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DIFFERENT THROMBOMODULIN INDUCTION IN MONOCYTIC, MACROPHAGIC AND NEUTROPHILIC CELLS DIFFERENTIATED FROM HL-60 CELLS

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Summary: Thrombomodulin (TM) antigen and its cofactor activity for thrombin-dependent protein C activation were not detected in the untreated HL-60 human promyelocytic cell line, but appeared in cells cultured with 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$: 10 - 1,000 nM) or phorbol 12-myristate 13-acetate (PMA: 0.1 - 10 nM) accompanied by an increase in TM mRNA levels. The induction of TM increased in parallel with the appearances of both nonspecific esterase activity, a typical marker of monocyte/macrophage lineages, and phagocytic activity. The TM antigen level induced in 1α,25$(OH)_2D_3$-treated cells was 8 times higher than that in PMA-treated cells. Trace amounts of TM antigen were induced in neutrophilic cells differentiated from HL-60 by treatment with retinoic acid. These results indicated that different levels of TM were induced in monocytic, macrophagic and neutrophilic cells differentiated from HL-60 cells.

Thrombomodulin (TM), a high affinity thrombin receptor, was first demonstrated on the surface of vascular endothelial cells (1,2). TM inhibits the procoagulant properties of thrombin by its complex formation with thrombin, and the resulting TM-thrombin complex in turn functions as an anticoagulant through activation of protein C (1,2). Expression of TM on the surface of endothelial cells is increased by exposure of the cells to dibutyryl cAMP (3,4) or retinoic acid (5,6), and is decreased by exposure to tumor necrosis factor-α (6-8) or interleukin-1β (4). TM antigen is not only present in endothelial cells, but also found in platelets, a megakaryoblastic cell line, and circulating monocytes and neutrophils (9-12).

It was reported that fetomodulin (FM), identified as mouse TM by gene cloning and functional assay, appeared in parietal endoderm cells when mouse teratocarcinoma F9 stem cells were differentiated into parietal endoderm cells by treatment with retinoic acid and cAMP (13). Imada et al. (14) also found that FM was localized not only in the vasculature, but also in tissues that were not directly exposed to body fluids in the mouse embryo, and they postulated the protein to be a multifunctional protein in embryonic development.

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Abbreviations: TM, thrombomodulin; 1α,25$(OH)_2D_3$, 1α,25-dihydroxy-vitamin D_3; PMA, phorbol 12-myristate 13-acetate; NBT, nitro blue tetrazolium; RT-PCR, reverse transcription polymerase chain reaction; HUVEC, human umbilical vein endothelial cells.

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The HL-60 cell line was established using the peripheral blood leukocyte of an adult human female with acute promyelocytic leukemia (15). HL-60 cells can be differentiated into monocytic, macrophagic, or neutrophilic cells by culture of the cells with 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), phorbol 12-myristate 13-acetate (PMA) or all-*trans* retinoic acid, respectively (16). Since any the relationship between TM induction and differentiation of human cells remains largely unknown, TM induction during differentiation of HL-60 cells was investigated in the present study.

MATERIALS AND METHODS

Materials; 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), phorbol 12-myristate 13-acetate (PMA) and other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan, unless otherwise indicated. RPMI 1640 medium, Dulbecco's modification of Eagle's medium (D'MEM) and fetal calf serum (FCS) were obtained from Flow Laboratories, Irvine, Scotland, U.K. All-*trans* retinoic acid (retinoic acid) and yeast particles (*Saccharomyces cerevisiae*) were purchased from Sigma, St. Louis, MO. Esterase stain kit was purchased from Muto Pure Chemicals Co. Ltd., Tokyo, Japan.

