Mechanisms That Regulate the Cell Cycle Status of Very Primitive Hematopoietic Cells in Long-Term Human Marrow Cultures. I. Stimulatory Role of a Variety of Mesenchymal Cell Activators and Inhibitory Role of TGF-β

By Johanne D. Cashman, Allen C. Eaves, Elaine W. Raines, Russell Ross, and Connie J. Eaves

Long-term marrow cultures (LTMC) allow the proliferation and differentiation of primitive human hematopoietic progenitor cells to be maintained for many weeks in the absence of exogenously provided hematopoietic growth factors. Previous investigations focused on defining various types of cells that are present in this culture system and on measuring the cycling behavior of the different subpopulations of colony-forming cells maintained within it. These studies suggested that mesenchymal stromal elements derived from the input marrow play a key role in regulating the turnover of the most primitive, high-proliferative potential erythroid and granulopoietic colony-forming cells that are found almost exclusively in the adherent layer of LTMC. In this study we show that the re-entry into S-phase of these primitive hematopoietic progenitors that occurs after each weekly medium change is due to an as yet undefined constituent of horse serum, which is absent from fetal calf serum. However, this effect is not unique to the factor present in horse serum. It is also elicited by the addition to LTMC of several well-defined growth regulatory molecules, ie, platelet-derived growth factor (PDGF), interleukin-1 (IL-1), transforming growth factor α (TGF-α), and IL-2. None of these was able to stimulate hematopoietic colony-forming cells in methylcellulose assays, although all have known actions on mesenchymal cells including, in some cases, the ability to increase production of growth factors that can stimulate primitive high-proliferative potential hematopoietic progenitors in clonogenic assays. Interestingly, a stimulating effect was not obtained after addition of endotoxin to LTMC. TGF-β, a direct-acting negative regulator that acts selectively on primitive hematopoietic progenitor cells if added to LTMC simultaneously with new medium or IL-1, blocked their stimulating activity. These results suggest a model in which indirect, local modulation of both positive and negative regulatory factors via effects on mesenchymal elements determines the rate of turnover of adjacent populations of very primitive hematopoietic cells that are normally maintained in a quiescent state in vivo.

CURRENT EVIDENCE suggests that hematopoiesis is maintained throughout adult life by the continuous recruitment into the cycle of cells from a primitive, largely quiescent stem cell pool.[1-3] Once recruited, each of these cells undergoes a series of amplifying divisions to produce a large cohort of differentiating progeny. Cells proceeding down various hematopoietic lineages can be detected by appropriate in vitro clonogenic assays,[4] and analysis of the specific molecular stimuli required for the development of different types of colonies has led to the identification of a variety of hematopoietic growth factors. Genes for more than a dozen of these factors have now been isolated and expressed as recombinant proteins, and their availability in pure form has led to the unequivocal demonstration that a variety of primitive and mature hematopoietic cells can respond directly to numerous factors either alone or in combination.[5] Availability of specific complimentary DNA (cDNA) probes for these growth factors has also enabled identification of the cells responsible for their production. For example, readily detectable levels of messenger RNAs (mRNAs) for macrophage-colony-stimulating factor (M-CSF), granulocyte (G)-CSF, GM-CSF, and interleukin-6 (IL-6) have been demonstrated in lymphocytes and/or macrophages, as well as in a variety of mesenchymal cell types of nonhematopoietic origin (eg, fibroblasts and endothelial cells) after their activation.[5-7] While this has suggested a number of possible direct and indirect progeny–precursor feedback regulatory loops, whether or how any of the known growth factors active on hematopoietic cells actually contribute to the local mechanisms that appear to regulate normal hematopoietic stem cell proliferation in vivo remains largely speculative.

One approach to investigating the role of specific growth factors in regulating hematopoiesis in vivo has been to assess the effect of growth factor administration on the number of clonogenic progenitors and/or mature blood cells later detectable at various times. Such studies have provided convincing evidence of the effectiveness of several direct-acting myelopoietic growth factors (including G-CSF, M-CSF, GM-CSF, and IL-3) in stimulating granulopoiesis in vivo.[5] On the other hand, other factors such as IL-1, which in vitro do not on their own appear to stimulate myeloid cell proliferation directly,[8] can stimulate granulopoiesis after administration in vivo.[9,10]

Analysis of how hematopoiesis is regulated in the long-term marrow culture (LTMC) system may offer an alternative in vitro strategy to investigating the role of various growth factors in vivo. In the LTMC system, a continuously dividing and differentiating population of hematopoietic cells is maintained for several months in the absence of exogenously added hematopoietic growth factors.[11] However, it is

From the Terry Fox Laboratory, British Columbia Cancer Research Centre, and the Departments of Medicine, Pathology, and Medical Genetics, University of British Columbia, Vancouver, Canada, and the Department of Pathology, University of Washington, Seattle.

