The effect of differentiation induction by a tumor-promoting phorbol diester, 12-0-tetradecanoylphorbol-13-acetate (TPA) on a clonal human megakaryoblastic cell line, MEG-01s, was investigated, and a prominent response was demonstrated. The cells became weakly adherent, developed conspicuous cytoplasmic blebs, and displayed mature megakaryocytic characteristics by light microscopy such as the development of azurophilic cytoplasmic granules and a mosaic pattern of oxyphilic patches. Multiplication of nuclei, and enhancement of the PAS reaction and alpha-naphthyl acetate esterase staining. Ultrastructural studies demonstrated the development of prominent cytoplasmic blebs, budding of blebs, and multiplication of nuclei. Numerous granules with central nucleoids that are similar to α granules had developed as well as granules with high electron density and clearly demarcated zones. Surface marker analysis revealed a moderate increase in IgG Fe receptor levels and a profound decrease in C3 receptor sites. By an immunofluorescent technique using monoclonal and polyclonal antibodies, a dramatic change in the expression of several megakaryocyte-platelet-specific proteins was demonstrated. All the proteins that have been expressed before induction such as platelet glycoprotein (GP) IIb/IIIa, fibrinogen, von Willebrand factor, factor XIIIa, β-thromboglobulin (β-TG), and HLA class 1 antigen were profoundly enhanced after induction by TPA. Induction by TPA led to the expression of fibronectin and factor V, which were not detected on nontreated cells. An ultrastructural immunoperoxidase study demonstrated platelet GPlb and GPllb/llla in both plasma membranes and protein synthesis areas such as perinuclear cisternae and endoplasmic reticulum after TPA induction. β-TG was also observed in some cytoplasmic granules of TPA-treated cells. TPA remarkably increased the secretion of β-TG into the culture medium of MEG-01s. Ploidy was also increased from 2C to 4C to 4C to 8C. Similar maturation of MEG-01s was induced by other phorbol diester analogues such as phorbol-12,13-dibutyrate, but not by phorbol itself. These results indicate that phorbol diester, TPA, can bring about differentiation and maturation of a human megakaryoblastic cell line (MEG-01s) and that MEG-01s cells will provide a useful model for studying megakaryocytic differentiation and numerous megakaryocyte-platelet-specific proteins.

**THE MECHANISMS** that result in stem cell commitment to megakaryocyte differentiation are poorly understood. In vitro megakaryocyte differentiation is known to be regulated by two factors: a megakaryocyte colony-stimulating factor, which is required for proliferation and appears to be identical to interleukin-3, and an auxiliary factor, megakaryocyte-potentiating factor, or thrombopoietin, which is necessary for full megakaryocyte development. Although most of this information has been obtained by the colony method, recent developments and studies of permanent megakaryoblastic cell lines in this and other laboratories appear to provide a useful model for the in vitro study of megakaryocyte maturation.

The tumor-promoting phorbol diesters have been shown to induce terminal differentiation of some human myeloid cell lines such as a promyelocytic cell line (HL-60), a myeloblastic line (KG-1), and myelomonoblastic lines (ML-1 and ML-3). Since phorbol diesters are well-characterized compounds, they may serve as good probes of the cellular mechanisms governing differentiation. The influence of 12-0-tetradecanoylphorbol-13-acetate (TPA) upon human megakaryocytic cell lines, however, is not well known. The establishment of a human megakaryoblastic cell line (MEG-01) prompted us to study the effect of phorbol diesters upon the differentiation of human megakaryocytes. In this paper, we have demonstrated that a clonal line, MEG-01s, has the capacity to differentiate morphologically, ultrastructurally, and biochemically into more mature megakaryocytes with the stimulation of phorbol diesters. A preliminary part of this study has been published in abstract form.

**MATERIALS AND METHODS**

**Cells**

The MEG-01 cells were established in our laboratory from a male patient in megakaryoblastic crisis of chronic myelogenous leukemia more than 3.5 years ago and have been maintained in continuous suspension culture for 340 passages. MEG-01 cells demonstrated many megakaryocytic characteristics and possessed no markers for B or T lymphocytes, myeloid cells, or erythroid cells. The cells were cultured in Falcon 3013 plastic flasks (Becton Dickinson, Oxnard, CA) in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS, 49

