Gene Expression during Platelet-Like Particle Production in Phorbol Myristate Acetate-Treated MEG-01 Cells

Yoshimasa ISAKARI,*b,∗ Shinji SOGO,1 Tatsuhiro ISHIDA,a Takuma KAWAKAMI,b Toshihide ONO,c Takao TAKI,a and Hiroshi KIWADAa

a Department of Pharmacokinetics and Biopharmaceutics, Subdivision of Biopharmaceutical Sciences, Institute of Health Biosciences, The University of Tokushima; 1–78–1 Sho-machi, Tokushima 770–8505, Japan; b Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd.; and c BioInfomatics Institute, Otsuka Pharmaceutical Co., Ltd.; 463–10 Kagasuno, Kawauchi-cho, Tokushima 771–0192, Japan.

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A comprehensive gene-expression analysis during platelet (PLT) production from megakaryocytes may give important information on genes involved in the PLT production process. However, the low abundance of primary megakaryocytes makes the gene expression analysis difficult. Therefore, we employed MEG-01 cells, a human megakaryocytic cell line, and confirmed that the cell line produces PLT-like particles by treatment with phorbol myristate acetate (PMA). After treatment of MEG-01 cells with PMA for 8 or 24 h, comprehensive gene expression analysis was carried out using a microarray and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). From the microarray analysis, 141 genes were up-regulated (>2-fold) and 164 genes were down-regulated (<1/2-fold). However, known PLT-related genes were not included in the up- or down-regulated genes. On the other hand, RT-PCR analysis detected increased expression of β1-tubulin, CD62P, gpIIb and gpIIbIII, which are related to PLT function and megakaryocyte differentiation, following PMA treatment for 24 h. These results indicate that the MEG-01 cell may be an alternative model to study the process of human PLT production from megakaryocytes. The gene-expression analysis might be a powerful tool for identifying genes related to PLT production, if the experimental conditions are optimized.

Key words platelet production; gene expression analysis; megakaryocyte

Circulating blood platelets (PLT) constitute the primary defense against bleeding and abnormalities in PLT or their function contribute to various hemorrhagic and thrombotic disorders. Pancytopenia, including neutropenia and thrombocytopenia, is one of limiting factors in dose escalated cancer chemotherapy, which can cause severe problems, such as fatal intracerebral bleeding. PLT transfusion is usually performed to avoid such bleeding. However, this increases the risk of infection and graft versus host disease.1,2 Enhancing the recovery of the peripheral PLT count is necessary to improve the quality of life for patients.

Megakaryocytes, which differentiate from hematopoietic stem cells, represent only 0.4% of the total bone marrow cells3 and produce PLTs through multiple stages.4 Although thrombopoietin (TPO) was identified as the only potential differentiation factor for megakaryocytopoiesis,5,6 it somewhat inhibits PLT production from mature megakaryocyte.7 Therefore, it has insufficient activity in cancer-chemotherapy-induced thrombocytopenia (CIT).

For the development or design of medications to achieve the recovery of PLT counts, we have focused on the production-mechanisms of PLT from megakaryocytes. Many studies have tried to identify molecules associated with PLT production, yet few, if any, have been defined. In transcription factor p45 subunit of nuclear factor erythroid 2 (NF-E2 p45) deficient mice, severe thrombocytopenia was observed.8 However, deficiencies of Thromboxane synthase (Txs) and β1-tubulin, known as the target molecules of NF-E2,9–11 did not result in complete attenuation of PLT production.12 Hence, it is accepted that those molecules are strongly associated with PLT function, but are not critical for PLT production.

