PLATELET-LIKE PARTICLES RELEASED BY INHIBITION OF DNA SYNTHESIS IN THE HUMAN-MEGAKARYOBLASTIC LEUKEMIA CELL LINE, MEG-01s

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Abstract: The human megakaryoblastic cell line, MEG-01s, released particles identified by a characteristic marginal microtubule bundle and by the localization of platelet-specific glycoprotein (GPIIb/IIIa) in the plasma membrane, detected by an immunofluorescence method. The fluorescence image and size of these particles were similar to these features in human blood platelets. To analyze the regulation of particle release by MEG-01s, the effects of DNA synthesis inhibitors, tumor promoters, and protein kinase inhibitors on these cells were examined by an immunofluorescence method. The release of particles from MEG-01s cells was enhanced by inhibitors of DNA synthesis: aphidicolin, 5-bromodeoxyuridine, 5-fluorodeoxyuridine, hydroxyurea, etoposide, and camptothecin. The particle release was specific to the MEG-01s cells, and did not occur in the myeloid leukemic cells, HL-60. These results suggest that the cessation of DNA synthesis may trigger terminal differentiation by synthesis of factors involving particle release by MEG-01s.

Key words: platelet-like particle, DNA synthesis inhibitor, differentiation, human megakaryoblastic leukemia cell, MEG-01s

INTRODUCTION

For many years, considerable efforts have been devoted to elucidate the mechanisms that control human megakaryocyte differentiation and the production of platelets from megakaryocytes. However, these questions remain unresolved, due to the scarcity of megakaryocytes for study. Examination of constituent protein synthesis and gene expression during megakaryocytopoiesis is especially difficult.

Established hemopoietic cell lines have been valuable for studies of hemopoietic cell differentiation and regulation1-3. The MEG-01 cell line, established by Ogura et al.4 from a chronic myelocytic leukemia patient, displays phenotypic properties that closely resemble those of megakaryoblasts. This cell line expresses characteristic platelet proteins, GPIIb/IIIa, My9, vWF, BA-1, GPIb, FVIII, CDW14, and CD3645, produces thrombomodulin, beta-TG, TGF-beta, and protein S6,7; and synthesizes the transcription factors NF-E1 and NF-E28. In addition, treatment of MEG-01 cells with phorbol 12-myristate 13-acetate (PMA), results in the expression of fibronectin and coagulation factor V9. In a recent immunofluorescence study,9 we previously found that MEG-01 released particles that were similar to platelets in terms of microtubule distribution and the presence of membrane glycoproteins (GPIIb/IIIa). The number of parti-
cles, however, was small under our culture conditions, indicating either that most MEG-01 cells do not release the particles, or that the particles may be degraded soon after they are released. The release of platelet-like particles has already been reported in four human cell lines of megakaryocytic lineage\(^{3-12}\), but these particles are not yet well characterized; the main cause for this may be the low frequency of particle release.

Under in vitro conditions, megakaryocytopoiesis is stimulated by several cytokines, namely, megakaryocyte-colony stimulating factor (MK-CSF)\(^{13,14}\), megakaryocyte-stimulating factor (MSF)\(^{13}\), granulocyte-macrophage-colony stimulating factor (GM-CSF)\(^{15,16}\), interleukin-3\(^{17}\), interleukin-6\(^{18,19}\), interleukin-11\(^{20}\), erythropoietin (Epo)\(^{21,22}\), and the mpl ligand\(^{23}\). Most of these cytokines do not, however, significantly enhance platelet production in vitro.

The differentiation and maturation of human promyelocytic leukemia (HL-60) cells\(^{24}\) and human neuroblastoma cells\(^{25}\) are induced not only by physiological agents but also by pharmacological agents. Treatment of some myeloid leukemia cell clones with such inhibitors of DNA synthesis as cytosine arabinoside\(^{26}\), 5-bromodeoxyuridine (BuDR)\(^{27}\), and mitomycin C\(^{27}\) has been shown to completely inhibit their proliferation and to induce their differentiation into mature macrophages or granulocytes. Similarly, BuDR\(^{25}\), mitomycin C\(^{25}\), and aphidicolin\(^{28}\) have been shown to induce neuroblastoma cells to differentiate into neuron-like cells. Recently, a human erythroleukemia cell line, K562, was reportedly induced to differentiate into hemoglobin-positive cells by aphidicolin\(^{29}\).