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Flow Laboratories, Stanmore, NSW, Australia) and aqueous penicillin G (100 U/mL) and streptomycin (50 μg/mL) at 37°C in 5% CO₂ and air in a humidified incubator. The original MEG-01 cells had a tendency to adhere to the plastic culture flask under the usual culture conditions. After cloning, clonal cells, named MEG-01s, were obtained. MEG-01s cells had almost the same characteristics as MEG-01 cells except for two properties: (a) MEG-01s cells grow in single suspension without attachment to the culture dish, and (b) the expression of platelet glycoprotein (GP) IIb/IIIa in MEG-01s cells is weaker than that in MEG-01 cells. Platelet GPIIb/IIIa was demonstrated in 25% to 40% of MEG-01s cells, whereas it was expressed in over 90% of MEG-01 cells. This clonal MEG-01s line has been maintained over 1.5 years for over 140 passages, and these characteristics have been stable. MEG-01s cells were used for all the following experiments.

Chemicals

TPA, the phorbol ester, was purchased from Paesel GmbH & Co (Frankfurt, West Germany). Stock solutions (10^-4 mol/L) of this compound were prepared in dimethyl sulphoxide (DMSO) (Dojin Chemical Institute, Kumamoto, Japan) and stored at -60°C. New stock solutions were prepared every 2 to 3 months. All stock solutions were diluted appropriately with RPMI 1640 medium just before use. The concentrations of DMSO present in the final solution of TPA (less than 0.1%) had no effect on cell growth and differentiation when added alone to the cells. Phorbol ester derivatives, such as phorbol-12,13-dibutyrate (PDBu) and phorbol, all from Sigma Chemical Co (St Louis), were also tested for their effect on cell proliferation and morphological changes in MEG-01s cells.

Cell Proliferation, Adhesion, and Morphological Studies

Cell proliferation was studied by placing 3 x 10⁴ cells/mL in Falcon 3013 plastic flasks containing 10 mL of culture medium supplemented with 10% FCS containing TPA. Cultures were harvested on days 1, 3, 5, and 7 for viable cell counts. Viable cell counts were examined by using trypan blue dye exclusion. Adhesion was examined by placing 3 x 10⁴ cells/mL in 2 mL of culture medium in flat glass tissue culture chambers (Lab-Tek Products; Miles Laboratories, Inc, Naperville, IL). After incubation for one to seven days at 37°C, the slides were gently washed with warm phosphate-buffered saline (PBS), and the nonadherent cells were collected. The adherent cells were removed by exposure to 0.25% trypsin and collected separately. Viable cells were counted.

Morphological observations of live cultured cells were made with an Olympus inverted microscope. Cytospin slide preparations of 0.2-mL aliquots of cell suspensions were prepared by using a Shandon Cytospin 2 centrifuge (Shandon Southern Products, Ltd, Cheshire, England) and stained with May-Grünwald-Giemsa (MGG). For histochemical studies, the cells were also stained by published procedures as follows: naphthol ASD chloroacetate esterase, alphanaphthyl butyrate esterase, alphanaphthyl acetate esterase, myeloperoxidase, leukocyte alkaline phosphatase, acid phosphatase, and the PAS reaction.

Ultrastructural Studies

Ultrastructural morphology. Cell samples harvested on day 5 after the addition of TPA were centrifuged at 1,200 rpm for ten minutes and then washed three times with 0.01 mol/L PBS. Ultrastructural morphology was analyzed as described previously. Control MEG-01s cells cultured in the absence of TPA were processed similarly. Ultrastructural platelet peroxidase (PPO) was studied as described earlier.

Immunoelectron microscopic observation. The indirect peroxidase-labeled antibody technique, similar to that described before, was used. MEG-01s cells (control cells and TPA-treated day 3 cells) were harvested and washed in 0.01 mol/L PBS. The cells were pelleted by centrifugation, fixed in chilled periodate-lysine-paraformaldehyde, and washed in increasing concentrations of sucrose in PBS. The fixed cells were embedded in Tissue-Tek OCT compounds (Miles), frozen in a dry ice-ethanol slurry, and sectioned (6 to 8 μm) in a cryostat. The sections, air-dried on glass slides, were reacted with an antiplatelet GPIIb/IIIa monoclonal antibody (MoAb) (HPL-3), an antiplatelet GPIIb MoAb (HPL-9), or rabbit anti-human β-thromboglobulin (β-TG) polyclonal antibody (PoAb) (Amersham Corp, Buckinghamshire, England) for 12 hours at 4°C. HPL-3 and HPL-9 were kindly provided by Dr K. Furukawa at the Department of Medicine, Nagoya University Branch Hospital. After washing, the sections were reacted with horseradish peroxidase (HRP)-labeled antimouse IgG rabbit antibody (Fab') or HRP-labeled antirabbit antibody (Fab') for six hours. Then the sections were postfixed in 0.5% glutaraldehyde in PBS, washed in PBS, and incubated sequentially in incomplete Karnovsky's solution. They were fixed in 2% osmium tetroxide in PBS, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections with or without lead stain were viewed under a Hitachi H-300 electron microscope.

