Long-Term Culture-Initiating Cell Expansion Is Dependent on Frequent Medium Exchange Combined With Stromal and Other Accessory Cell Effects

By Manfred R. Koller, Mahshid A. Palsson, Ilana Manchel, and Bernhard P. Palsson

Despite considerable effort, the expansion of long-term culture-initiating cells (LTC-ICs) in cultures of purified hematopoietic cells has not yet been achieved. In contrast, LTC-IC expansion has been attained in cultures of bone marrow mononuclear cells (MNC) using frequent medium exchange. The use of frequent medium exchange was, therefore, examined in cultures of CD34+ cells. In stromal-free, CD34-enriched cell cultures, medium exchange intervals ranging from 2 days to no feeding for 14 days gave similar results. Six different growth factor combinations, reported by other groups to give optimal expansion of CD34+ cells, were tested in comparison with the control combination of IL-3/GM-CSF/IL-7. None of the combinations resulted in improved colony-forming unit-granulocyte macrophage (CFU-GM) expansion or LTC-IC maintenance, although two were equivalent. All stromal-free cultures resulted in loss of LTC-IC to half of input. Because of the limited effect of medium exchange and growth factor variations on CD34+ cell cultures, the effect of preformed stroma was next examined. Preformed stroma increased cell (3-fold), CFU-GM (5-fold), and LTC-IC (3-fold) output, but only when the medium was exchanged every other day. Under these conditions, the number of LTC-IC was maintained near input level. The lack of LTC-IC expansion in CD34-enriched cell cultures prompted experiments to examine the effect of cell purification. Parallel cultures were performed at CD34+ cell purities of 20%, 40%, 70%, and 95%, with each well containing exactly 4,000 CD34+ cells in addition to the CD34+ accessory cells required to give the desired percentage. Also, MNC from the same source (~2% CD34+) were cultured at a concentration to give 4,000 CD34+ cells per well. As CD34+ cell purity was decreased from 95% to 2%, the output of cells, CFU-GM, and LTC-IC increased by threefold to fivefold. The loss of culture performance with purification was likely due to the removal of important accessory cells, because the levels of endogenously produced leukemia inhibitory factor and IL-6 were found to decline significantly with increasing CD34+ cell purity. In summary, preformed stroma abrogated the decrease in cell and CFU-GM output from cultured CD34-enriched cells and led to LTC-IC maintenance. In contrast, MNC inocula resulting in a growing stromal layer during the culture led to LTC-IC expansion (3.2-fold). The results suggest that LTC-IC expansion is dependent on accessory cells that are present in MNC, whose function is, in turn, dependent on frequent medium exchange.

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The expansion of human hematopoietic cells for clinical applications has recently been the focus of considerable research. Much of this effort has been directed towards the expansion of CD34+ cells because hematopoietic cells, from the stem through progenitor stages, are thought to express the CD34 antigen. Because of the rarity of CD34+ cells and the difficulty in obtaining them in large numbers, most cultures have been limited to a very small scale (eg, 96-well plates). These small-scale cultures of purified CD34+ cells, and even smaller subsets (eg, CD33+, CD38+), have yielded valuable information about hematopoietic stem cell biology. There is now very little doubt that the most prolific hematopoietic cells are contained within the CD34+ population and are concentrated in even smaller subsets of this compartment. This knowledge, along with the development of methods for large-scale enrichment of CD34+ cells, has led to the scale-up of cultures for the expansion of CD34-enriched cells. Although CD34-enriched cell cultures yield large expansions of total cells, colony-forming unit-granulocyte macrophage (CFU-GM) expansions have been considerably smaller and more variable. In addition, data on stem cell maintenance, in the form of long-term culture-initiating cell (LTC-IC) numbers, indicate that stem cell numbers often decline in stromal-free growth factor-supplemented cultures of CD34-enriched cells. The differentiation of CD34+ cells in these cultures has also been documented by flow cytometry, showing that the majority of cells rapidly lose CD34 expression and become positive for mature cell antigens.

