hematopoietic cell lines. However, it is not clear how these inhibitors are expressed during normal hematopoiesis. We examined various human hematopoietic colonies derived from cord blood CD34+ cells, bone marrow, and peripheral blood cells using a quantitative reverse transcription-polymerase chain reaction assay, immunocytochemistry, and/or Western blot analysis. p21 mRNA was expressed increasingly over time in all of the colonies examined (granulocytes, macrophages, megakaryocytes, and erythroblasts), whereas p27 mRNA levels remained low, except for erythroid bursts. Erythroid bursts expressed both p21 and p27 mRNAs with differentiation but expressed neither protein, whereas both proteins were expressed in megakaryocytes and peripheral blood monocytes. In bone marrow, p21 was immunostained almost exclusively in a subset of megakaryocytes and p27 protein was present in megakaryocytes, plasma cells, and endothelial cells. In megakaryocytes, reciprocal expression of p27 to Ki-67 was evident and an inverse relationship between p21 and Ki-67 positivities was also present, albeit less obvious. These observations suggest that a complex lineage-specific regulation is involved in p21 and p27 expression and that these inhibitors are involved in cell-cycle exit in megakaryocytes.

In the present study, we directed our attention to expression patterns of p21 and p27 in normal (primary) hematopoietic cells and terminally differentiated blood cells. The correlation of cell proliferation with p21 and/or p27 expression was given focus and the expression of Ki-67 antigen was also examined. We obtained evidence that the expression of p21 and p27 is regulated in a lineage-specific manner and that both proteins are highly expressed, particularly in megakaryocytes. In megakaryocytes, p27 and probably p21 proteins are involved in terminal exit from the cell cycle.

MATERIALS AND METHODS

Cytokines. Recombinant human granulocyte colony-stimulating factor (G-CSF) was provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan), recombinant human interleukin-6 (IL-6) was from Ajinomoto Co Ltd (Kawasaki, Japan), recombinant human stem cell factor (SCF) and recombinant human IL-3 were from Amgen Biologicals (Thousand Islands, USA). Recombinant human stem cell factor (SCF) and recombinant human IL-3 were from Amgen Biologicals (Thousand Islands, USA). Recombinant human stem cell factor (SCF) and recombinant human IL-3 were from Amgen Biologicals (Thousand Islands, USA). Recombinant human stem cell factor (SCF) and recombinant human IL-3 were from Amgen Biologicals (Thousand Islands, USA).

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was used at 1:25,000 dilution. Anti-p21 (6B6; Pharmingen, San Diego, CA), anti-p27 (F-8; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-p53 (DO-7; DAKOPA TTS A/S) monoclonal antibodies (MoAbs), which were used at 1:50 dilution. Rabbit anti-human von Willebrand factor (vWF; DAKOPA TTS A/S) was used at 1:3,200 dilution. Rabbit antihuman glycophorin A (JC159, DAKOPA TTS A/S) was used at 1:100 dilution. Rabbit antihuman von Willebrand factor (vWF; DAKOPA TTS A/S) was used at 1:400 dilution. Antihuman glycophorin A (JC159, DAKOPA TTS A/S) was used at 1:3,200 dilution. Rabbit antihuman von Willebrand factor (vWF; DAKOPA TTS A/S) was used at 1:25,000 dilution. Anti-p21 (6B6) and anti-p27 (G173-524) MoAbs were incubated in a 24-well or 48-well tissue culture plate for 1 hour. Nonadherent cells were used as lymphocytes. After four PBS washes, the plate was incubated at 4°C for 30 minutes with ice-cold MSP-E buffer supplied by the manufacturer. The adherent cells were detached by pipetting and used as monocytes. Cytochemically, the lymphocytes were 95% to 96% pure and monocytes were 88% to 89% pure (data not shown). PB granulocytes were prepared from the pellet of Ficoll-Paque density gradient centrifugation. Cells in the pellet were suspended in 3% dextran/PBS and left standing for 30 minutes at room temperature for erythrocyte sedimentation. Cells in the supernatant were resuspended in 0.2% NaCl for 1 minute on ice for hemolysis, followed by the addition of 1.6% NaCl. After pelleting, cells were collected in PBS. Granulocytes usually accounted for greater than 95%.

