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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; Deryugina 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

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formation (Kaushansky, 1995). However, the production of normal megakaryocytes and platelets by knockout mice deficient in the gene encoding TPO suggests that other cytokines are also important and sufficient to support megakaryocytopoieisis 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., 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 homogenous (>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; Mbalaviele et al., 1999); cell adhesion molecules such as VCAM-1, E-selectin, and activated leukocyte-cell adhesion molecule (ALCAM); and ECM proteins such as collagen I and fibronectin (Haynesworth et al., 1992; Mosca et al., 1996; Bruder et al., 1997). In addition, these marrow-derived, culture-expanded hMSCs maintain LTC-IC in long-term bone marrow culture with $CD34^+$ hematopoietic progenitor cells and support both myeloid and erythroid differentiation (Majumdar et al., 1998).

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 900*g* 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 900*g* 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-cm^2 flasks (Nunc) at a density of 3×10^7 MNC/flask and the cultures were incubated at 37°C in 5% CO_2 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-cm^2 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-cm^2 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: CCTCCTTGGGGCCCTGCAGAGCCT and 3′ primer: GGGTGGAGCTGGACCACAGGG (amplified product = 606 bp); IL-2 5′ primer: ATGTACAGGATGCAACTCCTGTCTT and 3′ primer: GTCAGTGTTG AGATGATGCTTTGAC (amplified product = 458 bp); IL-6 5′ primer: GTAGCCGCCCCACACAGACAGCC; and 3′ primer: GCCATCTTTGGAAGGTTCAGG (amplified product = 628 bp); SCF 5′ primer: CCTCTCGTCAAAACTGAAGGG; and 3′ primer: AGGAGTAAAGAGCCTGGGTTC (amplified product = 346 bp); Flt3/Flk2 ligand (FL) 5′ primer: TGGAGCCCAACAACCTATCTC and 3′ primer: GGGCTGAAAGGCACATTTGGT (amplified product = 333 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 in 2 volumes of the CATCH buffer containing 13.8 mmol/L sodium citrate, 0.2% glucose, 1 mmol/L adenosine, 1 mmol/L theophyline, 10 mmol/L HEPES, and 0.5% BSA in 1× Hank's-buffered salt solution (Life Technologies), and were recovered by centrifugation at 1,300 rpm for 5 min. Pelleted MNCs were then resuspended in 0.5 ml of the CATCH buffer plus 0.15 U/ml apyrase (Sigma) and 1 U/ml hirudin (Sigma) at a final density of 20×10^6 cells/ml. Primary mouse monoclonal antibodies (0.2 μg/10^6 cells) or an equal volume of PBS were added to the cell suspensions and the mixtures were placed at 4°C 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 anti-mouse IgG1 (sheep) antibody conjugated to M-450 Dynabeads (Dynal, Lake Success, NY) (final concentration of $1–2 \times 10^7$ 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% CO_2 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.

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 Biotec, Ausburn, 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 $CD34^+$.

Megakaryocytopoiesis assay

Cocultures were established in six-well tissue-culture plates with equal numbers ($1–2 \times 10^5$) 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, final concentration) 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 cm^2/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% CO_2 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, nonadherent 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 Ca^{++} or Mg^{++}), containing 0.5 mmol/L EDTA for 10 min. Most hMSCs were aggregated and were removed by a 30 μm nylon filter. Adherent and nonadherent cells were pooled and recovered by centrifugation. There was no significant loss of primitive $CD34^+$ cells during this procedure, since we obtained similar numbers of $CD34^+$ cells with or without the filtering when harvested cells were stained with a CD34 antibody and analyzed by FACS. The FACS analyses also indicated that we eliminated most of the hMSCs (displaying higher levels of forward and side scatter) by the filtering procedure. Cell aliquots (10 μl) were mixed with 10 μl of trypan blue solution (Sigma) and viable (smaller hematopoietic) cells were counted using a hemacytometer. Cell aliquots were assayed for CFU-Mk and CFU-C (Hogge et al., 1997) using assay kits purchased from StemCell Technologies, in addition to being analyzed by FACS to measure the presence of CD34 and CD41 markers.

Immunofluorescence

Cocultures used for immunofluorescence analysis were established in four-well chamber slides (Lab-Tek Nalgene, Naperville, IL) using $1–2 \times 10^3$ $CD34^+$ cells and 2×10^3 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 Immu-mount (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), equipped with a 15 mW Argon-ion laser, was used for these analyses. Selected events (1×10^4) 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).