Submitted June 8, 1989, accepted September 1, 1989.

Supported by an operating Grant from the National Cancer Institute of Canada (NCIC) and National Heart, Lung, and Blood Institute (Grant no. HL-18645) with core support from The British Columbia Cancer Foundation and the Cancer Control Agency of British Columbia. C.J.E. holds a Terry Fox Cancer Research Scientist award from the NCIC.

Address reprint requests to C.J. Eaves, PhD, Terry Fox Laboratory, 601 W 10th Ave, Vancouver, British Columbia, Canada V5Z 1L3.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

0006-4971/90/7501-0021$3.00/0

now clear that these are produced endogenously within the culture[12-14] at least in part by marrow-derived nonhematopoietic mesenchymal (stromal) cells, which constitute a major component of the heterogeneous population of adherent cells that are present in these cultures.[15-17] These mesenchymal cells appear to be essential for the maintenance and regulation of the more primitive types of hematopoietic progenitors that are also found almost exclusively within the adherent layer.[18]

We have previously identified two types of lineage-restricted hematopoietic progenitor subpopulations that, like pluripotent progenitors,[3] are normally quiescent in adult human marrow.[19] These progenitors can be distinguished in standard colony assays by their ability to generate very large colonies in vitro (more than 500 granulocytes and macrophages and more than eight clusters of erythroblasts, respectively). They are well-maintained in the adherent layer of human LTMC, where they undergo cyclic oscillations in their proliferative status triggered by each weekly change of the culture medium.[19] These observations suggested the potential of the LTMC system for further analysis of the molecular mechanisms mediating these changes in the cycling behavior of primitive progenitors. The experiments in this report support the hypothesis that both positive and negative factors are involved and that the net effect of these is determined by other regulatory molecules that act indirectly on hematopoietic cells by modulating marrow stromal cell accessory functions.

MATERIALS AND METHODS

Cells and reagents. Fresh normal bone marrow aspirate cells diluted in heparin were obtained with informed consent as leftover material from uninvolved diagnostic specimens or normal bone marrow transplant harvests. Human platelet-derived growth factor (PDGF) was purified on phenyl sepharose.[20] Antiserum to PDGF was prepared by immunization of a goat with purified human PDGF and purification of the plasma by Na_2SO_4 precipitation and DEAE-sephacel chromatography.[21] Medium conditioned by phytohemagglutinin-stimulated peripheral blood leukocytes (PHA-LCM) from normal individuals was prepared as described previously[22] and shown to contain a colony-stimulating activity equivalent to ~10 ng/mL of human GM-CSF. Pure recombinant human IL-1β (3×10^7 U/mg) and GM-CSF, both expressed in *Escherichia coli,* were provided by Biogen (Geneva, Switzerland). In a few experiments, human IL-1 obtained from Cistron (Pine Brook, NJ) was also used. As results with IL-1 from both sources were indistinguishable, they have not been indicated separately. The endotoxin used was from *E coli* (Sigma, St Louis, MO). Purified rat transforming growth factor-α (TGF-α) was purchased from Bachem (Torrance, CA); recombinant human IL-2 (3×10^6 U/mg, Amgen, Thousand Oaks, CA) was purchased from Amersham (Oakville, Ontario, Canada); and purified porcine TGF-β_1 was purchased from R and D Systems Ltd (Minneapolis, MN).

Long-term marrow culture. Each LTMC was initiated from a single inoculum of 2.5×10^7 unprocessed nucleated marrow aspirate cells suspended in 8 mL of long-term culture growth medium and placed in a 60-mm tissue culture dish. These cultures were placed in a humidified atmosphere containing 5% CO_2 in air at 37°C for the first 3 to 4 days and thereafter at 33°C. At weekly intervals, half of the medium and nonadherent cells were discarded and an equivalent volume of new medium added. When cultures were subjected to a "mock medium change," old medium and cells were removed and returned to the cultures with or without the addition of growth regulatory molecules, as indicated. LTMC growth medium was based on a slightly modified α-medium with 12.5% horse serum (HS), 12.5% fetal calf serum (FCS), 10^{-4} mol/L 2-mercaptoethanol, and 10^{-6} mol/L hydrocortisone sodiumhemisuccinate described in detail previously.[23] Adherent cells were detached and prepared as a single cell suspension for plating with trypsin.[23]