**Clonal cell culture.** For erythroid bursts, granulocyte colonies, and macrophage colonies, purified CD34+ cells were incubated at concentrations of 250 cells/mL in methylcellulose culture, as reported. In brief, 1 mL of culture mixture contained cells, α-medium (GIBCO BRL), Life Technologies, Inc, Grand Island, NY, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 30% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 1% crystallized and deionized fraction V bovine serum albumin (BSA; Sigma, St Louis, MO), 0.05 mmol/L 2-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), 100 ng/mL SCF, 20 ng/mL IL-3, 80 ng/mL IL-6, 10 ng/mL G-CSF, and 2 µL/mL EPO. For megakaryocyte colonies, 1 mL of culture mixture contained 1,000 cells, α-medium, 0.9% methylcellulose, 30% platelet-poor plasma from a healthy adult volunteer, 1% BSA, 0.05 mmol/L 2-mercaptoethanol, 100 ng/mL SCF, and 5 µg/mL TPO. Individual colonies, lifted under direct microscopic visualization, were suspended in 200 µL of phosphate-buffered saline (PBS) with 30% FBS. Half of the cell suspension was spun in a cytocentrifuge (Cytospin 2; Shandon Southern Instruments, Sewickley, PA) at 500 rpm for 5 minutes and processed for May-Grünwald Giemsa staining. The other half was directly subjected to RNA extraction.

**Suspension culture.** One milliliter of culture mixture containing 10,000 to 15,000 purified CD34+ cells, α-medium (GIBCO BRL), 20% heat-inactivated FBS (HyClone), 1% BSA, with or without 100 ng/mL SCF and 80 ng/mL IL-6 was incubated in a 24-well or 48-well tissue culture plate (Becton Dickinson Labware, Lincoln Park, NJ) at 37°C in a humidified atmosphere with 5% CO2.

Preparation of normal human peripheral blood (PB) monocytes, lymphocytes, and granulocytes and bone marrow (BM) MNCs. PB was obtained from healthy adult volunteers with informed consent. BM aspirates from patients with non-Hodgkin’s lymphoma, without BM involvement, were obtained after the acquisition of informed consent. MNCs of PB or BM were prepared by Ficoll-Paque density gradient centrifugation. PB MNCs were suspended in RPMI1640 medium (GIBCO BRL) supplemented with 10% of heat-inactivated FBS (BioWhittaker, Walkersville, MD) and 60 mg/L kanamycin (Meiji Seika Kaisha, Ltd, Tokyo, Japan) and incubated in an MSP plate (Japan Immunoresearch Laboratories, Co, Ltd, Takasaki, Japan) at 37°C in a humidified atmosphere with 5% CO2 for 1 hour. Nonadherent cells were used as lymphocytes. After four PBS washes, the plate was incubated at 4°C for 30 minutes with ice-cold MSP-E buffer supplied by the manufacturer. The adherent cells were detached by pipetting and used as monocytes. Cytochemically, the lymphocytes were 95% to 96% pure and monocytes were 88% to 89% pure (data not shown). PB granulocytes were prepared from the pellet of Ficoll-Paque density gradient centrifugation. Cells in the pellet were suspended in 3% dextran/PBS and left standing for 30 minutes at room temperature for erythrocyte sedimentation. Cells in the supernatant were resuspended in 0.2% NaCl for 1 minute on ice for hemolysis, followed by the addition of 1.6% NaCl. After pelleting, cells were collected in PBS. Granulocytes usually accounted for greater than 95%.

**CD34+ cell preparation.** Human umbilical cord blood was obtained from normal full-term deliveries, after the acquisition of written informed consent. Mononuclear cells (MNCs) were separated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation after depletion of phagocytes with silica (Immuno-Biological Laboratories Co, Ltd, Fujioka, Japan). CD34+ cells were purified from the MNCs, using Dynabeads M-450 CD34 and DETACHABLE BEAD CD34 (Dynal, Oslo, Norway). Flow cytometric analysis showed that 85% to 95% of the cells separated were CD34+.

**Table 1. Primers Used for PCR Amplification**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Primer Length (bp)</th>
<th>GC (%)</th>
<th>Tm (°C) (0.05 mol/L Na+)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>p27S</td>
<td>5'-CCCTCTGGCCCGCTGGGAC-3'</td>
<td>19</td>
<td>73.7</td>
<td>54.6</td>
</tr>
<tr>
<td>p21AS</td>
<td>5'-CGTTTCTAGAAGCGACGAA-3'</td>
<td>19</td>
<td>57.9</td>
<td>48.1</td>
</tr>
<tr>
<td>p27AS</td>
<td>5'-CTGTCTCAGAAACCGCGC-3'</td>
<td>20</td>
<td>63.2</td>
<td>50.3</td>
</tr>
<tr>
<td>βS</td>
<td>5'-AAGAGAGAGCCTCACCCTCA-3'</td>
<td>20</td>
<td>55.0</td>
<td>48.7</td>
</tr>
<tr>
<td>βASp27S</td>
<td>5'-CTCTCTGGCCCGCTGGGACAGG-3'</td>
<td>36</td>
<td>66.7</td>
<td>68.5</td>
</tr>
</tbody>
</table>