To induce the release of particles in MEG-01s cells, we treated the cells with various inhibitors of DNA synthesis. We found, via an immunofluorescence method, that inhibitors of DNA synthesis, such as aphidicolin, enhanced the production of platelet-like particles by these cells.

**MATERIALS AND METHODS**

**Chemicals.**

Aphidicolin, cycloheximide, hydroxyurea, 5-fluorodeoxyuridine (FUDR), chlorophenylthio-cyclic AMP (CPT cAMP), retinoic acid, dimethyl sulfoxide, PMA, and cytochalasin D were purchased from Wako Pure Chemical Co. (Osaka, Japan). Etoposide and camptothecin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Taxol was a gift from Dr. K. H. Groover (NCI, Bethesda, MD, USA).

**Cells.**

MEG-01s cells\(^5\), cloned from a human megakaryoblastic leukemia cell line (MEG-01, IFO 50151),\(^4\) were cultivated in Dulbecco’s modified Eagle’s MEM (DME, Nissui Seiyaku K. K., Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS; MAB Co., Rockville, MD, USA). The original MEG-01 cells tended to adhere to the plastic culture dish under the usual culture conditions, and are a mixture of adherent and suspended cell types\(^4\). MEG-01s cell line, a suspended type cloned from MEG-01 cells\(^5\), have almost the same characteristics as MEG-01 cells except that MEG-01s cells grow in single suspension without attachment to the culture dish. MEG-01s were used for all the following experiments. The cells were counted with a Coulter counter. Cell viability was determined by the dye exclusion method, using 0.02% trypan blue.

**Fractionation of MEG-01s particles.**

MEG-01s cells (3x10^5/ml) were cultured in DME supplemented with 1% FBS for 2 days. The cells were treated with 2 to 5 \(\mu g/ml\) of aphidicolin in culture medium. After 2 days, 1 \(\mu g/ml\) of taxol, for stabilizing the microtubule bundles\(^30\), was added to the culture medium, and the cells were incubated at 37°C for 1 hour. We then added a 1/30 volume of a solution of citrate-citric acid-glucose (final concentration: 14.2 mM sodium citrate, 10.8 mM citric acid, 1.67 g/L glucose, pH 6.7). The cell suspension was then centrifuged at 40xg for 10 min and
the supernatant was centrifuged at 400 x g for 10 min. The resultant pellet was suspended in Tyrode-HEPES buffer (8 g/L NaCl, 1.8 mM CaCl₂, 9 mM MgCl₂, 1 g/L glucose, 1 g/L NaHCO₃, 10 mM HEPES [pH 7.4]), containing 1 μg/ml taxol, and incubated at 37°C for 60 min. This suspension, termed the partially purified particle fraction, was used for the analysis of morphology by electron microscopy. All centrifugation procedures were done at 25-30°C.

Indirect immunofluorescence.

At an appropriate time after the seeding of MEG-01s, taxol (final concentration, 1 μg/ml) was added to the culture medium, and the cells and particles were fixed in 3.7% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 10 min. After being washed with PBS, the cells and particles were collected on a coverslip, using a Shandon Cytospin 3 centrifuge (Shandon Southern Products, Ltd, Cheshire, England), and treated with methanol at -20°C. They were then incubated with monoclonal antibody against alpha-tubulin (Amersham, Tokyo, Japan) at 37°C for 60 min. After being washed, they were visualized with Texas Red-conjugated anti-mouse Ig (Amersham) at 37°C for 60 min. For the double-staining of alpha-tubulin and GPIIb/IIIa, the specimens were incubated first with anti-tubulin and Texas Red-conjugated anti-mouse Ig and then with polyclonal anti-GPIIb/IIIa rabbit antibodies (a gift from Dr. K. Tanoue, Tokyo Metropolitan Institute of Science, Tokyo, Japan) at 37°C for 60 min and with FITC-conjugated anti-rabbit IgG (Cappel Worthington, Cooper Biomedical, Inc., Malvern, PA.) at 37°C for 60 min. Monoclonal antibody against glycoprotein GMP 140 (clone WGA-1) was purchased from Takara Shuzo Co. Ltd. (Otsu, Japan). The coverslips were then mounted in glycerol and examined with an epifluorescence microscope (BX2-50; Olympus, Osaka, Japan) equipped with x40 or x100 objective lenses. The fluorescence images were recorded on Kodak TMAX film and developed in a TMAX developer (Eastman Kodak, Rochester, N. Y., USA) at an ASA value of 1600.

