RAPID COMMUNICATION

Large-Scale Expansion of Human Stem and Progenitor Cells From Bone Marrow Mononuclear Cells in Continuous Perfusion Cultures

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There is a growing consensus that clinical practice in the areas of bone marrow (BM) transplantation and gene therapy will rely on the ex vivo expansion of hematopoietic cells. We report here on the development of continuously perfused culture systems (bioreactor systems) that expand human stem and progenitor cells from BM mononuclear cell (MNC) populations obtained without cell enrichment.

In three separate experiments, 10 bioreactors were each inoculated with $3 \times 10^7$ BM MNC from patients undergoing marrow harvest for autologous transplantation. At various times thereafter (between days 6 and 16), duplicate bioreactors were harvested and cells were analyzed. The bioreactors contained three cell populations that were analyzed separately: nonadherent cells; cells that were loosely adherent to the endogenously formed stromal layer; and an adherent cell layer that required trypsinization for removal. Total cell numbers increased continuously to give an overall 10-fold (range, 8- to 11-fold) expansion for removal. Total cell numbers increased continuously and expanded to more than $2 \times 10^7$ cells, but remained less than 6% of the total cell population. Colony-forming unit–granulocyte-macrophage (CFU-GM) numbers expanded 21-fold (range, 12- to 34-fold) by day 14 and, because this expansion was greater than that for total cells, CFU-GM were enriched by as much as fourfold by day 14. Burst-forming unit–erythroid (BFU-E) numbers peaked earlier than did CFU-GM numbers, with a 12-fold (range, 6- to 18-fold) expansion obtained on day 8. In contrast to CFU-GM, which were predominantly nonadherent, BFU-E were more evenly distributed between the three cell populations.

Stem cell activity was measured by the long-term culture-initiating cell (LTC-IC) limiting dilution assay. The number of LTC-IC per reactor consistently increased with time in all cultures, resulting in a 7.5-fold (range, 3.4- to 9.8-fold) expansion. In summary, more than 3 billion cells, containing 12 million CFU-GM, were reproducibly generated from the equivalent of a 10 to 15 ml BM aspirate. These data indicate that small numbers of BM MNC can be readily expanded ex vivo in continuous perfusion cultures, and that such ex vivo expansion may have direct applications in clinical and experimental BM transplantation.

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Submitted March 4, 1993; accepted April 14, 1993.

Supported in part by US Department of Commerce ATP Grant No. 70NANB2H1243.

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Blood, Vol 82, No 2 (July 15), 1993: pp 378-384
duce sufficient numbers of cells for transplantation or gene therapy.

MATERIALS AND METHODS

Medium and cytokines. LTBM C 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, 50 µg/mL penicillin and streptomycin (GIBCO, Grand Island, NY), and 10 µmol/L hydrocortisone (Sigma, St Louis, MO).

Growth medium was prepared by supplementing LTBM C medium with recombinant human (rh) growth factors. Growth factor concentrations were 2 ng/mL rh interleukin-3 (IL-3; R & D Systems, Minneapolis, MN), 5 ng/mL rh granulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA), 0.1 U/mL rh erythropoietin (Epo; Amgen, Thousand Oaks, CA), and 10 ng/mL rh c-kit ligand (Immunex).

Cells and cell separation procedure. Human BM cells were obtained after informed consent from patients undergoing marrow harvest for autologous transplantation at the University of Michigan. Cells used in these studies were derived from a 51-year-old female with non-Hodgkin's lymphoma (NHL), a 26-year-old male with testicular cancer, and a 68-year-old male with NHL. Cells were washed from Fenwal BM collection container filters after patient marrow harvesting, and these cells were pooled with 20 mL of the marrow aspirate product. Cells were diluted with Hank's Balanced Salt Solution (HBSS; GIBCO) containing 10% FBS, and were then layered onto Ficoll (1.077 g/mL; Pharmacia, Uppsala, Sweden). Cells from the interface band were collected after centrifugation at 300g for 25 minutes at 25°C. Cells were washed twice in HBSS containing 10% FBS and were then resuspended in growth medium. Cells were diluted in 10% cetrimide (Sigma, for MNC counting) and counted on a Coulter ZM cell counter (Coulter Electronics, Hialeah, FL).