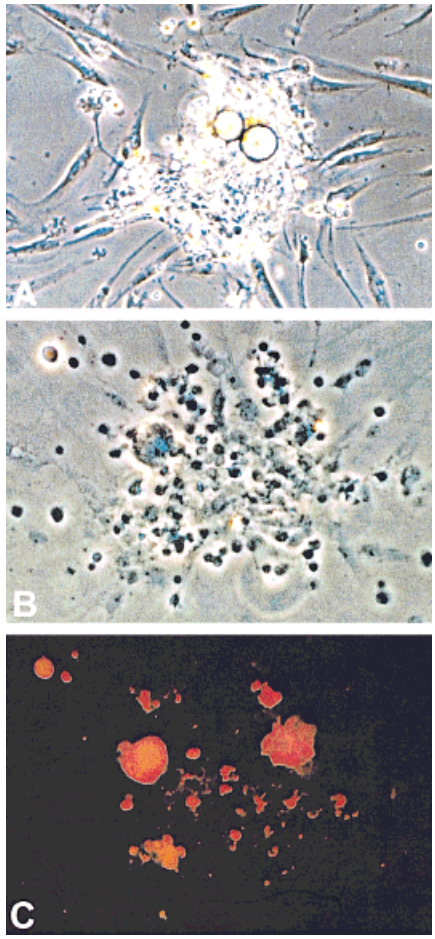

Fig. 1. Photomicrographs of $CD41^+$ megakaryocyte clusters present in hMSC primary cultures. Low-density mononuclear cells isolated from human bone marrow by Percoll gradient fractionation were plated onto plastic in a selective medium for optimal growth of hMSCs. **A:** A phase contrast image of a representative non-hMSC cell complex in primary culture of hMSCs (adherent fibroblastic cell) after 3 days of culture. **B:** A phase contrast image of another such complex. **C:** Anti-CD41-PE immunofluorescence staining of the complex seen in (B). Due to the loss of cells during the many manipulations used in staining shown in (B) and (C), these images underrepresent the total cellularity of the starting coculture.

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 density 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 (gpIIb/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

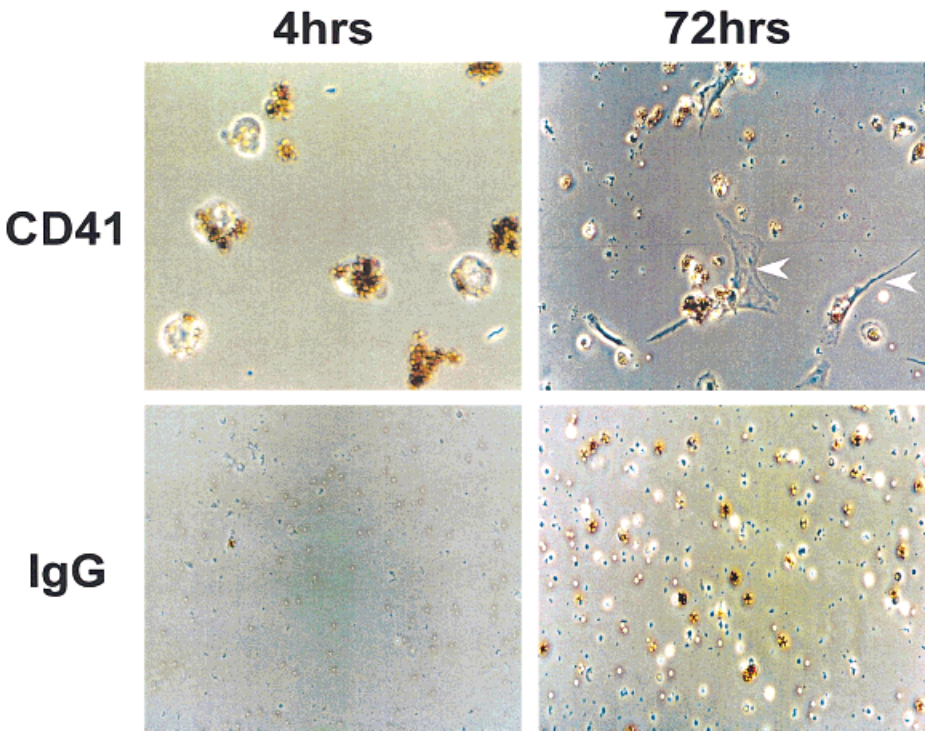

Fig. 2. Copurification of hMSCs using anti-CD41 monoclonal antibody. Percoll-fractionated mononuclear cells were incubated with either anti-CD41 (upper panels) or murine IgG1 (lower panels) monoclonal antibodies. After being washed, cells were incubated with paramagnetic beads conjugated with an antimurine IgG antibody. After collection with a magnet, selected cells were plated directly onto culture plastic in hMSC medium (without removing the paramagnetic beads). hMSC cultures were examined after 4 and 72 h. After 72 h, adherent fibroblastic cells (arrowheads) are seen in cultures of the CD41-selected (upper right) cells but not those selected with murine IgG (lower right).

