Stromal Cell-Associated Hematopoiesis: Immortalization and Characterization of a Primate Bone Marrow-Derived Stromal Cell Line

By S.R. Paul, Y-C. Yang, R.E. Donahue, S. Goldring, and D.A. Williams

An elucidation of the interaction between the bone marrow microenvironment and hematopoietic stem cells is critical to the understanding of the molecular basis of stem cell self renewal and differentiation. This interaction is dependent, at least in part, on direct cell to cell contact or cellular adhesion to extracellular matrix proteins. Long-term bone marrow cultures (LTMC) provide an appropriate microenvironment for maintenance of primitive hematopoietic stem cells and a means of analyzing this stem cell-stromal cell interaction in vitro. Although LTMC have been successfully generated from murine and human bone marrow, only limited success has been reported in a primate system. In addition, few permanent stromal cell lines are available from nonmurine bone marrow. Because the primate has become a useful model for large animal bone marrow transplant studies and, more specifically, retrovirally-mediated gene transfer analysis, we have generated immortalized bone marrow stromal cell lines from primate bone marrow using gene transfer of the Simian virus large T (SV40 LT) antigen. At least one stromal cell line has demonstrated the capacity to maintain early hematopoietic stem cells in long-term cultures for up to 4 weeks as measured by in vitro progenitor assays. Studies were undertaken to characterize the products of extracellular matrix biosynthesis and growth factor synthesis of this cell line, designated PU-34. In contrast to most murine bone marrow-derived stromal cell lines capable of supporting hematopoiesis in vitro that have been examined, the extracellular matrix produced by this primate cell line includes collagen types I, III, and V, and fibronectin rather than collagen type IV and laminin. Growth factor production analyzed through RNA blot analysis, bone marrow cell culture data, and factor-dependent cell line proliferation assays includes interleukin-6 (IL-6), IL-7, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, M-CSF, leukemia inhibitory factor, and a novel cytokine designated IL-11. This immortalized primate bone marrow stromal cell line may be useful in maintaining early progenitor cells for experimental manipulation without the loss of reconstituting capacity and as a potential source of novel hematopoietic growth factors.

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However, few stromal cell lines from human bone marrow have been characterized\textsuperscript{35,36} and none are available from primates. Such stromal cell lines could provide a unique resource for the isolation, characterization, and cloning of cytokines, which may affect hematopoietic cells.\textsuperscript{30} For example, interleukin-7 (IL-7) was identified from a murine stromal cell line isolated from long-term B-lymphoid cultures.\textsuperscript{30}

Work in our laboratory and by other investigators has shown that retrovirus vectors containing oncogenes can immortalize primary cells from several tissues of adult animals and humans.\textsuperscript{31-33} We hypothesized that an in vitro culture system could be established using immortalized cloned primate bone marrow stromal cells generated using such recombinant retrovirus vectors as previously described with murine bone marrow.\textsuperscript{32} We report here the immortalization of multiple primate bone marrow stromal cell lines using a recombinant retrovirus vector containing the SV40 LT antigen and neo phosphotransferase (Neo) gene. One such line, PU-34, was capable of maintaining primitive hematopoietic progenitor cells in long-term culture. This cell line was further characterized to establish the features responsible for this effect.

**MATERIALS AND METHODS**

*Generation of primate stromal cell lines.* Details of the recombinant retroviral vector, U19, have been previously reported.\textsuperscript{19} Briefly, the SV40 early region viral sequences from the Bgl I site to the Hpa I site were inserted into the BamHI site of the pZip Neo SV(X) 1 vector. An amphotropic producer line, U19BL, was generated by infection of \( \Phi \) am cells\textsuperscript{37} with ecotropic viral harvest from the U19-5 cell line\textsuperscript{19} and produces recombinant amphotropic SV40 large T virus at a titer of \( 5 \times 10^{7} \) G418 resistant CFU/mL when assayed on NIH/3T3 cells. The producer line was maintained in Dulbecco's modified Eagle medium (DME) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin (all from GIBCO, Grand Island, NY).