In contrast to the numerous static culture studies performed with purified cells, continuously perfused bioreactors inoculated with bone marrow (BM) mononuclear cell (MNC) populations have shown significant expansion of cells, CFU-GM, and also 5-week and 8-week LTC-IC from adult BM. A recent report has also described LTC-IC expansion from BMMNC in a stirred bioreactor system using cumulative counting over a 28-day culture period. These results show that purification of CD34+ cells is not required to achieve cell expansion. In fact, each stage of cell purification results in a loss of primitive cells that significantly decreases the net expanded cell yield from a given volume of BM aspirate. However, there are circumstances in which cell purification before expansion may occur. For example, tumor cell purging and gene therapy procedures may result in, or be facilitated by, populations that are enriched for stem cells. Therefore, the ability to expand enriched cell populations, while maintaining/expanding the stem cell compartment, is of significant interest.

In cultures of BMMNC, increased medium perfusion rates have been used to obtain stromal-dependent LTC-IC expansion. These data, and the reported inability to expand...
**Table 1. Growth Factor Combinations and Concentrations Tested**

<table>
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<th>Factors</th>
<th>A</th>
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LTC-IC in nonperfused, stromal-free cultures of purified CD34<sup>+</sup> cells, 10–17 raise a number of important questions that warrant study. First, the effect of frequent medium exchange on CD34-enriched cell cultures needs to be delineated. Second, the effect of preformed stroma on CD34-enriched cell cultures, in the context of frequent medium exchange, must be examined. Third, different growth factor combinations, each reported to be optimal for cell and CFU-GM expansion by different investigators, 5,9,10,27 need to be compared with respect to LTC-IC maintenance. Fourth, the effect of CD34<sup>+</sup> cell purity on culture output needs to be studied in a controlled fashion. The effect of cell purity is an important issue, because most large-scale purification techniques that would be used in clinical applications do not result in a consistently pure cell product. The present study addresses these four issues, with emphasis on the fate of the LTC-IC population under the different culture conditions.

**MATERIALS AND METHODS**

**Medium and Growth Factors**

Long-term bone marrow culture (LTBMC) medium was prepared by supplementing Iscove's modified Dulbecco's medium (IMDM) with 10% horse serum, 10% fetal bovine serum (FBS), 4 mmol/L L-glutamine, 100 μg/mL penicillin, and 100 U/mL streptomycin (all from GIBCO, Grand Island, NY), and 5 μmol/L hydrocortisone (Sigma, St Louis, MO).

Growth medium was prepared by supplementing LTBMC medium with recombinant human growth factors. The different combinations and concentrations of growth factors that were used are shown in Table 1. Some of these combinations have been reported as being beneficial for the expansion of CD34-enriched cells from either BM or mobilized peripheral blood. Growth factors used included: interleukin-1 (IL-1), IL-3, IL-6, and interferon-γ (all from R&D Systems, Minneapolis, MN); granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and IL-3 (all from Immunex, Seattle, WA); and erythropoietin (Epo) and granulocyte colony-stimulating factor (G-CSF, Amgen, Thousand Oaks, CA).

**Cell Source and Separation Procedure**

Human BM cells were obtained from iliac crest aspirates, or from BM processing screens (Fenwal, Deerfield, IL) obtained after the harvest of BM from normal donors. Cells were diluted with LTBMC medium and layered onto Ficol (1.077 g/mL; Pharmacia, Uppsala, Sweden). MNC from the interface band were collected after centrifugation at 300g for 20 minutes at 25°C. MNC were washed twice in LTBMC medium, and were counted on an electronic cell counter (Coulter Electronics, Hialeah, FL). A fraction of the MNC were set aside, and the remainder were processed with a MACS laboratory separation system (Miltenyi Biotec, Sunnyvale, CA) according to manufacturer instructions.

**Culture System With Frequent Medium Exchange**

The benefits of frequent medium exchange have been previously reported for BMMNC cultures. 5,27 This culture system was adapted here for studies of CD34-enriched cell expansion. Cells were cultured in 1 mL in 24-well plates (Costar, Cambridge, MA), both with and without the presence of irradiated preformed stroma. Preformed stroma was prepared by trypsinizing (GIBCO) adherent stromal cells from 2-week old primary human BM cultures in LTBMC medium. Cells were irradiated with 20 Gy from a ¹³³Cs source, and were immediately plated at 5 × 10⁴ per well in LTBMC medium. Stroma was maintained for up to 2 weeks with weekly medium exchange at 33°C before use. CD34-enriched cells in growth medium were added to either empty wells or wells with preformed stroma, and were harvested 14 days later. Nonadherent cells were removed, and each well was washed with 0.25 mL phosphate-buffered saline (PBS: GIBCO). Wash cells were pooled with the nonadherent cells, and 0.25 mL trypsin was added to each well. After 10 minutes, adherent cells were collected by vigorous pipetting of trypsin and washing each well with 0.25 mL LTBMC medium. Adherent cells were pooled with the nonadherent and wash fractions, and each tube was then centrifuged at 300g for 7 minutes at 4°C. Supernatant was discarded, and cells were resuspended in LTBMC medium for counting and assays.