*Tm was calculated using the formula: Tm (°C) = 81.5 + 16.6 (Log [molar Na+]) + 41 (%GC) – 675primer length.
MgCl₂, pH 8.3) with 200 µmol/L of each dNTP, 2 µCi [α-32P]dCTP, primers (p21AS, p27AS, βS, and p27S at 200 nmol/L and βASp27S at 2 nmol/L), and 0.5 U recombinant Taq DNA polymerase (Takara, Kyoto, Japan). Reaction parameters were 94°C for 1 minute (first cycle, 5 minutes), 62°C for 2 minutes, and 72°C for 3 minutes (last cycle, 10 minutes). After the indicated cycles were performed, 5 µL of each PCR product was separated on a 4.5% polyacrylamide gel followed by autoradiography. An optical scanner was used and densitometrical analysis was made using NIH Image software (NIH, Bethesda, MD). As negative controls, water instead of cDNA or the products of the RT reactions without reverse transcriptase were subjected to PCR and we confirmed no false-positive reaction.

**Northern analysis.** RNAs extracted from MEG-01s cells stimulated with 12-o-tetradecanoylphorbol-13-acetate (TPA; Sigma) at 10 nmol/L were subjected to Northern analysis, as described.28

**Immunocytochemical study.** For immunostaining of p21, p27, and Ki-67, cytosmears were fixed with 10% formaldehyde in PBS at room temperature for 10 minutes. After a wash in PBS, the cytosmears were treated with 100% methanol for 10 minutes at −20°C and washed twice with Tris-buffered saline (TBS). For immunostaining of CD41 and glycophorin A, cytosmears were air-dried for 2 hours, fixed with 100% acetone at 4°C for 1 minute, and washed with TBS twice. Primary antibody in 3% BSA/TBS was incubated overnight at 4°C in a humidified chamber. After washing with TBS, cells were stained with universal DAKO APAAP kit (DAKOPATTS A/S), as described by the manufacturer, and were counterstained with hematoxylin.

**Western blot analysis.** Cells were lysed with 1× sample buffer (60 mmol/L Tris-HCl, 2% sodium dodecyl sulfate [SDS], 0.1 mol/L dithiothreitol, pH 6.8) and boiled for 5 minutes. Protein concentration was determined by spectrophotometry using BCA Protein Assay Kit.
Reagent (Pierce, Rockford, IL). A total of 30 µg protein per lane was loaded on 15% SDS-polyacrylamide gel and subjected to Western blot analysis, as described.

Immunohistochemical study. Human BM aspirates were retrieved from the archives of the Department of Pathology, the Branch Hospital, University of Tokyo, School of Medicine. These materials had been fixed in formalin and embedded in paraffin using standard methods. Three-micrometer-thick sections were dried on MAS-coated glass slides (Matsunami Glass Ind, Ltd, Kishiwada, Japan), deparaffinized with xylene, and soaked in PBS. Slides were transferred to citrate buffer (pH 6.0) and heated in a microwave oven 5 times for 5 minutes. After cooling at room temperature for 1 hour and two washes in TBS, the slides were incubated overnight at 4°C with the indicated primary antibody diluted in 3% BSA/TBS in a humidified chamber. After washing with TBS, staining with universal DAKO APAAP kit was performed as described by the manufacturer. Nuclei were counterstained with hematoxylin. For negative controls, mouse IgG was used.

For p27, a human reactive lymph node sample served as a positive control.

Sequential double immunohistochemical staining. Sequential double immunohistochemical staining was performed using the alkaline-phosphatase/anti-alkaline-phosphatase (APAAP) procedure, followed by the avidin-biotinylated peroxidase complex (ABC) method. After the above-mentioned antigen retrieval, the slides were incubated for 10 minutes in a 3% H2O2 solution in PBS for blocking endogenous peroxidase. After the APAAP procedure, the slides were treated three times for 30 minutes with 0.1 mol/L glycine-HCl buffer (pH 2.2). For three washes in PBS, the slides were covered with normal serum contained in a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature and then incubated overnight at 4°C with the indicated antibody in a humidified chamber. The sections were stained using the Vectastain ABC kit, as described by the manufacturer. Peroxidase activity was visualized by applying diaminobenzidine chromogen, containing 0.015% H2O2. For negative controls, mouse IgG served as a substitute for the primary antibody or the primary antibody was omitted.