Adult macaques were anesthetized with 0.5 mL Ketamine HCl (Parke-Davis, Morris Plains, NJ) given intramuscularly and placed in a ventral recumbent position. The posterior superior iliac crest was identified, the hair was shaved, and the site prepared surgically with betadine solution and 100% ethanol. An 18-gauge disposable Jamshidi bone marrow needle (Baxter Care Corp, Valencia, CA) was inserted and 5 mL of bone marrow aspirated. These cells were subjected to Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation at 1,500 rpm for 30 minutes. LTMC were established from the mononuclear cells in Iscove modified Dulbecco medium (IMDM) with 10% horse serum, 10% FCS, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin (all from GIBCO) complete long-term culture media). The cultures were fed weekly by removal of 75% of medium and cells followed by addition of fresh medium. Cultures were maintained at 33°C and 5% CO\textsubscript{2}.

Spermantans containing the U19 virus were harvested from confluent plates of U19BL producer cells 18 hours after the addition of fresh medium, filtered through 0.45-\( \mu \)m filters (Nal-gene, Rochester, NY) and frozen at \(-70^\circ \)C until use. Adherent cells of primate LTMC were infected 7 and 10 days after establishment with 2 mL of viral stock in the presence of 8 \( \mu \)g/mL of polybrene (Aldrich Chemical Co, Inc, Milwaukee, WI) at 33°C for 2.5 hours. Subsequently, the cultures were fed in the usual manner. Simultaneous titer of the same viral stock was determined on NIH/3T3 as previously described.\textsuperscript{19} Beginning 14 days after establishment of long-term cultures, G418-resistant stromal cells were selected by addition of 0.5 mg/mL of G418 (dry powder) (GIBCO). Well-isolated G418-resistant colonies were picked on day 21 and expanded into multiwell plates in complete long-term culture medium. Once established, the cell lines were maintained at 37°C. One such cell line was extensively analyzed and has been designated PU-34.

*Analysis of support of hematopoiesis by stromal cell lines.* Confluent PU-34 cells were incubated with 10 \( \mu \)g/mL mitomycin C (Boehringer-Mannheim Co, Mannheim, Germany) for 2 hours at 37°C and 5% CO\textsubscript{2}. Subsequently, cells were trypsinized, washed extensively, and replated on 0.1% gelatin coated 25-cm\textsuperscript{2} tissue culture flasks (Corning Glass Works, Corning, NY) at a concentration of \( 1 \times 10^{6} \) cells/flask. Primate bone marrow mononuclear cells were depleted of adherent cells by two passages on fresh tissue culture flasks in 24 hours. These low-density bone marrow cells depleted of plastic-adherent cells were charged onto confluent mitomycin C-treated PU-34 cells. Nonadherent cells were removed at weekly intervals and assayed in semisolid medium for the presence of progenitor cells. Cultures were plated with \( 10^{5} \) nonadherent cells/mL in IMDM, 0.9% methocelullose, 1% bovine serum albumin (BSA), \( 10^{-4} \) mol/L \( \beta \)-mercaptoethanol, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin. Methylcellulose cultures were supplemented with 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 2 U/mL erythropoietin (supplied by Genetics Institute, Cambridge, MA). Cultures were plated in 1 mL volumes in 35-mm tissue culture plates in 5% CO\textsubscript{2}, 37°C and colonies were counted on day 12. Support of progenitors was calculated based on the number of CFU per \( 10^{5} \) cells and the total number of recovered nonadherent cells per flask at each weekly feeding. These data do not reflect adherent CFU present in or on the stromal cells.

