

Development of Novel Perfusion Chamber to Retain Nonadherent Cells and Its Use for Comparison of Human "Mobilized" Peripheral Blood Mononuclear Cell Cultures with and without Irradiated Bone Marrow Stroma

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Received May 10, 1995/Accepted December 28, 1995

Perfusion and static cultures of peripheral blood (PB) mononuclear cells (MNCs), obtained from patients following stem cell mobilization, were supplemented with interleukin-3 (IL-3), IL-6, granulocyte colony-stimulating factor (G-CSF), and stem cell factor (SCF) and compared with and without a preformed irradiated allogeneic bone marrow stromal layer. Perfusion cultures without a stromal layer effectively retained nonadherent cells through the use of a novel "grooved" perfusion chamber, which was designed with minimal mass transfer barriers in order to achieve a well-defined culture environment. The grooved chamber allowed easy and efficient culture inoculation and cell recovery. Average maximum expansion of CFU-GM (colony-forming unit granulocyte-macrophage) cells was observed on day 10 for all cultures. Perfusion cultures had a maximum CFU-GM expansion of 17- and 19-fold with and without a stromal layer, respectively. In contrast, static cultures had a maximum CFU-GM expansion of 18- and 13-fold with and without a stromal layer, respectively. Average long-term-culture initiating cell (LTC-IC) numbers on day 15 were 34% and 64% of input in stroma-containing and stroma-free perfusion cultures and 12% and 11% of input in stroma-containing and stroma-free static cultures, respectively. Thus, perfusion enhanced CFU-GM expansion and LTC-IC maintenance more for the stroma-free cultures than for stroma-containing cultures. This was surprising because analysis of medium supernatants indicated that the stroma-containing cultures were metabolically more active than the stroma-free cultures. In view of their equivalent, if not superior, performance compared to stroma-containing cultures, stroma-free perfusion cultures may offer significant advantages for potential clinical applications. © 1996 John Wiley & Sons, Inc.

Key words: perfusion chamber • bone marrow stroma • mononuclear cell cultures • hematopoietic cultures • cell retention

INTRODUCTION

Ex vivo culture of hematopoietic cells has potential applications for supplementing or replacing cells tradition-

ally used for bone marrow (BM) transplantation. Such potential applications could include stem and progenitor cell expansion in order to increase the number of eligible patients and available cells for transplantation,^{23,24} purging tumor cells for autotransplants,^{1,5,6} and committed progenitor cell expansion in order to potentially decrease the period of myelosuppression resulting from intensive radio/chemotherapy.¹⁹ The most commonly used measure for assessing the adequacy of cells for transplantation is the number of colony-forming units granulocyte-macrophage (CFU-GM).^{2,18} Peripheral blood (PB) mononuclear cells (MNCs) obtained from patients following stem cell mobilization is an important and promising source for hematopoietic cell expansion for use in BM transplantation therapies.^{5,9,23,24} In the remainder of this article we will use the term PB MNCs for PB MNCs obtained from patients following stem cell mobilization.

Cultures using a "feeder" layer of BM stroma (referred to here as "stroma-containing" cultures) have greater culture longevity and progenitor cell expansion, for both lymphoid^{11,34} and myeloid^{14,21} lineages, than cultures without a "feeder" layer of BM stroma (referred to here as "stroma-free" cultures). Nevertheless, stroma-free cultures using combinations of hematopoietic growth factors (HGFs) have achieved large progenitor cell expansions.^{27,29} Stromal layers support hematopoiesis through the production of HGFs and extracellular matrix (ECM) compounds, and through cell-to-cell contact.^{7,8,28} However, the use of a stromal layer is undesirable for many research and clinical applications. The effects and composition of stromal layers are poorly defined and variable.^{7,11,34} Stroma-containing cultures are more labor intensive and require enzymatic treatment (i.e., trypsinization) in order to harvest the cultures. In addition, PB and cord blood (CB; another promising source for hematopoietic cell expansion^{3,4}) MNCs typically do not form stromal layers themselves

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and, therefore, require an additional BM cell sample to establish the stromal layer. Although adherent layers have been formed from CB MNCs, this requires careful and specific culture conditions.³⁵

Frequent feeding^{25,26} and continuous perfusion^{13,15,16,20} enhance progenitor cell expansion over traditional stroma-containing static cultures. Consequently, perfusion cultures of hematopoietic cells may be potentially useful for clinical applications. In view of the aforementioned advantages of stroma-free hematopoietic cultures, a direct performance comparison between stroma-containing and stroma-free perfusion cultures would be very desirable. In fact, we are not aware of any published reports on stroma-free perfusion cultures. Thus, one of the two objectives of this work is to compare the expansion of PB MNCs in stroma-free and stroma-containing perfusion cultures.