Perfusion culture system. This group has previously published details on small-scale cell culture chambers for the expansion of human hematopoietic progenitors. These culture chambers were scaled-up 10-fold to determine the feasibility of producing a large dose of hematopoietic cells through ex vivo expansion. Ten such units were inoculated with 3 x 10^6 BM MNC each (on day 0), and were thereafter maintained at 37°C. After a 24-hour period without perfusion to facilitate stromal layer attachment, medium flow was initiated at 22.5 mL/d through a peristaltic pump (Watson-Marlow, Boston, MA). Medium was maintained at 4°C, and then was warmed to 37°C just before entering the bioreactors. Two bioreactors were harvested on each even numbered day between days 6 and 16. Nonadherent cells were collected with a syringe by aspirating the medium contained in the bioreactor. Wash cells were subsequently collected with two 15 mL washes of HBSS. These wash cells were analyzed separately because they were loosely adherent to the stroma, and therefore might have a different composition than either the nonadherent cells or the tightly adherent stromal cells. Finally, adherent cells were collected 10 minutes after the addition of 15 mL 0.05% trypsin to the bioreactor at 37°C, with two 15 mL washes of HBSS containing 10% FBS. Each sample was centrifuged and the pellets were resuspended in 10 mL LTBM C medium. MNC were counted in 10% cetrimide on a Coulter ZM cell counter.

Methylcellulose colony assays. Cells at ≈ 2 x 10^4/mL were inoculated in colony assay medium (Terry Fox Laboratories, Vancouver, British Columbia, Canada) containing 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, and 100 µmol/L 2-mercaptoethanol, to which 2 mmol/L L-glutamine, 20 ng/mL rhGM-CSF, 100 ng/mL rh c-kit ligand, and 2 U/mL rhEpo were added. 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 5% O₂ in N₂ at 37°C. Colonies of greater than 50 cells were scored with a darkfield stereo microscope (Nikon, Garden City, NY). In addition to CFU-GM and burst-forming unit-erythroid (BFU-E) colonies, discrete colonies of stromal cells were observed in these dishes, and these were also enumerated in some experiments.

LTC-IC assay. LTC-IC were determined by limiting dilution assay (LDA) of cells on irradiated stroma. BM MNC were plated in 75 cm² tissue culture flasks (Corning Glass Works, Corning, NY) in 20 mL LTBM C medium at 10^6 cells/cm². After 2 days at 37°C in a fully humidified atmosphere of 5% CO₂ in air, all the nonadherent cells were removed and 20 mL fresh LTBM C medium was added. Stromal cultures were maintained at 37°C and were fed weekly with a one-half medium exchange for up to 5 weeks. After the second week, stromal cells were trypsinized and were plated into the center 60 wells of 96-well plates at 2.5 x 10^4 cells/well in 100 µL LTBM C medium. The outer 36 wells were filled with sterile water to reduce evaporative losses from the wells containing cells. After 24 hours of incubation at 37°C in a fully humidified atmosphere of 5% CO₂ in air, plates were irradiated with 15 cGy from a Co source. Test cells were then added to these irradiated stromal layers at 3 or 4 different concentrations in 100 µL LTBM C medium per well (in 15 to 20 replicates each). In the various experiments, each test population was plated at concentrations ranging from 400 to 10^5 cells per well, but most often at concentrations between 1.5 x 10^4 and 6 x 10^4 cells per well. These cell concentrations were chosen based on a mathematical analysis of LDA. Briefly, the most informative range of LDA lies between multiplicities of 1 and 2.5. For BM LTC-IC, which have a reported frequency of 1 per 2 x 10^4, the most informative range is therefore between 2 x 10^4 and 5 x 10^4 cells per well. In our studies, these boundaries were expanded by 20% to 25% to allow for variation in LTC-IC frequency. The plates were then placed at 33°C (37°C in experiment 1) in a fully humidified atmosphere of 5% CO₂ in air, and the cultures were fed weekly by replacing 100 µL LTBM C medium per well with removal of approximately one-half of the nonadherent cells. At week 5, adherent and nonadherent cells were harvested from each well by removal of the medium and the addition of 30 µL trypsin for 10 minutes at 37°C. Trypsinized cells were washed from the well with 30 µL HBSS and were pooled with the nonadherent cells. The cells from each well were added directly to 0.25 mL of colony assay medium in nontissue culture-treated 24-well plates (Falcon, Lincoln Park, NJ). After 14 days under fully humidified conditions in an atmosphere of 5% CO₂ and 5% O₂ in N₂ at 37°C, wells were scored for colonies of greater than 50 cells. For each sample, the number of LTC-IC was determined through an iterative calculation procedure based on the maximum likelihood method. Confidence intervals were calculated as described and used to determine the level of statistical significance for the LTC-IC expansion values obtained.