RESULTS

Quantitative RT-PCR. To monitor p21 and p27 mRNA expression levels in a small amount of material such as a colony generated from a CD34+ cell, we first developed a quantitative RT-PCR assay for relative expression levels of p21 and p27 mRNAs with endogenously expressed β-actin used as an internal control. To circumvent difficulties in conventional competitive RT-PCR assays, coamplification of targets (p21 and p27) and control (β-actin) in a tube was designed (Fig 1A).

Simple coamplification of control and target DNAs can correct for variation in tube-to-tube amplification efficiency if the data are obtained during the exponential phase of the PCR reaction. However, β-actin is expressed at a level much higher than those of the target genes and comparable coamplification can be difficult because of rapid saturation of control reactions (data not shown). Therefore, we designed a PCR using a composite primer at a low concentration to reduce the β-actin signals at a constant ratio to the level comparable to the target signals.

We designed a common primer, p27S, derived from a homologous sequence between p21 and p27 and a composite primer, βASp27S, the 3’ part of which is derived from the sequence of β-actin and the 5’ part of which is the same as the common primer, p27S. The βASp27S primer is used in the amplification of β-actin cDNA, but not p21 or p27, because there is a large region of β-actin-specific sequence in the 3’ part. The three specific primers for p21, p27, and β-actin are p21AS, p27AS, and βS, respectively. Amplification of β-actin cDNA must be initiated with βS and βASp27S, and then p27S takes the place of βASp27S, because we use βASp27S at a lower concentration. Final products for β-actin were generated by virtue of p27S and βS. We confirmed the identity of the PCR products by direct sequencing (data not shown). We found exponential amplification phases of three genes overlapped to 26 cycles with a cDNA from cell lines used as a template (Fig 1B and C) and to 32 cycles with a cDNA from cells of colonies and PB used as a template (data not shown). Therefore, PCR was run for 21 cycles for analysis of cell lines and for 30 cycles for cells from colonies and PB.

To further confirm the quantitative nature of the PCR, we compared the RT-PCR assay with Northern analysis using RNAs extracted from MEG-01s cells stimulated with TPA. The addition of 10 nmol/L TPA upregulated p21 mRNA expression and induced cell cycle arrest at the G1-S boundary in MEG-01s cells. As shown in Fig 1D and E, the two methods gave similar results and the quantitative nature of the PCR was confirmed.

p21 and p27 expressions in colonies generated from human cord blood CD34+ cells. We monitored p21 and p27 mRNA expression levels in various colonies derived from CD34+ cells using the above-mentioned quantitative RT-PCR method. A total of 116 colonies were analyzed. Figure 2 shows the time course of relative expression levels of p21 and p27 mRNAs. In all four lineages (granulocytes, macrophages, megakaryocytes, and erythroblasts), p21 mRNA expression increased over time up to day 15. The increased levels of p21 mRNA in erythroid bursts and megakaryocyte colonies were similar to those observed in differentiated MEG-01s cells. p27 mRNA levels remained low during the observed period in all lineages except erythroid bursts. The gradual increase of p27 mRNA up to day 15 was evident only in erythroid bursts.

We also examined p21 and p27 mRNA levels in purified CD34+ cells. The p27 mRNA levels were low, but these cells expressed unexpectedly high levels of p21 mRNA that were decreased after 12 hours of incubation in suspension culture with or without additional cytokines (Fig 2E and J). Because the elevated levels of p21 mRNA might be related to the purification process, whether they reflect in vivo levels remains to be investigated.