Gene expression profiling using oligonucleotide microarrays has recently become established as a useful tool for seeking candidate target molecules. This technique could be applicable to identify the molecules that are closely related to PLT production. However, due to the low abundance of primary megakaryocytes, they are not preferred as a source for a comprehensive gene expression analysis using a microarray. MEG-01 cells, a human megakaryocytic leukemia cell line,13 is a popular cell line that has been used in megakaryocyte differentiation studies, in gene function validation studies in megakaryocytopoiesis, in studies of PLT-like particle production, ploidy, and for the study of in vivo tumorigenesis.14 It has been confirmed that MEG-01 cells are committed to the megakaryocytic lineage and can produce PLT-like particles.15 In addition, it was reported that treatment with phorbol myristate acetate (PMA) enhanced the differentiation of MEG-01 cells,16 leading to the possibility that PMA-treatment could enhance the production of PLT-like particles from the MEG-01 cells. Accordingly, MEG-01 cells may be a good source for identifying the critical genes for PLT production by means of a RT-PCR.

In this study, we treated MEG-01 cells with PMA to enhance the production of PLTs from the cells. We then performed a comprehensive gene expression analysis in order to define genes strongly related to PLT production. The results indicated that MEG-01 cells might be a suitable model to study the process of PLT production in thrombocytopoiesis and that the gene-expression analysis also may be a powerful tool to identify genes relating to PLT production.

MATERIALS AND METHODS

Materials Recombinant human thrombopoietin (rhTPO) was purchased from Peprotech (NJ, U.S.A.). Bovine serum albumin (BSA), EDTA, and phorbol myristate acetate (PMA)
were purchased from Sigma (MO, U.S.A.).

**Cell Lines** MEG-01 and U937 cells were purchased from American Type Culture Collection (VA, U.S.A.) and cultured in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (JRH, KS, U.S.A.), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, CA, U.S.A.) at 37 °C in a 5% CO₂ humidified atmosphere.

**PMA or rhTPO Treatment of MEG-01 Cells and Counting of Culture-Derived PLT-like Particles** MEG-01 cells (2.5×10⁴ cells) were seeded in a 25 cm² culture flask and incubated with PMA (10 nm) or rhTPO (10 ng/ml) for 1 to 5 d at 37 °C. After incubation, the number of PLT-like particles derived from MEG-01 cells was counted using a EPICS XL-MCL flow cytometer (Beckman Coulter, CA, U.S.A.).

**RNA Purification and GeneChip Analysis** MEG-01 and U937 cells (1×10⁶ cells) were seeded in a 25 cm² culture flask and incubated with PMA (10 nm) for 8 or 24 h at 37 °C. After incubation, the cells were collected by centrifugation (1710×g) for 10 min at 4 °C, and stained with FITC-conjugated anti-human CD41 (gpllb) monoclonal antibody (mAb) (TP-80, Nichirei, Tokyo, Japan). Twenty thousand units of Flow Count (Beckman Coulter, CA, U.S.A.) were added to each sample as an internal control. The derived PLT-like particles derived from MEG-01 cells was counted using a EPICS XL-MCL flow cytometer (Beckman Coulter, CA, U.S.A.).

**Production of PLT-like Particles from MEG-01 Cells by PMA Treatment** Following a 3 d-incubation of MEG-01 cells with or without PMA or rhTPO, the number of PLT-like particles per 1000 units of Flow Count were enumerated as CD41⁺ events with the same forward/side scatter properties as human peripheral blood PLTs.