Cell Surface Markers

Rosette markers. Sheep erythrocytes (SRBC), mouse erythrocytes (MRBC), SRBC sensitized with anti-SRBC rabbit IgG and IgM, and SRBC sensitized with IgM antibody complement were used for E, MRBC, EA, and EAC rosetting, respectively.

Markers detected by MoAbs and PoAbs. The expressions of human megakaryocyte-platelet–specific antigens were analyzed by using three kinds of MoAbs: (a) antiplatelet GPIIb/IIIa MoAb (HPL-3), (b) antiplatelet GPIIb MoAb (HPL-9), and (c) anti–von Willebrand factor (vWF) MoAb (Dakopatts, Glostrup, Denmark). The cells were also analyzed by the following three MoAbs: (a) anti-fibronectin MoAb (Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, MD), (b) anti-human Ia antigen MoAb (4D1), and (c) anti-HLA class I antigen MoAb (4E). Anti-vWF MoAb is the supernatant harvested from hybridoma cells, and other MoAbs are all ascites fluid.

Next, six rabbit PoAbs were also used: (a) anti-factor V (FV) PoAb (Dakopatts); (b) anti-vWF PoAb (Dakopatts); (c) anti-FXIla PoAb, (d) FXIIIa PoAb (Behringwerke AG, Marburg, West Germany), (e) antihuman fibrinogen PoAb (Dakopatts), and (f) antihuman fibrinectin PoAbs (Dakopatts).

Binding of these antibodies was tested by an indirect immunofluorescence (IF) technique as described previously. Cell samples were analyzed by flow cytometry (FACS IV, Becton Dickinson). Normal mouse serum and normal rabbit serum were used as negative controls of primary antibody for MoAb and PoAb, respectively. Some samples were resuspended in a minimal amount of medium, which was deposited with one or two drops of 50% buffered glycerol on a coverslip. They were examined and photographed under an Olympus fluorescence microscope.

Cytoplasmic Antigens

The six PoAbs described earlier and anti–β-TG PoAb were used for the study of cytoplasmic antigens of TPA-treated cells (day 3) and control MEG-01s cells. After washing three times in cold PBS, the cells were centrifuged and resuspended in a small amount of PBS, and cytospin glass slide preparations were prepared. The cells were fixed with 4% paraformaldehyde at 4°C for 30 minutes, washed three times in PBS, and incubated in 0.1% Triton X-100 in PBS at room temperature for 15 minutes. After being washed three times in PBS, the specimens were incubated with 5% normal goat serum in PBS for 15 minutes. After being gently rinsed, the specimens were
incubated with each primary antibody at 4°C overnight. A negative control for the primary antibody was performed as described earlier. After being washed three times in cold PBS, a fluorescein isothiocyanate-labeled second antibody, as described earlier, was reacted at 4°C for one hour. The samples were then washed three times in cold PBS and deposited in one drop of 50% buffered glycerol on a coverslip. They were examined, and positive cells per 200 cells were counted.

**Flow Cytometric DNA Analysis**

The cell suspensions (5 × 10⁵) of control and TPA-treated MEG-01s cells (days 1, 3, and 5) were mixed with normal human peripheral mononuclear cells (5 × 10⁵), and the mixtures were stained with 50 μg/mL propidium iodide (Sigma) in 0.1% sodium citrate containing 0.1% Nonidet P40 (Sigma) and treated with ribonuclease (1 mg/mL) (Sigma) simultaneously.12,22 There was no cell clumping in both control and TPA-treated cells. The DNA content per cell of up to 2 × 10⁶ cells was measured in a flow cytometer (Ortho Cytofluorograf 50H, Westwood, MA) after two to 24 hours of staining. Fluorescence of over 600 nm was measured at 200 mW and a wavelength of 488 nm. The internal standard was adjusted to 19 channels of 200 total channels to detect 16C, and the peak of the diploid cells was demonstrated in channel 25.