**Medium exchange interval.** To establish the optimal feeding protocol with this culture system, wells containing 5,000 CD34-enriched cells (both with and without preformed stroma) were fed with a 50% medium exchange at various intervals. These experiments were conducted with growth medium containing IL-3, GM-CSF, SCF, and Epo (3GM/SCF/Epo, column G in Table 1). An effort was made to remove as few cells as possible at each feeding by removing medium from the top of the meniscus. Inspection of the spent medium showed that very few cells were removed with this feeding technique.

**Effect of growth factors and preformed stroma.** Cultures were established as described above using growth medium containing the different growth factor combinations shown in Table 1. These experiments utilized 5,000 CD34-enriched cells per well (both with and without preformed stroma) and were fed with a 50% medium exchange every other day.

**Effect of CD34-enriched cell purity.** Because of the variability in CD34-enriched cell purity from the MACS column, the effect of cell purity on culture performance was investigated. Cells were plated so that each well contained exactly 4,000 CD34<sup>+</sup> cells as determined by flow cytometry (see below). For example, when CD34-enriched cells were 40% pure, 10,000 cells were plated containing 4,000 CD34<sup>+</sup> cells. From the same BM aspirate, the CD34<sup>+</sup> content of the MNC and CD34-depleted cells was also determined. MNC were plated to give 4,000 CD34<sup>+</sup> cells per well within the MNC mixture (eg, 200,000 MNC per well at 2% CD34<sup>+</sup> cell purity). To obtain purities higher than those provided by the CD34-enriched fraction, cells were sorted by flow cytometry (see below). To obtain purities lower than those provided by the CD34-enriched fraction, CD34-depleted cells were mixed with CD34-enriched cells to achieve the desired purity. The number of CD34<sup>+</sup> cells within the CD34-depleted fraction was included in calculating the 4,000 CD34<sup>+</sup> cells per well. In this manner, wells containing 4,000 CD34<sup>+</sup> cells at ~2% (MNC), 20%, 40%, 70%, and 95% purity were cultured in parallel from a single BM aspirate. In these experiments, cells were placed in growth medium containing...
3GM/SCF/Epo (both with and without preformed stroma), and wells were fed every other day.

**Flow Cytometry Analysis and Cell Sorting**

Cells to be analyzed were washed and resuspended in PBS containing 1% bovine serum albumin (BSA; Intergen, Purchase, NY). On melting ice, tubes containing 10^6 cells in 0.5 mL were stained with a cocktail of lineage (lin)-specific antibodies: FITC-Leu4 (anti-CD3), FITC-Leu16 (anti-CD20), FITC-LeuM1 (anti-CD15; all from Becton Dickinson, San Jose, CA), FITC-anti-CD11b (Serotec, Indianapolis, IN), and FITC-antilymphoglycopin A (Dako, Carpinteria, CA). The lin stain was combined with either PE-HPCa-2 (anti-CD34) or PE-IgG control monoclonal antibodies (Becton Dickinson). After 20 minutes, cells were washed and resuspended in 0.5 mL PBS/BSA for flow cytometry. FACS Vantage and FACScan flow cytometers (Becton Dickinson) were used in these studies.

In some experiments, CD34-enriched cells from the MACS column were further enriched for CD34 lin cells by fluorescence-activated cell sorting (FACS). Cells stained with the antibodies described above were sorted directly into 24-well plates using the Automated Cell Deposition Unit (ACDU). The ACDU was tested by sorting 1, 10, 20, or 50 fluorescent beads (Calibrite; Becton Dickinson) per well in a series of wells. The actual number of beads in each of the 248 wells was then counted with a fluorescent microscope (Nikon, Garden City, NY), and was found to be accurate in 94.8% of the wells. In the other 13 wells, 11 were off by just 1 bead and 2 were off by 2 beads. CD34'lin cells sorted by this technique were reanalyzed on the flow cytometer, and were found to be 95% to 97% pure.