Colony assays for hematopoietic progenitors. Assays for clonogenic cells were performed in an Iscove medium-based methylcellulose culture medium containing 3 U/mL human erythropoietin and 10% (vol/vol) agar-stimulated human LCM used in this laboratory for many years.[24] After 18 to 21 days' incubation of the cultures at 37°C, erythroid, granulopoietic, and multi-lineage colonies were sized and evaluated in situ to infer origin from primitive and mature burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) (greater than 8, 3 to 8, and 1 to 2 clusters of erythroblasts/colony, respectively), primitive and mature CFU-GM (more than and $\leq$500 granulocytes and macrophages/colony, respectively) and CFU-GEMM (colonies containing both erythroid cells and granulocytes, and/or macrophages or megakaryocytes).[19,24] Very few CFU-E and CFU-GEMM were detected in assays of cells from LTMC as previously reported[11,23] and therefore could not be used to generate meaningful thymidine suicide values (see below). In some experiments (Tables 1 through 3), recombinant human GM-CSF or other factors were substituted for agar-LCM to assay for their ability to stimulate colony formation. All assays of LTMC adherent layer cells involved plating cells at a maximum final concentration of 10^5 cells/mL. Fresh marrow buffy coat cells were plated at 2×10^5 cells/mL (Table 2).

Thymidine suicide procedure. The cell cycle status of different classes of clonogenic cells was determined by exposing washed cells suspended in serum-free Iscove's medium to 20 μCi/mL of high specific activity ^{3}H-thymidine for 20 minutes before plating, and then comparing the final colony counts with results obtained in parallel assays of cells treated identically, except that ^{3}H-thymidine was omitted from the medium in which the cells were suspended during the 20-minute incubation. Procedures and reagents were the same as described in detail previously.[19] For convenience, all negative percent kill values (^{3}H-thymidine-treated values greater than control values) have been expressed in the Tables as zeros.

Assays for PDGF activity. Levels of PDGF were determined by a PDGF radio-receptor assay as described elsewhere.[25]

RESULTS

Origin of the activity in fresh medium that stimulates primitive progenitors in LTMC. A first series of experi-

Table 1. Mesenchymal Cell Activators Have Little Effect on Colony Formation by Primitive Progenitors From 4-Week-Old Long-Term Culture-Adherent Layers

Addition (concentration)	Primitive BFU-E		Primitive CFU-GM	
	Exp 1	Exp 2	Exp 1	Exp 2
None	9	6	0	0
PDGF (5 ng/mL)	7	8	0	0
PDGF (15 ng/mL)	10	7	0	0
IL-2 (6.7 ng/mL)	9	4	0	1
TGF-α (50 ng/mL)	11	6	0	0
IL-1 (0.4 ng/mL)	14	14	17	3
Agar-LCM (10% vol/vol)	21	13	21	9
GM-CSF (8 ng/mL)	26	9	67	12

Values shown represent the sum of colonies derived from the class of progenitor indicated scored in two replicate 1.1-mL assay cultures, each containing 10^5 suspended adherent layer cells.

Table 2. TGF-β Selectively Inhibits Colony Formation by Primitive Progenitors: Assays of Fresh Human Marrow

Additions*	Primitive Progenitors		Mature Progenitors	
	BFU-E	CFU-GM	BFU-E + CFU-E	CFU-GM
Agar-LCM	88	1	273	63
Agar-LCM + TGF-β	3	2	59	56
GM-CSF	67	15	212	57
GM-CSF + TGF-β	1	0	37	68

*Agar-LCM at 10% (vol/vol), GM-CSF at 8 ng/mL and TGF-β at 5 ng/mL.

ments was undertaken to identify the component(s) of fresh medium responsible for the activation of the quiescent high proliferative potential, granulopoietic, and erythroid progenitors that are present almost exclusively in the adherent layer of human LTMC 3 or more weeks after their initiation. In earlier studies we found that after a previous uninterrupted incubation period of 7 days, replacement of half of the medium with fresh medium stimulates the high-proliferative potential hematopoietic progenitors in the adherent layer to re-enter S-phase, and that this activation requires the addition of some new molecular species (or dilution of a negative regulator); ie, it cannot be achieved by simply removing and returning the old medium back into the culture (mock medium change). These experiments also ruled out both glutamine and hydrocortisone (two labile components of growth medium) as the responsible agents.[19] Therefore we then evaluated the possible role of the two different types of sera present.