RESULTS

Cell expansion kinetics. The bioreactor systems consistently expanded BM MNC over a 14-day period (Fig 1). These high-density cell cultures consisted of nonadherent cells, cells that were loosely adherent to the endogenously formed stromal layer, and an adherent layer that required trypsinization for removal. These three cell populations were analyzed separately. By day 6, there was a twofold expansion in total cell numbers. The majority of cells (96.7%) were distributed between the nonadherent and loosely adherent fractions at a ratio of 2:1. The adherent fraction was quite small (3.3%), but a developing stromal
cell layer was visible under the microscope. By day 8, the adherent layer had grown to $5 \times 10^6$ cells per bioreactor, concomitant with a large increase in the loosely adherent cell compartment. After day 6, all cell populations continued to expand in a balanced fashion, with the nonadherent, loosely adherent, and adherent cells maintaining approximately a 12:7:1 ratio. By day 14, the adherent layer had grown to more than $20 \times 10^6$ cells, representing less than 6% of the total cell number. At this time, more than $340 \times 10^6$ cells were present in each bioreactor, representing an absolute $11.3$-fold expansion of BM MNC after 14 days of culture. If all 10 bioreactors had been operated for 14 days, more than 3.4 billion cells would have been produced from the equivalent of a 10 to 15 mL BM aspirate.

BM from three different patients undergoing harvest for autologous transplantation gave similar results. The cell counts obtained from each of the 30 bioreactors from the three separate experiments are shown in Fig 1B. Within each experiment, cell counts from duplicate bioreactors at each time point were usually within 5% of one another, indicating the reproducible results which are obtained under these well-controlled culture conditions.

**Progenitor expansion kinetics.** CFU-GM expansion kinetics (Fig 2A) were similar to total cell kinetics (Fig 1A), except that their expansion was more rapid. By day 6, CFU-GM numbers were expanded 3.2-fold as compared with the twofold expansion of total cells. As with total cells, the majority (99.5%) of CFU-GM were contained in the nonadherent and loosely adherent fractions. Although adherent CFU-GM were a very small population (0.47%) on day 6, they increased very rapidly (10-fold) between days 6 and 10. This observation correlates well with the emergence of an adherent stromal layer as described in Fig 1A. After day 6, all CFU-GM compartments continued to expand in a balanced fashion, with the nonadherent, loosely adherent, and adherent CFU-GM maintaining approximately a 36:14:1 ratio. This growth resulted in the production of $1.26 \times 10^6$ CFU-GM per bioreactor, representing an absolute $16.5$-fold expansion of CFU-GM from BM MNC after 14 days of culture. When compared with the $11.3$-fold expansion of total cells, we observe that progenitor cells were actually enriched by 46% during this culture.

Similar results were obtained in the replicate experiments, with CFU-GM numbers from duplicate bioreactors...
within an experiment being remarkably consistent (Fig 2B). Variability in CFU-GM expansion was observed between experiments, but even these numbers were reasonably consistent. For example, the average day 14 CFU-GM density was $1.18 \pm 0.21 \times 10^6$ per bioreactor in the three experiments. When expressed as a CFU-GM expansion ratio, an average of $21 \pm 7.9$-fold expansion was obtained.

Erythroid progenitors were also enumerated in these cultures. In contrast to CFU-GM numbers, BFU-E numbers peaked by day 8, and then declined to near input level by day 14 (Fig 3). The distribution of BFU-E progenitors between the three cell populations differed from that of CFU-GM. At day 6, when the adherent layer was relatively sparse, only 18% of BFU-E were loosely adherent or adherent. However, by day 12, when the adherent stromal layer had significantly developed, the majority of BFU-E were adherent (64%). This finding shows the preferential location of BFU-E in the adherent layer once it becomes available. A 6.3-fold expansion of BFU-E was obtained by day 8 in this experiment, and this was similar to the 6.7-fold expansion of CFU-GM at that time.