*Protein analysis.* Extracellular matrix components (ECM) and intermediate filament proteins were analyzed by two methods: immunofluorescence microscopy and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). PU-34 cells were grown to confluence in Labtek chambers (Nunc, Inc, Naperville, IL) and fixed in 3.7% formaldehyde for 4 minutes followed by 100% methanol for 1 minute. Cells were rinsed once each with water and phosphate-buffered saline (PBS), then 0.5 mL of the primary antibody was added and incubated for 1 hour at 37°C. The primary antibodies consisted of 1:50 dilution of Pab 419 (SV40 LT) (kindly provided by Dr Ed Harlow, Cold Spring Harbor Laboratory, NY); 1:100 dilution of anti-fibronectin; 1:50 dilution of anti-laminin; a 1:100 dilution of anti-desmin, anti-vimentin, and Pab 419 primary antibodies. Omission of the relevant first antibody with the secondary antibody for anti-fibronectin, anti-desmin, and anti-laminin; a 1:100 dilution of anti-type IV collagen (Collaborative Research, Bedford, MA); a 1:5 dilution of anti-vimentin; a 1:3 dilution of anti-neurofilament; a 1:50 dilution of anti-cytokeratin; a 1:5 dilution of anti-desmin (all Boehringer Mannheim); and a 1:5 dilution of anti-Factor VIII-associated antigen (Dako, Santa Barbara, CA). After 1 hour of incubation, the primary antibody was aspirated, the cells were washed thrice with PBS, and 0.5 mL of the appropriate rhodamine-conjugated secondary antibody was added and incubated with the cells for 1 hour at 37°C. Goat anti-rabbit IgG (Tago, Burlingame, CA; 1:100 dilution) was used as a secondary antibody for anti-fibronectin, anti-factor VIII-associated antigen, anti-laminin, anti-neurofilament, and anti-type IV collagen. Goat antispecies IgG (Tago; 1:100 dilution) was used as the secondary antibody for anti-desmin, anti-vimentin, and Pab 419 primary antibodies. Omission of the relevant first antibody with inclusion of 5% FCS served as controls for nonspecific staining. After incubation with the secondary antibody, slides were washed twice with PBS, once with water, then two drops of gelvatol added and cover slips were placed on each slide. Immunofluorescent-stained cells were viewed with a Zeiss fluorescence microscope using a 546-nm excitor filter with a 55-nm band path, FT 580 mirror, and 590-nm barrier filter.

SDS-PAGE analysis of metabolically labeled proteins was per-
formed as previously described. Briefly, PU-34 cells were plated at a density of 2 × 10^6 cells/dish in 3.5-cm tissue culture dishes. After 2 days, cells were incubated for 24 hours at 37°C in 5% CO₂ in IMDM without serum with 50 pg/mL P-aminopropionitrile, 50 pg/mL ascorbic acid, and 2 mmol/L glutamine (all Sigma Chemical Co, St. Louis, MO) with 25 μCi of L-[5-3H]-tryptophan (30 mCi/mmol; Amersham-Searle, Corp, Arlington Heights, IL). Labeled medium proteins were collected at 24 hours and analyzed by SDS-PAGE (5% acrylamide) with delayed reduction to distinguish alpha 1(I) from alpha 1(III) collagen chains. Fluorograms of the gels were prepared as described elsewhere.

Growth factor analysis. Poly A+ RNA was prepared by standard methods using the guanidinium isothiocyanate procedure from uninduced PU-34 cells and from PU-34 cells that had been induced for 24 hours with 2 μM recombinant human (rh) IL-1α (Cistron, Pinebrook, NJ). RNA samples were fractionated on a 2.2 mol/L formaldehyde-1% agarose gel, transferred to nylon filters (Gene Screen, New England Nuclear Research Products, Boston, MA), and hybridized with 32P-labeled cDNA probes for GM-CSF, G-CSF, IL-2, IL-3, IL-6, IL-9, and leukemia inhibitory factor (LIF) (DNAs supplied by Genetics Institute, Cambridge, MA). Hybridization, hybridization and posthybridization washes of filters were performed as recommended by the manufacturer. Filters were exposed to X-ray film in the presence of a calcium tungstate intensifying screen at -70°C.

Conditioned media (CM) from uninduced or induced PU-34 (IL-α for 48 hours) were tested for cytokine activity on a series of factor-dependent cell lines through analysis of tritiated thymidine incorporation. Factor dependent cell lines included the human megakaryoblastic leukemia cell line, Mo7E; a human erythroleukaemia cell line, TF1; two murine IL-3-dependent cell lines, DA2 and 32D; and a murine plasmacytoma cell line, T1165. The assays and culture conditions of these lines have been described in the individual references. Briefly, the Mo7E cell line is a human megakaryoblastic leukemia line dependent on IL-3, IL-4, IL-9, and GM-CSF. Mo7E cells were maintained in DME with 10% FCS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 200 μmol/L glutamine. The cells were resuspended in 96-well plates to a final concentration of 5 × 10^3 cells/well. Samples of PU-34 CM were added in the presence or absence of neutralizing antibody to human GM-CSF supplied by Genetics Institute, Boston, MA. Plates were wrapped in parafilm and incubated for 3 days. The wells were then pulsed with 0.5 μCi of tritiated thymidine (DuPont). After 4 hours of incubation the cells were harvested and counted.