As shown in Table 4, addition to established cultures of fresh growth medium in which the only modification was to substitute an additional 12.5% FCS for the usual HS component, failed to alter the cycling status of the primitive, quiescent progenitor cells present in the adherent layer. In contrast, in the same experiments, addition of new growth medium containing 25% HS and no FCS proved to be just as stimulatory as adding regular growth medium that contains HS and FCS in equal proportions. These findings show that all activity of the new medium can be attributed to a factor(s) that is present in HS and not in FCS.

Because it was possible that HS might generally contain higher levels of endotoxin than FCS, we measured these and also evaluated the effect of adding purified endotoxin from *E coli* to LTMC simultaneously with a mock medium change. Although the endotoxin level of the HS used in all of these experiments did prove to be higher than that present in the FCS used (0.4 ng/mL v <0.02 ng/mL by the E-toxate gel test; Sigma), the addition of up to 2,000 times more endotoxin than that present in a regular half-medium change failed to reproduce the effect of fresh HS in activating adherent layer progenitors (Table 4). It therefore seems unlikely that endotoxin contamination is the explanation for the activity present in HS.

Table 4. Horse Serum Is the Exclusive Origin of the Activity in Long-Term Culture Growth Medium That Induces Primitive Hematopoietic Progenitor Cycling

Treatment	Progenitor ^{3}H-Tdr Suicide Values (% kill after ^{3}H-Tdr)	
	Primitive BFU-E	Primitive CFU-GM
Pre-feed status (3 or 4 weeks)	1.0 ± 2.7(5)	7.0 ± 3.9(5)
2 to 3 Days after feeding:		
Regular medium change	45.0 ± 4.8(6)	51.8 ± 2.8(6)
Mock medium change	0 ± 2.6(5)	2.6 ± 2.9(5)
Regular medium change with 25% HS	38.0 ± 7.6(4)	46.0 ± 3.7(4)
Regular medium change with 25% FCS	5.3 ± 1.7(3)	7.0 ± 5.1(3)
Mock medium change plus endotoxin	0 ± 3.3(4)	0 ± 3.7(4)

Values shown are means ± 1 SEM from assessments of (n) experiments (each experiment representing a LTMC initiated with a marrow sample from a different individual). The four experiments with endotoxin used concentrations of 0.04 ng/mL (one experiment), 0.2 ng/mL (one experiment), and 0.1 μg/mL (two experiments). Results with all were similar and have been combined.

Effect of various mesenchymal cell activators on progenitor cycling in LTMC. In 1985, Delwiche et al[26] showed that PDGF, a well-known component of serum, could enhance the plating efficiency of suboptimally stimulated, clonogenic progenitors present in human marrow, but only by an indirect process that involved the activation of marrow mesenchymal elements to release colony-stimulating factors. This suggested that a similar indirect mechanism might explain the effects of HS in human LTMC. As a first test of this hypothesis, we evaluated the effect on progenitor cycling of adding either PDGF or IL-1β, another well-known mesenchymal cell-activating factor, to established LTMC 7 days after a previous regular half-medium change. In each case, cultures were given a mock medium change using the same protocol as in Table 4 and the growth regulatory molecule of interest (ie, PDGF or IL-1β) added at that time. Two to three days later, the cultures were sacrificed and the cycling status of the adherent clonogenic progenitor cells was then determined. As shown in Fig 1, addition of either PDGF (5 ng/mL) or IL-1 (0.4 ng/mL = 12 U/mL) mimicked the stimulating effect of a regular medium change. These doses

Table 3. TGF-β Selectively Inhibits Colony Formation by Primitive Progenitors: Assays of Cells From 4-Week-Old Long-Term Culture-Adherent Layers

Additions*	Primitive Progenitors				Mature Progenitors			
	BFU-E		CFU-GM		BFU-E		CFU-GM	
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Agar-LCM	21	13	21	9	10	2	69	34
Agar-LCM + TGF-β	0	0	6	1	0	0	36	27
GM-CSF	26	9	67	12	4	1	95	68
GM-CSF + TGF-β	0	0	0	0	0	0	14	55

*Agar-LCM at 10% (vol/vol), GM-CSF at 8 ng/mL, and TGF-β at 5 ng/mL.