**Analysis of β-TG Antigens**

β-TG antigen was analyzed by a radioimmunoassay (RIA) using a monospecific rabbit antihuman β-TG antibody (β-TG RIA pack, Amersham). MEG-01s cells were seeded at 2 × 10⁵/mL in 8 mL of RPMI 1640 medium containing 1% bovine serum albumin (BSA). Aliquots (2 mL) were removed every two to three days, and the same volume of fresh medium was replaced. Harvested medium was mixed with 2 μL of ethanol containing 10 mmol/L phenylmethylsulfonylfluoride (Sigma). The medium was then centrifuged, and the supernatant was frozen until use.

**RESULTS**

**Effect of TPA on Cell Proliferation, Adhesion, and Morphology**

Figure 1 shows the proliferation curve of MEG-01s cells in the presence of various concentrations of TPA. The cell numbers are the total of nonadherent and adherent cells. TPA at 10⁻⁵ to 10⁻⁹ mol/L inhibited the proliferation of the MEG-01s cell significantly. By the fifth day of TPA (10⁻⁷ to 10⁻⁸ mol/L) exposure, the net proliferation of cells decreased by approximately 28% (range, 24% to 39%), TPA at 10⁻¹⁰ mol/L, however, had almost no effect on cell proliferation.

A part of MEG-01s cell population began to adhere to the plastic surface 30 minutes after exposure to TPA. On day 1, the size of the cells began to increase, and cytoplasmic protrusions (blebs) became prominent. In contrast to profusely basophilic cytoplasm without any granules of control MEG-01s cells (Fig 2A), the cytoplasm of TPA-treated day 1 cells showed the development of azurophilic granules in the perinuclear zone, although the cytoplasm was still basophilic (data not shown). Multiplication and condensation of nuclear chromatin became evident on days 3 to 5, and numerous azurophilic granules of the cytoplasms had developed (Fig 2B). On day 7, the size of the cells increased, cytoplasmic azurophilic granules were more prominent, and a typical mosaic pattern of oxyphilic patches was observed (Fig 2C). The positivity of the PAS reaction and alphanaphthyl acetate esterase staining increased, and the strength of these reactions became more markedly intense than that of control cells (data not shown). Dual esterase staining of alphanaphthyl butyrate esterase and naphthol ASD chlonoacetate esterase, myeloperoxidase, and neutrophil alkaline phosphatase was completely negative (data not shown). The morphology of MEG-01s cells changed dramatically after exposure to TPA. On day 1, the size of the cells began to increase, and cytoplasmic protrusions (blebs) became prominent. In contrast to profusely basophilic cytoplasm without any granules of control MEG-01s cells (Fig 2A), the cytoplasms of TPA-treated day 1 cells showed the development of azurophilic granules in the perinuclear zone, although the cytoplasm was still basophilic (data not shown). Multiplication and condensation of nuclear chromatin became evident on days 3 to 5, and numerous azurophilic granules of the cytoplasms had developed (Fig 2B). On day 7, the size of the cells increased, cytoplasmic azurophilic granules were more prominent, and a typical mosaic pattern of oxyphilic patches was observed (Fig 2C). The positivity of the PAS reaction and alphanaphthyl acetate esterase staining increased, and the strength of these reactions became more markedly intense than that of control cells (data not shown). Dual esterase staining of alphanaphthyl butyrate esterase and naphthol ASD chlonoacetate esterase, myeloperoxidase, and neutrophil alkaline phosphatase was completely negative during the seven-day observation period. PDBu showed equal effects on the cell proliferation, cell morphology, and the histochemical pattern. In contrast, phorbol induced no change (data not shown).