**Methylcellulose Colony Assays**

Cells were inoculated in colony assay medium containing 0.9% methylcellulose (Dow, Midland, MI), 30% FBS, 1% BSA, 100 μg/ml L-2-mercaptoethanol (Sigma), 2 mM/l glutamine (GIBCO), 5 ng/ml PIXY321, 5 ng/ml G-CSF, and 10 U/ml Epo. CD34-enriched cells were plated at 200 to 600 per ml because of their high CFU-GM cloning efficiency (average 14%). Fresh MNC and all expanded cells were plated at 1 to 2 X 10^5 per ml depending on their expected clonogenicity. Aliquots of 1 mL were plated in duplicate in gridded 35-mm dishes (Nunc, Naperville, IL) for 14 days under fully humidified conditions in an atmosphere of 5% CO2 and air at 37°C. CFU-GM-derived colonies of greater than 50 cells were scored with a darkfield stereo microscope (Nikon).

**LTC-IC Assay**

LTC-IC were determined by culture on irradiated stroma with modification of the previously described technique.23 Irradiated stromal layers were prepared in 24-well plates as described above. CD34-enriched cells were added at 2,000 to 10,000 cells per well (depending on their purity), MNC were added at 2 to 5 X 10^5 cells per well (depending on their purity), and cultured cells were added at 2 to 10 X 10^5 cells per well (3 to 6 replicates each). Plates were maintained at 33°C in a fully humidified atmosphere of 5% CO2 in air, and cultures were fed weekly by replacing 0.5 mL LTBMG medium per well. At week 5, adherent and nonadherent cells were harvested from each well as described above. All cells from each well were plated directly into methylcellulose colony assay as described above. For each sample, the number of secondary colonies was enumerated and used as a measure of the number of LTC-IC present in the sample.30

**Measurement of Growth Factor Production**

Growth factor concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to manufacturer instructions. Sensitivities reported by the manufacturer were 0.35 pg/ml and 2.0 pg/ml for the IL-6 and leukemia inhibitory factor (LIF) assays, respectively. Standard concentration curves with 8 points were generated with each ELISA kit run. The standard curves were very consistent and curvefits typically had high regression coefficients (not shown). Samples were measured in duplicate, and were properly diluted to ensure that measured values were within the standard concentration curve range.

**Expansion Variability, Normalization of Data, and Statistical Analysis**

In the course of these studies, BM from 25 normal donors was CD34-enriched and expanded in vitro. The CD34-enrichment technique was variable, yielding an average of 62% purity (range, 32 to 91). The level of cell expansion obtained also varied considerably from donor to donor, and an average of 417-fold expansion (range, 63 to 1,110) was obtained under the standard control conditions of every other day medium exchange, without stroma, with 3/GM/SCF/Epo. The number of cells generated in culture did not correlate with the purity of cells used to initiate the culture.11 Within a given experiment, the relative change in cell expansion as a function of the independent variable being tested was reproducible. Therefore, to compare data from different experiments, results within each experiment were normalized with respect to the common control group. This normalization of data showed consistent relative effects of different variables on cell expansion. Therefore, data are reported as normalized values, and a value of 1 corresponds to the results obtained from CD34-enriched cells cultured in 3/GM/SCF/Epo without stroma and fed every other day. The actual number of cells, CFU-GM, and LTC-IC that correspond to the normalized value of 1 are given in each figure legend.

Normalized data were analyzed using Student's t-test. Points which were significantly different from control within each figure are marked with either 1 (P < .05), 2 (P < .01), or 3 (P < .001) asterisks.

**RESULTS**

**Effect of Medium Exchange Interval and Stroma**

CD34-enriched cells were cultured in growth medium containing 3/GM/SCF/Epo, both with and without preformed stroma. Fifty percent of the medium in each well was exchanged at intervals of 2, 3, or 7 days, or was not exchanged at all (ie, 14-day interval). Without stroma, CD34-enriched cell expansion was not significantly affected by the medium exchange interval within the range tested (Fig 1A). Similarly, production of CFU-GM and LTC-IC from CD34-enriched cultures was not influenced by the medium exchange interval (Fig 1B and C, respectively).