Quantitative analysis.

The number of particles with ring-shaped microtubules was determined by direct counting of particles stained with anti-tubulin antibody at 400x magnification. The fields observed with the fluorescence microscope were 5 x 10⁻² mm² in area, and each contained 100 to 500 MEG-01s cells. Values shown in the Figures and Table are means ± S. D. of 9 to 10 determinations.

Confocal laser scanning microscopy.

Confocal images were obtained with an Olympus OSM-GB200 microscope (Olympus) equipped with an argon/neon laser.

RESULTS

Microtubule patterns in particles observed by fluorescence microscopy.

When human platelets were stained with antitubulin antibody, the fluorescence image showed the characteristic ring structure in the cytoskeleton, consisting of a circumferential band of microtubules just inside the plasma membrane. This structure was easily detected as a closed brightly fluorescent ring. We have previously reported that the particles released from MEG-01 cells have an anti-tubulin staining pattern similar to that in the microtubules. The particles with a clear ring structure were, however, very few. We therefore attempted to bring about the release of more particles from MEG-01s cells by chemical treatment. MEG-01s cells were cultured in DME containing 1% FBS for 2 days after being seeded. Two μg/ml of aphidicolin was then added to the medium and the cells were cultured for two days. The MEG-01s cells and particles in the culture medium were then incubated in 1 μg/ml of taxol for 1 hour, and fixed with paraformaldehyde. Samples were placed on a glass coverslip by Cytospin and were stained with anti-tubulin antibody and Texas Red-conjugated anti-mouse Ig. Figure 1 shows the fluorescence image of the particles with a ring structure. On the addition of aphidicolin, we observed many parti-
Fig 1. Fluorescence micrograph of MEG-01s cells and particles released from the cells. MEG-01s cells and particles in the culture medium spun onto coverslip, stained with anti-tubulin antibody. (A) shows cells (arrow) and particles (arrowhead) on immunofluorescence microscopy and (B) the same field on phase contrast microscopy. Note the closed fluorescent rings and extended filaments in the microtubules of some of the particles. Bar, 10 μm.

cles with a brightly stained ring. Some rings extended long filaments to the outside. To clarify the details of the ring structure we analyzed the structure with a confocal microscope (Fig. 2). A particle shown by Nomarski differential interference contrast (DIC) microscopy was circular and had a 3 μm diameter. The Texas Red image showing antitubulin staining of the same particle showed a clear ring structure, as was observed in platelets. When the fluorescent particle ring in these optical sections was cut horizontally to the coverglass plane, brighter rings were seen in the central planes (Fig. 2A). When cut vertically, the fluorescence patterns showed bright dots at both sides of the optical sections (Fig. 2B). These findings indicated that the microtubules were located at the equatorial regions of the particle in a circumferential band.