Cell culture; The human promyelocytic leukemia cell line HL-60 was kindly donated by Dr. D. Mizuno (Biotechnology Research Center, Teikyo University, Kanagawa, Japan). Cells were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated FCS, 10 mM HEPES, 72 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5 % CO_2. For the induction of differentiation in HL-60 cells, the cells were seeded at initial concentration of 2 x 10^5 or 5 x 10^5 cells/ml and incubated with various concentrations of 1α,25$(OH)_2D_3$ or retinoic acid or PMA, respectively. After each incubation period, cell viability was determined by trypan blue exclusion (17). In all cases, trypan blue exclusion indicated that >90% of cells remained viable. Human umbilical vein endothelial cells (HUVEC) were harvested according to the method of Jaffe et al. (18) and cultured in D'MEM supplemented with 20% heat-inactivated FCS, 72 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5 % CO_2. Passages of the HUVEC were performed by the method previously described (6), and cells were used for the present experiments within three passages.

Assay of differentiation markers in cells; Phagocytic activity was measured by phagocytosed yeast particles which were heat-killed and opsonized with fresh human serum as described by Nojiri et al. (19). Nonspecific esterase activity was determined in cytocentrifuged preparations using an esterase stain kit with α-naphthyl butyrate as substrate (20). Nitro blue tetrazolium (NBT) reduction was assayed as reported previously (21). The percentage of nonspecific esterase activity, phagocytosing cells, and NBT reduction positive cells were determined by counting at least 200 cells. Adherent cells were recovered by scraping with rubber policeman after removing nonadherent cells.

Assay for TM antigen levels and the cofactor activity; After incubation of HL-60 cells with 1α,25$(OH)_2D_3$, PMA, or retinoic acid, the cells were washed 3 times with Hanks' balanced salt solution (pH 7.4), and TM was extracted from the cells with 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 0.5 % Triton X-100, and 1 mM benzamidine hydrochloride for 1 hr at 4°C. The TM antigen levels were measured by enzyme immunoassay using monoclonal antibodies (TMmAb 2, 11 and 20) as previously described (22). TM cofactor activity for thrombin-dependent protein C activation was measured by the method described previously (23).

Determination of TM mRNA levels by reverse transcription polymerase chain reaction (RT-PCR); Total RNA was prepared by the guanidium thiocyanate method (24) from HL-60 cells (10^7 cells) treated with or without 1α,25$(OH)_2D_3$, PMA, or retinoic acid. Reverse transcription and PCR were performed by the method previously described (6). The PCR product count from TM cDNA was normalized to that from the β-actin cDNA. When PCR samples were prepared without transcriptase in the reverse transcription reaction mixture, no radioactive products were obtained with this PCR procedure.

RESULTS

Differentiation of HL-60 cells into monocytic, macrophagic and neutrophilic cells and TM induction

The differentiation of HL-60 cells cultured with $1\alpha,25(OH)_2D_3$ or PMA into monocytic or macrophagic cells was confirmed by the appearance of nonspecific esterase, phagocytic, and NBT reduction activity and cellular adhesiveness (Fig. 1 and 2). In the $1\alpha,25(OH)_2D_3$-treated HL-60 cells, all of these activities were markedly induced in a dose- and time-dependent manner, whereas the adhesive property was only slightly induced (Fig. 1). The appearance of nonspecific esterase activity (a marker of monocytes and macrophages) was observed in 70 % of the cells cultured with 1 μM $1\alpha,25(OH)_2D_3$ for 6 days, indicating that about 70% of HL-60 cells were differentiated into monocytic cells during the treatment (Fig. 1). In the PMA-treated HL-60 cells, the nonspecific esterase and phagocytic activities were also induced in a dose- and time-dependent manner, but no induction of NBT reduction was observed (Fig. 2). Adherent