During colony assay scoring in early experiments, we observed a dramatic increase in discrete stromal cell colonies (possibly CFU-fibroblast [CFU-F]) as the culture progressed. Therefore, in later experiments, these putative CFU-F were enumerated. At day 6, only 37% of the stromal cell colonies were recovered in the adherent fraction, and the remainder were still in the nonadherent and loosely adherent fractions. However, at subsequent times, more than 98% of the stromal cell colonies were adherent, indicating that the culture system maintained attachment of stromal layer elements. A greater than 100-fold increase in adherent cell colonies was observed by day 14, indicating the rapid stromal layer development supported by this culture system.

**LTC-IC expansion kinetics.** LTC-IC were determined by LDA analysis. The total numbers of LTC-IC per bioreactor are shown in Fig 4A through C. In the first experiment, the number of LTC-IC per bioreactor increased from 90 to 886 (9.8-fold, $P < .0005$) between days 6 and 14. The inoculum LTC-IC measurement was not obtained in this experiment. However, based on the other two experiments (Fig 4B and C) and on previous data from continuously perfused systems, LTC-IC numbers are very likely to increase during the first 6 days of perfusion culture. This finding suggests that LTC-IC numbers were expanded by a minimum of
9.8-fold in the first experiment. In the second experiment, LTC-IC numbers were expanded from 1,034 to 3,546 (3.4-fold, \( P < .05 \)) between days 0 and 12. In the final experiment, LTC-IC numbers were expanded from 616 to 5,801 (9.4-fold, \( P < .00005 \)) between days 0 and 14. The expansion obtained in experiment 2 was somewhat lower than that obtained in the other two experiments, but the final measurement in experiment 2 was on day 12. A measurement on day 14 may have yielded a result more similar to the other two experiments. Nevertheless, these data show a significant, reproducible expansion of primitive LTC-IC from BM MNC through continuous perfusion culture.

**DISCUSSION**

We show here the ability to expand human hematopoietic LTC-IC and progenitor cells from BM MNC by culture under conditions of continuous perfusion. After 14 days, total cell numbers were expanded 10-fold (range, 8- to 11-fold) and CFU-GM were expanded 21-fold (range, 12- to 34-fold). More importantly, primitive LTC-IC were expanded 7.5-fold (range, 3.4- to 9.8-fold). It is important to note that the expansions reported here are not measured by cumulative counting, or by counts corrected for cell removal at previous feedings, but represent the actual cell numbers that are present in the bioreactors on day 14. The large scale of these cultures shows the feasibility of producing a transplantable dose of cells from a small marrow aspirate or stem cell collection.

The total cell numbers (3.03 ± 0.36 \( \times 10^8 \) per bioreactor) and CFU-GM numbers (1.18 ± 0.21 \( \times 10^6 \) per bioreactor) obtained at day 14 from the different patients, who ranged from 26 to 68 years in age, were remarkably similar. This reproducibility suggests that bioreactor expansion of BM cells is generally applicable. The CFU-GM expansion ratio of 21 (± 7.9)-fold was more variable than the total CFU-GM number, but this was due to a large variation in CFU-GM inoculum densities. For example, input CFU-GM numbers varied from 3.3 \( \times 10^4 \) per reactor in experiment 1 to 8.2 \( \times 10^4 \) per reactor in experiment 2. The lower input CFU-GM number gave the highest expansion number (as high as 34-fold). Therefore, the final CFU-GM density was more consistent than the expansion ratio. The total number of CFU-GM produced is more relevant than the expansion ratio if cells are to be transplanted, and it was encouraging that this number was reproducible from patient to patient. Furthermore, in all experiments the fraction of progenitor cells in the cultures actually increased, indicating selective expansion of progenitors was obtained. The selective expansion of CFU-GM in this system results in a cell population that is significantly more enriched for progenitors than fresh marrow. Given the current literature, the selective expansion of progenitors is a unique result, and it is routinely observed in these perfused cultures.