However, on preformed stroma, decreasing the medium exchange interval to every other day increased the production of total cells, CFU-GM, and LTC-IC (Fig 1). Total cell production increased by 60% (P < .005; Fig 1A) as compared with unfed cultures. No exchange of medium during the 14 days was found to give the same level of expansion as feeding once at day 7 (P = .71). The effect of medium exchange interval on CFU-GM expansion was similar to that for total cell expansion (Fig 1B), whereas LTC-IC were more dramatically affected by the medium exchange interval (Fig 1C). On preformed stroma, every other day feeding resulted in a 1.7-fold increase (P < .05) in LTC-IC numbers as compared with unfed cultures.

The effect of preformed stroma was significant at all me-
Effect of feeding interval and preformed stroma on CD34-enriched cell culture output. Data obtained in each of seven independent experiments were normalized by the values obtained from CD34-enriched cells grown in 3/GM/SCF/Epo without stroma and fed every other day, and then combined. Average normalized (A) total cells, (B) CFU-GM, and (C) LTC-IC numbers are shown. A normalized value of 1 corresponded to 1.23 x 10^6 cells (246-fold expansion), 1,939 CFU-GM (4.7-fold), and 22 LTC-IC (0.4-fold) per well.

Effect of Growth Factor Combination

A number of different optimal growth factor combinations for the growth of CD34-enriched cells have been reported. Different growth factor combinations (summarized in Table I) were therefore compared, using the 3/GM/SCF/Epo combination as the normalizing control within each experiment. A wide range of cell output was observed (Fig 2A), with the lowest in 1/3/6/SCF/Epo/IFN (25% of control, P < .005) and the highest in 3/6/SCF/Epo (330% of control,
Removal of IFN from the lowest performing combination resulted in an increase in cell output to 145% of control ($P < .01$). Cell output using the PIXY/SCF combination was 37% of control ($P < .005$). The combinations of 1/3/6/GM/G/SCF and 1/3/6/GM/G/SCF/Epo were not significantly different than control.

CFU-GM numbers generated by the different growth factor combinations were surprisingly similar (Fig 2B). In fact, except for the 1/3/6/SCF/Epo/IFN combination (10% of control, $P < .001$), no combinations were significantly different from the control combination. However, there were differences observed in the quality of CFU-GM colonies formed from cells cultured in the different growth factor combinations. In general, combinations resulting in higher cell expansions had the poorest quality colonies, in terms of both size and diversity of lineages present (not shown). The different growth factor combinations varied in their ability to support LTC-IC maintenance (Fig 2C). The 1/3/6/SCF/Epo/IFN, PIXY/SCF, and 1/3/6/GM/G/SCF combinations resulted in LTC-IC numbers that were significantly less than control. The 1/3/6/GM/G/SCF/Epo combination gave LTC-IC numbers that were 68% of control, but the result did not reach statistical significance ($P = .12$). In contrast, the combination of 3/6/SCF/Epo gave LTC-IC maintenance that was very similar to control. Importantly, LTC-IC numbers declined from input in all of these CD34-enriched cell cultures, regardless of the growth factor combination used.

Effect of Stroma With Different Growth Factor Combinations

Stroma was observed to have a significant positive effect on the output of cells, CFU-GM, and LTC-IC using the control growth factor combination (Fig 1), whereas other growth factor combinations did not increase CFU-GM or LTC-IC numbers above control (Fig 2). The effect of stroma was, therefore, tested with the best combination of Fig 2 (3/6/SCF/Epo) in addition to the control combination (3/GM/SCF/Epo). Cell output on preformed stroma (~320% of the control combination without stroma) was identical with both growth factor combinations (Fig 3A). Therefore, stroma increased cell expansion with the control combination, but offered no additional increase with the 3/6/SCF/Epo combination.