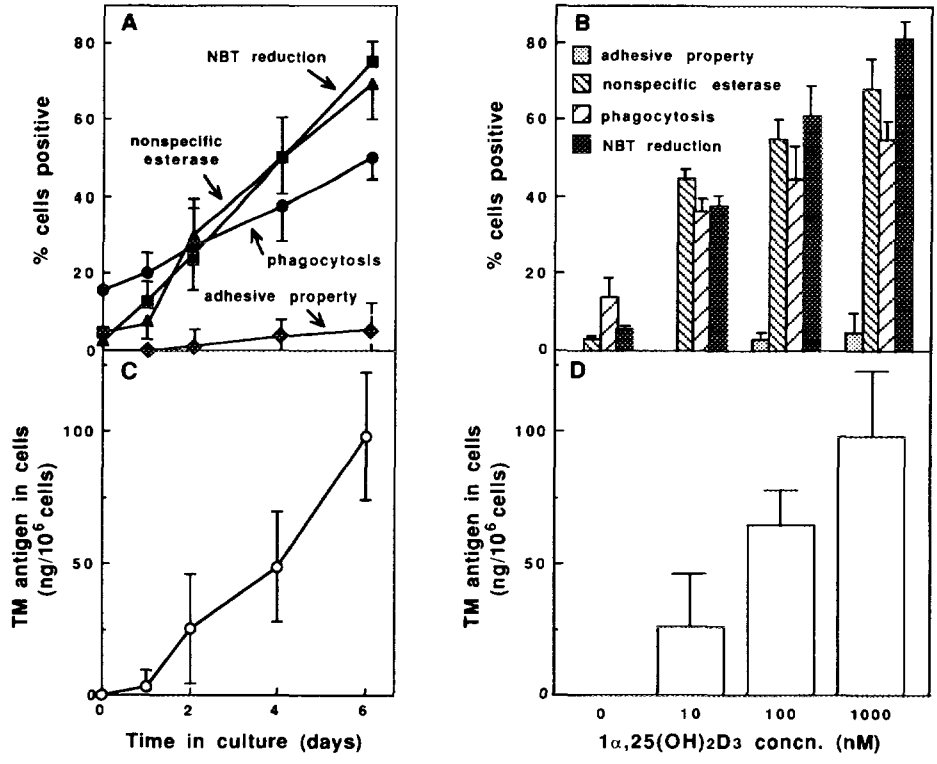

Fig.1. Differentiation of HL-60 cells into monocytic cells and TM induction by treatment of the cells with $1\alpha,25(OH)_2D_3$. HL-60 cells were cultured with 1μM $1\alpha,25(OH)_2D_3$ for various periods (A,C) or various concentrations of $1\alpha,25(OH)_2D_3$ for 6 days (B,D). After each incubation period, phagocytic, nonspecific esterase, and NBT reduction activities and adhesive property were determined as differentiation markers. TM antigen levels were measured by the methods described in Materials and Methods. Results are shown as mean ± S.D. of 4 different experiments.

