Soluble Factor(s) Produced by Human Bone Marrow Stroma Increase Cytokine-Induced Proliferation and Maturation of Primitive Hematopoietic Progenitors While Preventing Their Terminal Differentiation

By Catherine M. Verfaillie

We have recently shown that conservation and differentiation of primitive human hematopoietic progenitors in in vitro long-term bone marrow cultures (LTBMC) occurs to a greater extent when hematopoietic cells are grown separated from the stromal layer than when grown in direct contact with the stroma. This finding suggests that hematopoiesis may depend mainly on soluble factors produced by the stroma. To define these soluble factors, we examine here whether a combination of defined early-acting cytokines can replace soluble stroma-derived biologic activities that induce conservation and differentiation of primitive progenitors. Normal human Lineage$^-$/CD34$^+$/HLA-DR$^-$ cells (DR$^-$) were cultured either in the absence of a stromal layer ("stroma-free") or in a culture system in which DR$^-$ cells were separated from the stromal layer by a microporous membrane ("stroma-noncontact"). Both culture systems were supplemented three times per week with or without cytokines. These studies show that culture of DR$^-$ cells for 5 weeks in a "stroma-free" culture supplemented with a combination of four early acting cytokines (Interleukin-3 [IL-3], stem cell factor [SCF], leukemia-inhibitory factor [LIF], and granulocyte colony-stimulating factor [G-CSF]) results in a similar cell expansion as when DR$^-$ cells are cultured in "stroma-noncontact" cultures supplemented with the same cytokines. However, generation of committed progenitors and conservation of the more primitive long-term bone marrow culture initiating cells (LTBMC-IC) was far superior in "stroma-noncontact" cultures supplemented with or without IL-3 than in "stroma-free" cultures supplemented with IL-3 alone or a combination of IL-3, LIF, G-CSF, and SCF. These studies indicate that human BM stroma produces soluble factors that can either alone or in synergy with defined cytokines (1) conserve primitive LTBMC-IC, (2) induce early differentiation of a fraction of the primitive progenitors, and (3) prevent their terminal differentiation. We show here that these stroma-derived factors are not likely to be the known early acting cytokines IL-3, SCF, LIF, or G-CSF. Characterization of the stroma-derived factor(s) may have important implications for clinically relevant studies, such as in vitro stem cell expansion in cancer treatment and gene therapy.

HEMATOPOIESIS is a complex process in which totipotent hematopoietic stem cells can self-replicate but also differentiate into lineage-committed progenitors. These committed progenitors undergo further multiplications before they terminally differentiate into mature blood elements. In vivo, this process usually occurs in close proximity with the bone marrow (BM) microenvironment. The factors that regulate both self-replication and the initial differentiation process of primitive uncommitted hematopoietic progenitors are still largely unknown. Studies in mice have shown that self-replication, proliferation, and differentiation of totipotent hematopoietic stem cells can be supported in vitro in a stroma-dependent long-term BM culture (LTBMC) system that closely mimics the BM microenvironment.[1-3] Moreover, short-term culture (10 days) of BM from patients with leukemia in a similar culture system may at least preserve human transplantable hematopoietic stem cells, as suggested by the durable engraftment seen in patients who received such cultured BM after lethal irradiation.[4,5] Therefore, evaluation of factors produced by the stromal cells in LTBMC may uncover mechanisms underlying the self-replication and initial differentiation steps of the human hematopoietic stem cell.

We have recently shown that primitive, lineage-noncommitted CD34$^+$/HLA-DR$^-$ cells can differentiate and can be maintained when cocultured with stromal layers but separated from the stromal layers by a 0.4 μm microporous membrane.[6] Additional studies show that DR$^-$ cells can differentiate and proliferate when cultured without a stromal layer but are supplemented daily by media conditioned by normal allogeneic BM stromal layers (manuscript in preparation). These studies suggest that soluble factors derived from the BM stromal layer are capable of inducing differentiation of primitive human hematopoietic cells and conserve at least a fraction of more primitive progenitors.

An important role of the stromal cells in stroma-dependent cultures may be to provide a combination of cytokines that promote differentiation and proliferation of primitive hematopoietic progenitors.[7-11] Long-term cultures can indeed be established from primitive hematopoietic progenitors in the absence of an adherent stromal layer when defined cytokines are repeatedly added.[12-15] Cytokines thought to be important in the induction of differentiation and/or proliferation of these primitive hematopoietic progenitors include recombinant human granulocyte colony-stimulating factor (rhuG-CSF),[16] rhu interleukin-1 (IL-1),[17,18] rhuIL-6,[17,18] rhuIL-3,[16-18] rhuIL-11,[19,20] leukemia-inhibitory factor (LIF),[21-23] stem cell factor (SCF),[12-15,24,25] and basic

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fibroblast growth factor (bFGF).[26,27] Although mRNA transcripts for almost all these cytokines are constitutively expressed or can be induced in stromal cells,[10,11] detection of cytokines in stroma conditioned media with either immunologic methods or bioassays has been limited to IL-6, G-CSF, GM-CSF, and SCF.[7,11] The exact role of cytokines in the hematopoiesis occurring in LTBMC remains uncertain.