In the presence of stroma, CFU-GM output (~470% of the control combination without stroma) was very similar in the different growth factor combinations (Fig 3B). Therefore, preformed stroma increased both cell and CFU-GM output, whereas the combination of 3/6/SCF/Epo increased only cell output. Furthermore, the quality of CFU-GM colonies was always better from cultures containing preformed stroma (not shown). This effect was not due to the presence of stromal cells in the colony assay, because the direct addition of stromal cells to colony assays of cells derived from stromal-free cultures did not have a significant effect (not shown). The effect of stroma on LTC-IC numbers was similar to the effect on CFU-GM (Fig 3C). Preformed stroma had a significant positive effect (~3-fold) on LTC-IC numbers, regardless of the growth factor combination used. This increase over stromal-free cultures resulted in the maintenance of LTC-IC in cultures on preformed stroma.

Effect of CD34+lin Cell Purity on Culture Performance

Because of the large variability in CD34+lin cell purities obtained from the purification procedure, and the lack of
LTC-IC expansion obtained in cultures of CD34-enriched cells, the effect of cell purity on culture performance was directly investigated. Flow cytometry was used to sort 4,000 CD34"lin^{-} cells into each well, and varying numbers of CD34-depleted cells were added back to the cultures to give CD34 purities ranging from 20% to 95%. In addition, MNC from the same four donors were plated to give 4,000 CD34"lin^{-} cells in each well (at an average purity of 2%). These cultures were performed with 3/GM/SCF/Epo, both with and without preformed stroma.

The cultured cell output decreased as the initial purity of the CD34"lin^{-} cells was increased, even though the number of CD34"lin^{-} cells within each well was initially the same in all cultures (Fig 4A). Purification to only 20% CD34"lin^{-} cells resulted in a 50% decline in cell output ($P < .01$), and this decline increased to a 65% decline at 95% cell purity ($P < .01$). The calculated cell expansion ratio was greater from the 95% purity group (425-fold) than from the 2% (MNC) group (24-fold). However, because all groups initially contained the same number of CD34"lin^{-} cells, the MNC group gave the greatest output per initial CD34"lin^{-} cell present. Therefore, the calculated expansion ratio is not an appropriate measure for comparing culture output from cell populations with different initial compositions.

The effect of increasing CD34"lin^{-} purity on CFU-GM output was more pronounced than the effect on cell output. CFU-GM output decreased by 58% at 20% purity ($P < .005$) and by 72% at 95% purity ($P < .005$, Fig 4B). The influence of CD34"lin^{-} cell purity on LTC-IC output was even more dramatic (Fig 4C). Purification to only 20% resulted in a 66% decline in LTC-IC output ($P < .01$), and this decline enlarged to 78% at 95% cell purity ($P < .0001$). The output of LTC-IC from the MNC cultures represented a net LTC-IC expansion (threefold), whereas the most purified cell cultures experienced a 30% decline in LTC-IC numbers.

Preformed stroma altered the effects of CD34-enrichment. The addition of preformed stroma abrogated the loss in culture performance with respect to cell and CFU-GM output (Fig 4A and B). However, LTC-IC output was only partially restored by the addition of preformed stroma (Fig 4C). Therefore, cell purification resulted in a loss of the ability to expand LTC-IC, and preformed stroma increased LTC-IC output only to the level of LTC-IC maintenance.

Cell Purification Eliminated Endogenous Growth Factor Activity

The results above suggested that CD34-enrichment was accompanied by a loss of accessory cell function, thereby resulting in reduced culture output. To further examine this hypothesis, spent media from the different cultures were analyzed for growth factor content using ELISA. Endogenous production of IL-6 and LIF was significant in the MNC cultures (Figs 5A and 6A), and showed a characteristic profile that changed with time as previously described.\(^\text{31}\) As CD34"lin^{-} cell purity was increased, endogenous IL-6 production decreased, and was actually undetectable from sorted CD34"lin^{-} cells. LIF production was below the level of detection (2 pg/mL) from all cultures of CD34-enriched cells. Therefore, CD34-enrichment resulted in elimination of endogenous growth factor production by accessory cells. In contrast to both the MNC and CD34-enriched cell cultures, cultures with preformed stroma produced very high levels of both IL-6 and LIF (Figs 5B and 6B). By this measure, the culture environment provided by preformed stroma differed significantly from that provided by MNC.