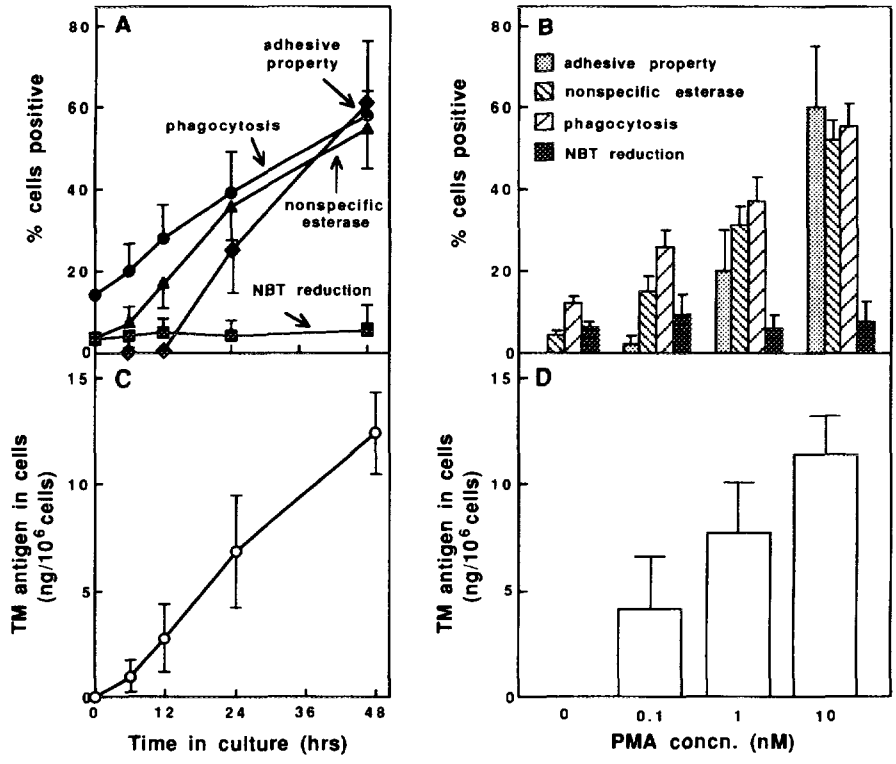

Fig.2. Differentiation of HL-60 cells into macrophagic cells and TM induction by treatment of the cells with PMA. HL-60 cells were cultured with 10 nM PMA for various periods (A,C) or various concentrations of PMA for 2 days (B,D). After each incubation period, phagocytic, nonspecific esterase, and NBT reduction activities and adhesive property were determined as differentiation markers. TM antigen levels were measured by the methods described in Materials and Methods. Results are shown as mean ± S.D. of 4 different experiments.

cells and nonspecific esterase positive cells were observed at 60 % and 55 %, respectively, of the total cells cultured with 10 nM PMA for 48 hrs, indicating that about 55 - 60 % of the HL-60 cells differentiated into macrophagic cells (Fig. 2).

TM antigen was not detected in untreated HL-60 cells as measured by enzyme immunoassay, but appeared in parallel with the appearance of nonspecific esterase and phagocytic activity in HL-60 cells cultured with $1\alpha,25(OH)_2D_3$ or PMA (Fig. 1 and 2). The antigen levels were 98.1 ± 25.2 ng and 12.1 ± 1.8 ng/10^6 cells in the cells cultured with 1 μM $1\alpha,25(OH)_2D_3$ for 6 days and 10 nM PMA for 2 days, respectively (Fig. 1 and 2), as compared with 285 ± 45 ng/10^6 cells in cultured human umbilical vein endothelial cells (HUVEC). In contrast, when about 80 % of HL-60 cells were differentiated into neutrophilic cells by treatment of the cells with 10 μM retinoic acid for 4 days, only a trace amount of TM antigen (< 1.0 ng/10^6 cells) was detected in the cells (data not shown). The differentiation into neutrophilic cells was confirmed by the appearances of cells with phagocytic (80 %) and NBT reduction (85 %) activity and no appearances of nonspecific esterase or increased adhesive property.

The cofactor activity of TM for thrombin-dependent protein C activation was measured in cell lysates after culture with 1 μM 1α,25(OH)$_2$D$_3$ for 6 days, 10 nM PMA for 2 days, or 10 μM retinoic acid for 4 days (Table 1). Cofactor activity was not detected in untreated HL-60 cells nor in cells cultured with retinoic acid. The total cofactor activity in the cell lysates was 0.44 and 0.08 pmol of protein C activated/min/10^6 cells in 1α,25(OH)$_2$D$_3$- and PMA-treated cells, respectively, as compared with 0.96 pmol/min/10^6 cells in HUVEC.

TM mRNA levels in monocytic, macrophagic and neutrophilic cells

Total RNA was extracted from HL-60 cells cultured with 1 μM 1α,25(OH)$_2$D$_3$ for 6 days, 10 nM PMA for 2 days, or 10 μM retinoic acid for 4 days and TM mRNA levels in these cells were measured using the RT-PCR method (Fig. 3). Marked increases in TM mRNA were observed in the 1α,25(OH)$_2$D$_3$- or PMA-treated cells, whereas it was very low in untreated HL-60 cells or the retinoic acid-treated cells. The mRNA level in 1α,25(OH)$_2$D$_3$-treated cells was higher than that in PMA-treated cells.