To identify biologically active molecules required for support of hematopoietic stem cell functions, we compared the proliferation and differentiation of primitive DR^- cells cultured in the presence of defined cytokines but in the absence of a stromal layer ("stroma-free" culture) with DR^- cells cultured in a "stroma-noncontact" culture system in the absence or presence of exogenously added cytokines. The cytokine combinations used in both culture systems included IL-3, G-CSF, LIF, and SCF. G-CSF and IL-3 have recently been shown to increase the proliferation and differentiation of primitive hematopoietic progenitors in stroma-dependent culture systems.[28] LIF was chosen because it can increase retroviral integration into murine hematopoietic stem cells[21] and hence induce their proliferation. SCF can stimulate differentiation and proliferation of very primitive hematopoietic progenitors when used in association with IL-3 and may have the capacity of affecting the earliest hematopoietic stem cell.[12-15,24,25]

We show that a similar cell expansion can be obtained by culturing very primitive human hematopoietic progenitors in a "stroma-free" culture supplemented with four early acting cytokines as in "stroma-noncontact" cultures supplemented with the same cytokines. However, generation of committed progenitors and conservation of the more primitive LTBMC-initiating cells (IC) is far superior in "stroma-noncontact" cultures both in the presence and absence of IL-3 than in "stroma-free" cultures supplemented with IL-3, LIF, G-CSF, and SCF. Therefore, these studies indicate that optimal ex vivo expansion of hematopoietic progenitors may require potentially novel stroma-derived growth-promoting factors.

MATERIALS AND METHODS

Cell Separations

BM was aspirated from the posterior iliac crest in heparin from healthy young volunteers after informed consent. BM mononuclear cells (BMMNC) were separated after Ficoll-Hypaque centrifugation (specific gravity, 1.077). BMMNC were purified further in an initial counterflow elutriation step.[17,29] BMMNC resuspended in phosphate-buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) and 0.01% EDTA were injected into an elutriator system (Beckman Instruments, Irvine, CA) with standard separation chamber primed with Iscove's modified Dulbecco's medium-fetal calf serum (IMDM-FCS)-EDTA. Rotor speed and temperature were maintained at 1,950 rpm and 10°C. After loading, 200 mL effluent was collected at a flow rate of 14 mL/min. The rotor was then stopped and the remaining BMMNC flushed from the separation chamber. Cells collected in fraction 14 were then depleted of T lymphocytes and natural killer (NK) cells by sheep erythrocyte rosetting.[21] Further depletion of committed lymphoid and myeloid/monocytic cells was obtained using immunomagnetic beads and CD11b, CD19 (Becton Dickinson, Mountain View, CA), MY8 (Coulter Cytometry, Hialeah, FL) and glycophorin-A antibodies (AMAC Inc, Westbrook, MA). The resultant lineage negative cells were labeled with anti-CD34 and anti-HLA-DR antibodies. Cells were sorted on a FACS-Star-Plus (Becton Dickinson, Mountain View, CA) laser flow cytometry system equipped with a consort 40 computer. Cells were selected for low vertical and very low/low horizontal light scatter properties and expression of CD34 and HLA-DR antigens using mouse IgG1-phycoerythrin (PE) and mouse IgG2a-fluorescein isothiocyanate (FITC) antibodies as control.[30]

Long-Term Culture Systems

All culture media consists of IMDM with 12.5% FCS (Hyclone, Logan, UT), 12.5% horse serum (Terry Fox Laboratories, Vancouver, CA), 2 mmol/L L-glutamine (GIBCO Laboratories, Grand Island, NY), penicillin at 1,000 U/mL, streptomycin at 100 U/mL (GIBCO Laboratories), and 10^{-6} mol/L hydrocortisone.[30]

"Stroma-free" cultures. DR^- cells were suspended in transwell inserts (2×10^3/mL for 24-well plates or 1 to 4×10^3/mL for 6-well plates) in nonstroma containing wells.

"Stroma-contact" cultures. Irradiated stromal cells were subcultured in 6-well (1.75×10^6 cells suspended in 5 mL) or 24-well (0.35×10^6 cells suspended in 1 mL) plates. DR^- cells (2×10^3/mL for 24-well plates or 1 to 4×10^3/mL for 6-well plates) were plated onto the irradiated allogeneic stromal layers as described.[30]

"Stroma-noncontact" cultures. Transwell cultures consisted of allogeneic irradiated stromal cells derived from the same donors as the stromal cells used in "stroma-contact" cultures subcultured in the bottom well of 6-well (1.75×10^6 cells suspended in 3 mL) or 24-well (0.35×10^6 cells suspended in 0.8 mL) plates. A collagen-treated transwell insert (0.4 μm microporous filter) was then placed on top of the stromal layer, and sorted DR^- cells plated in the upper wells (2×10^3 cells/0.2 mL for 24-well plates, or 1 to 4×10^3 DR^- cells/2 mL for 6-well plates).

Maintenance of cultures. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. At weekly intervals, "stroma-contact" cultures were fed by removing half of the cell-free supernatant and replacing it with fresh complete media. In "stroma-noncontact" and "stroma-free" cultures, half the cell-free media from the bottom wells only was removed and replaced by fresh complete media.

Enumeration of cell number. Cells recovered from "stroma-free" or "stroma-noncontact" cultures were recovered at week 5 from the transwell inserts after vigorous washing. Cells were resuspended in IMDM +20% FCS and an aliquot obtained for enumeration under hemocytometer.

Enumeration of committed colony-forming cells (CFC) by methylcellulose culture. Cells harvested at week 5 from transwell inserts of "stroma-free" and "stroma-noncontact" cultures or from adherent and nonadherent layers (after collagenase treatment) of "stroma-contact" cultures were plated in methylcellulose (final concentration, 1.12%; Fisher Scientific, Fair Lawn, NY) with IMDM (GIBCO Laboratories) supplemented with 30% FCS (HyClone Laboratories), antibiotics (penicillin at 1,000 U/mL and streptomycin at 100 U/mL; GIBCO), 5×10^{-5} mol/L 2-mercaptoethanol, 3 IU recombinant erythropoietin (Epo; Amgen, Thousand Oaks, CA), and 10% conditioned media from the bladder carcinoma cell line 5637. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 14 days. The cultures were assessed at day 18 of culture for the presence of secondary CFC as previously described.[21]

Enumeration of the absolute number of LTBMC-IC by limiting dilution assays (LDA). At day 0, DR^- cells (22 replicates per concentration; 400, 150, 50, 15 DR^- cells/well) were plated onto 3×10^4 irradiated allogeneic stromal cells subcultured in 96-well plates.