We next immunocytochemically examined p21 and p27 protein expression in these colonies on day 15 of the culture from 7 and 6 subjects, respectively. We confirmed cell lineages by May-Grünwald Giemsa staining and immunostaining with anti-CD41 and anti-glycoporphin A antibodies (Table 2). As shown in Fig 3 and Table 2, some megakaryocytes and macrophages were positive for p21, although there were few p27-positive erythroblasts and granulocytes. p21 staining was always restricted to nuclei and with none in the cytoplasm. Some megakaryocytes were positive for p27 (Fig 3), although there were few p27-positive erythroblasts, macrophages, and granulocytes. Therefore, the proteins expression did not necessarily correlate with their mRNA levels.

p21 and p27 expressions in PB and BM MNCs. To confirm the correlation of cells in the colonies and PB cells, we examined PB cells of each lineage by RT-PCR and Western blot analysis using PBs from 4 normal individuals (Fig 4). Lympho-
cytes expressed both p21 and p27 mRNAs, but only a substantial amount of p27 protein as reported by other workers. Monocytes expressed mainly p21 mRNA and much less p27 mRNA (Fig 4A) and expressed both proteins, albeit weakly (Fig 4B). Consistent with the results of Western analysis, immunocytochemistry showed that both proteins were present in monocytes and that PB lymphocytes expressed only p27 protein (Fig 4D through F and I through K and Table 3). Both proteins were detected in nuclei. In PB granulocytes, p21 or p27 was not detected immunocytochemically (Fig 4G and L).

**Immunohistochemistry of human BM.** Finally, we immunohistochemically examined p21 and p27 expression in in vivo BM cells from 6 different individuals. Typical results of BM are shown in Figs 5, 6, and 7. Because of rarity of megakaryocytes...
in normal BM, we also used BM from essential thrombocytemia (ET) patients for analysis of megakaryocytes. p21 immunoreactivity was present in the nuclei of a subset of megakaryocytes (Figs 5A and D and 6A). Most cells comprising BM, namely erythroid and myeloid cells, did not stain for p21 antigen. According to double staining using anti-p21 and anti-vWF antibodies, p21 positivity in megakaryocytes in 2 ET patients (31.4% to 42.4%) was similar to 32.0% to 44.7% (n = 2) in normal BM. When we immunohistochemically investigated p53 protein expression in normal BM, we found that normal BM cells, including megakaryocytes, were p53 negative (data not shown). Therefore, p21 may be expressed in megakaryocytes through p53-independent mechanisms, as noted in other cell types.4,19-22,24,40

Weak to moderate p27 immunoreactivity was evident in nuclei of a subset of megakaryocytes (Figs 5B and E and 7A). Weak cytoplasmic staining in some megakaryocytes was also visible when using the MoAb, G173-524. However, it was not reproducible with another antibody, F-8. Although it is not clear whether the cytoplasmic staining was specific, there might be a significance of cytoplasmic localization of p27 protein.41 Double staining using anti-p27 and anti-vWF antibodies (data not shown) showed that approximately half the number of megakaryocytes expressed p27 protein, both in normal BM (51.35 to 77.9%, n = 2) and in BM from ET patients (42% to 52%, n = 2), although the proportions were somewhat overestimated because of endothelial cells (see below). Some small cells in BM showed strong nuclear staining (Fig 5B and E). Because p27 mRNA levels increased exclusively in erythroid bursts, we examined p27 positivity in erythroid cells. However, glycoporphin A-positive cells were negative for p27 (data not shown). Double staining using anti-p27 and anti-α- and -λ light chains antibodies showed the small p27-strongly positive cells to be plasma cells (data not shown); these cells were p21-negative and no Ki-67–positive plasma cells were found, as reported.42 p27 is involved in inhibiting normal B-cell proliferation,11,43 but to our knowledge, there are no reports regarding a role for p27 in normal plasmacytic maturation or in proliferation. It will be of interest to determine if tumor cells of plasma cell dyscrasias show aberrant p27 expression. Vascular endothelial cells and lymphoid aggregates were also p27-positive (data not shown). Findings in the subset of the cells with nuclear staining of p27, using either of the two MoAbs (G173-524 and F-8), were consistent.

We used the anti–Ki-67 antibody, MIB1, as a marker of proliferation. This antibody recognizes an antigen present in the nuclei of continuously cycling cells in G1, S, G2, and M phases, but not in G0 phase,44,45 and is a reliable marker of cell cycling in BM samples.42,46 Most of the cells in BM were positive for Ki-67 (Figs 5C and F, 6B, and 7B), as noted by other investigators.42 To clarify the relationship between cell cycling status and expression of p27 or p21 in vivo megakaryocytes, serial sections of BM from an ET patient were stained alternately for Ki-67 and p21 (Fig 6) or for Ki-67 and p27 (Fig 7). We scored p21, p27, and Ki-67 immunostaining of each megakaryocyte by the intensity ([-] none, [+] weak, [+]+ moderate, or [+] strong). At least 200 cells identified in more than three consecutive sections were counted. Regardless of p21 expression levels, approximately half the number of megakaryocytes expressed no Ki-67 antigen. However, the percentage of Ki-67 strongly positive megakaryocytes decreased with the increase in p21 intensity (Fig 6); thus, there was inverse relationship between p21 and Ki-67 expression in a subset of megakaryocytes. A clear inverse relationship was also noted between p27 and Ki-67 expression (Fig 7). Almost all of the p27-positive megakaryocytes were negative or weakly positive for Ki-67, and almost all of the p27-negative megakaryocytes were moderately or strongly positive for Ki-67. These observations strongly suggest that p27 and probably p21 expression is tightly linked to cell cycling status in megakaryocytes.