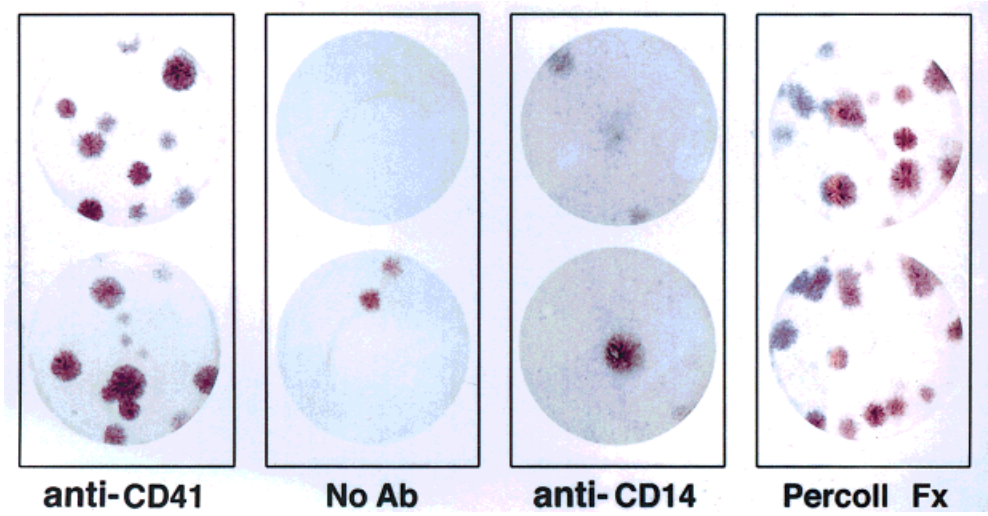

Fig. 3. MSC colony formation from marrow cells selected by anti-CD41 antibody. Percoll-fractionated marrow cells were selected by an anti-CD41 antibody and immunomagnetic beads as in Fig. 2. Selected cells were cultured in hMSC medium for 14 days and then stained with crystal violet. Images of duplicated culture wells are shown for each of four groups. Immunoselection was performed on 1×10^6 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 (1×10^5) 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).

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

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 megakaryocytopoiesis 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).

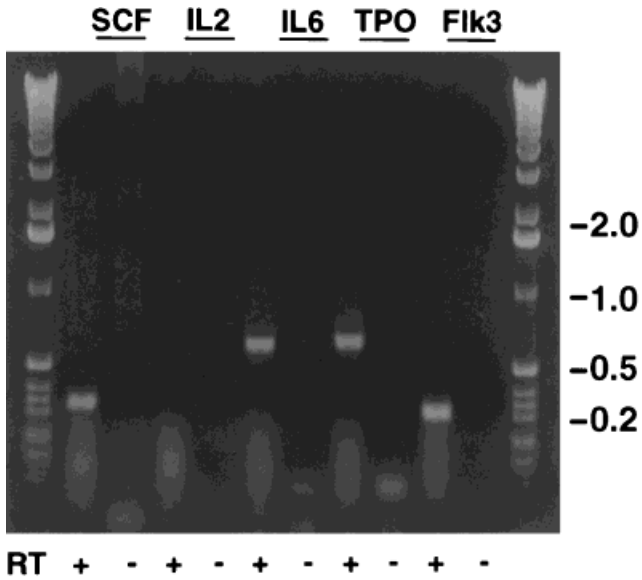

Fig. 4. A TPO transcript is expressed in hMSCs. RT-PCR was performed on total RNA isolated from culture-expanded hMSCs (see Materials and Methods for specific primer pairs). PCR was performed with (+) and without (−) the addition of reverse transcriptase (RT) and the amplified products were analyzed on 1% agarose gel containing ethidium bromide. Each target mRNA (SCF, IL-2, IL-6, TPO, and FL) is indicated at the top of the figure. Size markers (in kilobases) are indicated on the right side of the gel.

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$^+$ premature 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$^+$