Likewise, cells recovered after 5 weeks from "stroma-contact," "stroma-noncontact," and "stroma-free" cultures initiated at day 0 with 13,530 DR^- cells were plated in LDA (cell number = the equivalent of 400, 150, 50, or 15 DR^- cells at day 0). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and fed weekly with 75 μL fresh media. At week 5, all media was removed and the stromal layers overlayed with methylcellulose-containing media supplemented with Epo (3 IU/mL) and supernatant of the bladder carcinoma cell line 5637 (10%). Wells were scored for the presence or absence of secondary CFC at day 14. The absolute number of LTBMC-IC present in the different cell populations was calculated as the reciprocal of the concentration of test cells that gives 37% negative cultures using the Poisson statistics[31] and the weighted mean method.[32]

Cytokines

rhuIL-3 (a generous gift from Dr G. Wong, Genetics Institute, Cambridge, MA) was added to cultures 3 times per week to obtain a final concentration of 5 or 20 ng/mL (as indicated in the result section). rhuIL-6 (a generous gift from Dr G. Wong, Genetics Institute, Cambridge, MA) was added to cultures 3 times per week to obtain a final concentration of 5 ng/mL. rhuIL-1 was kindly provided by Dr M.B. Widmer, Immunex Biologicals, and was added to cultures 3 times per week to obtain a final concentration of 100 U/mL. rhuSCF was kindly provided by Dr Kris Szebo (Amgen) and was added to cultures 3 times per week to obtain a final concentration of 10 to 100 ng/mL (as indicated in the Results). rhuG-CSF (Philgastrim), obtained from Amgen Biologicals, was added to cultures 3 times per week to obtain a final concentration of 5 ng/mL. rhu granulocyte-macrophage-CSF (GM-CSF) (Leukine), obtained from Immunex Biologicals, was added to cultures 3 times per week to obtain a final concentration of 200 U/mL. rhuLIF, obtained from R&D systems (Minneapolis, MN), was added to cultures 3 times per week to obtain a final concentration of 20 ng/mL.

Statistics

Results of experimental points obtained from multiple experiments were reported as the mean ±1 standard error of the mean (SEM). Significance levels were determined by two-sided nonpaired Student's *t*-test analysis.

RESULTS

Cell Expansion Induced by Single Cytokines in "Stroma-Free" and "Stroma-Noncontact" Cultures

Primitive DR^- cells were suspended in FCS, horse serum, and hydrocortisone-containing media and plated either in transwell inserts without a stromal layer ("stroma-free") or in transwell inserts above allogeneic stromal layers ("stroma-noncontact"). Cultures were supplemented with cytokines 3 times per week as indicated in the method section and in the legend for Fig 1.

As we and others have previously shown,[6,12] DR^- cells cultured in "stroma-free" cultures to which no cytokines were added failed to proliferate over a 5-week culture period (Fig 1A). The addition of IL-1 (100 U/mL), IL-6 (5 ng/mL), LIF (20 ng/mL), or SCF (20 ng/mL) alone to "stroma-free" cultures, cytokines that are capable of stimulating primitive hematopoietic progenitors, particularly in combination with cytokines such as IL-3 and GM-CSF (synergistic factors), did not induce cell growth as expected.[17] Addition of either G-CSF (5 ng/mL) or GM-CSF (50 U/mL) to "stroma-free" cultures resulted in a variably increased recovery of cells after 5 weeks of culture. IL-3 (5 ng/mL) was the only cytokine that consistently resulted in a significant cell expansion when added as a single cytokine to "stroma-free" cultures.

In accordance with our previous studies,[6] a significant cell expansion (32-fold ± 6-fold [n = 16]) was seen when DR^- cells were cocultured with stromal layers in the absence of exogenous cytokines using transwell inserts that prevent direct hematopoietic cell-stroma contact (Fig 1B). This cell