**RT-PCR** MEG-01 cells were seeded at a density of 5×10⁴ cells/ml and treated with PMA (10 nm) for 1, 2, 4, 8, 24, 48 or 72 h at 37 °C. After treatment, the cells were collected by centrifugation (1710×g, 10 min, 4 °C), and total RNA was isolated using an RNeasy mini kit according to the manufacturer’s protocol. Total RNA extracted from each cell pellet was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, CA, U.S.A.) and random primers (Takara, Shiga, Japan) in a 20 μl mixture and diluted to 100 μl with distilled water. Subsequent PCR amplification (50 μl) was performed with 5 μl of cDNA solution using TaKaRa Ex Taq in the presence of specific primer pairs. Each cycle of PCR consisted of 30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C. The following oligonucleotides were used as primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer, 5’-TGAAGTCCGAGTCAACGGATTTGTTG-3’, antisense primer, 5’-CATGTGCCATGAGTCCACCAC-3’. TXS (thromboxane synthase) sense primer, 5’-CTCTTGTGGTTGGATAGACAGAACC-3’, antisense primer, 5’-TCACGCTAGTTAGACACAAGAACC-3’. CD62P (P-selectin), sense primer, 5’-GAACCTTAAAGCCGCTGCTCTTTGACC-3’, antisense primer, 5’-CTCAATCTCGCTTGATAGACACAAGAACC-3’. CD42b (gpIb-α), sense primer, 5’-AGATGCGAAAGCTCACCACCA-3’, antisense primer, 5’-TAGCTCTACTATATGCTTTG-3’, maK, sense primer, 5’-ATGACGACTATCCTCAACACCG-3’, antisense primer, 5’-CTAGGTGCAAGGCACAAGGG-3’. PAI-I, sense primer, 5’-GACACAGGAATGACGAGACAC-3’, antisense primer, 5’-ATCTAGACGACTTGTTGATAGG-3’. CD20 (gplla), sense primer, 5’-AAGCTGAGAAGCTCAGTCTGG-3’, antisense primer, 5’-CTCTTACTGAGATCAGTCTGG-3’.

**Statistics** Two-way analysis of variance and Dunnett’s multiple comparison test was used in order to analyze the differences between the control group and the PMA treated, or TPO-treated group. The level of significance was set at p<0.05.

**RESULTS**

**Production of PLT-like Particles from MEG-01 Cells by PMA Treatment** Following a 3 d-incubation of MEG-01 cells with or without PMA or rhTPO, the number of PLT-like particles (cells that could be stained with fluorescence-labeled anti-human CD41 Ab) in the medium was determined by flow cytometry. For PLT-like particle gating, a region 1 (R1) was designated by the loading of human peripheral PLTs following staining with fluorescence-labeled anti-human CD41 Ab (data not shown). The designated region shown in Fig. 1A was loaded with unstained control. In the absence of PMA, slight staining of PLT-like particles was detected in the designated region (Fig. 1B). The number of PLT-like particles was increased by treatment with PMA (Fig. 1C), while the number was not increased by treatment with rhTPO (Fig. 1D).

The effect of time of incubation with PMA on the production of PLT-like particles from MEG-01 cells was investigated. The number of PLT-like particles in the incubation media increased with increasing time of incubation in the
presence of PMA (Fig 2). The number of PLT-like particles was 1.5-fold higher than those in the presence of rTPO and in the absence of PMA (control). It appears that MEG-01 cells can produce PLT-like particles without PMA treatment, which is consistent with previously reported observations. The PMA treatment obviously accelerated the rate of PLT-like particle production from MEG-01 cells. It has been reported that rhTPO promotes the differentiation of megakaryocytes, but does not enhance PLT production from megakaryocytes and our results confirm this observation. These results strongly suggest that a PMA-treated MEG-01 cell line is a good source for analyzing genes related to PLT production.

Comprehensive Gene Expression Analysis by Means of Microarray

The comprehensive gene expression analysis was performed at 8 and 24 h of incubation with PMA by means of microarrays, Human Genome U133A and B GeneChips, to detect genes that were affected during PLT-like particle production in MEG-01 cells. To exclude non-megakaryocyte-related genes, U937 cells, a human monocytic cell line, were employed as a control cell line. The conditions for selecting up-or down-regulated genes are shown in the Materials and Methods. As shown in a Venn diagram (Fig. 3), genes that were up-or down-regulated genes only in MEG-01 cells were 141 and 164 genes, respectively. The differentially expressed genes were classified into different categories on the basis of their functions (Table 1). The results indicated that the expressions of genes relating to cell proliferation and differentiation were affected by PMA treatment. However, genes relating to megakaryocyte differentiation or PLT-function (such as NF-E2, β1-tubulin, gpIIb (CD41), gpIbα (CD42b), gpIIa (CD61), gpIX, mafK, CD62P and TXS (18, 19) did not appear to be differentially expressed.