TF-1 cells, a human erythroleukemia line dependent on IL-3, IL-4, IL-5, IL-6, GM-CSF, and LIF, were maintained in RPMI supplemented with 5% FCS, 100 U/mL penicillin, 100 U/mL streptomycin, and 200 μmol/L glutamine. Samples of PU-34 CM were added into 96-well plates containing 5 × 10^4 TF-1 cells/well in the presence or absence of neutralizing antibody to human GM-CSF or human IL-6 (both supplied by Genetics Institute). Plates were wrapped in parafilm and incubated for 3 days. The wells were then pulsed with 0.5 μCi of tritiated thymidine. After 4 hours of incubation, the cells were harvested and counted.

T1165 cells, a murine plasmacytoma line dependent on murine IL-1, IL-6, murine GM-CSF, and tumor necrosis factor (TNF), were maintained in RPMI supplemented with 10% FCS, 2 mmol/L glutamine, 100 mg/mL penicillin, 100 μg/mL streptomycin, 5 × 10^−4 mol/L β-mercaptoethanol and rh IL-6. T1165 cells were plated at 5 × 10^3 cells/well in 96-well tissue culture plates with samples of PU-34 (CM) in the presence or absence of antibody to human IL-6 (supplied by Genetics Institute). Cells were incubated for 2 days, then pulsed for 4 hours with tritiated thymidine. Labeled cells were harvested and counted.

Human bone marrow cells were obtained from normal donors in accordance with the guidelines of the Human Investigation Committee of the Children's Hospital, Boston, MA. Human bone marrow progenitor assays were performed with mononuclear cells isolated using Ficoll-Paque separation and depleted of plastic-adherent cells by two passages on fresh tissue culture flasks over 24 hours. Mononuclear cells, 10^3, were plated in IMDM with 0.3% agarose, 20% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10^−4 mol/L β-mercaptoethanol at 37°C and 5% CO₂. These cultures were plated with various dilutions of PU-34-conditioned media and colonies were evaluated after 10 days of incubation. Analysis of constitutive expression of M-CSF was performed using murine CFU-M assay. Freshly isolated C57/HeJ mouse bone marrow was plated at 1 × 10^6/mL in IMDM methylcellulose (0.9%) containing 20% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ^−4 mol/L β-mercaptoethanol. Colonies containing greater than 50 cells were counted after 10 days at 37°C and 5% CO₂.

Erythroid activity was evaluated through burst promoting activity (BPA) assays. Peripheral blood progenitor cells from leukoplas were acquired from patients undergoing platelet pheresis in the blood component lab of the Children's Hospital in Boston, MA, in accordance with guidelines of the Human Investigation Committee. Peripheral cells were separated over a Ficoll-Paque gradient. The mononuclear cells were then washed and resuspended at 5 × 10^5 cells/mL and placed on ice. Cold-induced aggregates (Ag⁺) were collected, layered on FCS, and allowed to settle by unit gravity through the FCS at 4°C. The resulting cells were incubated with carbonyl iron and phagocytic cells magnetically separated. These Fe⁺ cells were further depleted of monocytes/macrophages by adherence depletion (Ad⁺) on tissue culture plates at 37°C for 1 to 2 hours. The resulting Ag⁺, Fe⁺, Ad⁺ cells were plated at 10^3 cells/mL in IMDM, 0.9% methylcellulose, 30% FCS, 10^−4 mol/L β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Conditioned media from IL-1α-induced PU-34 cells were added at dilutions of 1:10 and 1:50 and burst forming units-erythroid (BFU-E) were counted at 12 days.