**Utrastructural Changes Induced by TPA**

**Ultrastructural morphological effect.** On day 5 after exposure to 10⁻⁷ mol/L TPA, cytoplasmic blebs became prominent, and clusters of glycogen and “budding” were clearly seen (Fig 3A through D). The shape of the nucleus became irregular, and multiplicate. Cytoplasmic organelles were strikingly developed, and numerous granules with dense nucleoids, similar to α granules formed. Other granules with high electron density were also occasionally observed. In some cells, clearly demarcating zones were observed, but
Fig 2. (A) MGG staining of control MEG-01s cells with diameters of about 30 to 40 μm. The deeply basophilic cytoplasmas without any azurophilic granules and round to ovoid nuclei are demonstrated. (B) MGG staining of day 5 cells treated with TPA (10⁻⁷ mol/L) with diameters of about 80 to 80 μm. The enlargement of cell size, the multiplication of nuclei and the increase in azurophilic granules are evident. (C) MGG staining of day 7 cells treated with TPA (10⁻⁷ mol/L). The diameter of the cells is more than three times that of the control cells. The azurophilic granules are diffusely distributed, and a mosaic pattern of the oxyphilic patches can be observed. Cytoplasmic basophilicity is not observed (original magnification ×4,500).
whether these are typical demarcation membranes is unknown. PPO was expressed in the rough endoplasmic reticulum and perinuclear cisternae and not in the Golgi apparatus. Both control and TPA-treated MEG-01s cells showed a PPO reaction (data not shown).

Ultrastructural immunoperoxidase staining. Platelet GPIIb/IIIa was clearly demonstrated on the surface of cytoplasmic membranes, especially on the cytoplasmic blebs as HRP particles of both control cells and TPA-treated day 3 cells (data not shown). Endoplasmic reticulum, however, became labeled only in TPA-treated day 3 cells. Platelet GPIb was faintly demonstrated in the perinuclear space of control MEG-01s cells. In day 3 cells treated by TPA, however, this antigen was demonstrated not only in the perinuclear space, but also in the plasma membrane (data not shown). Negative control sections showed no specific staining. fl-TG was demonstrated in the rough endoplasmic reticulum of both control (Fig 4A) and TPA-treated cells (Fig 4B). However, positive staining of the perinuclear space and a few granules became evident only after TPA exposure.

Effect of TPA on Cell Surface Markers

Rosetting markers. E rosette, MRBC rosette, and EA tests for IgM Fc receptors were uniformly negative in both control cells and cells exposed to TPA (Table 1). The EAC test and EA tests for IgG Fc receptors were positive in 75% and 8% of control MEG-01s cells, respectively. After exposure to TPA, the positivity of the C3 receptor (EAC) rapidly decreased, whereas the positivity of the EA test for IgG Fc receptors increased.

Cell surface markers analyzed by antibodies. After exposure to TPA, the positivity of platelet GPIIb/IIIa and HLA class I antigen, vWF, and FXIIIa was dramatically increased (Tables 2 and 3). Although fibronectin and FX were not detected on the control MEG-01s cells, these antigens were clearly demonstrated after treatment with TPA. Fibrinogen levels were mildly increased following the exposure to TPA. These positivity rates were maintained during the seven-day observation period. Platelet GPIb, HLA-DR (human la-like) antigen, and FXIIIa were uniformly negative before and after TPA exposure. The increase in the expression of these proteins on TPA-treated cell surfaces is not due to the increased binding of these proteins to cell surfaces from the medium because similar changes were induced by TPA in MEG-01s cells cultured in serum-free medium (data not shown). Nonspecific binding of antibodies to the increased numbers of Fc receptors on TPA-treated MEG-01s cells was also unlikely since control experiments were negative that used, as primary antiserum, normal mouse or rabbit serum that contained the same amounts of IgG as did the specific antibodies. The positivity rate of cell surface antigens, as described earlier in control MEG-01s cells, was constant during the seven-day observation period without medium change.

Effect of TPA on Cytoplasmic Antigens

The positivity of vWF FXIIIa, β-TG, and fibrinogen was remarkably increased following exposure to TPA (Table 4). Although FV and fibronectin were not detected in control cells during the seven days of culture, these antigens were clearly demonstrated in TPA-treated cells. FXIIIa was not detected in control or TPA-treated cells.

Effect of TPA on Polyploidization

In control day 1 and day 3 cells, the pattern of DNA content was almost constant, and the peak of ploidy was in 2C (43%) and 4C (15.1%) (Fig 5A and B). After exposure to TPA, the peak of 4C increased to 29.3%, and small numbers of cells with 8C (8.4%) and 16C (7.4%) appeared.
Fig 3. (A) Electron microscopic photograph of control MEG-01s cells. The round nucleus and relatively developed cytoplasmic organelles such as mitochondria and rough endoplasmic reticulum can be observed. No demarcation membranes and α granules are found (original magnification ×7,800). (B) Electron microscopic photograph of day 5 MEG-01s cells treated with TPA (10^{-7} mol/L). Peripherally located and multiplicated nuclei are evident. Clearly demarcated zones of the cytoplasm are demonstrated (arrowheads). Granules with central nucleoids are also evident (original magnification ×3,900). (C) Electron microscopic photograph of day 5 MEG-01s cells treated with TPA (10^{-7} mol/L) (magnification of part of Fig 3B). Cytoplasmic granules and demarcating zones (arrowheads) are observed (original magnification ×6,110). (D) Electron microscopic photograph of day 5 MEG-01s cells treated with TPA (10^{-7} mol/L). Prominent cytoplasmic blebs (arrowheads), demarcating zones, and a small granule with a central nucleoid are evident (original magnification ×14,500).