DISCUSSION

It is widely accepted that HL-60 cells differentiate into monocytic, macrophagic, or neutrophilic cells by culture of the cells with 1α,25(OH)$_2$D$_3$, PMA, or retinoic acid, respectively. The differentiation of the cells was confirmed by the characteristic appearance of nonspecific esterase, phagocytic, and NBT reduction activities and adhesive property, and a similar extent of differentiation into the monocytic, macrophagic or neutrophilic cells was obtained by treatment of the cells with 1 μM 1α,25(OH)$_2$D$_3$ for 6 days, 10 nM PMA for 2 days, or 10 μM retinoic acid for 4 days. The present studies demonstrated that TM with cofactor activity for thrombin-dependent protein C activation appeared in 1α,25(OH)$_2$D$_3$- or PMA-treated HL-60 cells, whereas neither TM antigen nor cofactor activity were observed in

Table 1. TM cofactor activity in endothelial cells and monocytic, macrophagic, or neutrophilic cells differentiated from HL-60 cells

Cells	TM cofactor activity (pmol of protein C activated/min/10^6 cells)
HL-60 cells	
untreated	N.D.
1α,25(OH)$_2$D$_3$	0.44 ± 0.12
PMA	0.08 ± 0.05
retinoic acid	N.D.
HUVEC	0.96 ± 0.11

HL-60 cells were cultured with or without 1 μM 1α,25(OH)$_2$D$_3$ for 6 days, 10 nM PMA for 2 days, or 10 μM retinoic acid for 4 days. TM was extracted from the HL-60 cells or human umbilical vein endothelial cells (HUVEC) and the cofactor activities were measured as described in Materials and Methods. N.D., not detectable. Results are shown as mean ± S.D. of 4 different experiments

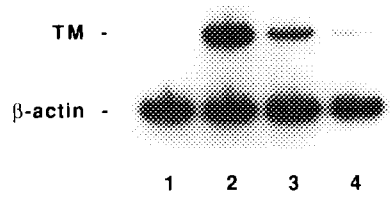

Fig.3. TM mRNA levels in HL-60 cells treated with 1α,25(OH)$_2$D$_3$, PMA, or retinoic acid. Total RNA was prepared from untreated HL-60 cells (lane 1) or HL-60 cells treated with 1μM 1α,25(OH)$_2$D$_3$ for 6 days (lane 2), 10 nM PMA for 2 days (lane 3), or 10 μM retinoic acid for 4 days (lane 4), and then reverse transcription and PCR were performed. Results shown are representative of 3 different experiments.

untreated HL-60 cells. The appearance of TM in the cells treated with 1α,25(OH)$_2$D$_3$ or PMA increased in parallel with the extent of differentiation into monocytic or macrophagic cells as monitored by nonspecific esterase and phagocytic activities. It is highly probable that TM biosynthesis was initiated in these cells, because increases in TM mRNA levels were also observed in both cell systems. However, the levels of TM antigen and cofactor activity were different between the differentiated monocytic and macrophagic cells. McCachren et al. (11) recently reported that immunohistochemical staining of TM in circulating monocytes was convincingly more intense than that for macrophages lining synovial tissue. Therefore, the different levels of TM expression during differentiation of HL-60 cell may reflect a different TM content between circulating monocytes and macrophages.

We reported that TM antigen and its cofactor activity in HUVEC were markedly increased with an accompanying increase in TM mRNA by treatment of the cells with 10 μM retinoic acid (5,6). When HL-60 cells were differentiated into neutrophilic cells by treatment with same concentration of retinoic acid, trace amounts of TM antigen were observed, but without detectable cofactor activity. Conway et al. (12) reported that human circulating neutrophils contain TM antigen which does not possess TM cofactor activity. However, treatment of HUVEC with 1 μM 1α,25(OH)$_2$D$_3$ did not affect on the TM content of these cells (data not shown), whereas treatment of HL-60 cells with the same concentration of compound markedly increased TM biosynthesis accompanying increase in the mRNA (Fig. 1 and 3). These findings imply that TM expression in blood cells including monocytes, macrophages, and neutrophils is regulated by mechanisms different from HUVEC and the mechanism of regulation may be different in each cell species.

The present paper is the first to demonstrate TM induction in human cell differentiation. Different levels of TM induction in the differentiation of HL-60 cells could provide significant information to investigate the regulation mechanisms for induction and function of TM.

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