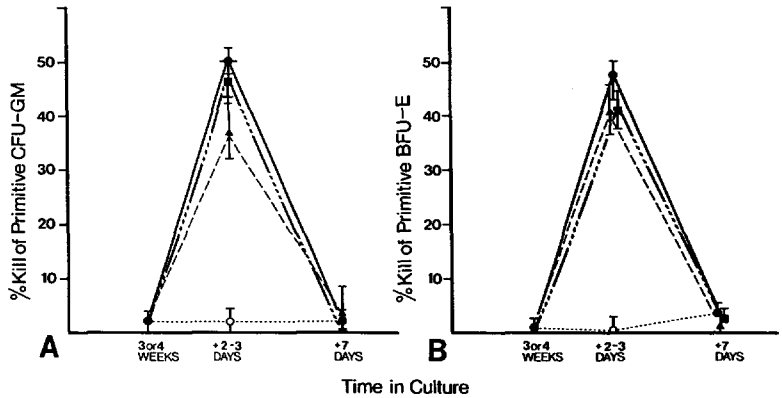

Fig 1. Thymidine suicide values for primitive BFU-E (A) and primitive CFU-GM (B) in the adherent layer of LTMC assessed at various times after feeding. Cells were suspended by trypsinization, immediately exposed to ^{3}H-thymidine (or not), and then plated in methylcellulose as described in Materials and Methods. Each point represents the mean of values from 3 to 13 experiments (each experiment being set up with a different marrow). (····○····), mock medium change; (— ●—), regular medium change; (--▲--), mock medium change plus 0.4 mg/mL IL-1; (····■····) mock medium change plus 5 ng/mL PDGF. All treatments at 3 or 4 weeks after initiation of LTMCs.

of PDGF and IL-1 were chosen because they have been shown to be sufficient for maximal or near-maximal stimulation of mesenchymal cells in other studies. The effect with both PDGF and IL-1, like that obtained with a regular medium change, although readily and consistently detected, was only transient and all primitive progenitors returned to a quiescent state 7 days later.

Assessment of the levels of PDGF in three of the batches of HS and three of the batches of FCS used in these experiments showed these to be 0.15 ± 0.04 ng/mL and 1.5 ± 0.6 ng/mL, respectively. Three days after a regular medium change, there was no detectable PDGF in the culture medium (<0.005 ng/mL, three experiments), and addition of a highly specific anti-PDGF antibody to cultures given a regular medium change at a concentration sufficient to inactivate 5 ng/mL of PDGF as shown in parallel controls failed to neutralize the stimulating effect of the fresh HS added (data not shown).

Two other growth regulatory molecules to which mesenchymal cells are known to respond, ie, TGF-α[27] and IL-2,[28] were then tested using the same protocol and found to be similarly effective in stimulating primitive hematopoietic cells in the LTMC system (Table 5).

In all of these experiments, there was no significant effect of any of the treatments on absolute progenitor numbers (either primitive progenitors or total progenitors) except where 0.1 μg/mL of endotoxin was added. (In this case, the total number of granulopoietic progenitors was frequently increased 2 to 7 days later, particularly in the nonadherent fraction; data not shown.) Neither was there any effect on the cycling status of the more mature progenitor cell types also present in the adherent layer of the same cultures. However, this would not have been readily detectable by this type of ^{3}H-thymidine suicide assay since such progenitors never showed a low percent kill at any time (less than 20%) (eg, see Table 6), as noted previously.[19]

Table 5. TGF-α and IL-2 Stimulation of Primitive Hematopoietic Progenitor Cycling in Long-Term Culture-Adherent Layers

Treatment	Progenitor ^{3}H-Tdr Suicide Values (% kill after ^{3}H-Tdr)		
	Primitive BFU-E	Primitive CFU-GM	No. of Experiments
Mock feed	3 ± 3	5 ± 2	3
Regular feed	42 ± 5	51 ± 5	3
Mock feed + TGF-α	44 ± 2	50 ± 8	3
Mock feed	2 ± 2	5 ± 2	7
Regular feed	42 ± 2	57 ± 3	7
Mock feed + IL-2	45 ± 4	66 ± 4	7

TGF-α was added to give a final concentration of 50 ng/mL and IL-2 was added to give a final concentration of 6.7 ng/mL. All LTMC were 3 or 4 weeks old at the time the treatments listed were tried, and ^{3}H-Tdr measurements were performed on clonogenic cells in the adherent layer harvested and assayed 2 to 3 days after the treatment specified. Each experiment refers to a series of LTMC set up with marrow from a different individual so that the effects of the various treatments listed could be compared.

Lack of effect of mesenchymal cell activators on progenitors in methylcellulose assays. Each of the four factors that had been shown to stimulate primitive quiescent progenitors in the adherent layer of LTMC was then tested for its potential to substitute for GM-CSF (or other hematopoietic growth factors present in agar-LCM) in stimulating colony-formation by the same progenitors after their suspension at a relatively dilute cell concentration in methylcellulose cultures. The results of two such experiments are shown in Table 1. It can be seen that none had colony-stimulating activity when tested at the same factor concentration in methylcellulose cultures as had proven stimulatory in the LTMC system.