The culture chamber in our previously described perfusion system employed a "feeder" layer of irradiated BM stroma.¹³ MNCs were retained in this chamber through the adherence of primitive hematopoietic cells to the adherent stromal layer.^{17,30} In view of our first objective, our second objective was to modify the perfusion chamber design in order to allow stroma-free cultures. We set the following goals for a modified perfusion chamber: (1) efficient cell retention in the absence of a stromal layer without using membranes in the culture chamber, (2) efficient cell recovery upon culture termination, (3) easy culture inoculation, (4) a well-defined culture environment with minimal concentration gradients, and (5) simple construction. These goals were met by placing rectangular grooves in the lower surface of our perfusion chamber, perpendicular to the direction of bulk medium flow. The original and modified perfusion chambers are referred to as "smooth" and "grooved" chambers, respectively.

We compared stroma-containing and stroma-free cultures of PB MNCs, obtained from patients following stem cell mobilization with G-CSF and cyclophosphamide, using serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. Stroma-free cultures were conducted in the grooved perfusion chamber and static petri dish, while stroma-containing cultures were conducted in the smooth perfusion chamber and static petri dish. These cultures were conducted primarily to evaluate cell, CFU-GM, and long-term-culture initiating cell (LTC-IC) numbers and to evaluate the grooved chamber in regards to our design objectives. Media supernatants were analyzed for IL-6, GM-CSF, tumor necrosis factor- α (TNF- α), glucose, lactate, glutamine, ammonia, and pH in order to potentially elucidate the results obtained. In addition, we compared stroma-free cultures using the grooved and smooth perfusion chambers and static petri dish using CB MNCs in serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. These cultures were conducted primarily to compare cell retention in perfusion cultures using the

grooved and smooth chambers. CFU-GM numbers and photographic observations were performed to support the cell number results.

MATERIALS AND METHODS

Cells and Cell Separation Procedures

These studies were approved by the Institutional Review Board, and patient samples were obtained after informed consent. PB MNCs were obtained by apheresis from patients undergoing therapy for nonhematological cancers following stem cell mobilization with G-CSF and cyclophosphamide and were provided by Response Technologies (Memphis, TN). The apheresis products were transported in plasma overnight at room temperature, were used the same day as arrival, and will be further referred to as PB MNCs. Human BM cells were obtained from BM collection filters made available by the University of Chicago Medical School (Chicago, IL). BM collection filters were shipped overnight on ice and used the same day as arrival. Human CB was obtained from full-term, normal newborns from St. Therese Hospital (Waukegan, IL). CB was collected immediately after delivery, mixed with 5.0 mL anticoagulant citrate dextrose solution (ACD formula A; Baxter Healthcare Corporation, Deerfield, IL), stored at room temperature, and used within 3 days of collection. Excess red cells were separated from CB and PB MNC samples by Ficoll/Histopaque (1.077 g/mL, Pharmacia, Uppsala, Sweden) density gradient centrifugation and from BM cells by water lysis for 30 s.¹⁴

Culture Media and Reagents

Human long-term medium (HLTM) was prepared by supplementing McCoy's 5A medium with 1mM sodium pyruvate, 2 mM L-glutamine, 1% minimal essential medium (MEM) amino acid solution, 1% MEM nonessential amino acid solution, 1% MEM vitamin solution, 100 μ M monothioglycerol, 1 μ M hydrocortisone, 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 50 μ g/mL gentamicin sulfate (Gibco, Grand Island, NY), 12.5% fetal bovine serum (FBS), and 12.5% horse serum as previously described.¹² For hematopoietic cultures, HLTM was supplemented with 0.25 ng/mL IL-3 (R&D Systems, Minneapolis, MN), 40 ng/mL IL-6 (R&D systems), 50 ng/mL stem-cell factor (SCF) (Amgen, Thousand Oaks, CA), and 1.5 ng/mL G-CSF (R&D Systems). A different mixture of growth factors was used for the experiment of Fig. 7 as noted in its legend. Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO).