**DISCUSSION**

We made use of quantitative RT-PCR to evaluate p21 and p27 mRNA levels in greater than 100 colonies. The results were reproducible and comparable to findings in Northern analysis. Because each assay is performed in a tube and includes an internal control, a control signal confirms the appropriateness of

**Table 2. Positivity of Immunostaining in Cells of Colonies on Day 15**

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>p21</th>
<th>p27</th>
<th>Ki-67</th>
<th>GpA</th>
<th>CD41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage colony</td>
<td>33.7 ± 5.8 (7)</td>
<td>3.1 ± 0.5 (6)</td>
<td>13.6 ± 3.8 (4)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Granulocyte colony</td>
<td>4.2 ± 0.5 (5)</td>
<td>1.2 ± 0.6 (5)</td>
<td>17.0, 20.2 (2)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Erythroid burst</td>
<td>1.3 ± 0.5 (7)</td>
<td>2.1 ± 0.9 (6)</td>
<td>27.2 ± 6.1 (4)</td>
<td>84.6, 85.6 (2)</td>
<td>ND</td>
</tr>
<tr>
<td>Megakaryocyte colony</td>
<td>11.0, 15.6 (2)</td>
<td>12.6, 41.7 (2)</td>
<td>21.0 (1)</td>
<td>ND</td>
<td>82.1, 98.4 (2)</td>
</tr>
</tbody>
</table>

**Values are percentages. The mean ± standard error is shown in case of more than 2 experiments. Numbers of successful experiments are denoted in parentheses. Abbreviation: ND, not done.**
Fig 3. p21 and p27 expression in cells of colonies. Cells of colonies on day 15 of the culture generated from human cord blood CD34+ cells were immuno-
stained using anti-p21 antibody, 6B6 (A through E), and anti-p27 antibody, F-8 (F through J). Immunostaining was performed using the APAAP method as described in Materials and Methods. Nuclei were counterstained with hematoxylin. Positive cells showed red nuclear staining. (A and F) megakaryocytes, (B and G) erythroblasts, (C and H) macrophages, (D and I) granulocytes, and (E and J) MEG-01s cells harvested 48 hours after the addition of 10 nmol/L TPA (positive control; original magnifications: [A] and [F], ×200; [B] through [E] and [G] through [J], ×50).

Fig 4.
samples and reaction. The method will be applicable to other genes in multiple samples of small quantities.

According to our assay, p21 mRNA levels were elevated over time in all colonies of the lineage examined. The increased levels were comparable to those of differentiated and cell-cycle-arrested MEG-01s cells (Figs 1D and 2). Half lives of p21 transcripts were not altered during differentiation of MEG-01s cells (T.T. and T.M., unpublished data). In other differentiation models, mechanisms for elevation of p21 mRNA were often transcriptional and p53-independent.14,19,22-24,30 Accordingly, normal BM cells never immunostained for p53. Therefore, p21 mRNA expression during hematopoiesis may be regulated in a p53-independent and lineage-non-specific manner.

Both p21 and p27 proteins were expressed in a subset of megakaryocytes in normal human BM. An inverse relationship between p27 and Ki-67 suggests that p27 protein is expressed in cell-cycle arrested megakaryocytes. However, reciprocal expression of p21 to Ki-67 was not so obvious as that of p27, although the relationship between p21 and Ki-67 strong positivities was clearly inverse. The presence of p21-negative and Ki-67–negative megakaryocytes suggests that p21 expression is transient compared with continuous p27 expression in cell-cycle arrested cells. The proportion of p21-positive cells among Ki-67 moderately and strongly positive cells was larger than that of p27-positive cells, an observation that may reflect p21 expression earlier than p27 during cell-cycle exit. The hypothetical timing of p21 and p27 expression along the cell-cycle exit of megakaryocytes is shown in Fig 8. p21 may act early and transiently in the course of cell-cycle exit, and p27 may be expressed in a subset of megakaryocytes immediately adjacent to the proliferative compartment, the expression is decreased in terminally differentiated primary keratinocytes.49,50

