiation of K562 and SHI-1 cells remains to be extensively investigated.

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