Human Mesenchymal Stem Cells Support Megakaryocyte and Pro-Platelet Formation From CD34^+ Hematopoietic Progenitor Cells

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Megakaryocytopoiesis and thrombocytopoiesis result from the interactions between hematopoietic progenitor cells, humoral factors, and marrow stromal cells derived from mesenchymal stem cells (MSCs) or MSCs directly. MSCs are self-renewing marrow cells that provide progenitors for osteoblasts, adipocytes, chondrocytes, myocytes, and marrow stromal cells. MSCs are isolated from bone marrow aspirates and are expanded in adherent cell culture using an optimized media preparation. Culture-expanded human MSCs (hMSCs) express a variety of hematopoietic cytokines and growth factors and maintain long-term culture-initiating cells in long-term marrow culture with CD34^+ hematopoietic progenitor cells. Two lines of evidence suggest that hMSCs function in megakaryocyte development. First, hMSCs express messenger RNA for thrombopoietin, a primary regulator for megakaryocytopoiesis and thrombocytopoiesis. Second, adherent hMSC colonies in primary culture are often associated with hematopoietic cell clusters containing CD41^+ megakaryocytes. The physical association between hMSCs and megakaryocytes in marrow was confirmed by experiments in which hMSCs were copurified by immunoselection using an anti-CD41 antibody. To determine whether hMSCs can support megakaryocyte and platelet formation in vitro, we established a coculture system of hMSCs and CD34^+ cells in serum-free media without exogenous cytokines. These cocultures produced clusters of hematopoietic cells atop adherent MSCs. After 7 days, CD41^+ megakaryocyte clusters and pro-platelet networks were observed with pro-platelets increasing in the next 2 weeks. CD41^+ platelets were found in culture medium and expressed CD62P after thrombin treatment. These results suggest that MSCs residing within the megakaryocytic microenvironment in bone marrow provide key signals to stimulate megakaryocyte and platelet production from CD34^+ hematopoietic cells. J. Cell. Physiol. 184:58–69, 2000. © 2000 Wiley-Liss, Inc.

Megakaryocytopoiesis is initiated with the terminal differentiation of pluripotent hematopoietic progenitor cells (HPCs) along a pathway that results in the expression of the lineage-specific surface marker CD41, endomitosis, and subsequent cytoplasmic fragmentation–producing platelets, which are released into the blood circulation (Hoffman, 1989; Ellis et al., 1995; Gewirtz, 1995). This process is regulated by the action of numerous substances including cytokines (Kaushansky et al., 1986; Ikebuchi et al., 1987; Bruno and Hoffman, 1989; Bruno et al., 1989; Avraham et al., 1992; Burstein et al., 1992; Kaushansky, 1995), growth factors (Han et al., 1992), chemokines (Keller et al., 1994), and extracellular matrix molecules (ECMs) (Mossuz et al., 1997), many of which are produced by stromal cells within the marrow microenvironment (Eaves et al., 1982; Deruygina et al., 1990; Dorshkind, 1990).

Among these megakaryocytic cytokines/growth factors are thrombopoietin (TPO), Interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), and stem cell factor (SCF). TPO has been shown to be the primary regulator of megakaryocyte differentiation and platelet...
formation (Kaushansky, 1995). However, the production of normal megakaryocytes and platelets by knock-out mice deficient in the gene encoding TPO suggests that other cytokines are also important and sufficient to support megakaryocytepoiesis and platelet formation in vivo in the absence of TPO (Bunting et al., 1997). Although these gene knockout studies may define the necessity of individual cytokines for megakaryocyte differentiation, they do not provide insight into the interactions between different cytokines or between cytokines and cell adhesion molecules/ECM that are also produced by stromal cells.

The characterization of specific stromal cells involved in this process and the establishment of a coculture system with defined stromal cells and HPCs is critical to understanding the role that the stromal cell plays in the regulation of megakaryocyte and platelet production. To this end, HPCs have been characterized extensively (Eaves et al., 1982; Szilvassy and Hoffman, 1995) and marrow stromal fibroblasts have been shown to be derived from pluripotent mesenchymal stem cells (MSCs) (Caplan, 1991; Prockop, 1997; Pittenger et al., 1999). MSCs also give rise to osteoblasts (Jaiswal et al., 1992; Eaves et al., 1982; Szilvassy and Hoffman, 1995) and macrophage contamination (Majumdar et al., 1998; Pittenger et al., 1999), and cells that produce tendon (Young et al., 1999), adipocytes (Pittenger et al., 1997), chondrocytes (Johnstone et al., 1998), myocytes (Wakatani et al., 1995), adipocytes (Pittenger et al., 1999), and cells that produce tendon (Young et al., 1998). We have developed methods for the isolation and culture of MSCs from human bone marrow aspirates and have demonstrated that these cultured cells display a fibroblastic morphology and retain their pluripotentiality following extensive culture expansion (Haynesworth et al., 1996; Majumdar et al., 1998; Pittenger et al., 1999). After two passages (approximately 14 cell divisions), culture-expanded human MSCs (hMSCs) are morphologically and phenotypically homogeneous (>99%) and essentially free (<0.5%) of monocyte/macrophage contamination (Majumdar et al., 1998; Pittenger et al., 1999).