Gene Expression Analysis by Means of RT-PCR

Gene expression analysis in MEG-01 cells relating to PLT production was also performed at 1, 2, 4, 8, 24, 48 and 72 h of incubation with PMA by means of RT-PCR (Fig. 4). The enhancement of expression of β1-tubulin, CD62P, TXS and PAI-1 were increased by PMA treatment.
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<th>Down-regulated genes</th>
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Table 1: Classification of Up- and Down-Regulated Genes as a Function of Their Biological Roles
DISCUSSION

Megakaryocytes, among the rarest of hematopoietic cells, serve the essential function of producing PLTs. Mechanisms of thrombopoiesis are of considerable interest in hematology and cell biology. PLT production is especially important because it is the main method for quick recovery in severe thrombocytopenia. One of the barriers to the clarification of the mechanism of PLT production has been the difficulty in obtaining sufficient numbers of megakaryocytes, because mature megakaryocytes are only 0.4% of the total bone marrow cells. In this study, we employed MEG-01 cells, a human megakaryocytic leukemia cell line, as an alternative source of megakaryocytes. The cell line has been shown to produce PLT-like particles, and it has also been reported that PMA treatment enhances the differentiation of MEG-01 cells. However, there has been no report indicating that PMA-treatment accelerates the production of PLT-like particles from MEG-01 cells. Therefore, we attempted to use PMA to enhance PLT production from MEG-01 cells. As shown in Figs. 1 and 2, we confirmed the production of PLT-like particles from MEG-01, and showed that PMA accelerated the production. Thus the PMA-treated MEG-01 cells are a good system for studying human PLT production.

We then performed a comprehensive gene expression analysis on PMA-treated MEG-01 cells using microarrays. We hoped that this genetic study would provide insights into the molecular and transcriptional regulation of thrombopoiesis. We detected many genes that were up- or down-regulated specifically in the PMA-treated MEG-01 cells (Fig. 3). Most of those are similar to those reported by Raslova et al. for human megakaryocytes derived from CD34+ cells. Unfortunately, changes in PLT-related gene expressions were not detected under these experimental conditions. Initially, we expected to identify genes associated with the early phase of PLT production. Figure 4 changes in the expression of genes (β1-tubulin, CD62P, TXS etc.) associated with PMA function following 8 or 24 h-incubation with PMA. This suggests that the selected time points (8, 24 h) might be too early to detect the change in mRNA levels relating to PLT-production, even though the cells were stimulated by PMA treatment. Further studies are required to optimize the experimental conditions for microarray analysis.

RT-PCR study indicated enhanced gene expression of PLT-related markers such as β1-tubulin, CD62P and TXS and megakaryocyte differentiation markers such as gpIbα (CD42b) and gpIIIa (CD61), starting at least 24 h after incubation with PMA (Fig. 4). In addition, the study showed that those expression levels tended to reach a maximum around 48 h (Fig. 4). β1-tubulin is considered to be important for the late phase of megakaryocyte differentiation and the early phase of PLT production. Thus the result of RT-PCR study suggests that the gene expression levels relating to PLT-production increase following 48 h-incubation with PMA. In addition, the pattern of gene expressions (Fig. 4) is consistent to the pattern of PLT-production described in Fig. 2. This might mean that the PMA-treated MEG-01 cells are an alternative model system for studying the process of PLT production from megakaryocytes.

One function of bone marrow megakaryocytes (Mks) is the controlled release of PLTs into the circulation (thrombopoiesis). Over the past half decade, the molecular mechanism that contributes to PLT production has begun to be elucidated. Several fundamental questions and hypotheses on the molecular mechanism of Mks commitment, the precise role of some transcription factors and the endomitotic process still need to be addressed. Determination of the detailed transition from Mks to PLTs and, more precisely, the characterization of the molecules related to PLT release will be also extremely important. Knowledge of genes relating to PLT production will provide the basis for novel therapeutic approaches for modulation of PLT number and function. Recently, we established a gene-transfer system for MEG-01 cells. These powerful tools, including the gene delivery system and this comprehensive gene expression analysis of PMA-treated MEG-01 cells, will help future studies of, and search for, novel molecules related to PLT production.

REFERENCES