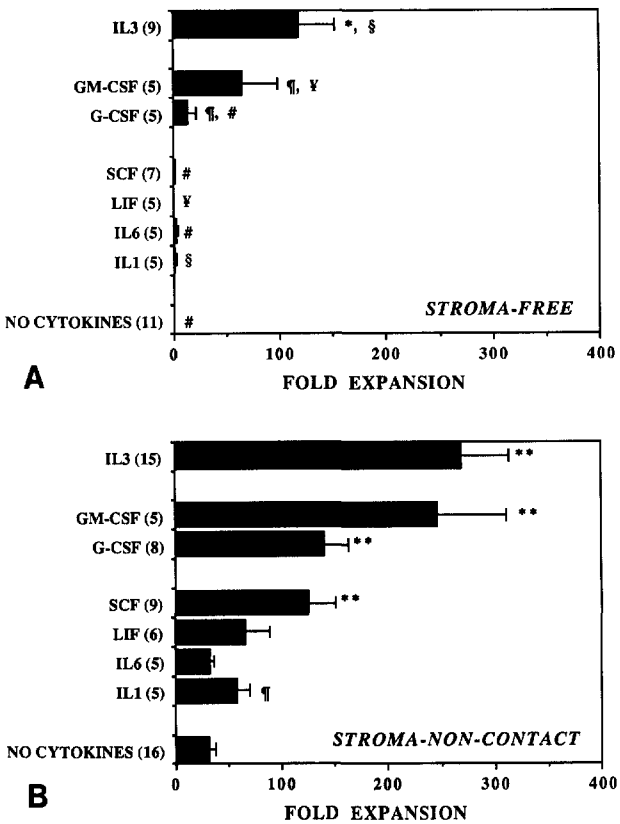

Fig 1. Expansion of DR^- cells is significantly greater in "stroma-free" than in "stroma-noncontact" cultures supplemented with single cytokines. Freshly sorted DR^- cells were suspended in transwell inserts (2×10^3/mL) in either non–stroma-containing wells ("stroma-free") or stroma-containing wells ("stroma-noncontact"). Cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2 and weekly fed by replacing half the cell-free media from the bottom wells only by fresh complete media. Each week on days 0, 2, and 4, cytokines diluted in PBS/BSA or IMDM/BSA were added to the bottom well only. Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Cytokines used: rhuIL-1: final concentration, 100 U/mL; rhuIL-6: final concentration, 5 ng/mL, specific activity, 4×10^6 U/mg; SCF: final concentration, 20 ng/mL; LIF: final concentration, 20 ng/mL; G-CSF: final concentration, 5 ng/mL; GM-CSF: final concentration, 50 U/mL; IL-3: final concentration, 5 ng/mL, specific activity, 2.5×10^6 U/mg. Statistical analysis: comparison between "stroma-free" cultures or "stroma-noncontact" cultures without cytokines and "stroma-free" cultures or "stroma-noncontact" cultures with cytokines: $^{**}P \leq .001$; $^{*}P \leq .01$; ¶$P \leq .05$. Comparison between "stroma-free" (A) and "stroma-noncontact" (B) cultures: #$P \leq .001$; §$P \leq .01$; ¥$P \leq .05$.

expansion was significantly greater than that observed in "stroma-free" cultures to which no cytokines were added, or which were supplemented with single cytokines (SCF, IL-1, IL-6, LIF, G-CSF, or GM-CSF). The addition of synergistic factors IL-1 or LIF to "stroma-noncontact" cultures did result in a small but significantly increased recovery of cells at 5 weeks of culture. When IL-3, G-CSF, or GM-CSF were added, factors known to directly induce proliferation of immature and/or more committed progenitors, a fivefold to eightfold increased number of cells was seen at the end of 5 weeks of culture compared with "stroma-noncontact" cultures, to which no cytokines were added. Similarly, addition of the synergistic factor SCF to "stroma-noncontact" cultures resulted in the generation of 5 times more cells than in cytokine free "stroma-noncontact" cultures.

Cell Expansion Induced by Multiple Cytokines in "Stroma-Free" and "Stroma-Noncontact" Cultures

Several studies have indicated the need for multiple cytokines to induce cell proliferation in "stroma-free" cultures of very primitive human hematopoietic progenitors.[12-15] In accordance with these studies, we show that, when IL-3 was combined with SCF both at relatively low concentrations (5 and 20 ng/mL, respectively), IL-3 and SCF, both at high concentrations (20 and 100 ng/mL, respectively), or IL-3 with SCF, G-CSF, and LIF (5, 20, 5, and 20 ng/mL, respectively), a progressively greater number of cells was generated in "stroma-free" cultures (Fig 2A). Somewhat surprisingly, the addition of multiple cytokines to "stroma-noncontact" cultures resulted in an only minimal further increase in cell number at week 5 of culture (Fig 2B). "Stroma-free" and "stroma-noncontact" cultures supplemented with multiple cytokines (either high-dose SCF + IL-3 [$P = .4$] or a combination of IL-3, G-CSF, SCF, and LIF [$P = .3$]) generated a similar number of cells at week 5, suggesting that a combination of defined cytokines with FCS and horse serum may be used to replace stromal layers in the induction of proliferation and differentiation of human hematopoietic progenitors.

Generation of Clonogenic Cells by Single Cytokines in "Stroma-Free" and "Stroma-Noncontact" Cultures

To be useful, ex vivo culture of primitive human hematopoietic progenitors should ideally yield not only a large numbers of cells but also of clonogenic cells. Therefore, we evaluated cells recovered at week 5 from transwell inserts of "stroma-free" or "stroma-noncontact" cultures supplemented with or without cytokines for the presence of clonogenic cells. An aliquot was replated in methylcellulose progenitor culture for 14 days and CFC were enumerated.

In accordance with our previous studies,[6] no clonogenic cells were present in "stroma-free" cultures at week 5. When cells recovered from "stroma-free" cultures supplemented with single cytokines were evaluated for their clonogenic cell content, we observed that IL-3 was the only cytokine that, when used alone, was capable of consistently inducing small numbers of CFC in "stroma-free" cultures (Fig 3A).