The number and size of the particles with ring structures were examined in fractionated particle samples. MEG-01s cells were incubated in 5 μg/ml aphidicolin for 2 days and the particles were separated from MEG-01s cells by centrifugation, as described in Materials and Methods. The 400 xg pellet fraction (partially...
purified particle fraction) was stained with anti-tubulin antibody and Texas Red-conjugated anti-mouse Ig. Figure 3A shows the Nomarski DIC image of the fractionated samples; the fluorescence image in the same field is shown in Fig. 3B. Most of the fractionated particles were circular in shape and had a smooth surface and clear outlines (Fig. 3A), but only 20%–30% of the particles had fluorescent rings (Fig. 3B). The size of particles with fluorescent rings was measured from the Nomarski DIC images of the particle. The particles ranged from 1.8 to 5.0 \( \mu m \) in diameter, and 40% of them were 2.6–3.1

\( \mu m \), as shown in Fig. 4. The size distribution of these particles, was heterogeneous, as has been shown in human platelets

We have previously shown that the particles released from MEG-01 cells contained the platelet-specific surface glycoprotein GpIib/IlIa. The particles obtained from MEG-01s with aphidicolin treatment also had this marker as revealed by double-staining with antibodies against tubulin (Fig. 5A) and platelet GpIib/IlIa (Fig. 5B). In addition, the particles were stained with antibody against glycoprotein GMP 140, which is localized in platelet granules and translocates to the cell surface upon activation. The staining of particles by anti-GMP 140 antibody revealed an overall cellular distribution, as is observed in permeabilized resting platelets (data not shown).

Effects of DNA synthesis inhibitors on particle release.

MEG-01s cells proliferated exponentially in the absence of aphidicolin until 5 days after the culture was split; the cells then began to die gradually (Fig. 6A, B). Aphidicolin inhibited MEG-01s cell proliferation in a dose-dependent manner and caused arrest of cell growth, at con
Fig 5. Double-labelled immunofluorescence of tubulin and platelet-specific membrane glycoprotein (GPIIb/IIIa) in particles.
MEG-01s particles were permeabilized with methyl alcohol after fixation and stained with anti-tubulin (A) and anti-GPIIb/IIIa (B). GPIIb/IIIa antibody stained the particle periphery brighter than the interior. Bar, 10 μm.

Fig 6. Effects of aphidicolin on proliferation of MEG-01s.
MEG-01s cells (1 x 10⁵) were cultured and incubated in 1 ml of medium containing : 20 μg/ml (e), 2 μg/ml (d), 0.2 μg/ml (c), and 0.02 μg/ml (b) of aphidicolin or without additions (a) on day 2 (arrow) after seeding. The total number of cells (A) and the number of viable cells (B) were determined with a Coulter counter and by a dye exclusion method, respectively. Values are means ± S.D. for 3 estimations. The S. D. is included in each symbol.
centrations exceeding 0.2 \( \mu g/ml \), on the day after incubation. The viability of the cells was 90\%-97\% until day 2 of the treatment (Fig. 6B). MEG-01s cells began to release particles in the absence of aphidicolin two days after culture splitting, but particles with the ring structure stained by anti-tubulin antibody were few. Figure 7 shows the numbers of particles with the ring structure released in the presence of various concentrations of aphidicolin. Aphidicolin caused a marked increase in particle numbers. On day 3 of treatment with 0.2 \( \mu g/ml \) of aphidicolin, the maximum increase was 5- to 6-fold compared with that without aphidicolin. The particle number decreased with further increases in the aphidicolin concentration, and the particle release was maximal on days 1 and 2 of the treatment.

To ascertain whether the enhanced particle release induced by aphidicolin was actually due to the inhibition of DNA synthesis, we cultured MEG-01s cells with other inhibitors of DNA synthesis (FUdR, hydroxyurea, etoposide, and camptothecin) for 2 days. All these compounds also increased the number of particles on day 2 of treatment (Table 1). More than a 10-fold increase was observed upon treatment with 10 \( \mu M \) FUdR, 0.13 mM hydroxyurea, 2 \( \mu g/ml \) aphidicolin, 0.2 \( \mu g/ml \) etoposide, and 0.5 \( \mu g/ml \) camptothecin compared to the value in control cultures without inhibitors. Thus, not only aphidicolin but also other inhibitors of DNA synthesis enhanced the release of particles with a ring structure composed of microtubules from MEG-01s.