Perfusion and Static Culture

Perfusion and static cultures were conducted with slight modifications to the methods described.^{14,23} Briefly, both

stroma-containing and stroma-free perfusion cultures were conducted at $37 \pm 0.5^\circ\text{C}$ in three parallel polycarbonate culture chambers (each holding 15 mL of culture medium under flow conditions), which were each perfused at 2.5 mL/min (10 mL/min for the experiment of Fig. 7) with medium equilibrated with ambient oxygen and with the pH controlled at 7.35 ± 0.05 . There is no gas phase in the culture chambers. Smooth chambers designed for use with stromal "feeder" layer were previously described.¹³ For stroma-free cultures, we used a culture chamber with a modified bottom surface with grooves perpendicular to the direction of flow (Fig. 1). Stromal "feeder" layers were never used in the grooved chamber. Cells were effectively retained in the grooved chambers at flow rates in excess of 20 mL/min.²² Both the smooth and grooved chambers had a gross surface area of ca. 30 cm². In the grooved chamber, only the 15-cm² area occupied by the grooves is available to the cultured cells due to medium flow. Static cultures were conducted in 100-mm-diameter polycarbonate petri dishes (with polycarbonate blocks covering part of the dish, such that the surface area was 30 cm²) and incubated at $37 \pm 0.5^\circ\text{C}$ under 5% CO₂, and 5% O₂ for PB MNC cultures or 20% O₂ CB MNC cultures.

The perfusion rate used (see below) was chosen to

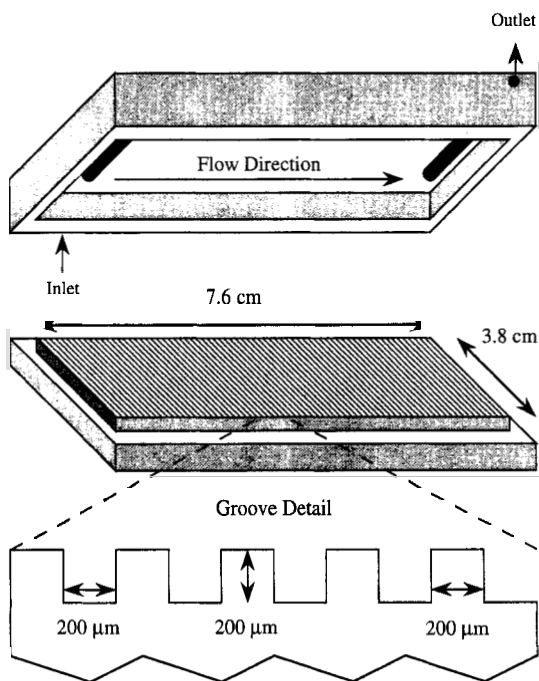


Figure 1. Schematic diagram of the grooved culture chamber. The chamber is constructed of two pieces of polycarbonate. Medium is perfused through the chamber through two slits, which are shown in black in the upper chamber surface. Cells are retained through the use of rectangular grooves in the lower chamber surface perpendicular to the direction of bulk medium flow. A blow-up of the lower chamber surface shows a cross section of the grooved surface. The individual grooves are 200 μm across and 200 μm deep and are separated from one another by 200 μm .

provide 100 times more oxygen than the cells were anticipated to consume in order to minimize concentration gradients in the perfusion chamber. The perfusion rate was determined for the grooved chamber assuming an oxygen consumption rate of 1 nmol/min 10⁶ cells, a cell density of 1.7×10^6 cells/cm² (the maximum we typically observed in 24-well plate cultures) and an O₂ solubility of 1.01×10^{-6} mol/cm³ in saline at 37°C. The perfusion rate is different than the feeding rate, which is the rate at which recirculating medium is replaced with fresh medium. The feeding rate was chosen to be greater in the perfusion cultures in order to minimize changes in the recirculating medium composition. The O₂ concentration in the grooves (200 μm deep) is estimated to change by less than 2% of air saturation assuming simple Fick's law diffusion through stagnant saline at 37°C using the above cell density and oxygen consumption rate, and an O₂/saline diffusivity of 2.5×10^{-5} cm²/s. This is an upper limit to the change in O₂ concentration due to secondary flows in the grooves caused by the bulk fluid flow over the groove.¹⁰ Theoretical calculation of the O₂ concentration profile in the perfusion chamber, based upon the convective-diffusion and Navier-Stokes equations using a zero-order oxygen consumption rate, indicate that the O₂ concentration should exceed 60% of air saturation at the bottom of the last groove before the chamber exit.