RESULTS

Generation of cell lines. Infection of primate LTMC with the amphotropic U19 virus and selection with G418 led to the appearance of multiple macroscopic G418-resistant colonies. Such colonies consistently led to the establish-
Table 1. Immunofluorescence Analysis of PU-34 Stromal Cell Line

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Fluorescence</th>
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<tbody>
<tr>
<td>Pab 419</td>
<td>+</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>+</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>-</td>
</tr>
<tr>
<td>Desmin</td>
<td>-</td>
</tr>
<tr>
<td>Laminin</td>
<td>-</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>-</td>
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<tr>
<td>Factor VIII-associated Ag</td>
<td>-</td>
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</table>

ment of permanent cell lines and 15 cell lines were easily established from the initial infection of primate LTMC. All of the cell lines established exhibited nuclear-specific SV40 LT antigen immunofluorescence staining using Pab 419 antibody (Table 1). The cell lines demonstrated fibroblast-like morphology with large round nuclei and prominent nucleoli on phase microscopy. Figure 1 demonstrates the morphology of a representative line, PU-34. None of these cell lines accumulated obvious fat vacuoles in culture, even in the presence of hydrocortisone. Three cell lines were chosen for preliminary analysis based on growth velocity (PU-22, PU-27, and PU-34). PU-34 supported hematopoietic cells for prolonged periods in vitro during this initial screening and was extensively studied.

Support of hematopoiesis in long-term cultures by stromal cell line PU-34. Confluent flasks of mitomycin C-treated PU-34 cells were charged with primate bone marrow cells depleted of plastic-adherent cells. Treatment with mitomycin C prevents further proliferation while maintaining cellular function of the PU-34 stromal cells. In a typical experiment, 860 progenitors (535 myeloid, 325 erythroid) were plated onto PU-34 (Fig 2A). Subsequently, nonadherent cells continuously shed into the media in these cultures included multilineage, myeloid, and erythroid progenitors and mature granulocytes and monocytes. Assayed in meth...

Fig 2. Support of primate progenitor CFU by PU-34 cell line. Nonadherent cells produced during long-term cultures initiated on PU-34 were harvested weekly and assayed in methylcellulose cultures for myeloid (N) and erythroid (○) progenitors (A) or myeloid progenitors only with cultures sacrificed and replated at week 3 (B). Colonies (>50 cells) were counted 10 to 12 days after plating. These results represent two representative and independent experiments.

Fig 1. Photomicrograph of PU-34 immortalized stromal cell line. Cells display a fibroblastic morphology with a large round nucleus (N) and prominent nucleoli. Note absence of fat vacuoles. (Original magnification ×250.)
ylcellulose, large numbers of myeloid and erythroid progenitor-derived colonies were present in the nonadherent cells for up to 4 weeks after initiation, with a cumulative increase in colony-forming cells in each culture. As seen in Fig 2A, from the initially plated 860 progenitors, 555 were recovered at week 1 in the nonadherent fraction. Subsequently, an additional 400 were recovered by week 4, with erythroid colonies present throughout the culture period. In addition, characteristic refractile hematopoietic cells were present in the adherent fraction of the cultures.

Morphologically, these cultures appeared indistinguishable from standard human LTMC, with refractile hematopoietic cells scattered throughout the stroma rather than in prominent nests (cobblestone areas) seen in murine LTMC. In some experiments (Fig 2B), the adherent cells were trypsinized, hematopoietic cells separated by Ficoll-Paque density centrifugation, and the low-density cells replated in methylcellulose cultures. The adherent cells from cultures represented in Fig 2B gave rise to a mean of 21 colonies/flask, representing approximately 30% of the total CFU/flask at week 3. Individual progenitor colonies plucked from methylcellulose and stained with Wright-Giemsa stain after cytocentrifugation consisted of granulocytic and monocytic cells. Some multilineage colonies containing erythroid and megakaryocytic cells were also evident up to 2 weeks in culture (Fig 3A and B). These data suggest that PU-34 supported the survival of multilineage, myeloid, and erythroid progenitor cells as well as sustained myeloid differentiation in vitro. Similar attempts to maintain colony forming progenitor cells in primary primate

![Image](https://example.com/fig3.png)