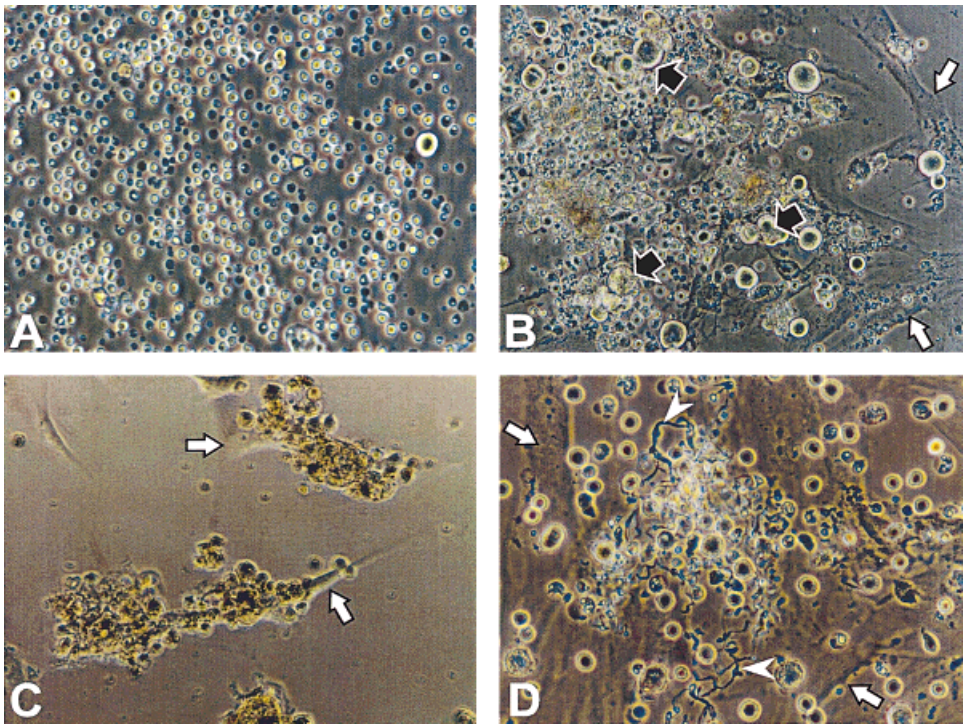

Fig. 5. Megakaryocytopoiesis and pro-platelet formation in coculture of hMSCs and $CD34^+$ cells. **A:** $CD34^+$ cells alone in the absence of hMSCs. **B–D:** Selected fields in coculture of $CD34^+$ cells on hMSCs. **C:** Arrows point out dense cell clusters formed in the $CD34^+$ cell/hMSC coculture. **D:** Pro-platelets with extended cytoplasmic processes (arrowheads) are observed at high magnification.

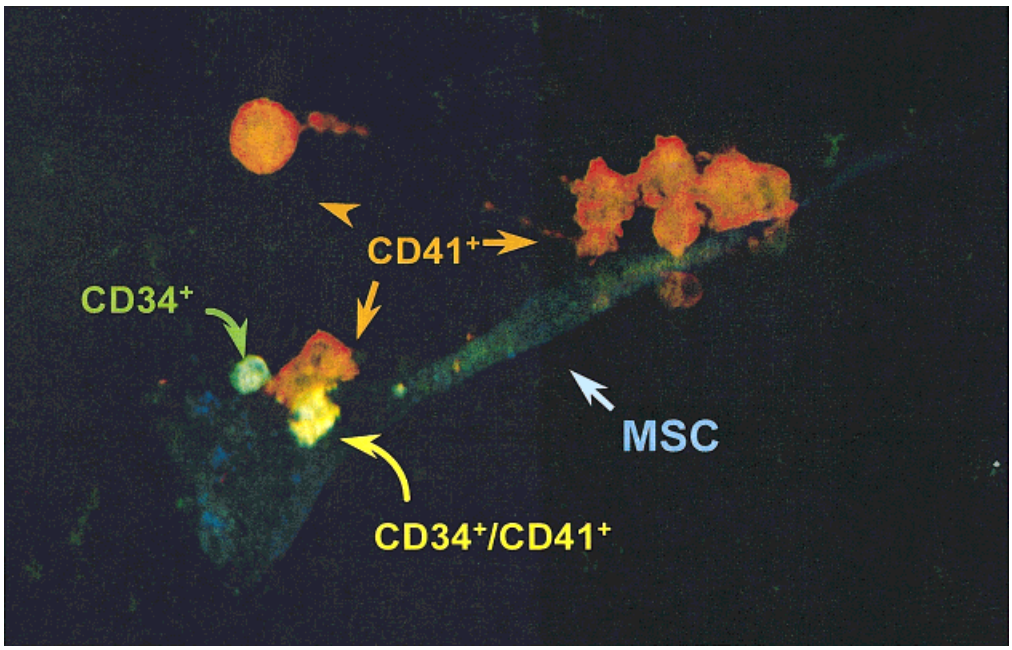

Fig. 7. Immunofluorescence analysis of hMSC supported megakaryocytopoiesis. Adherent cells in cocultures of hMSCs and $CD34^+$ cells were fixed and incubated with SH-3 (biotin-conjugated), anti-CD41 (R-PE–conjugated), and anti-CD34 (FITC-conjugated) monoclonal antibodies that recognize hMSCs, megakaryocytes/platelets, and $CD34^+$ cells, respectively. Streptavidin-conjugated cascade blue was used as the fluorochrome for SH-3. Therefore, hMSCs appear blue, $CD34^+$ cells green, and $CD41^+$ cells red. The double-positive $CD34^+/41^+$ cells, which are labeled by both R-PE and FITC, appear yellow in color.