The particle release, however, was not enhanced by treatment with 100 \( \mu M \) CTP or cAMP, with 1% dimethyl sulfoxide, or with 1 \( \mu M \) cytochalasin D, but was decreased by treatment with 100 nM phorbol 12-myristate 13-acetate (PMA), 10 \( \mu g/ml \) genistein, 1 \( \mu M \) staurosporin, 5 mM N, N’-hexamethylene bisacetamide, 0.5 \( \mu M \) okadaic acid, 1 \( \mu M \) retinoic acid, 1 \( \mu M \) ionophore 23187, and 10 \( \mu g/ml \) cycloheximide. Before the addition of aphidicolin, PMA treatment for 2 days completely inhibited the particle release, but when PMA was added at the same time as aphidicolin, PMA had little inhibitory effect on particle release (Table 1).

Cytokines with proliferative activity on megakaryocytes, such as erythropoietin, interleukin-6, and interferon alpha, showed only a weak effect in enhancing the particle release (data not shown).

Similar experiments with nonmegakaryocytic cell lines, the myeloid cell line, HL-60, and the fibrobrastic cell line, BALB/c 3T3, indicated that effect of aphidicolin on particle
Table 1  Effects of eight inhibitors of DNA synthesis on the release of particles with ring-shaped microtubules. Experimental conditions were the same as those described in Fig. 7. Numbers of particles with ring-shaped microtubules are means plus S. D. of 9 estimations.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>No. of Particles/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1 control</td>
<td></td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>2 μg/ml</td>
<td>10.1±1.2</td>
</tr>
<tr>
<td>FUdR</td>
<td>10 μM</td>
<td>18.1±0.9</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>0.13mM</td>
<td>7.3±2.9</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.2 μg/ml</td>
<td>39.6±2.0</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>0.5 μg/ml</td>
<td>46.9±9.8</td>
</tr>
<tr>
<td>Exp. 2 control</td>
<td></td>
<td>8.8±0.4</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>1 μg/ml</td>
<td>19.5±2.3</td>
</tr>
<tr>
<td>Ap + PMA</td>
<td>1 μg/ml +10⁻⁷M</td>
<td>0</td>
</tr>
<tr>
<td>Exp. 3 control</td>
<td></td>
<td>10.4±4.1</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>1 μg/ml</td>
<td>30.8±4.7</td>
</tr>
<tr>
<td>PMA → Ap*</td>
<td>10⁻³ M →1μg/ml</td>
<td>4.8±1.9</td>
</tr>
</tbody>
</table>
* No. of particles with ring-shaped microtubules.  
** Pretreatment with PMA for 2 days before the addition of Ap. (aphidicolin).

release was specific to megakaryocytic MEG-01s cells.

DISCUSSION

Particles similar to platelets have been obtained at low yield from cells of the human megakaryoblastic cell lines, MEG-01\(^9\) and MEG-01s. In resting platelets, the cytoskeleton, composed of microtubules, is present in a circumferential band just inside the plasma membrane, which is clearly revealed as a closed brightly fluorescent ring by anti-tubulin antibody staining. Platelets are characterized by such an orderly marginal microtubular bundle. Many reports \(^{31,33}\) support the concept that the discoid shape of the platelet is dependent on the integrity of the circumferential band. In the present study, the fluorescence images of some particles with anti-tubulin antibody staining revealed a characteristic ring structure, as was observed in platelets (Figs. 1, 2, 3B, and ref. 9). The distinctive morphology of microtubule organization and the presence of a protein important for platelet function, GPIIb/IIIa, were the first indications that the particles were similar to platelets\(^9\). It was evident that the ring structure was located at the equatorial region of the particle (Fig. 2A). Thus, we concluded that the microtubules did not localize as the membrane cytoskeleton in the subplasmalemmal region of the particles, but were localized in a coil only in the equatorial region. Most of the particles released did not produce a ring image on anti-tubulin staining. These particles may have immature microtubules that are not yet organized as a ring, or, alternatively, the ring structure of the microtubule may have dissociated due to some instability. When the particles were incubated at a low temperature or at 37°C for several hours, the microtubule ring structure disappeared and the fluorescence images were diffused throughout the cytoplasm.