We previously reported that MEG-01s treated with TPA showed megakaryocytic differentiation and expressed both p21 and p27 proteins in association with a G1-phase arrest.28 Both p21 and p27 were present in cyclin E-associated complexes, the histone H1 and Rb kinase activities of which were then inactivated. In this differentiation model, p21 protein expression preceded that of p27 in analysis of synchronized cell population.29 It remains to be elucidated whether p21 and p27 are indeed associated with any cyclin/CDK complex in the course of cell-cycle arrest of normal megakaryocytes.

Various studies using leukemic cell lines showed that p21 and p27 may be involved in differentiation and polyploidization of megakaryocytes. For example, the ectopic expression of p21 or p27 leads to induction of megakaryocytic differentiation of CMK cells.26 Overexpression of p21 results in an increase in polyploidy of UT-7 cells, which suggests that p21 may be implicated in polyploidization via suppression of cdc2 activity at mitosis.27 However, reciprocal expression of p21 and p27 to Ki-67 in vivo megakaryocytes does not support these views. Recent studies using normal megakaryocytes showed that polyploidization of megakaryocytes is due to abortive mitosis due to alterations in the regulation of mitotic exit49,51 and that cdc2 is active in endoreduplicating megakaryocytes.51 Our observations together with these reports suggest that suppression of cdc2 kinase activity by p21 (or p27) is unlikely to be the mechanism of polyploidization in megakaryocytes and that p21 and p27 have functions after endoreduplication is completed.

In mice lacking p21, red and white blood cells in PB are normal,52 although the absolute numbers of marrow progenitors are significantly decreased.53 Meanwhile, in mice lacking p27, complete blood count is normal, although the numbers of hematopoietic progenitors are increased significantly.54 Although either p21 or p27 may be dispensable for hematopoietic differentiation, it remains to be determined how hematopoiesis, especially megakaryocytogenesis, would be affected by knock-out of both p21 and p27.

p27 mRNA levels remained low during the observed period in megakaryocyte colonies, even though p27 protein was expressed in a subset of megakaryocytes. This finding is consistent with knowledge that the p27 protein level is regulated mainly by protein degradation steps through the ubiquitin-dependent proteolytic pathway and that p27 mRNA and protein levels do not coincide in many situations.55-57 On the contrary, the elevation of p27 mRNA in erythroid bursts, without detectable proteins in BM erythroblasts, is intriguing. Although p27 regulation is frequently posttranslational, p27 mRNA is

### Table 3. Positivity of Immunostaining in PB Cells

<table>
<thead>
<tr>
<th>Proteins</th>
<th>p21</th>
<th>p27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>$0.0 \pm 0.0 (5)$</td>
<td>$66.1 \pm 2.4 (4)$</td>
</tr>
<tr>
<td>Monocytes</td>
<td>$39.9 \pm 2.3 (5)$</td>
<td>$63.6 \pm 3.9 (4)$</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>$0.0 \pm 0.0 (5)$</td>
<td>$0.0 \pm 0.0 (4)$</td>
</tr>
</tbody>
</table>

The mean ± standard error is shown. Numbers of individual B cells analyzed are denoted in parentheses.

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**Fig 5.** p21, p27, and Ki-67 staining of BM. Normal BM (A, B, and C) and BM from an ET patient (D, E, and F) was immunostained using anti-p21 antibody, 686 (A and D); anti-p27 antibody, G173-524 (B and E); and anti–Ki-67 antibody, MIB1 (C and F). Immunostaining was performed using the APAAP method, as described in Materials and Methods. Positive cells showed red nuclear staining. Although weak cytoplasmic staining in association with a G1-phase arrest.28 Both p21 and p27 were present in cyclin E-associated complexes, the histone H1 and Rb kinase activities of which were then inactivated. In this differentiation model, p21 protein expression preceded that of p27 in analysis of synchronized cell population.29 It remains to be elucidated whether p21 and p27 are indeed associated with any cyclin/CDK complex in the course of cell-cycle arrest of normal megakaryocytes.