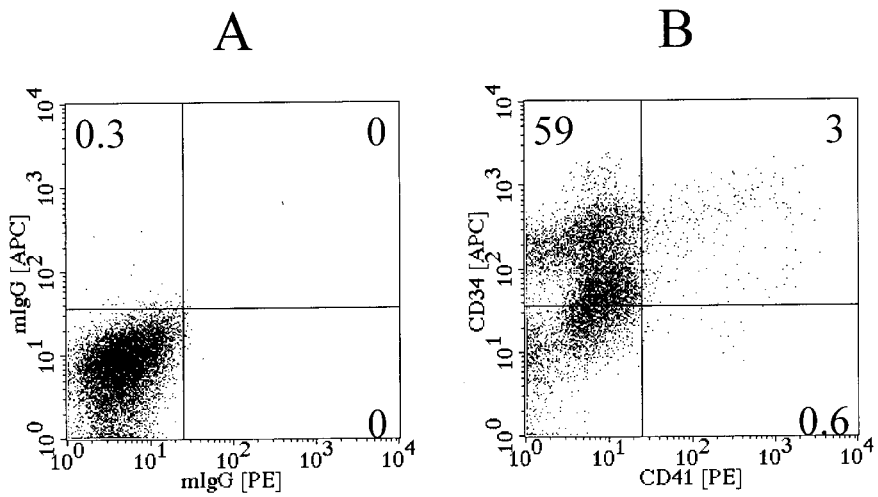

Fig. 6. $CD41^+$ megakaryocyte differentiation from $CD34^+$ cells cocultured with hMSCs. Hematopoietic cells harvested 7 days after $CD34^+$ cells were plated onto hMSCs in the absence of exogenous cytokines. Cells were stained with anti-CD41 and anti-CD34 antibodies (**B**) or isotype control antibodies (**A**), and analyzed by flow cytometry. Nonviable cells and hMSCs were excluded from dot plots. The data are from a representative experiment. Similar results were obtained in at least two other experiments.

(red) cells, consistent with their developmental stages in megakaryocytopoiesis.

The presence of $CD34^+/CD41^-$ cells and $CD34^+/CD41^+$ 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 $CD34^+$ 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, which is associated with platelet activation. After 14 days of coculture on hMSCs, numerous pro-platelets were observed. Platelet-sized particles from these culture supernatants were treated with thrombin to activate 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. The percentage of $CD41^+/CD62P^+$ platelets increased significantly upon activation with thrombin (Fig. 8, bottom right panel). A small population of platelet-like particles in culture supernatants expressed CD41, and the majority of these $CD41^+$ platelets also expressed the CD62P marker on the surface upon activation with thrombin (Fig. 8, top right panel). In addition, 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. This was also true for blood-derived platelets processed under the same conditions. 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

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

Assayed cell populations	No. of total cells (fold)	Progenitor frequencies		No. of progenitors (fold)	
		CFU-Mk (in 5,000 cells)	CFU-C (in 400 cells)	CFU-Mk	CFU-C
$CD34^+$ cells before culture	(1×)	30.5 ± 4.5	56.5 ± 11.5	(1×)	(1×)
$CD34^+$ cells cultured on hMSCs	0.57 ± 0.20×	47.5 ± 4.5	101 ± 0	1.0×	0.9×
$CD34^+$ cells cultured on plastic	0.02 ± 0×	N/A[2]	N/A	N/A	N/A

[1]BM-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 (BIT) medium in the absence of exogenous cytokines and hMSCs, few cells survived after 7 days, preventing further analyses for progenitor cells.
[2]N/A, not applicable.

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; Guerriero 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, Guerriero 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 megakaryocytopoiesis and platelet formation, which can be further enhanced by TPO. The first hypothesis is based on the notion that TPO is absolutely essential to megakaryocytopoiesis 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 megakaryocytopoietic 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 megakaryocytopoiesis 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 megakaryocytopoiesis 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 synergistic 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.,