Effect of TPA on the Secretion of β-TG

The concentration of β-TG in the medium increased slightly with time from <1 ng/mL on day 0 to 11 ng/mL on day 7 in the control culture without TPA (Fig 6). TPA remarkably enhanced the accumulation of secreted β-TG in the culture medium, and the quantity of secreted β-TG was dependent on the concentration of added TPA. When TPA was added at the concentrations of 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} mol/L, the quantities of β-TG on day 7 were 12, 37, 140, and 149 ng/mL, respectively.

DISCUSSION

The present paper demonstrates that TPA (10^{-7} mol/L) can stimulate a human megakaryoblastic cell line (MEG-01s) to differentiate into more mature megakaryocytes as judged by several morphological and biochemical criteria. Similar maturation of MEG-01s was induced by other phorbol diester analogs such as PDBu, but not by phorbol itself. Since our study was completed, a similar study on the effect of phorbol esters on another human megakaryocytic cell line (EST-IU) has appeared in abstract form. These results are consistent with recent observations of Long et al in in vitro marine megakaryocyte colony formation. They showed that phorbol diesters can bring about murine megakaryocyte differentiation by substituting for megakaryocyte potentiator activity.

The inhibition of growth of MEG-01s cells by TPA was similar to that of other human leukemic cell lines in which
the differentiation was induced by TPA; the concentrations of TPA used in our experiments are similar to those reported in other cell lines. The sequence of maturation of the bone marrow megakaryocytes has been divided into three stages: promegakaryoblast or megakaryoblast (stage I), promegakaryocyte or basophilic megakaryocyte (stage II), and granular megakaryocyte or mature megakaryocyte (stage III). The morphological process of cytoplasmic maturation of MEG-01s after TPA stimulation, as examined by MGG staining, resembles the process of in vivo human megakaryocyte maturation as follows: (a) control MEG-01s cells, megakaryoblast; (b) day 1 to day 3 cells treated with TPA, promegakaryocyte; (c) day 5 to day 7 cells, granular megakaryocyte. The PAS reaction, which is positive in
Fig 4. (A) Electron micrograph of day 3 control MEG-01s cells reacted with anti-β-TG PoAb. Rough endoplasmic reticulum (rER) is positive for β-TG. Filament-like cytoplasmic protrusions are observed. (B) Electron micrograph of day 3 MEG-01s cells treated with TPA (10⁻⁷ mol/L) reacted with anti-β-TG PoAb. In addition to more developed rough endoplasmic reticulum (rER) and perinuclear space, a few granules are positive for β-TG (open arrowheads). Cytoplasmic blebs are evident (closed arrowheads). Original magnification x11,000.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control Cells*</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<td>EAC (C3 receptor)</td>
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<td>3 ± 1</td>
<td>2 ± 0</td>
<td>3 ± 0.57</td>
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<tr>
<td>EA</td>
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Values (percentage of positive cells) are the means ± SD of three separate experiments.

*Values for control cells are the mean positivity rates of the control cells on each harvesting day.
DIFFERENTIATION OF A MEGAKARYOCYTIC CELL LINE

Table 2. Effect of TPA on Cell Surface Antigens. I. Analyzed by MoAbs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Control Cells</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
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<td>HPL-3</td>
<td>Platelet GPIIb/IIIa</td>
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<td>76 ± 2</td>
<td>97 ± 0.57</td>
<td>96 ± 1.5</td>
<td>40 ± 2.6</td>
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<td>HPL-9</td>
<td>Platelet GPIb</td>
<td>Neg†</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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<tr>
<td>Anti-vWF (Dakopatts)</td>
<td>vWF</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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<tr>
<td>Anti-fibronectin (BRL)</td>
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<td>16 ± 1.15</td>
<td>12 ± 0.57</td>
<td>14 ± 1</td>
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<td>4D1</td>
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<td>Neg</td>
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<td>4E</td>
<td>HLA class I antigen</td>
<td>60 ± 2</td>
<td>93 ± 1.52</td>
<td>97 ± 2</td>
<td>94 ± 1</td>
<td>94 ± 1.52</td>
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</table>

Values (percentage of positive cells) are the means ± SD of three separate experiments.
*Values for control cells are the mean positivity rates of the control cells on each harvesting day.
†Negative, <8% of the cells were positive under conditions in which the negative control showed a 5% positive background.