The multilineage potential of individual hMSCs isolated from adult marrow and expanded to colonies was clearly demonstrated recently by Pittenger et al. (1999). We and others have shown that these culture-expanded cells express various hematopoietic cytokines including IL-6, IL-11, LIF, SCF, and Flt3/Flk2 ligand (FL) (Haynesworth et al., 1996; Majumdar et al., 1998; Pittenger et al., 1999). After two passages (approximately 14 cell divisions), culture-expanded human MSCs (hMSCs) are morphologically and phenotypically homogeneous (>99%) and essentially free (<0.5%) of monocyte/macrophage contamination (Majumdar et al., 1998; Pittenger et al., 1999).

In this study, we examined the role of hMSCs in megakaryocyte differentiation in vitro. We first identified an association between hMSCs and megakaryocytes in human bone marrow. To address the function of MSCs, we used coculture of hMSCs and CD34+ HPCs to demonstrate that hMSCs support megakaryocyte differentiation, pro-platelet formation, and platelet release in the absence of exogenous cytokines.

**MATERIALS AND METHODS**

**Bone marrow aspirates**

Bone marrow samples used in these studies were either collected from healthy human donors at The Johns Hopkins University Oncology Center under an Institutional Review Board–approved protocol or purchased from Poietic Technologies, Inc. (Gaithersburg, MD).

**Human MSC isolation**

MSCs were isolated and culture-expanded according to the method described by Majumdar et al. (1998). Briefly, heparinized bone marrow was mixed with an equal volume of phosphate-buffered saline (PBS; Life Technologies, Gaithersburg, MD) and centrifuged at 900g for 10 min at 25°C. Washed mononuclear cells (MNCs) were resuspended in PBS to a density of 2 × 10^7 cells/ml. Aliquots (10 ml) were layered over 20 ml of a 1.073 g/ml Percoll (Pharmacia, Piscataway, NJ) solution and the tubes were centrifuged at 900g for 30 min at 25°C. MNCs at the interface were recovered, diluted with 5 volumes of PBS, recovered by centrifugation, and finally resuspended in hMSC medium composed of Dulbecco’s modified Eagle’s medium (DMEM) with low glucose (DMEM-LG) (Life Technologies), selected lot of 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), and 1% antibiotic-antimycotic stock solution (Life Technologies). Cells were plated into 185-cm² flasks (Nunc) at a density of 3 × 10^5 MNC/flask and the cultures were incubated at 37°C in 5% CO₂ in air and 95% relative humidity. The medium was exchanged after 48 h and every 3 to 4 days thereafter. When the cultures reached approximately 90% of confluence, hMSCs were recovered by the addition of trypsin-EDTA solution (Life Technologies) and replated into passage culture at a density of 1 × 10^6 cells per each 185-cm² flask. For the colony-formation assay, aliquots (1.6 × 10^5 cells/well) were plated into six-well culture dishes for 14 days after which they were washed, fixed with 1% gluteraldehyde solution (Sigma Chemicals, St. Louis, MO), and stained with 0.1% solution of crystal violet (Sigma) for 30 min.

**RNA preparation and RT-PCR analysis**

Total RNA was extracted from monolayers of culture-expanded hMSCs (passages 2–3) in 185-cm² flasks by modification of the method of Chirgwin et al. (1979). Briefly, hMSCs were lysed by the addition of 7 ml of a solution containing 4 M guanidinium isothiocynate (Sigma), 0.03 M sodium acetate, and 0.4 g/ml of cesium chloride (Life Technologies). Lysates were layered over a 3-ml pad of 5.7 M solution of CsCl and centrifuged overnight at 35,000 rpm in a Beckman Ti70 rotor. The pelleted RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and precipitated at room temperature after the addition of 3 M sodium acetate and 2.5 volumes of absolute ethanol. RNA was recovered by centrifugation and dissolved in DEPC-treated water. The RNA concentration was determined by absorbency at 260 nm, and the volume was adjusted to bring the final RNA concentration to 0.5 mg/ml.