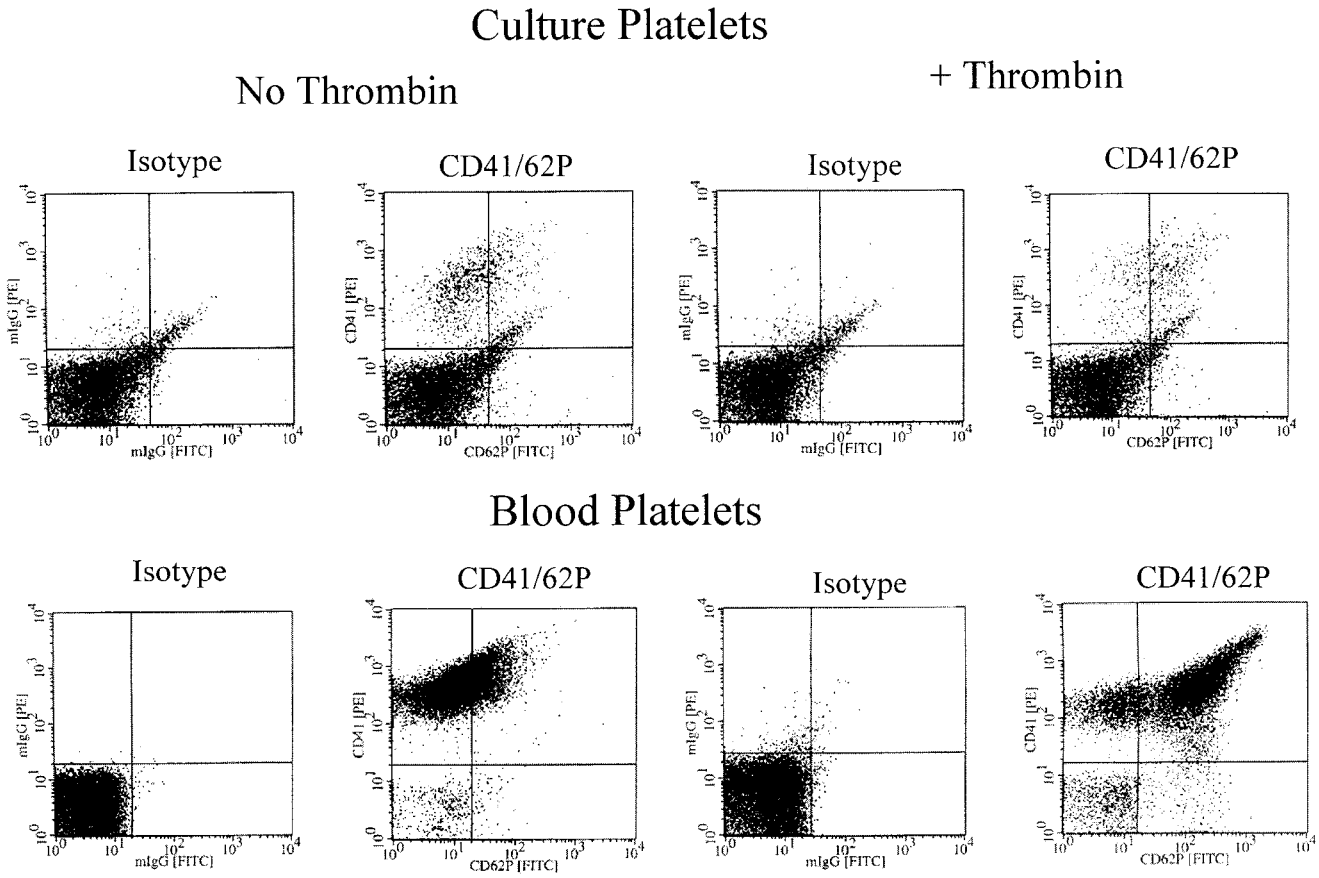

Fig. 8. Expression of CD62P and CD41 markers by platelets derived from coculture. $CD34^+$ cells and hMSCs were cocultured for 14 days. Activation of platelets was performed by incubating with 2 U/ml of thrombin for 10 min at 37°C. Platelets released in culture media and the remaining cell fraction were collected seperately. Mature platelets in peripheral blood were also collected as a control and similarly activated with thrombin. The treated platelets were then stained with PE-conjugated anti-CD41 and FITC-conjugated anti-CD62P antibodies. FACS analyses were performed by setting the side and forward scatter gates for platelet-like small particles using blood platelets as a standard. Culture-derived platelets ± thrombin are shown in the top panel with matching isotype controls. Blood platelets are shown in the bottom panel. Similar results were obtained in one other experiment, although the percentage of platelets obtained in culture varied.

1997; Schiffer et al., 1998). 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 megakaryocytopoietic 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

Araneda M, Krishnan V, Hall K, Krishnan K. 1998. Serum thrombopoietin (TPO) levels in disorders of thrombocytopenia: implications for future use of recombinant TPO in clinical practice. Blood 92(Suppl):31a.

Avraham H, Vannier E, Cowley S, Jiang S, Chi S, Dinarello CA, Zsebo KM, Groopman JE. 1992. Effects of stem cell factor, c-kit ligand, on human megakaryocytic cells. Blood 79:365–371.

Bertolini F, Battaglia M, Pedrazzoli P, Da Prada GA, Lanza A, Soligo D, Caneva L, Sarina B, Murphy S, Thomas T, Robustelli della Cuna G. 1997. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. Blood 89:2679–2688.

Beveridge R, Schuster M, Waller E, Stuart R, Abboud C, Cruickshank S, Menchaca D, Macri M, Connaghan DG. 1997. Randomized, double-blind, placebo-controlled trial of PEGylated recombinant human megakaryocyte growth and differentiation factor in breast cancer patients following autologous bone marrow transplantation. Blood 90(Suppl):580a.