Composition of Culture Inoculum Determined the Fate of LTC-IC in Culture

An ex vivo culture system may result in one of three outcomes with respect to LTC-IC production: loss, mainte-
DISCUSSION

The ability to expand primitive hematopoietic stem and progenitor cells in culture is of considerable current interest. Factors which affect the expansion of CD34-enriched cells were studied in a controlled fashion in an effort to obtain culture conditions that result in expansion of cells and CFU-GM while maintaining/expanding LTC-IC. Four factors that influence culture performance were examined: the medium exchange interval, the growth factor combination, the presence of preformed stroma, and the CD34+lin- cell purity of the inoculum. Several important findings result from this study. (1) Increasing the rate of medium exchange significantly improved culture performance of CD34-enriched cells, but only when cells were cultured on preformed stroma. (2) Manipulation of the growth factor combination significantly affected the expansion of total cells, but most of the combinations gave CFU-GM expansions that were not significantly different from one another. All of the growth factor combinations resulted in a decline of LTC-IC numbers in stromal-free culture. (3) The use of preformed stroma resulted in higher numbers of CFU-GM and LTC-IC, regardless of the growth factor combination used. (4) Increasing the purity of CD34+lin- cells negatively affected culture productivity. It appeared that the loss of accessory cell function was partly responsible, as judged by decreased levels of endogenous growth factor production. Preformed stroma completely abrogated the negative effect of cell purity, or expansion. During the course of these studies, it became apparent that the composition of the culture inoculum and the medium exchange interval was related to the fate of LTC-IC in culture. This result is further demonstrated by the frequency distribution of LTC-IC expansion ratios for a large number of experiments using CD34-enriched cells, CD34-enriched cells on preformed stroma, and MNC as the culture inocula (Fig 7). In CD34-enriched cell cultures, LTC-IC number typically declined to a value that was below input. Only 3 of 21 experiments resulted in >1-fold "expansion" (1.1-, 1.2-, and 1.6-fold), whereas 11 experiments resulted in <30% maintenance of LTC-IC. Overall, a distribution of LTC-IC expansion ratios was measured with an average of 0.48-fold. The addition of preformed stroma shifted this distribution, resulting in LTC-IC maintenance (average 0.98-fold, Fig 7B). The distribution of LTC-IC expansion ratios was further shifted by the use of MNC inocula, resulting in net LTC-IC expansion (average 3.2-fold, Fig 7C). Using MNC, expansion ratios of greater than 1 were obtained in 25 of 32 experiments.
least two mechanisms for this effect have been demonstrated. First, medium perfusion stimulates the production of growth factors by BM stromal cells.\textsuperscript{13} These endogenously produced growth factors are numerous,\textsuperscript{31} and are likely to contribute to culture performance. Second, medium perfusion results in a stable culture environment that more closely resembles the in vivo state. Depletion of nutrients and build-up of metabolic waste products is prevented with medium perfusion.\textsuperscript{14} The results of the current study extend the previous results to include cultures of purified cells on preformed stroma. Purified cells without stroma did not benefit from a constant level of frequent medium exchange. This result is likely related to the lack of accessory cells that require perfusion for stimulation. Also, because of the very low inoculum density of CD34-enriched cells, perfusion may not be beneficial until the latter stages of these cultures.

Growth factor combinations that had been previously optimized by others\textsuperscript{5,9,10,21,27} were examined in the context of frequent medium exchange. Altering the growth factor combination had significant effects on total cell expansion, but had relatively little effect on CFU-GM output. There have been a number of previous reports studying the effect of growth factor combinations on purified cell expansion.\textsuperscript{5,9,15,17,20,21,35-38} Although each study found a different optimal combination, there is consensus on the importance of several growth factors, such as IL-3, IL-6, and SCF for the generation of cells and progenitors. Considerably less information is available on the question of stem cell maintenance/expansion from cultures of purified cells.\textsuperscript{17} Although recent reports have described the stromal-free expansion of primitive cells defined phenotypically by flow cytometry,\textsuperscript{5,35} the functionality of these cells is not certain. In fact, stromal-free murine cultures which yield 1,000-fold expansion of primitive cells measured by flow cytometry (Sca-1\textsuperscript{+}, WGA\textsuperscript{-}) result in a 30% loss of primitive cells as measured in vivo by the competitive repopulating unit (CRU) assay.\textsuperscript{39} Therefore, phenotyping by flow cytometry does not appear to accurately assess primitive cell functionality.\textsuperscript{11} One possible reason is that the fidelity of the stem cell phenotype as measured by flow cytometry may not be maintained after cell expansion. In contrast, the in vitro biological LTC-IC assay has shown results similar to the in vivo model. Namely, that primitive cell numbers often decline during stromal-free in vitro expansion of purified cells, even with the use of various growth factor combinations.\textsuperscript{12,17} In the present study, the different growth factor combinations differed in their ability to support LTC-IC, but none were better than the control combination of 3/GM/SCF/Epo. Importantly, even the best growth factor combinations maintained less than half of the input LTC-IC. Consequently, the expansion of LTC-IC from purified populations of adult tissue has not been shown to date.