Reverse transcriptase (RT)-coupled polymerase chain reaction (RT-PCR) was performed using the RNA PCR Kit (Perkin–Elmer, Foster City, CA), according to
the manufacturer's instructions. Total RNA (0.5–1.0 μg) was used as a template for the RT-PCR assay and the RT-PCR products were visualized by ethidium bromide staining, following electrophoresis through a 1% agarose gel.

The sequences (5’ to 3’) of oligonucleotide primer pairs (Operon, Alameda, CA) used in RT-PCR analyses are listed here. TPO 5’ primer: CCTCCTTTGGGC-CCTGCAAGCCT and 3’ primer: GGGTGAGGCTG-GACCAAAGGG (amplified product = 606 bp); IL-2 5’ primer: ATGFGACAGTAGCCTCCCCTTCTT and 3’ primer: GTCAAGTGGTG AGATGATCCTGTCG (amplified product = 458 bp); IL-6 5’ primer: GTAGCCGC-CCCACACAGACGCC; and 3’ primer: GCCATCTTT-GGAAGGTTCCAGG (amplified product = 628 bp); SCF 5’ primer: CCTCTCGTCAAACGTGAAGG; and 3’ primer: AGGAGTAAAGAGCCTGGTTC (amplified product = 346 bp); Flt3/Flik2 ligand (FL) 5’ primer: TGGAGCCCAACAACCTATCT and 3’ primer: GGGTGAGCTG-GACCAAAGGG (amplified product = 458 bp). RT was omitted in one set of PCR reactions to confirm that DNA products were exclusively derived from mRNA and not genomic DNA.

**Selection of megakaryocyte/MSC complexes by an anti-CD41 antibody**

Immunoselection was performed on MNCs fractionated by Percoll density sedimentation as described earlier. The MNCs collected at the interface were diluted by Percoll density sedimentation as described earlier. The MNCs collected at the interface were diluted by Percoll density sedimentation as described earlier. The MNCs collected at the interface were diluted by Percoll density sedimentation as described earlier. The MNCs collected at the interface were diluted by Percoll density sedimentation as described earlier.

**Selection of CD34+ bone marrow cells**

CD34+ cells in bone marrow aspirates of healthy donors were either isolated in our laboratory using the CD34+ progenitor cell selection system (Miltenyi Biotech, Auburn, CA) following the manufacturer’s instructions, or purchased from either Poietic Technologies, Inc. or AllCells LLC (San Mateo, CA) after selection with the same method. Isolated CD34+ cells were analyzed by flow cytometry (see below) to measure purity of CD34+ cells and the presence of the CD41 cell-surface antigen. By this method, purity of CD34+ cells was found to be 94 ± 3% (n = 5) and percentages of CD41+ cells were 2 ± 1% (n = 3). Most of the CD41+ cells were also CD34+.

**Megakaryocytepoiesis assay**

Cocultures were established in six-well tissue-culture plates with equal numbers (1–2 × 10^5) of purified CD34+ cells and passage-2 or -3 hMSCs (approximately 14 days or seven cell divisions for each cell culture passage). Cells were cultured in IMDM medium supplemented with BIT (10 mg/ml BSA, 10 μg/ml human insulin, and 200 μg/ml human transferrin) from StemCell Technologies (Vancouver, British Columbia, Canada) plus 100 μmol/L 2-mercaptoethanol (Sigma) and 40 μg/ml low-density lipoproteins (Sigma). hMSCs were preplated in six-well dishes (9.4 cm²/well) in the hMSC medium and grown to confluency. hMSCs were washed twice with the complete serum-free (BIT-containing) medium before CD34+ cells were added. Cells were cultured for up to 21 days at 37°C in 5% CO₂ in air and 95% relative humidity. If CD34+ cells were plated in the absence of hMSCs, few cells (less than 3% of input cells, either adherent or in suspension) survived after 7 days under the previous culture conditions. To characterize hematopoietic cells in the coculture, non-adherent hematopoietic cells were collected and pooled with the subsequent washes. Hematopoietic cells adherent to hMSCs were recovered by incubating the cell layer with PBS (without Ca²⁺ or Mg²⁺) for 30 min. An anti-CD41a antibody (clone HIP8) and a control mouse antibody (clone 107,3) were purchased from PharMingen (San Diego, CA) and an anti-CD14 antibody (clone My4), from Immunotech (Westbrook, ME). After incubation with primary antibodies (0.2 μg/10^6 cells) or 4 μg/ml for 30 min, cells were recovered by centrifugation, washed with the CATCH buffer, pelleted, and resuspended in 1 ml of the CATCH buffer containing 5% normal goat serum (NGS; Life Technologies). A suspension of antimouse IgG1 (sheep) antibody conjugated to M-450 Dynabeads (Dynal, Lake Success, NY) (final concentration of 1–2 × 10^5 beads/ml) was added to the cell suspensions and the mixtures were rotated at 4°C for 60 min. The cell/bead complexes were washed twice with CATCH buffer and collected with a magnet (Dynal). After the final wash, cell complexes were recovered by centrifugation and suspended in 0.5 ml of CATCH buffer. Aliquots of cells were plated into six-well tissue-culture plates containing 2 ml of the hMSC medium and the selected cells were cultured at 37°C in 5% CO₂ in air and 95% relative humidity. The medium was exchanged after 48 h and every 3 to 4 days thereafter. MSC colony formation was assessed on day 14 of culture as described earlier.