Stromal layers for the stroma-containing cultures were prepared with slight modifications to the methods described.^{13,14} Briefly, stromal cells, obtained after trypsinizing the adherent layer from a 2- to 5-week-old BM MNC culture, were irradiated as a single-cell suspension with a dose of 2000 rad from a ¹³⁷Cs source (662 keV, Mark I irradiator; J. L. Sheppard and Associates, San Fernando, CA). Stromal layers were prepared by inoculating 2×10^6 freshly irradiated cells in 20 mL HLTM on a 3.8 cm \times 7.2-cm tissue culture treated polystyrene slide (Thermanox; Nunc, Naperville, IL) in a 100-mm petri dish. Slides were then incubated overnight at 37°C under 5% CO₂ and 5% O₂ and were transferred to polycarbonate petri dishes or smooth chambers immediately prior to inoculation with PB MNCs.

Unless otherwise noted, perfusion and static cultures were inoculated with either 2×10^6 PB or 5×10^6 CB MNCs. Cells were added in 10 mL HLTM directly to the petri dishes or injected directly into the perfusion chambers. The cells in perfusion cultures were allowed to settle for 15 min before the perfusion rate was gradually stepped up to 2.5 mL/min over a period of 1.5 h. Short-term cell retention experiments indicated that greater than 97% of the cells are retained in the smooth chamber in excess of 2 h, providing time for primitive cells to become adherent.²² Negligible number of cells were observed in the cell trap at any time for cultures in the grooved chamber or for stroma-containing cultures in the smooth chamber, but not for stroma-free cultures in the smooth chamber.²² The initial total medium volume in the perfusion-culture system with

three chambers was 120 mL. After each chamber was harvested, the medium reservoir volume was decreased by 30 mL. Perfusion cultures were fed 3 times per week by replacing one-half (one-third for the experiment of Fig. 7) of the medium with fresh HLTM and HGFs. The culture volume was 20 mL (each) for static cultures. Static cultures were fed every 5 days by slowly removing one-half of the medium and replacing with fresh HLTM and HGFs. The associated cell depopulation in the stroma-free static cultures was $19 \pm 19\%$, as determined by nuclei counts on the medium removed. Static cultures were not corrected for this cell depopulation.

One of three parallel cultures was sacrificed every 5 days to assess cell viability and the number of cells, CFU-GM, and LTC-IC present, as described below. Stroma-containing cultures were harvested by trypsinization.¹³ Stroma-free cultures were harvested by removing the cell suspension from the culture chamber or petri dish, rinsing with 10 mL of Ca^{2+} and Mg^{2+} free phosphate-buffered saline (CMF-PBS), rinsing with 10 mL cell dissociation solution (Sigma, #C-5789), and rinsing a second time with 10 mL CMF-PBS. After harvest, the cells were washed and resuspended in HLTM. Cell counts and viability were determined using a hemocytometer with trypan blue dye exclusion.

Harvest efficiency was obtained by dividing the number of cells remaining in either the perfusion chambers or petri dish by the total number of cells recovered. For this calculation, cell counts on the harvested cells were performed by nuclei counts. Remaining cell numbers were determined by carefully rinsing the perfusion chambers or petri dish with 10 mL cetrimide solution (Sigma) and performing nuclei counts. Cells were easily and efficiently harvested from all stroma-free cultures without trypsinization, with recoveries typically around 97%. In addition, microscopic examination of the cultures after harvesting revealed few cells remaining in the perfusion chambers or petri dishes.

Culture Assays

Colony assays were conducted with slight modifications to the methods described.^{14,23} Briefly, test cells were plated in duplicate at three different cell densities, ranging between 1000 and 18,000 cells/mL for fresh MNCs and between 1000 and 27,000 cells/mL for cultured MNCs. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) containing 0.8% methylcellulose (Dow, Midland, MI), 30% FBS, 2% bovine serum albumin (BSA) (Intergen, Purchase, NY), 100 μM 2-mercaptoethanol, 50 $\mu\text{g}/\text{mL}$ gentamicin sulfate, 0.25 ng/mL IL-3, 40 ng/mL IL-6, 4.0 ng/mL granulocyte-macrophage CSF (GM-CSF, R&D Systems), 1.5 ng/mL G-CSF, and 83 ng/mL erythropoietin (Epo, Ortho Biotech, Raritan, NJ). CB MNC cultures were also supplemented with 50 ng/mL SCF. Aliquots of 1.0 mL were plated in duplicate in 35-mm suspension

culture dishes (Nunc, Naperville, IL) and incubated for 13–15 days at 37°C under 5% CO_2 and 5% O_2 . Colonies containing greater than 50 cells were scored as CFU-GM (nonhemoglobinized; white), BFU-E (fully hemoglobinized; red or pink), or CFU-Mix (clusters of hemoglobinized and nonhemoglobinized cells).