Bruder SP, Horowitz MC, Mosca JD, Haynesworth SE. 1997. Monoclonal antibodies reactive with human osteogenic cell surface antigens. Bone 21:225–235.

Bruno E, Hoffman R. 1989. Effects of interleukin-6 on in vitro megakaryocytopoiesis: its interaction with other cytokines. Exp Hematol 17:1038–1043.

Bruno E, Miller ME, Hoffman R. 1989. Interacting cytokines regulate in vitro human megakaryocytopoiesis. Blood 76:671–677.

Bruno E, Briddell RA, Cooper RJ, Hoffman R. 1991. Effects of recombinant interleukin-11 on human megakaryocyte progenitor cells. Exp Hematol 19:378–381.

Bunting S, Widmer R, Lipari T, Rangell L, Steinmetz H, Carver-Moore K, Moore M, Keller G-A, de Sauvage FJ. 1997. Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. Blood 90:3423–3429.

Burstein SA, Me R-L, Henthorn J, Friese P, Turner K. 1992. Leukemia inhibiting factor and interleukin-11 promote maturation of murine and human megakaryocytes in vitro. J Cell Physiol 153: 305–312.

Caplan AI. 1991. Mesenchymal stem cells. J Ortho Res 9:641–650.

Cheng L, Mbalaviele G, Liu X, Novelli E, Vanguri P, Mosca J, Deans R, Civin CI, Thiede MA. 1998. Human mesenchymal stem cells support proliferation and multilineage differentiation of human hematopoietic stem cells. Blood 92(Suppl):57a (abstract 228).

Chirgwin JM, Pryzbyla AE, MacDonald RJ, Rutter WJ. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.

Choi E, Nichol J, Hokom M, Hornkohl A, Hunt P. 1995. Platelets generated in vitro from proplatelets displaying human megakaryocytes are functional. Blood 85:402–413.

Deryugina EI, Muller-Sieburg CE. 1993. Stromal cells in long-term cultures: keys to elucidation of hematopoietic development? Crit Rev Immunol 13:115–150.

Dorshkind K. 1990. Regulation of hematopoiesis by bone marrow stromal cells and their products. Annu Rev Immunol 8:111–137.

Du X, Williams DA. 1997. Interleukin-11: review of molecular, cell biology, and clinical use. Blood 89:3897–3908.

Eaves CJ, Cashman JD, Kay RJ, Dougherty GJ, Otsuka T, Gaboury LA, Hogge DE, Landsorp PM, Eaves AC, Humphries RK. 1991. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long term human bone marrow cultures. II: Analysis of positive and negative regulators produced by stromal cells within the adherent layer. Blood 78:110–117.

Ellis MH, Avraham H, Groopman JE. 1995. The regulation of megakaryocytopoiesis. Blood 9:1–6.

Gewirtz AM. 1995. Megakaryocytopoiesis: the state of the art. Thromb Haemost 74:204–209.

Guerriero A, Worford L, Holland HK, Guo GR, Sheehan K, Waller EK. 1997. Thrombopoietin is synthesized by bone marrow stromal cells. Blood 90:3444–3455.

Han ZC, Belucci S, Wan HY, Caen JP. 1992. New insights into the regulation of megakaryocytopoiesis by hematopoietic and fibroblast growth factors and transforming growth factor beta-1. Br J Haematol 81:1–5.

Hassan HT, Zander A. 1996. Thrombocytopenia after high-dose chemotherapy and autologous stem cell transplantation: an unresolved problem and possible approaches to resolve it. J Hematother 5:407–414.

Haynesworth SE, Baber MA, Caplan AI. 1992. Cell surface antigens on human marrow derived mesenchymal cells are detected by monoclonal antibodies. Bone 13:69–80.

Haynesworth SE, Baber MA, Caplan AI. 1996. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL1α. J Cell Physiol 166:585–592.

Hoffman R. 1989. Regulation of megakaryocytopoiesis. Blood 74:1196–1212.

Hogge H, Fanning SF, Bockhold K, Petzer A, Lambie K, Eaves A, Eaves CJ. 1997. Quantitation and characterization of human megakaryocyte colony forming cells using a standardized serum-free assay. Br J Haematol 96:790–800.

Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M. 1987. Interleukin-6 enhancement of interleukin-3 dependent proliferation of multipotential hematopoietic progenitors. Proc Natl Acad Sci USA 84:9035–9039.

Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. 1997. Osteogenic differentiation of purified culture expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312.

Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. 1998. In vitro chondrogenesis of bone marrow derived mesenchymal progenitor cells. Exp Cell Res 238:265–272.

Kaushansky K. 1995. Thrombopoietin: the primary regulator of platelet production. Blood 86:419–431.