**Immunofluorescence**

Cocultures used for immunofluorescence analysis were established in four-well chamber slides (Lab-Tek Nalgene, Naperville, IL) using 1–2 × 10^4 CD34+ cells and 2 × 10^5 hMSCs (passages 2–3) per well. Prior to
this analysis, adherent cell layers were carefully washed with PBS, fixed by the addition of ice-cold acetone (5 min), and washed a final time with ice-cold PBS. The fixed cells were incubated for 30 min with a 5% NGS solution. The NGS solution was discarded and the appropriate antibody was added (with 5% NGS). The cocultures were stained with three different antibodies. Fixed cultures were first incubated in the dark with biotinylated SH-3 antibody (a marker for hMSCs), 2 μg/ml ATCC HB-10744 (Haynesworth et al., 1992) for 1 h on ice, after which a 1:200 dilution of Streptavidin conjugated to the fluorochrome cascade blue was added for 30 min on ice. The cocultures were then washed with ice-cold PBS and incubated for 30 min simultaneously with anti-CD41-PE (2 μg/ml) and anti-CD34-FITC (2 μg/ml) (PharMingen) monoclonal antibodies. The assays were finally washed for 10 min with ice-cold PBS. All incubations were completed on ice for 30 min in the dark. Cell complexes were visualized after mounting the slides with coverslips in Immumount (Shandon, Pittsburgh, PA). Immunofluorescence analysis of the hMSC/megakaryocyte cell-complexes in primary culture was performed with monoclonal antibody anti-CD41a-PE (2 μg/ml), as described earlier.

Flow cytometric analysis

Cells harvested from cocultures were pooled, washed twice in FACS buffer (2% BSA, 2 mmol/L EDTA, and 0.1% Azide in PBS), and suspended in 100 μl FACS buffer containing 2 mg/ml human IgG (to block nonspecific IgG binding). APC-conjugated anti-CD34 antibody (2 μg/ml; Clone HPCA-2, mouse IgG1) from Becton Dickinson (Mountain View, CA) and 2 μg/ml R-PE–conjugated anti-CD41a antibody (PharMingen) were added. The mixtures were incubated on ice for 30 min. Cells were then washed once in the FACS buffer and resuspended in 0.4 ml of FACS buffer containing PI. The appropriate conjugated mouse IgG1 control antibodies were used to establish nonspecific staining. Nonviable cells containing PI were excluded from cell analysis. A FACS Calibur flow cytometer (Becton Dickinson) was used for these analyses. Selected events (1 × 10⁴) were collected for each sample and analyzed using the CellQuest software (Becton Dickinson).

Platelet activation with thrombin

The supernatants of the cocultures were collected after 14 to 21 days, and these were considered as the platelet fractions. The platelets were collected by centrifugation of the supernatants at 3,000 rpm. Activation with thrombin (Sigma) was performed by incubation with 2 U/ml thrombin at 37°C for 10 min as described by Norol et al. (1998). The platelets were washed with PBS, suspended in FACS buffer, and stained with anti-CD41-PE (Immunotech) and anti CD62P-FITC (PharMingen). The adherent cell fraction of the cocultures was also collected and activated with thrombin. As a control, platelets from peripheral blood were collected, similarly activated, and then stained with antibodies to CD41 and CD62P. Flow cytometry was performed as described earlier, except that unstained peripheral blood platelets were used to establish the gate (selecting platelet-like particles based on their characteristic forward and side scatters).