In contrast to "stroma-free" cultures, culture of DR^- cells

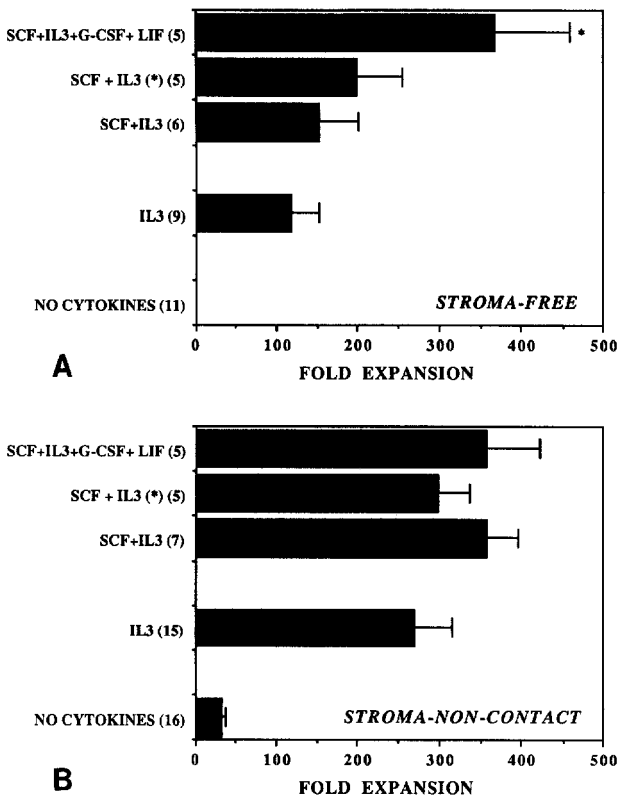

Fig 2. Expansion of DR^- cells is similar in "stroma-free" as in "stroma-noncontact" cultures supplemented with multiple cytokines. Freshly sorted DR^- cells were "stroma-free" or "stroma-noncontact" as described in Fig 1. Each week on days 0, 2, and 4, cytokines diluted in PBS/BSA or IMDM/BSA were added to the bottom well only. Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Cytokines used: IL-3: final concentration, 5 ng/mL; IL-3 + SCF: final concentration, 5 and 20 ng/mL, respectively; IL-3 + SCF (*): final concentration, 20 and 100 ng/mL, respectively; IL-3 + SCF + G-CSF + LIF: final concentration, 5, 20, 5, and 20 ng/mL, respectively; Statistical analysis: comparison between "stroma-free" cultures or "stroma-noncontact" cultures with IL-3 alone and "stroma-free" cultures or "stroma-noncontact" cultures with IL-3 in combination: **$P \leq .001$; *$P \leq .01$; ¶$P \leq .05$. Comparison between "stroma-free" (A) and "stroma-noncontact" (B) cultures: #$P \leq .001$; §$P \leq .01$; ¥$P \leq .05$.

in a "stroma-noncontact" system without exogenous cytokines resulted in the generation of 199 ± 58 CFC/2,500 DR^- cells (n = 11) at week 5 (Fig 3B). The addition of LIF and SCF to "stroma-noncontact" cultures consistently resulted in a twofold greater number of CFC present in "stroma-noncontact" cultures (Fig 3B), whereas the addition of the synergistic cytokines IL-1 and IL-6 to "stroma-noncontact" cultures resulted in a minimal increase in CFC present at week 5 (Fig 3B). Cell expansion and generation of CFC were induced to the same degree in each of these cultures, as illustrated by the cloning efficiency of cells recovered at week 5 (Table 1). The addition of GM-CSF to "stroma-noncontact" cultures failed to result in a significantly greater number of CFC at week 5 (Fig 3B), although an eightfold increase in cell number was seen (Fig 1B). Simi-

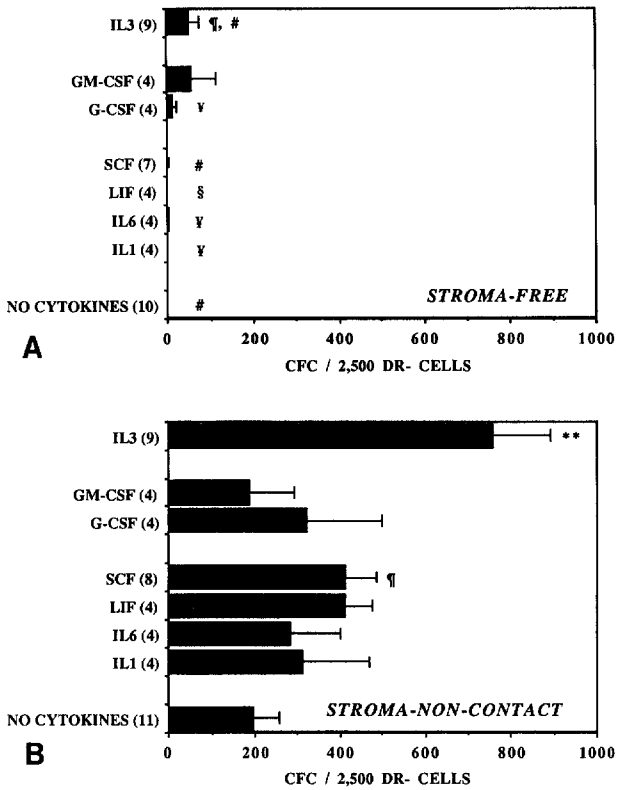

Fig 3. Generation of CFC at week 5 of culture from 2,500 DR⁻ cells is significantly lower in "stroma-free" than in "stroma-noncontact" cultures supplemented with single cytokines. Freshly sorted DR⁻ cells were cultured in "stroma-free" or "stroma-noncontact" cultures as described in Fig 1. Each week on days 0, 2, and 4, cytokines diluted in PBS/BSA or IMDM/BSA were added to the bottom well only. Concentrations of cytokines used are described in the legend to Fig 1. Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Statistical analysis: comparison between "stroma-free" cultures or "stroma-noncontact" cultures without cytokines and "stroma-free" cultures or "stroma-noncontact" cultures with cytokines: **$P \leq .001$; *$P \leq .01$; ¶$P \leq .05$. Comparison between "stroma-free" (A) and "stroma-noncontact" (B) cultures: #$P \leq .001$; §$P \leq .01$; ¥$P \leq .05$.