Ability of TGF-β to prevent primitive progenitor cell activation in LTMC and methylcellulose assays. A number of groups have reported that TGF-β inhibits colony-formation by normal human hematopoietic progenitors, particularly under conditions that allow more primitive progenitor types to proliferate.[29,30] This suggested a parallelism with the effect of stromal cell containing adherent layers

Table 6. TGF-β Selectively Inhibits Cycling of Primitive Hematopoietic Progenitors in Long-Term Culture-Adherent Layers

Treatment	Percent Kill After ^{3}H-Thymidine			
	Primitive BFU-E	Primitive CFU-GM	Mature CFU-GM	No. Experiments
Mock feed	3 ± 3	2 ± 3	48 ± 8	3
Mock feed + TGF-β	2 ± 5	0 ± 5	49 ± 3	3
Regular feed	43 ± 2	58 ± 3	53 ± 3	5
Regular feed + TGF-β	0 ± 1	0 ± 4	42 ± 3	5
Mock feed + IL-1	46 ± 4	53 ± 3	45 ± 4	3
Mock feed + IL-1 + TGF-β	0 ± 4	0 ± 2	43 ± 3	3
Mock feed + IL-2	42 ± 4	62 ± 3	58 ± 4	3
Mock feed + IL-2 + TGF-β	0 ± 3	8 ± 3	45 ± 3	3

IL-1 at 0.4 ng/mL, TGF-β at 5 ng/mL, and IL-2 at 6.7 ng/mL. Other experimental details were as described in the footnote to Table 5.

on primitive (high proliferative potential) colony-forming cells in LTMC. We had previously shown that the selective, but reversible, suppression of cycling of this particular type of colony-forming cell was dependent on the presence of a stromal cell containing adherent layer.[18] To further investigate this possible parallelism, we first evaluated the ability of TGF-β to inhibit the formation in standard methylcellulose cultures of colonies of different sizes. As shown in Tables 2 and 3, the same types of primitive progenitors whose cycling can be down-regulated in the adherent layer of LTMC, as occurs normally in the marrow in vivo, were more susceptible to the inhibitory activity of TGF-β than were more mature classes of colony-forming cells, distinguished by their lower proliferative potential. Moreover, this differential effect of TGF-β was seen both for progenitors in fresh marrow aspirates and for progenitors harvested from the adherent layer of LTMC. These findings suggest that susceptibility to the negative regulatory effects of TGF-β decreases as primitive hematopoietic progenitor cells differentiate and lose their ability to become quiescent as their proliferative potential declines.

Next we investigated whether addition of TGF-β to LTMC could override or prevent the ability of a regular medium change, or IL-1 or IL-2, to stimulate primitive quiescent progenitors to enter S-phase. Results of such experiments are summarized in Table 6. TGF-β at 5 ng/mL totally blocked the activation of primitive progenitors in the adherent layer, and this was not due to an irreversibly toxic action of TGF-β on these cells as their numbers remained unchanged 2 to 3 days after the TGF-β was added (Table 7). Also shown in the experiments presented in Table 6 was a complete lack of effect of TGF-β on the cycling of the more mature hematopoietic progenitors present in the adherent layers of the same LTMCs.

DISCUSSION

We used the LTMC system to examine mechanisms regulating the proliferative status of very primitive hematopoietic progenitors that are present in normal adult human marrow as a predominantly quiescent population. Such progenitors can be readily identified as a high proliferative potential subgroup of the erythropoietic (BFU-E) and granulopoietic (CFU-GM) colony-forming cells detected in standard methylcellulose assays by the application of appropriate colony-sizing criteria to distinguish large and small colonies after 18 to 21 days of colony growth.[19] We previously showed that very few progenitors of the largest colonies are found in the nonadherent layer of established human LTMC, but are retained in substantial numbers in the adherent layer.[23] There they undergo a periodic oscillation in their cycling behavior that correlates with the timing of the routine weekly medium change.[19] In suspension cultures initiated with an adequate source of primitive hematopoietic cells, but not containing any stromal elements or their precursors, progenitors of large hematopoietic colonies may persist in detectable numbers for a few weeks. However, under these conditions they remain continuously in cycle as their numbers decline.[18] These results suggest that an adherent layer containing stromal cells may be required for both the maintenance and proliferation control of high proliferative potential progenitors in the LTMC system.