RESULTS

Identification of cell complexes of hMSCs and megakaryocytes in primary cultures of human bone marrow

The preparation of hMSCs from bone marrow includes the enrichment of a low-density fraction of cells using Percoll gradient sedimentation followed by adherence and growth on tissue-culture plastic in a selective medium. In primary cultures of hMSCs, we have consistently noted the presence of adherent cell complexes composed of fibroblastic MSCs and a central cluster of cells (Fig. 1A), some of which resemble megakaryocytes. Immunofluorescence, by the use of an anti-CD41 (gpIb/IIIa complex) monoclonal antibody, demonstrated the presence of megakaryocytes in these complexes (Fig. 1B, C). It is also clear from the immunostaining (Fig. 1B, C) that hMSCs present in the coculture do not express the CD41 marker. The lack of
CD41 expression on hMSCs was confirmed by FACS analyses of hMSCs harvested after one or two more passages (data not shown).

If MSCs and megakaryocytes are in proximity with each other in the marrow, we predicted that MSCs could be co-isolated with megakaryocytes selected from Percoll-fractionated cells with either anti-CD41 (first panel) or anti-CD14 (third panel) antibodies, or in the absence of a primary antibody (No Ab, second panel). As a control, 10-fold fewer (1 × 10⁶) Percoll-fractionated (Percoll Fx) cells were plated without any selection. Approximately 15% of colony-forming hMSCs were coselected with the anti-CD41 antibody.

CD41 expression on hMSCs was confirmed by FACS analyses of hMSCs harvested after one or two more passages (data not shown).
the Percoll fraction of human marrow using an anti-CD41 monoclonal antibody and immunomagnetic beads. Cells selected by using the anti-CD41 antibody were cultured in hMSC medium to optimize attachment and proliferation of hMSCs. After 72 h in culture, fibroblastic cells appeared in cultures of CD41-selected cells, whereas no such fibroblastic cells were observed in cultures prepared from cells selected with an isotype-matched control antibody (Fig. 2). The derived fibroblastic cells were stained positive by SH-3, a monoclonal antibody that recognizes hMSCs (Haynesworth et al., 1992). Anti-CD41–selected cultures were maintained for 14 days in hMSC medium to demonstrate the formation of discrete fibroblastic colonies (Fig. 3). Only a few colonies were generated from cells isolated in the absence of a primary antibody or in the presence of a control (anti-CD14) antibody. Expanded progeny from the anti-CD41–selected cultures maintained the ability to undergo osteogenic, adipogenic, and chondrogenic differentiation in vitro under the differentiation-inducing conditions (Pittenger et al., 1999), as did cells isolated in the initial Percoll fraction in the absence of anti-CD41 selection (data not shown).

**hMSCs support megakaryocyte differentiation of CD34+ cells**

We and others have previously shown that culture-expanded hMSCs express a variety of hematopoietic cytokines including IL-6, IL-11, and LIF (Majumdar et al., 1998). In the course of this work, we found that hMSCs also express an RNA transcript encoding TPO (Fig. 4), an important regulator of megakaryocyte growth and differentiation. Transcripts for SCF and IL-6, but not IL-2, were also found in the same preparation from hMSCs, consistent with the published data (Majumdar et al., 1998). To examine whether the hMSCs could support megakaryocyte differentiation in culture, purified CD34+ progenitor cells from bone marrow cells were cocultured with preformed monolayers of culture-expanded hMSCs in a defined serum-free medium (supplemented with insulin and transferrin), in the absence of exogenous hematopoietic cytokines. Under this condition, monolayers of hMSCs maintained their fibroblastic morphology and survived for up to 3 weeks. As seen in Fig. 5, cocultures of CD34+ cells and hMSCs produced clusters of megakaryocyte-like cells on hMSC surfaces in addition to myeloid-like cells (Fig. 5B). Cultures of CD34+ cells alone in the absence of hMSCs did not produce such clusters (Fig. 5A), and only 3% of input CD34+ cells survived after 7 days (data not shown). After 7 days of coculture, adherent hMSCs were covered with dense clusters of hematopoietic cells (Fig. 5C), and some clusters contained structures that resembled pro-platelets (Fig. 5D) as described by others (Choi et al., 1995; Norol et al., 1998). The numbers of these pro-platelet networks or strings increased in the next 2 weeks. We also observed that the addition of recombinant TPO (at 10–20 ng/ml) in the cocultures enhanced megakaryocytepoiesis and platelet formation from BM CD34+ cells (data not shown).