Table 3. Effect of TPA on Cell Surface Antigens. II. Analyzed by PoAbs

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<th>Antibody</th>
<th>Specificity</th>
<th>Control Cells</th>
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<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
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<td>Neg</td>
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<td>23 ± 1</td>
<td>34 ± 1</td>
<td>21 ± 1.52</td>
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<td>vWF</td>
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<td>91 ± 2.08</td>
<td>81 ± 1</td>
<td>78 ± 1.52</td>
<td>64 ± 3.05</td>
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<td>34 ± 3.6</td>
<td>68 ± 3.05</td>
<td>65 ± 2.08</td>
<td>67 ± 2</td>
<td>48 ± 2</td>
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<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>Antifibronogen (Dakopatts)</td>
<td>Human fibrinogen</td>
<td>29 ± 1.52</td>
<td>32 ± 2.52</td>
<td>30 ± 1</td>
<td>40 ± 1.73</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Antifibronectin (Dakopatts)</td>
<td>Human fibronectin</td>
<td>Neg</td>
<td>27 ± 1</td>
<td>25 ± 1.52</td>
<td>31 ± 2</td>
<td>21 ± 1</td>
</tr>
</tbody>
</table>

Values (percentage of positive cells) are the means ± SD of three separate experiments.
*Values for control cells are the mean positivity rates of the control cells on each harvesting day.

Normal megakaryocytes and platelets, becomes more intense after the addition of TPA.

Ultrastructural changes in MEG-01s cells following TPA stimulation such as the formation of α granules and demarcation membranes are similar to those observed in the human megakaryocyte maturation in an in vitro colony. Ultrastructural immunoperoxidase studies showed that the synthesis of GPIIb/IIIa and GPIb was enhanced by TPA. The discrepancy in the detection of GPIb by different techniques may be due to the weak concentration of GPIb antigen in contrast with that of GPIIb/IIIa. β-TG, one of the platelet-specific proteins, is well known to be localized in the α granules of megakaryocytes and platelets and reported to be synthesized and secreted from MEG-01 cells. TPA remarkably enhanced the positivity of cytoplasmic β-TG and the secretion of β-TG in culture medium. Immunoelectron microscopic analysis revealed that after the addition of TPA β-TG appeared in a few granules in addition to the more developed rough endoplasmic reticulum and perinuclear space. This appears to be unequivocal evidence for cytoplas-

Table 4. Effect of TPA on Cytoplasmic Antigens

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Specificity</th>
<th>Control Cells†</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-FV</td>
<td>FV</td>
<td>0</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>Anti-vWF</td>
<td>vWF</td>
<td>43 ± 3</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Anti-FXIIIa</td>
<td>FXIIIa</td>
<td>29 ± 1.15</td>
<td>66 ± 1.52</td>
</tr>
<tr>
<td>Anti-FXIIIb</td>
<td>FXIIIb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Antifibronectin</td>
<td>Fibronectin</td>
<td>0</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>Anti-β-TG (Amersham)</td>
<td>β-TG</td>
<td>34 ± 2</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>Antifibrinogen</td>
<td>Fibrinogen</td>
<td>16 ± 3</td>
<td>46 ± 1</td>
</tr>
</tbody>
</table>

At least 200 cells were examined with the fluorescent microscope.
* Rabbit polyclonal antibody.
†Values for control cells are values of control MEG-01s cells cultured for 3 days under the same conditions except for the addition of TPA.

Fig 5. (A) The pattern of the ploidy of control MEG-01s cells. The high peak of 2C and moderate peak of 4C are evident. (B) The pattern of the ploidy of day 5 MEG-01s cells treated with TPA (10^-7 mol/L). The increase in the peak of 4C and the new small peak of 8C are demonstrated.
Fig 6. Time course of the accumulation of $\beta$-TG antigen in the control culture medium (A), culture medium with TPA at $10^{-10}$ mol/L (B), $10^{-9}$ mol/L (C), $10^{-8}$ mol/L (D), and $10^{-7}$ mol/L (E). MEG-01s cells were seeded at $2 \times 10^5$/mL in 8 mL of RPMI 1640 medium containing 1% BSA. Values are expressed as nanograms of $\beta$-TG antigen per milliliter of medium and plotted as means ± SD of three separate flasks.