The release of platelet-like particles has already been reported in four human cell lines of megakaryotic lineage\(^9\) - \(^12\). MEG-01\(^9\) and MEG-01s cells spontaneously release the particles, although only a few are seen in these cells. Normal megakaryocytes become polyploid by allowing many rounds of DNA replication without completion of the intervening mitosis; and DNA synthesis is stopped by an unknown mechanism, the cells undergo cytoplasmic maturation and ultimately produce platelets. The present study was performed to determine whether cessation of DNA synthesis was correlated with the induction of differentiation, determined by particle release from MEG-01s cells. Aphidicolin, which prevents DNA replication by inhibiting DNA polymerase-alpha in competition with deoxypyrimidine\(^34\), and is not incorporated into the DNA, blocked the proliferation of MEG-01s cells and increased the number of particles with ring-shaped microtubules released from the cells (Figs. 6, 7). Another inhibitor of DNA replication, FUdR, an analogue of deoxypyrimidine that inhibits thymidylate synthetase, resulting in thymidine starvation, also enhanced
the particle release (Table 1). Inhibitors of topoisomerase I (camptothecin) and topoisomerase II (etoposide) also stimulated the release of the particles. Therefore, we concluded that the inhibition of DNA replication, and not the inhibition of DNA polymerase itself, may lead to the maturation of these cells into megakaryocytes and to the subsequent release of particles.

The number of particles released from MEG-01s cells, however, changed only slightly with differing concentrations of aphidicolin (Fig. 7). These findings indicate the following possibilities: (i) Inhibition of DNA synthesis in MEG-01s cells may induce the expression of a factor, or factors, that are required for particle release in the cells, by blocking the synthesis of inhibitory regulator(s). The target of such factor(s) would be only committed MEG-01s cells which undergo cytoplasmic maturation under the influence of such factors. The effects of aphidicolin on particle numbers would be observed only in MEG-01s cells that were responsive to the factor(s). Thus, the number of particles released would depend on the number of committed MEG-01s cells, but not on the aphidicolin concentration. (ii) Aphidicolin induced cell damage and cell death in a dose-dependent manner (Fig. 6). As higher concentrations of aphidicolin strongly induced cell damage and cell death before cytoplasmic maturation, the number of particles released from surviving MEG-01s cells would decrease with higher concentrations of aphidicolin. Increases in aphidicolin concentration appeared to shorten the time required for cytoplasmic maturation after the inhibition of DNA synthesis (Fig. 7). The underlying mechanism here is not known, and further study is required.

PMA activates protein kinase C (PKC), which then initiates various signal transductions and modulates many cellular responses, including platelet secretion, smooth muscle contraction, and cell proliferation and differentiation. PMA induced the differentiation of normal megakaryocytes$^{35}$ and MEG-01s$^{60}$ cells to the next stage, although terminal differentiation was not observed. Similarly, we did not observe particle release as an indication of MEG-01s terminal differentiation when MEG-01 or MEG-01s were treated with PMA. These results suggest that other pathways or factors are required for particle release. On the other hand, aphidicolin seems to operate in a particle release pathway that is not linked with the PKC pathway. However, our findings that sequential treatment of MEG-01s cells with PMA and aphidicolin decreased particle release (Table 1), and the findings of Murate et al.$^{26}$ that aphidicolin blocked the PMA-induced differentiation of MEG-01 cells, may explain some effects of aphidicolin on both the PKC pathway and the non-PKC-linked pathway. At present, little is known about the events that follow the aphidicolin-induced cessation of DNA synthesis by MEG-01s cells. It is important to identify the factor(s) induced after aphidicolin treatment. The release of particles from MEG-01s was inhibited by cycloheximide (data not shown), suggesting that this release requires protein synthesis.

In a recent study, thrombopoietin$^{23}$, which is probably the major humoral regulator of thrombocytopoiesis, was isolated in recombinant form and was shown to be highly potent in increasing platelet numbers in mice. No expression of c-mpl, however, was observed in MEG-01 cells$^{10}$. It will be of interest to determine the effects of thrombopoietin and other cytokines on MEG-01s differentiation and maturation. We are now conducting further studies on the biological activities of these particles.

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