**Fig 3.** (A) Progenitor-derived mixed primate colony grown in methylcellulose from nonadherent cells of long-term culture established on PU-34. The darker cells of the main colony (at arrow head) include hemoglobinized cells and erythroid precursors by Wright-Giemsa and benzidine staining of cytospin preparations. (Original magnification ×40.) (B) Cytospin preparation of primate progenitor colony derived from nonadherent cells of long-term culture established on PU-34. Stained with Wright-Giemsa. G, granulocytes; M, macrophages; Mk, megakaryocyte, E, erythroid precursor. (Original magnification ×1,000.)
LTMC were unsuccessful due to premature peeling of the adherent stromal cells. Experiments performed with nonadherent mononuclear cells from both human and rhesus primate bone marrow aspirates showed virtually identical support of cells capable of colony formation for 3 weeks (data not shown). Plating of nonadherent cells from these cultures yielded predominantly myeloid colonies and some erythroid and mixed colonies.

Phenotypic analysis of primate stromal cell line, PU-34.

The phenotypic analysis of stromal cell lines included demonstration of intermediate filament proteins, extracellular matrix components, and constitutive and induced growth factor production. Intermediate filaments and the components of the ECM were evaluated through immunofluorescence microscopy and SDS-PAGE analysis. Immunofluorescence studies demonstrated the presence of vimentin and fibronectin and the absence of cytokeratin, desmin, laminin, collagen type IV, and Factor VIII-related antigen (Table 1). SDS-PAGE was used to analyze the types of collagen present in the ECM. Collagen types I, III, and V were clearly demonstrated and the absence of collagen type IV (as suggested by the immunofluorescence data) was confirmed by SDS-PAGE analysis (Fig 4). As seen in Fig 4, digestion of the medium procollagens with pepsin followed by SDS-PAGE with delayed reduction made it possible to distinguish alpha 1(I), alpha 2(I), and alpha 1(III) collagen chains. Faint bands representing alpha 1(V) and alpha 2(V) are also demonstrated. No band with mobility suggestive of collagen type IV could be detected. These findings demonstrate that PU-34 expresses a fibroblastic phenotype consistent with a mesenchymal origin.

Growth factor production by PU-34.

Growth factor production was evaluated by Northern blot analysis and by biologic assays. PU-34 constitutively produced M-CSF, LIF, and small amounts of IL-6 and GM-CSF by bioassay (Table 2). The expression of G-CSF, GM-CSF, IL-6, IL-7, and LIF were demonstrated by the appearance of appropriate size cDNA-hybridizing transcripts noted on Northern blot analysis of poly A+ RNA obtained from IL-1α-induced PU-34 (Fig 5). The expression of G-CSF and LIF were confirmed by bioassay using DA-2 cells in the presence and absence of antibody to human G-CSF (Fig 6A). The expression of GM-CSF was confirmed by bioassay using TF-1 cells and Mo7-E cells in the presence or absence of antibody to human GM-CSF (Fig 6B and C). The expression of IL-6 was confirmed by bioassay using T1165 cells in the presence or absence of antibodies to human IL-6 (Fig 6D). No expression of IL-3, IL-4, IL-5, IL-9, or TNFα was noted by RNA analysis. We did not evaluate IL-7 through bioassays.

IL-1α-induced PU-34 CM also stimulated the growth of CFU-GM colonies in human progenitor assays (Table 3A) and BFU-E colonies in human erythroid cultures (Table 3B), even in the presence of excess antibodies to GM-CSF, the only demonstrated source of BPA in PU-34. Stimulation of BFU-E by PU-34 CM was equivalent to that seen with addition of optimal doses of rhGM-CSF, but nearly 50% of BFU-E colonies remained after treatment with equivalent amounts of neutralizing antibody to GM-CSF. No further neutralization of BFU-E formation was demonstrated with larger amounts of neutralizing antibody to GM-CSF. In contrast, addition of anti-GM-CSF antibody completely abrogated the growth of BFU-E stimulated by rhGM-CSF. Antibody-resistant BFU-E demonstrates residual BPA-like activity in PU-34 CM and may represent another novel cytokine activity. Dilutions of PU-34 CM also stimulated the growth of murine megakaryocyte colonies (data not shown), which could be accounted for by combinations of the known hematopoietic growth factors present in PU-34 CM (LIF, IL-6, IL-11).