Mic $\alpha$-granule formation in MEG-01s cells following TPA exposure.

Megakaryocytic maturation of the nucleus involves nuclear endoreduplication, a process in which nuclear material reduplicates itself without nuclear division. In humans, 65% of the recognizable megakaryocytes were reported to be of the 8N type as judged by microscopic nuclear counts. However, cytoplasmic maturation and development of polyploidy are neither totally concurrent nor consecutive processes; the appearance of cytoplasmic organelles begins during ploidization and continues after DNA synthesis has stopped. In our study, the cells of 4C to 8C moderately increased in number after TPA stimulation, which suggests the appearance of a new subpopulation. These findings may suggest that the DNA endoreduplication process may be vestigial to cytoplasmic maturation of megakaryocytes. The moderate increase in ploidy may be also due to the leukemic nature of MEG-01s cells or to forced nonphysiological induction by TPA.

Rabellino et al reported the expression of IgG Fc receptors (IgG-FcR), complement receptors (CR), and Ia antigen as hematopoietic differentiation markers in megakaryocytes and platelets as follows: (a) IgG-FcR were found on over 90% of isolated human megakaryocytes, (b) CR were not found, and (c) Ia antigens were demonstrated on only small proportions of human megakaryocytes. In our study, TPA stimulation of MEG-01s cells resulted in similar changes in these markers (Tables 1 and 2), which suggests that MEG-01s cells differentiate to a mature stage.

Several coagulation factors including FV, vWF, FXIIIa, protein S, fibrinogen; platelet-specific proteins including $\beta$-TG platelet factor 4; and platelet GP such as platelet GPIb and GPIb/IIIa were shown to be localized in megakaryocytes and platelets, and some of them were demonstrated to be synthesized by megakaryocytes. Therefore, these proteins have been used as markers of differentiation and maturation of megakaryocytes. The GPIb/IIIa complex and GPIb were shown to be early antigenic markers of megakaryocytic maturation, although GPIb was detected in a lower percentage than was GPIb/IIIa. and vWF was detected in promegakaryoblasts or megakaryoblasts. More recently, it was reported that erythroid cell lines (K562 and HEL) expressed several megakaryocytic markers including GPIb and GPIIIb and that phorbol ester led to the expression of vWF factor, platelet factor 4, and thrombospondin. In the present study, the expression of these coagulation factors or platelet-specific proteins remarkably increased after the exposure of MEG-01s cells to TPA, which suggests that TPA induced MEG-01s cells to differentiate along the normal megakaryocytic pathway. It is noteworthy that the expression of FXIIIa in MEG-01s cells may be used as one of the early markers of megakaryocytic lineage. In TPA-treated day 7 cells, the positivity of platelet GPIb/IIIa was decreased to 40% ± 2.6%. This may be due to the more fragile cytoplasmic membranes with numerous buddings. However, the cause of this decrease remains to be investigated since HLA antigen levels were not decreased in the same cells. MEG-01s cells had HLA class I antigen on their cell’s surfaces, and TPA enhanced the expression of this antigen. Although the origin of HLA antigen on platelets has been a subject of debate, our observation may suggest the possibility that the HLA class I antigen of platelets may not be acquired from plasma but may be synthesized by megakaryocytes.

Our investigation will be useful for studying the biosynthesis of proteins unique to human megakaryocyte-platelets and the process of controlling megakaryopoiesis. It should be emphasized, however, that TPA induction of MEG-01s may be of limited use as a model for normal maturation mechanisms since MEG-01s cells are of leukemic origin and in vitro differentiation under these conditions does not appear to be complete in that shedding of platelets was not confirmed. Although the mechanisms by which TPA induced the differentiation of MEG-01s is not known, recent studies suggest that protein kinase C may play a role in mediating cellular membrane signals and may serve as a TPA receptor. If the effect of TPA is related to protein kinase C activation, the induction of MEG-01s differentiation by TPA will be also a useful model to elucidate general mechanisms of cellular differentiation.

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Functional and morphological differentiation induction of a human megakaryoblastic leukemia cell line (MEG-01s) by phorbol diesters

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