Megakaryocytic differentiation in this coculture system was also determined by flow cytometry using anti-CD41 as well as anti-CD34 monoclonal antibodies. The input CD34+ bone marrow cells were typically 94 ± 3% pure (n = 5) and contained 2 ± 1% (n = 3) CD41+ cells, most of which were also CD34+, numbers consistent with observations of other researchers (Williams et al., 1998). After 7 days in coculture, both hematopoietic cells and hMSCs were harvested by the addition of a cell-dissociation buffer, and filtered through 30-μm nylon mesh. Most of the hMSCs were still aggregated to each other and eliminated by this filtration. Harvested hematopoietic cells were enumerated and then analyzed by flow cytometry. As shown in Fig. 6B, a population of CD41+ cells (3% of total hematopoietic cells representing 5% of CD34+ population) was present and most of these CD41+ cells were also CD34+ cells. Interestingly, the CD34+ population remained as a relatively high percentage (approximately 60%) of the cells during the coculture on hMSCs in the absence of exogenous cytokines (Fig. 6B). In addition, CD14- and/or CD15-positive myeloid cells were also observed (data not shown).

The cell composition of the coculture was also examined using immunofluorescence microscopy (Fig. 7). Human MSCs, megakaryocytes, and hematopoietic progenitor cells were identified in the coculture by their reactivity to SH-3, anti-CD41, and anti-CD34 antibodies, respectively. The photomicrograph shown in Fig. 7 demonstrates the physical association between hMSCs (blue), CD34+ (green), and CD41+ (red) cells in the coculture, after extensive washing before the immunostaining. CD34+ /CD41+ immature megakaryocytes appeared yellow (green + red) in coculture (Fig. 7). Interestingly, we observed that sizes of cultured hematopoietic cells increased from CD34+ /CD41+ (green) cells, CD34+ /CD41+ (yellow) cells, to CD34+ /CD41+ (blue) cells.
Fig. 5. Megakaryocytopoiesis and pro-platelet formation in coculture of hMSCs and CD34<sup>+</sup> cells. A: CD34<sup>+</sup> cells alone in the absence of hMSCs. B–D: Selected fields in coculture of CD34<sup>+</sup> cells on hMSCs. C: Arrows point out dense cell clusters formed in the CD34<sup>+</sup> cell/hMSC coculture. D: Pro-platelets with extended cytoplasmic processes (arrowheads) are observed at high magnification.

Fig. 7. Immunofluorescence analysis of hMSC supported megakaryocytopoiesis. Adherent cells in cocultures of hMSCs and CD34<sup>+</sup> cells were fixed and incubated with SH-3 (biotin-conjugated), anti-CD41 (R-PE–conjugated), and anti-CD34 (FITC-conjugated) monoclonal antibodies that recognize hMSCs, megakaryocytes/platelets, and CD34<sup>+</sup> cells, respectively. Streptavidin-conjugated cascade blue was used as the fluorochrome for SH-3. Therefore, hMSCs appear blue, CD34<sup>+</sup> cells green, and CD41<sup>+</sup> cells red. The double-positive CD34<sup>+</sup>/CD41<sup>+</sup> cells, which are labeled by both R-PE and FITC, appear yellow in color.
(red) cells, consistent with their developmental stages in megakaryocytopoiesis.

The presence of CD34^1/CD41^2 cells and CD34^1/CD41^1 premature megakaryocyte cell populations led us to examine whether significant numbers of these progenitor cells were maintained in the 7-day coculture without exogenous cytokines. Progenitors for erythroid/myeloid cells were assayed as colony-forming unit-cells (CFU-C) in semisolid methylcellulose medium and megakaryocytic (Mk) progenitors were assayed as CFU-Mk in a collagen-based semisolid medium, before and after coculture. As shown in Table 1, frequencies of CFU-Mk and CFU-C in cultured hematopoietic cells (on hMSCs) were slightly increased as compared with those of the input cells, whereas the total numbers of harvested hematopoietic cells were slightly decreased. The data indicated that hMSCs maintained progenitor populations while supporting megakaryocyte and myeloid differentiation in the absence of exogenous cytokines.