Table 7. TGF-β Addition to Long-Term Marrow Cultures Has No Effect on Primitive Hematopoietic Progenitor Numbers

Treatment	No. of Primitive Progenitors/Adherent Layer	
	BFU-E	CFU-GM
Mock feed	122	101
Mock feed + TGF-β (5 ng/mL)	96	99
Regular feed	62	22
Regular feed + TGF-β (5 ng/mL)	101	71

Data from one representative LTMC experiment. Other experimental details were as described in the footnote to Table 5.

Experiments reported here indicate that the proliferation of primitive progenitors in LTMC is normally dependent on the addition of fresh HS. However, a variety of growth-regulatory molecules, all with known abilities to activate metabolic events in fibroblasts and/or endothelial cells, were found to mimic the effects of fresh HS on primitive progenitor cell cycling in established LTMC after their addition in concert with a mock medium change. The defined factors shown here to stimulate the entry into S-phase of primitive, quiescent hematopoietic cells in LTMC were PDGF, IL-1, TGF-α, and IL-2. Because the levels of PDGF in fresh HS and FCS did not correlate with the progenitor activating effects of the latter in the LTMC system, and since neutralizing anti-PDGF antibody did not inhibit the effect of fresh HS, it does not seem likely that the effect of HS is due to its content of PDGF. On the other hand, since we have now shown that multiple factors can mediate the activation of progenitors in this system, establishment of the identity of the activity in HS may be a difficult task.

None of the mediators shown to stimulate primitive hematopoietic progenitors in the adherent layer of LTMC appeared to act directly, as shown by their lack of colony-stimulating ability in methylcellulose assays (Table 1). One possibility is that their stimulatory action in the LTMC system is mediated by an ability to alter the growth factor output profile of other cells in the culture. PDGF and IL-1 are known to cause dramatic increases in hematopoietic growth factor gene expression in a number of mesenchymal cell types.[6,7] Evidence that such changes correlate with alterations in the cycling activity of the most primitive hematopoietic progenitor cell types will be presented later.[14] Corresponding information for TGF-α and IL-2 is not yet available. Since T cells are known to persist in LTMC of human origin for many weeks,[31] it is possible that the effect seen with IL-2 is mediated by these cells rather than by IL-2 activation of mesenchymal cells. Additional experiments with more defined populations of regulatory and responder cells will be required to define the cell types involved in each case.

This study suggests that the cycling status of the primitive hematopoietic progenitors in the LTMC system at any given time reflects the overall balance of the positive and negative regulatory factors present at that moment. TGF-β appears to be able to exert its negative regulatory effect on primitive hematopoietic cells directly and, if present at a high enough concentration, can reversibly block the stimulation that

would otherwise be initiated in the LTMC system by even the most potent mesenchymal cell activators tested (Table 6).

In summary, we have described how the LTMC system can be exploited to examine the regulatory network that may control the recruitment of primitive hematopoietic progenitors to allow subsequent production of large cohorts of mature cells. Data suggest that this may be initiated by a variety of agents that influence mesenchymal cell metabolism resulting in an alteration in the balance of positive and negative regulators in the microenvironment surrounding adjacent primitive hematopoietic cells. After their activation and subsequent differentiation, the progeny of these cells may be less able to re-enter a G_0 state. Regulation of their capacity for mature cell output would then be more simply achieved by provision or deprivation of essential positive growth factors leading to a corresponding increase or decrease in the viability and continuing differentiation of these cells along a particular pathway.

ACKNOWLEDGMENT

The authors thank K. Lambie and D. Reid for excellent technical assistance and C. Freer for typing the manuscript. The generous gift of IL-1β and GM-CSF from Biogen is also gratefully acknowledged.

REFERENCES

1. Becker AJ, McCulloch EA, Siminovitch L, Till JE: The effect of differing demands for blood cell production on DNA synthesis by hematopoietic colony-forming cells of mice. Blood 26:296, 1965

2. Pietrzyk ME, Priestly GV, Wolf NS: Normal cycling patterns of hematopoietic stem cell subpopulations: An assay using long-term in vivo BrdU infusion. Blood 66:1460, 1985

3. Messner HA, Fauser AA, Lepine J, Margin M: Properties of human pluripotent hemopoietic progenitors. Blood Cells 6:595, 1980

4. Golde DW, Takaku F: Hematopoietic Stem Cells. New York, NY, Dekker, 1985

5. Seiler FR, Schwick HG: Colony Stimulating Factors–CSF–, vol 83. Marburg, West Germany, Die Medizinische Verlagsgesellschaft mbH, 1988