The presence of preformed stroma was found to significantly improve culture performance, increasing cell, CFU-GM, and LTC-IC numbers by threefold to fivefold. The effect of stroma on primitive cell maintenance has been well documented,\textsuperscript{30,40-45} and it was long thought that direct contact of primitive cells with stroma was required.\textsuperscript{46} Recent reports have suggested that recombinant growth factors can replace the requirement for stroma,\textsuperscript{5,9,10,27} or that stroma separated
from purified cells by a microporous membrane improves primitive cell maintenance. The implication is that soluble factors alone are capable of supporting primitive cells. Unfortunately, the factors that are responsible remain to be defined. The present report indicates that LTC-IC expansion does not occur in stromal-free cultures of CD34-enriched cells, and this is in agreement with a number of published reports. The addition of preformed stroma to the cultures resulted in LTC-IC maintenance, but not expansion. Further, although LTC-IC were maintained in cultures of CD34-enriched cells on preformed stroma, inefficiencies in the purification procedure result in a net loss of LTC-IC relative to the starting MNC population.

Because of the donor-to-donor variability in CD34+ cell purity obtained from the enrichment procedure, the effect of cell purity on culture performance was directly examined. When a fixed number of CD34+lin- cells were cultured, there was very little change in culture output within the range of 20% to 95% purity. However, the level of performance was significantly greater from MNC (at ~2% CD34+lin- cell purity). The number of cells, CFU-GM, and LTC-IC obtained from MNC was threefold to fivefold higher than from an equivalent number of highly purified CD34+lin- cells. Given a fixed number of CD34+lin- cells, in the context of frequent medium exchange, proliferation was obtained regardless of the number of CD34+ cells that were present. However, within the MNC population, the accessory cell effect (reflected in part by the increased level of endogenous growth factor production) increased the proliferation of the CD34+lin- cells, resulting in the increased cell yield from an equivalent number of CD34+lin- cells (4.3 × 10^6 from MNC v 1.7 × 10^6 cells from purified CD34+lin- cells). However, the large number of nonproliferating CD34+ cells in the inoculum resulted in a lower calculated expansion ratio (24-fold v 425-fold), even though the CD34+lin- cells within the MNC mixture proliferated to a greater extent. Consequently, the calculated cell expansion ratio does not give an accurate indication of the degree of CD34+lin- cell proliferation that occurs in cultures initiated with starting populations of different purity.

Taken together, the results of this study confirm that the expansion of LTC-IC is achievable in vitro. Of the culture conditions examined here, only frequent medium exchange with MNC inocula reproducibly gave expansion of LTC-IC. The culture of CD34-enriched cells on irradiated, preformed stroma with frequent medium exchange led to the maintenance of LTC-IC numbers, whereas stromal-free cultures of CD34-enriched cells led to the loss of LTC-IC in culture. A recent report comparing the long-term survival of irradiated mice transplanted with cells from cultures initiated with MNC versus purified Thy-1+ lin- cells has also shown that primitive cells are rapidly lost from purified cell cultures, but not from MNC cultures. These results suggest that cell populations present in MNC are capable of mediating primitive cell expansion. Identification of these accessory cell population(s) would greatly facilitate the definition of factors required to obtain primitive cell expansion.

ACKNOWLEDGMENT

We thank Brian S. Newsom and Robert J. Maher for excellent technical assistance, and Drs Albert Deisseroth (M.D. Anderson, Houston, TX), Melissa Fenner (University of Michigan, Ann Arbor), and Voravit Ratanaatharoth and Joseph Uberti (Harper Hospital, Detroit, MI) for bone marrow specimens.

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Long-term culture-initiating cell expansion is dependent on frequent medium exchange combined with stromal and other accessory cell effects

MR Koller, MA Palsson, I Manchel and BO Palsson