**hMSCs support thrombocytopoiesis in vitro**

Pro-platelet formation in cocultures of CD34^1 bone marrow cells and hMSCs (Fig. 5D) suggests that hMSCs support terminal differentiation of megakaryocytes into platelets. To further characterize hMSC-supported platelet formation, flow cytometry was used to determine the presence of CD41^+ platelets released into media and the presence of the CD62P marker on the surface of activated platelets. Platelets from peripheral blood were used to establish the gates for FACS analysis and as a positive control for CD41 and CD62P staining (Fig. 8). As expected, the majority of blood-derived platelets expressed CD41, and the majority of these CD41^+ platelets also expressed the CD62P marker on the surface upon activation with thrombin (Fig. 8, bottom right panel). A small percentage of CD41^+ platelets remained attached to the cells and was not represented in the supernatants shown in Fig. 8. The percentage of platelets released and activated was significantly increased when TPO was included in the cocultures (data not shown). In addition, we found that the culture-derived platelets were already activated and expressed the CD62P marker in some experiments. Together, these data indicate that CD41^+ platelets were generated by mature megakaryocytes, released into media, and expressed the CD62P activation marker.

**DISCUSSION**

In this study we used defined culture conditions to show that culture-expanded pluripotent hMSCs support megakaryocytic differentiation and pro-platelet formation from CD34^+ hematopoietic progenitor cells. Together with the evidence of physical association between primary megakaryocytes and hMSCs, these results suggest functional roles of hMSCs within BM microenvironment for megakaryocytopoiesis and platelet formation.

It has been shown that the stages of megakaryocyte development are regulated by the action of numerous cytokines. For example, IL-3, GM-CSF, and SCF have been shown to stimulate CFU-Mk formation (Kaushansky et al., 1986; Ikebuchi et al., 1987; Avraham et al., 1992). Cytokines such as IL-6, IL-11, and LIF regulate megakaryocyte maturation, endomitosis, and subsequent platelet release (Ikebuchi et al., 1987; Burstein et al., 1992), whereas TPO has been shown to act at all levels of megakaryocyte differentiation (Kaushansky, 1995). Culture-expanded hMSCs express transcripts for IL-6, IL-11, LIF, and SCF, but not IL-3 (Majumdar et al., 1992).
et al., 1998). In this study, we demonstrated that hMSCs also express a transcript for TPO. However, the TPO protein, which is primarily made in the liver and to a lesser extent in the kidney and bone marrow (Kaushansky, 1995; Guerrero et al., 1997; Qian et al., 1998), was undetectable in media conditioned with hMSCs (the detection limit of the ELISA kit we used is ~15 pg/ml). In contrast, IL-6 (2–10 ng/ml), IL-11 (0.5–2 ng/ml), and LIF (13–180 pg/ml) were readily detected by ELISA in hMSC-conditioned media prepared similarly, consistent with published data (Haynesworth et al., 1996; Mbalaviele et al., 1999). Consistent with our observation, Guerrero et al. (1997) recently demonstrated that only a fraction of marrow stromal cell clones (which were established by a different method) produced low levels of the TPO protein, although most of these cell clones expressed a TPO transcript. Using different antibodies in ELISA, they were able to detect TPO protein at levels below the detection of the assay we used in our study.

The ability of hMSCs to support megakaryocyte and pro-platelet formation in the absence of exogenous cytokines or serum suggests two possibilities. First, hMSCs may produce very low, yet efficacious quantities of TPO, which synergizes with other MSC-produced cytokines such as IL-6, IL-11, and LIF. Alternatively, cytokines and other molecules produced by hMSCs are sufficient to support megakaryocytogenisis and platelet formation, which can be further enhanced by TPO. The first hypothesis is based on the notion that TPO is absolutely essential to megakaryocytogenesis and platelet formation, which is inconsistent with the fact that megakaryocyte- and platelet production are observed in TPO-deficient mice (Bunting et al., 1997). It remains to be determined whether physiological levels of TPO can be detected, and therefore whether more sensitive methods are needed to measure TPO protein production by hMSCs. Similarly it is important to delineate whether TPO (if it is indeed produced at a low level) or other megakaryocytogenetic cytokines (IL-6, IL-11, and LIF) produced by hMSCs play a primary role in our system. Since cytokines such as IL-11 can be readily detected in cultured supernatants of hMSCs (0.5 to 2 ng/ml) and other stromal cell systems (Kawashima et al., 1991; Paul et al., 1991), and support