Kaushansky K, O'Hara RJ, Berkner K, Segal GM, Hagen FS, Adamson JW. 1986. Genomic cloning, characterization and multilineage growth promoting activity of human granulocyte-macrophage colony stimulating factor. Proc Natl Acad Sci USA 83:3101–3105.

Kawashima I, Ohsumi J, Mita-Honjo K, Shimoda-Takano K, Ishikawa H, Sakakibara S, Miyadai K, Takiguchi Y. 1991. Molecular cloning of cDNA encoding adipogenesis inhibitory factor and identity with interleukin-11. FEBS Lett 283(2):199–202.

Keller JR, Bartelmez SH, Sitnicka E, Ruscetti FW, Ortiz M, Gooja JM, Jacobson SEW. 1994. Distinct and overlapping direct effects of macrophage inflammatory protein-1α and transforming growth factor-β on hematopoietic progenitors/stem cells growth. Blood 84: 2175–2181.

Lazarus HM, Winton EF, Williams SF, Grinblatt D, Campion M, Cooper BW, Gunn H, Manfreda S, Isaacs RE. 1995a. Phase I multicenter trial of interleukin 6 therapy after autologous bone marrow transplantation in advanced breast cancer. Bone Marrow Transplant 15:935–942.

Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS and Caplan AI. 1995b. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications in therapeutic use. Bone Marrow Transplant 16:557–564.

Liu X, Rapp N, Cheng L. 1998. Human mesenchymal stem cells enhance *ex vivo* expansion of human megakaryocyte, erythroid and myeloid progenitors from purified cord blood $CD34^+$ cells. Blood 92(Suppl):725a (abstract 2977).

Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. 1998. Phenotype and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 17:57–66.

Mbalaviele G, Jaiswal N, Meng A, Cheng L, van den Bos C, Thiede M. 1999. Human mesenchymal stem cells promote human osteoclast differentiation from CD34+ bone marrow hematopoietic progenitors. Endocrinology 140(8):3736–3743.

Mosca JD, Majumdar MK, Hardy WB, Pittenger MF, Thiede MA. 1996. Initial characterization of the phenotype of the human mesenchymal stem cells and their interaction with cells of the hematopoietic lineage. Blood 88(Suppl):186a.

Mossuz P, Schweitzer A, Molla A, Berthier R. 1997. Expression and function of receptors for extracellular cellular matrix molecules in the differentiation of human megakaryocytes in vitro. Br J Haematol 98:819–827.

Norol F, Vitrat N, Cramer E, Guichard J, Burstein SA, Vainchenker W, Debili N. 1998. Effects of cytokines on platelet production from blood and marrow $CD34^+$ cells. Blood 91:830–843.

Paul SR, Yang YC, Donahue RE, Goldring S, Williams DA. 1991. Stromal cell-associated hematopoiesis: immortalization and characterization of a primate bone marrow-derived stromal cell line. Blood 77(8):1723–1733.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, and Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Prockop DJ. 1997. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74.

Qian S, Fu F, Li W, Chen Q, de Sauvage FJ. 1998. Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. Blood 91:2189–2191.

Schiffer CA, Miller K, Larson RA, Stone R, Amrein P, Antin JH, Zani VJ. 1998. A double blind, placebo controlled trial evaluating PEGylated recombinant human megakaryocyte growth and differentiation factor (MGDF) as an adjunct to induction and consolidation therapy in patients with acute myeloid leukemia (AML). Blood 92(Suppl):313a.

Szilvassy SJ, Hoffman R. 1995. Enriched hematopoietic stem cells: basic biology and utility. Biol Blood Transplant 1:3–17.

Tepler, I, Elias, L, Smith JW III, Hussein M, Rosen G, Chang AY-C, Moore JO, Gordon MS, Kuca B, Beach KJ, Loewy JW, Garnick MB, Kaye JA. 1996. A randomized placebo-controlled trial of recombinant human interleukin 11 in cancer patients with severe thrombocytopenia due to chemotherapy. Blood 87:3607–3614.

Vadhan-Raj S, Verschraegen C, McGarry L, Bueso-Ramos C, Kudelka A, Freedman R, Edwards C, Bevers M, Levenback C, Gershenson D, Jones D, Yang T, Bast RC, Kavanagh J. 1997. Recombinant human thrombopoietin attenuates high-dose carboplatin-induced thrombocytopenia in patients with gynecologic malignancy. Blood 90(Suppl):580a.

Wakatani S, Saito T, Caplan AI. 1995. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426.

Williams JL, Pipia GG, Datta NS, Long MW. 1998. Thrombopoietin requires additional megakaryocytic-active cytokines for optimal ex vivo expansion of megakaryocyte precursor cells. Blood 91:4118–4126.

Young RG, Butler DL, Weber W, Caplan AI, Gordon, SL, Fink DJ. 1998. The use of mesenchymal stem cells in a collagen matrix for achilles tendon repair. J Orthop Res 16:406–413.