Unlike animal stem cells, the long-term in vivo reconstitution potential of primitive human stem cells cannot be directly assayed. As a result, long-term in vitro reconstitution assays have been developed to detect very primitive human hematopoietic cells.\(^{19,22}\) It is encouraging to note that long-term in vivo reconstitution has been correlated with long-term in vivo reconstitution for murine cells.\(^{25-23}\) There has been an increasing acceptance of the LTC-IC assay as a measure of stem cell activity.\(^{7,23,26,27}\) If this interpretation is accurate, the significant expansion of LTC-IC in this culture system represents the first published quantitative evidence of human stem cell expansion in a BM culture system. Qualitative evidence for human stem cell expansion had been obtained earlier in cultures with rapid medium exchange.\(^{28}\) Another report has documented in vitro murine stem cell self-renewal, but this was accompanied by an 85% net decline in the total number of stem cells by week 4 of culture.\(^{22}\) Therefore, although it has been shown that some murine stem cells undergo self-renewal in vitro with a decrease in total numbers, the present work shows ex vivo self-renewal of human stem cells accompanied by a net expansion of their numbers. These results have obvious implications for the transplantation applications of ex vivo hematopoietic cultures, and also for gene therapy. Because active cell cycling is required for retroviral integration,\(^{29}\) a culture system that stimulates the proliferation of stem cells is required for gene transfer protocols.

Previous studies of human hematopoietic cell expansion have focused on the expansion of CD34-selected cells. Based on those results, it has been stated that hematopoietic cells must be enriched before culture for the expansion to succeed.\(^1\) However, this view is inconsistent with in vivo hematopoiesis, in which stem and progenitor cells proliferate in close association with the support cells of the marrow stroma, and in the presence of numerous CD34\(^+\) cells. Therefore, it seems that the failure of BM cells to expand ex vivo is a failure of the culture systems used, and is not due to the inoculum composition. We reasoned that a culture system that mimics the in vivo environment would provide a prolific ex vivo hematopoietic system that supports the concurrent development of stroma, stem cells, progenitors, precursors, and mature cells. The tissue-like density of cells in these bioreactors reconstructs the close association of all the cell types, much like the situation in vivo. In fact, there is significant endogenous production of growth factors by the support cells in these cultures (manuscript in preparation), and this may reduce the requirements for exogenous growth factor addition. It has been previously shown that growth factor production by human stroma is increased with increased medium exchange schedules.\(^{30}\) The proportion of stromal cells observed in these bioreactors is similar to the in vivo situation, but is in stark contrast to what occurs in static LTBMC. Static LTBMC composition often becomes overwhelmingly stromal,\(^{7,31,32}\) and irradiation is frequently required to suppress stromal layer overgrowth and detachment. This report shows that a continuously perfused system can in fact support the full spectrum of marrow cell development ex vivo, and also is able to selectively expand stem and progenitor cell numbers to transplantable levels. In addition, this expansion is performed in a sealed system without periodic manipulations, thereby limiting the risks of contamination.

The major clinical application of LTBMC to date has been leukemic cell purging, although no cell expansion was obtained with the culture systems used.\(^{33,34}\) The extent of
leukemic cell purging in an expanding culture system remains to be investigated. If successful, this would provide purging cultures that also offer the benefit of significant cell expansion for autologous transplantation. These cultures did provide a significant depletion of the T-lymphocyte population (data not shown), which may be useful in the allogeneic transplant setting for reducing both graft-versus-host disease and the required size of the marrow harvest.

In the continuously perfused cultures, BFU-E numbers peaked by day 8, and then declined to near input level by day 14. This pattern is in contrast to all other cell populations that continued to expand. This result may be due to a feedback inhibition mechanism from the large number of mature erythroid cells that accumulated in the cultures. Alternatively, the decrease in BFU-E formation may have been due to the absence of cytokines, which did not impact CFU-GM development. For example, it may be necessary to add other growth factors such as IL-9, which acts on very early erythroid progenitors, to sustain BFU-E development. Interestingly, the erythroid forming potential of cells in the bioreactors was not lost, because a significant number of erythroid colonies were observed from these cells during LTC-IC assay scoring (data not shown).

In summary, more than 3 billion cells, containing 12 million CFU-GM, were reproducibly generated from the equivalent of a 10 to 15 mL BM aspirate from patients undergoing marrow harvest for autologous transplantation. The cultures also resulted in a 7.5-fold expansion of primitive LTC-IC numbers. The potential clinical applications of such cells would include the augmentation or supplantation of BM or mobilized peripheral blood transplant therapies, as well as gene therapy. In addition, the availability of multiple infusions of expanded cells between multiple cycles of chemotherapy would allow dose and schedule intensification of current anticancer therapies. The further scale-up of these systems for various applications is well underway.

ACKNOWLEDGMENT

We thank Dr Samuel M. Silver and Judith W. Douville at the University of Michigan Bone Marrow Transplant Unit for bone marrow samples. We also acknowledge the excellent technical support provided by Shannon Bradley.

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