**TABLE 1. Numbers of hematopoietic progenitor cells after 7 days of coculture**

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<thead>
<tr>
<th>Assayed cell populations</th>
<th>No. of total cells (fold)</th>
<th>Progenitor frequencies</th>
<th>No. of progenitors (fold)</th>
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<tr>
<td></td>
<td></td>
<td>CFU-Mk (in 5,000 cells)</td>
<td>CFU-C (in 400 cells)</td>
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<tr>
<td>CD34⁺ cells before culture</td>
<td>(1×)</td>
<td>30.5 ± 4.5</td>
<td>56.5 ± 11.5</td>
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<tr>
<td>CD34⁺ cells cultured on hMSCs</td>
<td>0.57 ± 0.20×</td>
<td>47.5 ± 4.5</td>
<td>101 ± 0</td>
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<tr>
<td>CD34⁺ cells cultured on plastic</td>
<td>0.02 ± 0×</td>
<td>N/A²</td>
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1BM-derived hematopoietic cells before and after coculture with hMSCs were assayed for their CFU-Mk and CFU-C contents using StemCell kits. The relative numbers of input (progenitor and total) cells are defined as 1× (shown in parenthesis) for a simple calculation. Numbers of progenitors (fold) are calculated as the progenitor frequency after coculture divided by progenitor frequency before coculture, multiplied by the number of total cells (fold). Values of average ± SD (standard deviation) of two sets of duplicates in a representative experiment are shown here. Similar results were observed in another independent experiment. When cultured on plastic in serum-free (BIF) medium in the absence of exogenous cytokines and hMSCs, few cells survived after 7 days, preventing further analyses for progenitor cells.

2N/A, not applicable.

megakaryocytogenisis in culture (Bruno et al., 1991; Burstein et al., 1992), it is more likely to be a major cytokine in our experimental system. The hMSC-based coculture system we described here may aid in elucidation of these factors and mechanisms that support megakaryocytogenesis and platelet formation.

hMSCs have the capacity to maintain LTC-IC and expand lineage-specific colony-forming units from CD34⁺ marrow cells in long-term bone marrow culture (Majumdar et al., 1998). In addition, hMSCs can support B-cell lineage commitment from sorted bone marrow or cord blood CD34⁺/CD19⁻ cells (Cheng et al., 1998). Moreover, our data indicate that hMSCs, together with cytokines such as TPO, may be more efficient at expanding CD34⁺/CD41⁺ megakaryocyte progenitors from CD34⁺ cells than TPO alone or cytokine combinations such as SCF, FL, IL-3, IL-6, and TPO (Cheng et al., 1998; Liu et al., 1998). Therefore, it is plausible that an adherent hMSC culture could provide the appropriate milieu for efficient expansion of progenitors for megakaryocyte and other hematopoietic lineages for the transplant setting (Bertolini et al., 1997). It remains to be determined whether synergetic activities of hMSCs attributed to known cytokines (such as IL-11, which is known to have pleiotropic effects at multiple stages and lineages of hematopoiesis) and other undefined molecules produced by hMSCs, have such a role.

Prolonged thrombocytopenia following myeloablative therapy for cancer remains an unmet clinical challenge (Hassan and Zander, 1996). Allogeneic platelet transfusions are widely used clinically for treating thrombocytopenia; however, it is associated with additional costs and risks. Posttransplant injection of cytokines such as IL-6, IL-11, and TPO have been evaluated clinically for thrombocytopenia (Lazarus et al., 1995a; Tepler et al., 1996; Beveridge et al., 1997), and treatment with IL-11 offers clinical benefits to the recovery of patients' platelets and other affected cells/tissues (Du and Williams, 1997). Although TPO or its variant MGDF are effective in increasing platelet production in healthy donors and in reducing thrombocytopenia in myelosuppressed patients, they are less effective in reducing thrombocytopenia in patients undergoing myeloablative therapy (Vadhan-Raj et al.,
One possibility is that the TPO level in these patients may not be the rate-limiting factor for platelet recovery. This notion is consistent with the observations that serum TPO levels were significantly elevated (10-fold) in patients after chemotherapy (Araneda et al., 1998). Infusion of hMSCs, which produce several megakaryocyteopoietic cytokines and proved safe in a clinical trial (Lazarus et al., 1995b), together with transplantation of CD34\(^+\) hematopoietic progenitor cells, may provide a viable alternative to cytokine therapy and platelet transfusions for reducing thrombocytopenia in the transplant setting.

In conclusion, these data have provided evidence for the physical association between primary megakaryocytes and hMSCs. They have also demonstrated that, under defined culture conditions, hMSCs are sufficient to support megakaryocyte differentiation and platelet formation from CD34\(^+\) hematopoietic progenitor cells. This defined coculture system can now be exploited to elucidate the cytokine(s) and mechanism(s) by which megakaryocyte differentiation and platelet formation are regulated.

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**LITERATURE CITED**