larly, the addition of G-CSF, known to act both at the immature progenitor level as a synergistic factor for IL-3[16] and at the more differentiated colony-forming unit-granulocyte macrophage (CFU-GM) and CFU-G level as a differentiation inducing factor, resulted in a twofold increased number of CFC at week 5 of culture (Fig 3B), whereas fivefold more cells were present compared with cytokine-free "stroma-noncontact" cultures. Therefore, addition of either GM-CSF or G-CSF to "stroma-noncontact" cultures resulted in a significantly lower cloning efficiency of cells recovered at 5 weeks as compared with control "stroma-noncontact" cultures without supplemental cytokines (Table 1). These findings indicate that GM-CSF and, to a lesser extent, G-CSF, but not the synergistic factors IL-1, IL-6, SCF, or LIF, induce terminal differentiation of DR⁻ cells without preserving clonogenic cells. As can be seen in Fig 3B, the most potent single cytokine at inducing generation of clonogenic cells in "stroma-noncontact" cultures was IL-3. When IL-3 was added to "stroma-noncontact" cultures, 759 ± 132 CFC/2,500 DR⁻ cells were generated, which was significantly greater than what we observed for "stroma-noncontact" cultures supplemented without cytokines or with any other single cytokine. These results are similar to studies performed in "stroma-contact" cultures in which the addition of GM-CSF fails to increase the clonogenic cell output, whereas the addition of G-CSF and even moreso, IL-3, results in a twofold increase in CFC[28] (unpublished observations, March 1993). However, the total number of CFC recovered after 5 weeks of culture is significantly lower in "stroma-contact" compared with "stroma-noncontact" cultures.

Generation of Clonogenic Cells by Multiple Cytokines in "Stroma-Free" and "Stroma-Noncontact" Cultures

Because combinations of different cytokines may be required for the maximal generation of CFC from "stroma-free" cultures initiated with primitive hematopoietic progenitors, we compared the clonogenic cell production in "stroma-free" and "stroma-noncontact" cultures supplemented with combinations of IL-3 and SCF. The addition of IL-3 to "stroma-free" cultures in combination with either SCF (low-dose IL-3 and SCF; high-dose IL-3 and SCF) or multiple cytokines (IL-3+ G-CSF, SCF, and LIF) resulted in a significantly greater production of CFC than when IL-3 was used alone (Fig 4A). In contrast to "stroma-free" cultures, combined addition of either low or high concentrations of SCF or a combination of G-CSF, SCF, and LIF with IL-3 did not result in a further increase in the number of

Table 1. Cloning Efficiency of Cells Recovered at Week 5 From "Stroma-Free" Cultures Is Significantly Lower Than That From "Stroma-Noncontact" Cultures

Cytokine	"Stroma-Free"	"Stroma-Noncontact"	$P =$
None	0 ± 0 (11)	0.34 ± 0.84 (11)	.006
IL-1	0.005 ± 0.005 (4)	0.24 ± 0.1 (4)	.028
IL-6	0.003 ± 0.003 (4)	0.40 ± 0.19 (4)	.014
SCF	0.007 ± 0.007 (7)	0.16 ± 0.30 (8)	.001
LIF	0 ± 0 (4)	0.36 ± 0.24 (4)	.05
G-CSF	0.032 ± 0.022 (4)	0.073 ± 0.03 (4)	NS
GM-CSF	0.029 ± 0.021 (4)	0.037 ± 0.20 (4)	NS
IL-3	0.009 ± 0.007 (9)	0.11 ± 0.20 (9)	.000
IL-3 + SCF	0.050 ± 0.027 (5)	0.13 ± 0.24 (6)	.02
IL-3 + SCF*	0.003 ± 0.002 (3)	0.11 ± 0.25 (5)	.03
IL-3 + SCF + G-CSF + LIF	0.016 ± 0.011 (3)	0.12 ± 0.70 (4)	.005

Cloning efficiency = colonies enumerated/cells cultured × 100%. Cells recovered at week 5 from transwell inserts of "stroma-free" and "stroma-noncontact" cultures with or without supplemental cytokines were enumerated under hemocytometer before replating in methylcellulose clonogenic assays. The total number of CFC was determined to calculate the cloning efficiency. Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Concentrations of cytokines used are described in the legend to Figs 1 and 2. Comparison between "stroma-free" and "stroma-noncontact" cultures are shown in the table.

Abbreviation: NS, not significant.

* IL-3 20 ng/mL and SCF 100 ng/mL 3 times per week.