DISCUSSION

The microenvironment that supports HSC renewal both in vitro and in vivo is composed of multiple cell types with a complex extracellular matrix. LTMC provide an in vitro environment that closely mimics the HSC-HM interactions seen in vivo. We have previously shown that recombinant retrovirus vectors provide an efficient and rapid method of immortalizing stromal cells from multiple tissues of an adult mouse, including adult bone marrow. Because primary LTMC have been problematic in the primate system, we hypothesized that immortalized cell lines may...
provide a means of studying primate HSC-stromal cell interactions in vitro. In addition, such cell lines would be useful for support of stem cells into which foreign genetic sequences had been inserted via retrovirus vectors. Successful infection of reconstituting HSC of larger species is a critical goal of current experiments before the application of gene transfer technology toward human somatic gene therapy. A major obstacle in the extension of gene transfer

![Fig 5. Northern blot analysis of poly-A + RNA from IL-1-induced PU-34 cells. ²P-labeled probes used are shown at top of each lane. Arrows show location of hybridizing transcripts and molecular size markers are shown at left.](image)

![Fig 6. Tritiated thymidine incorporation by-factor dependent cell lines in response to CM from PU-34 cells. Response of (A) DA-2 cells in the presence or absence of antibody to G-CSF; (B) TF-1 cells in the presence or absence of antibody to GM-CSF; (C) Mo7-E cells in the presence or absence of antibody to GM-CSF; and (D) T1165 cells in the presence or absence of antibodies to IL-6. The dilution of PU-34 CM is noted on the abscissa and the counts incorporated on the ordinate. (-----) PU-34, (-----) PU-34 + antibody.](image)
experiments from the murine system to larger species (dog, primate, and presumably human) is the inefficiency of infection of long-term reconstituting stem cells. The use of drug-resistant stromal cell lines, such as PU-34, may address this limitation by providing an optimal environment for reconstituting stem cells during prolonged in vitro manipulation required for infection and selection.17,18

Although many murine bone marrow stromal cell lines have been generated and characterized, few stromal cell lines are available from human bone marrow and none from primate bone marrow. The few human stromal cell lines available have been generated using transfection of SV40 LT antigen by DNA-mediated transfection methods.28,29 The technique using recombinant retrovirus vectors used in these studies has proven to be a more efficient means of gene transfer into mammalian cells making up the HM.32-49

Table 3. Support of (A) Myeloid and (B) Erythroid Progenitor Growth by PU-34 CM

<table>
<thead>
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<th></th>
<th>A. CSF*</th>
<th>CFU-GM/10^5†</th>
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<tr>
<td>PU-34 (1:16)</td>
<td>15,14</td>
<td></td>
</tr>
<tr>
<td>PU-34 (1:16) + anti-G-CSF</td>
<td>12,14</td>
<td></td>
</tr>
<tr>
<td>PU-34 + anti-G-CSF + anti-GM-CSF</td>
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<td></td>
</tr>
<tr>
<td>B.</td>
<td></td>
<td>BFU-E/10^9‡</td>
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<td>rHGM-CSF (5 ng/mL)</td>
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<td>4,8,12</td>
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<td>PU-34 (1:10)</td>
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<tr>
<td>(1:50)</td>
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<tr>
<td>(1:50) + anti-GM</td>
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*Conditioned media (dilutions) or growth factor (concentration) added.
†Assayed on human bone marrow cells.
‡Assayed on peripheral blood mononuclear cells.

The stromal line PU-34 was generated from a randomly picked G418-resistant colony derived from primate LTMC infected with a defective recombinant retrovirus expressing the SV40 LT antigen by DNA-mediated transfection methods.28,29 The technique using recombinant retrovirus vectors used in these studies has proven to be a more efficient means of gene transfer into mammalian cells including HSC30 and cells making up the HM.32-49

The interaction of the microenvironment and hematopoietic cells may be dependent, at least in part, on local secretion of growth factors. In LTMC, reentry into the S phase of primitive hematopoietic progenitors occurs after each weekly medium change.51 Recent work suggests that this may be due to a yet undefined component of horse serum inducing cytokine expression by stromal cells. Precedent for such a model is suggested by the demonstrated capacity of regulatory molecules, such as platelet-derived growth factor (PDGF), IL1-α, and transforming growth factor β (TGF-β) to modulate cytokine production by mesenchymal cells.50,51,56,57 Therefore, there are likely to be cultures on PU-34, we postulate that reconstituting primate and human HSC will survive in vitro on PU-34 for prolonged periods.