6. Nathan CF: Secretory products of macrophages. J Clin Invest 79:319, 1987

7. Clark SC, Kamen R: The human hematopoietic colony-stimulating factors. Science 236:1229, 1987

8. Stanley ER, Bartocci A, Patinkin D, Rosendaal M, Bradley TR: Regulation of very primitive, multipotent, hemopoietic cells by hemopoietin-1. Cell 45:667, 1986

9. Moore MAS, Warren DJ: Synergy of interleukin 1 and granulocyte colony-stimulating factor: In vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc Natl Acad Sci USA 84:7134, 1987

10. Neta R, Sztein MB, Oppenheim JJ, Gillis S, Douches SD: The in vivo effects of interleukin 1. I. Bone marrow cells are induced to cycle after administration of interleukin 1. J Immunol 139:1861, 1987

11. Eaves AC, Cashman JD, Gaboury LA, Eaves CJ: Clinical significance of long-term cultures of myeloid blood cells. CRC Crit Rev Oncol Hematol 7:125, 1987

12. Gordon MY, Riley GP, Watt SM, Greaves MF: Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326:403, 1987

13. Eaves AC, Eaves CJ: Maintenance and proliferation control of primitive hemopoietic progenitors in long-term cultures of human marrow cells. Blood Cells 14:355, 1988

14. Humphries RK, Kay RJ, Dougherty GJ, Gaboury LA, Eaves AC, Eaves CJ: Growth factor mRNA in long-term human marrow cultures before and after addition of agents that induce cycling of primitive hemopoietic progenitors. Blood 72:121a, 1988 (abstr, suppl 1)

15. Dukes PP, Izade P, Ortega JA, Shore NA, Gompertz E: Inhibitory effects of interferon in mouse megakaryocytic progenitor cells in culture. Exp Hematol 8:1048, 1980

16. Perkins S, Fleischman RA: Hematopoietic microenvironment: Origin, lineage, and transplantability of the stromal cells in long-term bone marrow cultures from chimeric mice. J Clin Invest 81:1072, 1988

17. Simmons PJ, Przepiorka D, Thomas ED, Torok-Storb B: Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature 328:429, 1987

18. Eaves AC, Cashman JD, Gaboury LA, Kalousek DK, Eaves CJ: Unregulated proliferation of primitive chronic myeloid leukemia progenitors in the presence of normal marrow adherent cells. Proc Natl Acad Sci USA 83:5306, 1986

19. Chasman J, Eaves AC, Eaves CJ: Regulated proliferation of primitive hematopoietic progenitor cells in long-term human marrow cultures. Blood 66:1002, 1985

20. Raines EW, Ross R: Platelet-derived growth factor. I. High yield purification and evidence for multiple forms. J Biol Chem 257:5145, 1982

21. Cashman J, Henkelman D, Humphries K, Eaves C, Eaves A: Individual BFU-E in polycythemia vera produce both erythropoietin dependent and independent progeny. Blood 61:876, 1983

22. Raines EW, Dower SK, Ross R: Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science 243:393, 1989

23. Coulombel L, Eaves AC, Eaves CJ: Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. Blood 62:291, 1983

24. Eaves CJ, Eaves AC: Erythropoietin (Ep) dose-response curves for three classes of erythroid progenitors in normal human marrow and in patients with polycythemia vera. Blood 52:1196, 1978

25. Bowen-Pope DF, Ross R: Methods for studying the platelet-derived growth factor receptor. Methods Enzymol 109:69, 1985

26. Delwiche F, Raines E, Powell J, Ross R, Adamson J: Platelet-derived growth factor enhances in vitro erythropoiesis via stimulation of mesenchymal cells. J Clin Invest 76:137, 1985

27. Derynck R: Transforming growth factor-α. Cell 54:593, 1988

28. Frasier-Scott K, Hatzakis H, Seong D, Jones CM, Wu KK: Influence of natural and recombinant interleukin 2 on endothelial cell arachidonate metabolism: Induction of de novo synthesis of prostaglandin H synthase. J Clin Invest 82:1877, 1988

29. Sing GK, Keller JR, Ellingsworth LR, Ruscetti FW: Transforming growth factor β selectively inhibits normal and leukemic human bone marrow cell growth in vitro. Blood 72:1504, 1988

30. Ottmann OG, Pelus LM: Differential proliferative effects of transforming growth factor-β on human hematopoietic progenitor cells. J Immunol 140:2661, 1988

31. Touw I, Lowenberg B: Production of T-lymphocyte colony-forming units from precursors in human long-term bone marrow cultures. Blood 64:656, 1984