Various studies using leukemic cell lines showed that p21 and p27 may be involved in differentiation and polyploidization of megakaryocytes. For example, the ectopic expression of p21 or p27 leads to induction of megakaryocytic differentiation of CMK cells.26 Overexpression of p21 results in an increase in polyploidy of UT-7 cells, which suggests that p21 may be implicated in polyploidization via suppression of cdc2 activity at mitosis.27 However, reciprocal expression of p21 and p27 to Ki-67 in vivo megakaryocytes does not support these views. Recent studies using normal megakaryocytes showed that polyploidization of megakaryocytes is due to abortive mitosis due to alterations in the regulation of mitotic exit49,51 and that cdc2 is active in endoreduplicating megakaryocytes.51 Our observations together with these reports suggest that suppression of cdc2 kinase activity by p21 (or p27) is unlikely to be the mechanism of polyploidization in megakaryocytes and that p21 and p27 have functions after endoreduplication is completed.

In mice lacking p21, red and white blood cells in PB are normal,52 although the absolute numbers of marrow progenitors are significantly decreased.53 Meanwhile, in mice lacking p27, complete blood count is normal, although the numbers of hematopoietic progenitors are increased significantly.54 Although either p21 or p27 may be dispensable for hematopoietic differentiation, it remains to be determined how hematopoiesis, especially megakaryocytogenesis, would be affected by knock-out of both p21 and p27.

p27 mRNA levels remained low during the observed period in megakaryocyte colonies, even though p27 protein was expressed in a subset of megakaryocytes. This finding is consistent with knowledge that the p27 protein level is regulated mainly by protein degradation steps through the ubiquitin-dependent proteolytic pathway and that p27 mRNA and protein levels do not coincide in many situations.55-57 On the contrary, the elevation of p27 mRNA in erythroid bursts, without detectable proteins in BM erythroblasts, is intriguing. Although p27 regulation is frequently posttranslational, p27 mRNA is
Fig 5.

Fig 6.

Fig 7.
protein expression in megakaryocytes.

nied by protein expression. 58-60 The elevation of p27 mRNA indeed upregulated in some situations and is usually accompa-

58-60 The elevation of p27 mRNA expression. However, as p27 protein was absent, it may be dispensable in erythropoiesis.

Recently, Dao et al61 reported that CD34+ cells from human BM expressed p27 protein and that reduction in levels of p27 protein using antisense oligonucleotides to p27 coupled with transforming growth factor-β (TGF-β) neutralization induces cell-cycle entry and increases retroviral transduction of primitive human hematopoietic cells. We detected only low levels of p27 mRNA in cord blood CD34+ cells. The difference may be due to posttranslational regulation again. We suppose that primitive hematopoietic cells have elevated p27 protein levels, which are decreased in cycling cells and increased again with cell-cycle exit especially in megakaryocytes, although the mRNA levels remain low.

Steinman et al30 reported that myeloid maturation of umbilical cord blood CD34+ cells is associated with an increase in p21 expression at RNA and protein levels. We observed that p21 mRNA expression increased with time up to day 15 in granulocyte colonies, findings consistent with their report. However, in our system, neither BM myeloid lineage cells nor PB granulocytes were positive for p21 protein and only a few p21-positive cells were detected in granulocyte colonies. Although one would need to exclude the possible expression of p21 protein in granulocytes, p21 protein levels in myeloid cells are much lower than in megakaryocytes.

p21 mRNA expression increased with time in macrophage colonies and some of the macrophages immunostained for p21 antigen. Accordingly, PB monocytes expressed p21 mRNA and protein. Consistent with these observations, several cell lines are seen to express p21 mRNA and protein during monocytic differentiation19,21,25,62 and some alveolar macrophages of the lung are p21-positive.63 p27 protein levels are also elevated during monocyctic differentiation in some cell lines.23,29 However, the p27 protein level was low in PB monocytes in our study and p27 protein was not detected in macrophages in in vitro colonies.

By way of summary, although p21 mRNA levels increased over time in any lineage of hematopoietic colonies, p21 protein levels were elevated only in limited lineages (megakaryocyte and monocyte/macrophage). p27 protein was detected only in megakaryocytes, monocytes, and lymphoid cells (lymphocytes and plasma cells), whereas p27 mRNA levels were elevated only in erythroid bursts. These observations suggest that complex lineage-specific regulation mechanisms are involved in p21 and p27 protein expression in human hematopoiesis. In addition, reciprocal expression of p21 and p27 to Ki-67 suggests that, in megakaryocytes, both p21 and p27 may be involved in terminal exit from the cell cycle.

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Expression of p21Cip1/Waf1/Sdi1 and p27Kip1 Cyclin-Dependent Kinase Inhibitors During Human Hematopoiesis

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