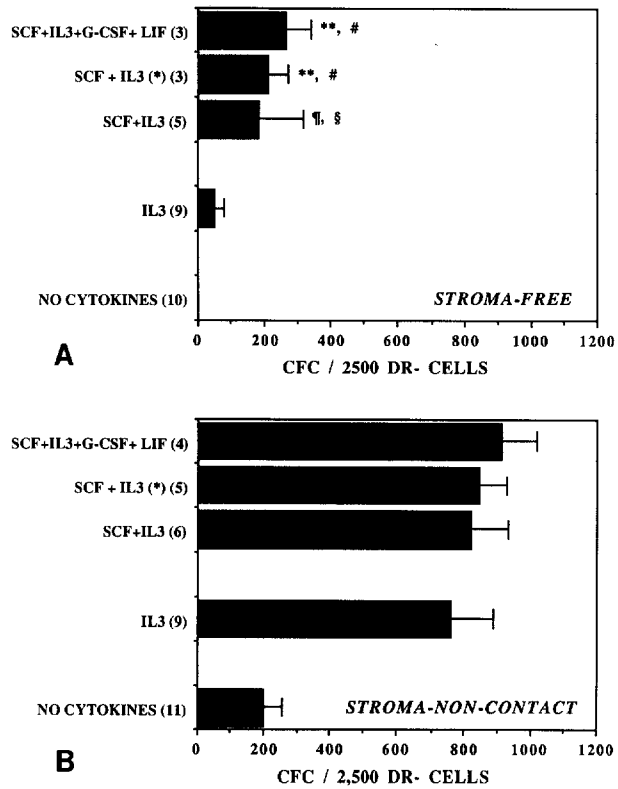

Fig 4. Generation of CFC at week 5 of culture from 2,500 DR⁻ cells is significantly lower in "stroma-free" than in "stroma-noncontact" cultures supplemented with multiple cytokines. Freshly sorted DR⁻ cells were cultured in "stroma-free" or "stroma-noncontact" cultures as described in Fig 1. Each week on days 0, 2, and 4, cytokines diluted in PBS/BSA or IMDM/BSA were added to the bottom well only. Concentrations of cytokines used are described in the legend to Fig 2. Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Statistical analysis: comparison between "stroma-free" cultures or "stroma-noncontact" cultures with IL-3 alone and "stroma-free" cultures or "stroma-noncontact" cultures with IL-3 in combination: **$P \leq .001$; *$P \leq .01$; ¶$P \leq .05$. Comparison between "stroma-free" (A) and "stroma-noncontact" (B) cultures: #$P \leq .001$; §$P \leq .01$; ¥$P \leq .05$.

CFC present at week 5 of "stroma-noncontact" cultures (Fig 4B).

Of interest, culture of DR⁻ cells in "stroma-free" cultures supplemented with any of these combinations did not result in the generation of significantly more CFC than when DR⁻ cells were cultured in "stroma-noncontact" cultures in the absence of exogenous cytokines. Moreover, significantly less CFC were generated in the absence of stroma irrespective of the combination of cytokines used. Because levels of G-CSF (1 to 2 ng/mL), LIF (50 to 100 pg/mL), SCF (100 to 300 pg/mL), and IL-3 (0 ng/mL) in stroma-conditioned media [7,11] (personal unpublished observations, March 1993) are significantly lower than those used in these experiments, these studies indicate that growth-promoting factors, different from IL-3, SCF, G-CSF, or LIF, present in stromal supernatants are likely responsible for the observed production of CFC in "stroma-noncontact" cultures. As shown in Fig 2A and B, cell expansion in "stroma-free" cultures supplemented with IL-3 + SCF or IL-3 + SCF + G-CSF + LIF was not significantly different than that observed in "stroma-noncontact" cultures. The lower cloning efficiency of cells recovered from "stroma-free" cultures than from "stroma-noncontact" cultures (Table 1) suggests that, in addition to growth-promoting activities, stromal supernatants may contain soluble factor(s) that can inhibit terminal differentiation of DR⁻ cells induced by supra-physiologic amounts of growth-promoting cytokines.

Recovery of LTBMC-IC From Cytokine-Supplemented "Stroma-Free" and "Stroma-Noncontact" Cultures

To further examine the possibility that stroma-derived factor(s) can prevent cytokine-induced terminal differentiation of DR⁻ cells, we examined the cells recovered at week 5 of initial culture for the presence of more primitive progenitors that can reinitiate in vitro LTBMC. Freshly sorted DR⁻ cells and cells recovered from "stroma-free" cultures and "stroma-noncontact" cultures supplemented with or without cytokines were plated in limiting dilution onto irradiated stromal layers subcultured in 96-well plates. At 5 weeks, stromal layers were overlayed with methylcellulose media containing FCS, supernatant of the bladder carcinoma cell line 5637 and Epo. Two weeks later, wells containing hematopoietic colonies were enumerated and the absolute number of LTBMC-IC calculated using Poisson statistics[31] and the weighted mean method.[32]

In accordance with previously published observations,[6,28] we show that the absolute number of LTBMC-IC present in the originally sorted population of DR⁻ cells is 0.96% ± 0.18% (Fig 5). On culture for 5 weeks in a classical "stroma-contact" culture, the absolute number of LTBMC-IC decreased to 20% of the initial LTBMC-IC number. When DR⁻ cells were cultured in "stroma-free" cultures not supplemented with cytokines, no cells, and therefore no LTBMC-IC, remained after 5 weeks of culture. The addition of IL-3 + SCF ± G-CSF ± LIF to "stroma-free" cultures, the most powerful cytokine combinations at inducing differentiation of DR⁻ cells to the committed cell stage, resulted in a 10% recovery of the initial LTBMC-IC number. In contrast, culture of DR⁻ cells for 5 weeks in "stroma-noncontact" cultures in the absence or presence of IL-3 resulted in a 40% to 50% recovery of the initial number of LTBMC-IC. However, addition of a combination of SCF, G-CSF, and LIF to IL-3 containing "stroma-noncontact" cultures resulted in a significantly greater loss of LTBMC-IC (20% recovery) over a 5-week culture period compared with either control "stroma-noncontact" cultures without supplemental cytokines ($P = .001$) or cultures supplemented with IL-3 alone ($P = .002$).

The recovery of significantly less LTBMC-IC from "stroma-free" cultures supplemented with IL-3 + SCF ± G-CSF ± LIF than from control "stroma-noncontact" cultures without supplemental cytokines suggests that stromal supernatants may contain soluble factor(s) that cannot only induce proliferation and/or conservation of LTBMC-IC but may also inhibit their terminal differentiation induced by supra-physiologic amounts of growth-promoting cytokines.