Multiple cell types, including fibroblasts, have been shown to comprise the adherent population in LTMC. Although bone marrow explants have given rise to adherent fibroblasts in short-term culture (CFU-F),50,51 Perkins and Fleischman52 have recently shown that the progeny of murine CFU-F are endothelial-like cells. In addition, Zucker et al have demonstrated an extensive extracellular network of fibronectin, laminin, and collagen IV throughout the life of adherent stroma of primary murine LTMC.53 The cell type giving rise to permanent cell lines that support hematopoiesis expresses collagen type IV and laminin, markers associated with endothelial lineages.28,32 In contrast, analysis of the extracellular matrix products of PU-34 shows the presence of collagen types I, III, and V with no collagen type IV or laminin, findings consistent with a fibroblastic phenotype. Nevertheless, this primate cell line is capable of the support of hematopoiesis and replacement of the complex LTMC microenvironment. Lim et al have reported that cells expressing collagen type IV can be grown out of human bone marrow using specialized culture conditions and termed the progenitor cell for these colonies CFU-RF.54 It is intriguing that the lines generated from primate bone marrow long term cultures are uniformly fibroblast-like. These results raise the important possibility that the cells in the microenvironment that are the critical determinant of the stroma's capacity to support hematopoiesis may be different in murine and human or primate LTMC. This difference could explain the lack of cobblestone areas in human and primate cultures as well as the differences in duration of hematopoiesis noted when human cultures are compared with murine cultures. Alternatively, the support of hematopoiesis may not depend on interactions with specific stromal cell types. Although results of Roberts et al55 would support this interpretation, the localization of hematopoiesis in the medullary cavity in adults suggests some specificity to this microenvironment. Studies are currently underway to address these questions using immortalized cell lines derived from CFU-RF colonies. In addition, systematic analysis of immortalized stromal cell lines derived from adult mice in our laboratory has shown that lines derived from the bone marrow, but not skin, lung, or kidney, are capable of maintaining CFU-S populations in vitro.60
soluble regulatory factors that influence the activities of hematopoietic cells indirectly through effects on cellular components of the HM. Many of these known growth regulating factors are produced by PU-34, such as G-CSF, IL-6, LIF, GM-CSF, and IL-7. However, novel growth factors may also play a positive or negative regulatory role in the support of hematopoiesis. Residual BPA activity not accounted for by known cytokines is present in IL1α-induced PU-34-conditioned media. In addition, a residual proliferative activity was noted at saturating concentrations of anti-IL-6-antibody in the T1165 bioassay. This residual activity provided a useful biologic assay for cloning this cytokine activity produced by PU-34. The cDNA encoding T1165 mitogenic activity was isolated by mammalian expression cloning methods and has been designated IL-11 and has been reported in detail in a separate communication.8 This cytokine can stimulate murine B cell and megakaryocyte development in synergy with IL-3. Although murine megakaryocyte stimulatory activity has been demonstrated using PU-34 CM, the combination of LIF, IL-6, and the novel cytokine described above may account for the observed support of murine megakaryocytic colonies. Once neutralizing antibodies to LIF and the novel cytokine described here are available, the potential presence of novel megakaryocyte stimulatory activity in PU-34 CM may be addressed more fully.

We have immortalized multiple primate bone marrow stromal cell lines and demonstrated that one line is capable of supporting primate and human multipotent, myeloid, and erythroid progenitor cells for up to 3 weeks in vitro. In addition, this stromal cell line has also provided a source for the molecular cloning of a novel hematopoietic growth factor. The role of this factor in the capacity of this cell line to support hematopoiesis as well as the full spectrum of its biologic activity has not been established. The use of immortalized stromal cell lines could have important implications for somatic gene therapy in large animal models because the efficiency of gene transfer into primitive HSC appears to be markedly lower in larger animal species when compared with the mouse.17,18 In addition, the use of retroviral vectors containing immortalizing oncogenes provides a novel and efficient approach to developing cell lines that might help to dissect the mechanisms by which stromal cell interactions regulate HSC self renewal.

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