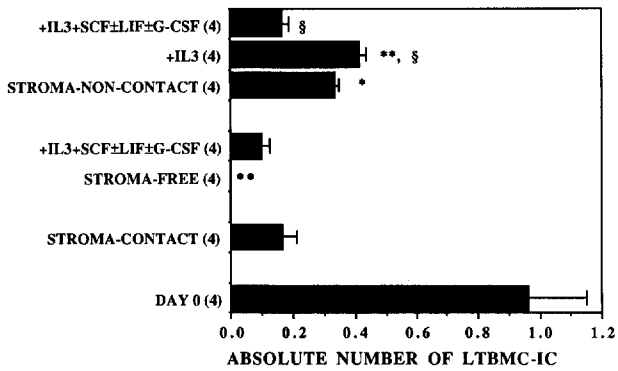

Fig 5. Significantly more LTBMC-IC are recovered from "stroma-noncontact" cultures without supplemental cytokines or with IL-3 than from "stroma-contact" cultures or "stroma-free" cultures supplemented with combinations of cytokines including IL-3 and SCF. DR^- cells were plated in "stroma-free" cultures, "stroma-noncontact" cultures or "stroma-contact" cultures without cytokines or in the presence of the indicated cytokines for 5 weeks. Freshly sorted DR^- cells (day 0) and cells recovered from 5-week-old "stroma-free", "stroma-noncontact", or "stroma-contact" cultures supplemented with or without cytokines were plated in limiting dilution onto irradiated stromal layers subcultured in 96-well plates. At 5 weeks, stromal layers were overlayed with methylcellulose media containing FCS, supernatant of the bladder carcinoma cell line 5637, and Epo. Two weeks later, wells containing hematopoietic colonies were enumerated and the absolute number of LTBMC-IC calculated using Poisson statistics (*) and the weighted mean method (*). Numbers in parentheses next to the cytokine mixtures represent the number of replicate experiments. Concentrations of cytokines used are described in the legend to Fig 2. Statistics: comparison between "stroma-contact" and all other cultures: #$P \leq .001$; §$P \leq .01$; ¥$P \leq .05$. Comparison between "stroma-noncontact" culture without cytokines and "stroma-noncontact" cultures with cytokines or "stroma-free" cultures: **$P \leq .001$; *$P \leq .01$; ¶$P \leq .05$.

In favor of this last hypothesis is the observation that addition of IL-3 alone to "stroma-noncontact" cultures resulted in a slightly greater recovery of LTBMC-IC at 5 weeks compared with control cytokine-free "stroma-noncontact" cultures, whereas addition of multiple cytokines at supra-physiologic concentrations, which may overwhelm the antidifferentiation effect of stromal supernatants, resulted in a decreased recovery of LTBMC-IC at week 5.

DISCUSSION

We show that "stroma-free" cultures supplemented with a combination of four early acting cytokines, IL-3, SCF, G-CSF, and LIF, yield at most equivalent numbers of clonogenic cells as cultures in which primitive progenitors are cultured in a stroma-dependent culture but suspended in a transwell insert that does not allow contact between the hematopoietic cells and the stromal layer. Addition of the same cytokines to "stroma-noncontact" cultures resulted in a consistently greater generation of clonogenic cells than in cultures without cytokines or in "stroma-free" cultures supplemented with this cytokine combination. Because levels of G-CSF, LIF, SCF, and IL-3 in stroma-conditioned media are significantly lower than those used in these experiments, this suggests that soluble stroma-derived factors different from IL-3, G-CSF, LIF, or SCF can induce differentiation of primitive hematopoietic cells and synergize with defined cytokines in this process. The higher clonogenicity of cells recovered from "stroma-noncontact" cultures both in the absence or presence of cytokines compared with cytokine supplemented "stroma-free" cultures suggests that, in addition to growth promoting activities, stromal supernatants may contain soluble factor(s) that can inhibit terminal differentiation of DR^- cells induced by supra-physiologic amounts of growth-promoting cytokines. This notion is further corroborated by our observations that significantly more primitive LTBMC-IC could be recovered from cytokine-free or IL-3 supplemented "stroma-noncontact" cultures than from cytokine supplemented "stroma-free" cultures. Therefore, these observations indicate that optimal ex vivo expansion of hematopoietic progenitors may require supra-physiologic concentrations of defined cytokines in association with thus far unidentified stroma-derived growth-promoting factors. Additional studies will be required to determine if the increased production of CFC on addition of exogenous cytokines to "stroma-noncontact" cultures is the result of a direct effect of these cytokines on hematopoietic progenitors or may in part result from the induction of (unknown?) growth-promoting substances in the stromal microenvironment.

These studies confirm and extend previous data from our group indicating that human BM stromal layers produce soluble factors capable of (1) inducing early differentiation of very primitive human hematopoietic cells, (2) inducing proliferation of a fraction of the primitive progenitors (manuscript in preparation), and (3) preventing their terminal differentiation. We show here that these factors are likely not the known early acting cytokines IL-3, SCF, LIF, or G-CSF. Candidate molecules involved in these events may be known growth-promoting factors such as IL-11[19,20] and factors such as transforming growth factor-β (TGF-β),[33] macrophage inflammatory protein-1α,[34,35] and other molecules of the latter group, known to inhibit proliferation of primitive human or murine primitive progenitors. Alternatively, novel cytokines or possibly other stroma-derived molecules, such as extracellular matrix components[36-38] or proteoglycans,[39-41] may be responsible for the observed growth-promoting and differentiation-inhibitory effects. Studies using either blocking antibodies in a "stroma-noncontact" culture setting or using additional defined cytokines/extracellular matrix components in a "stroma-free" culture setting are now underway to determine if thus far untested growth-promoting or inhibitory cytokines can explain the supportive function of stromal supernatants or if novel biologically active (macro)molecules are involved. Further characterization of these "missing" factors may have far reaching implications for in vitro stem cell expansion in cancer treatment, gene therapy, and the in vitro creation of different types of blood cell products.

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