The LTC-IC assays were conducted with slight modifications to the methods described.^{13,14,23} Briefly, test cells were plated onto 2×10^5 irradiated BM stroma cells in duplicate at four different cell densities, ranging between 2.5×10^4 and 2.0×10^5 cells per well for fresh MNCs, and at two different cell densities, ranging between 5.0×10^4 and 2.0×10^5 cells per well for cultured MNCs. The cultures were incubated at 33°C under 5% CO_2 and 5% O_2 , and were fed weekly by

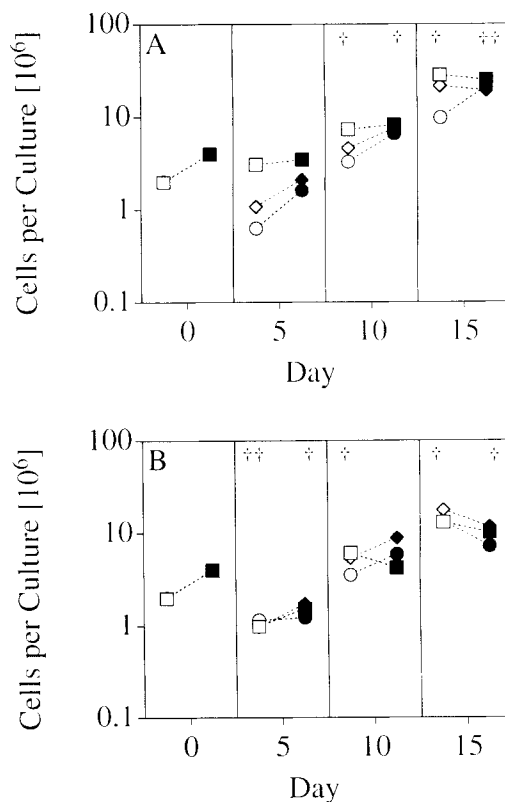


Figure 2. Total viable cells from stroma-containing and stroma-free PB MNC cultures in (A) perfusion culture system and (B) static petri dishes using serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. Stroma-containing cultures had $(2.00 \pm 0.0) \times 10^6$ irradiated allogeneic bone marrow stromal cells. Stroma-containing perfusion cultures were conducted in the smooth chambers, while stroma-free perfusion cultures were conducted in the grooved chambers. Cultures were inoculated with $(2.06 \pm 0.11) \times 10^6$ PB MNCs containing $(10.5 \pm 3.0) \times 10^3$ CFU-GM and $(3.5 \pm 1.3) \times 10^3$ LTC-IC. Three separate experiments are indicated by \circ , \square , and \diamond . Solid symbols represent stroma-containing cultures and open symbols represents stroma-free cultures. Dashed lines connect the separate experiments for stroma-containing and stroma-free cultures established with the same sample for the given time point. Symbols are used consistently for a given experiment in Figures 2 to 4. Significant differences between day 0 and the indicated day are indicated by \dagger ($p < 0.05$) and $\dagger\dagger$ ($p < 0.01$).

replacing 1 mL HLTM per well. At week 5, adherent and nonadherent cells were harvested from each well, combined, and plated into duplicate colony assays at 1.5×10^4 cells/mL.

Medium Analysis

Supernatants, including nonadherent cells, were removed from cultures at various times and stored at -70°C . In the perfusion system, medium samples were taken from the cell trap after purging once to remove dead cells. Frozen samples from different cultures were then simultaneously thawed and analyzed with enzyme-linked immunosorbent assay (ELISA) kits to determine the levels of IL-6 (BioSource International, Camarillo, CA), GM-CSF (Innogenetics, Zwijnaarde, Belgium), and TNF- α (BioSource International). Assay sensitivities stated by the manufacturers were 2, 8, and 1 pg/mL for the IL-6, GM-CSF, and TNF- α assays, respectively.

A second set of frozen medium samples was analyzed to determine glucose, lactate, glutamine, glutamic acid, and ammonia concentrations. Glutamine and glutamic acid concentrations were determined using a glutamine/glutamate determination kit (GLN-1, Sigma), and ammonia concentrations were determined using an ammonia assay kit (171-UV, Sigma). Glucose and lactate concentrations were analyzed on a Hitachi 911 (Boehringer Mannheim, Indianapolis, IN).

Data Analysis

Statistical analysis for comparison of total cells, CFU-GM, and LTC-IC was performed by taking the logarithm of the results and then using a two-tailed paired Student's *t*-test. Data are reported as the mean plus or minus the standard deviation (SD).

RESULTS

Stroma-Containing vs. Stroma-Free PB MNC Cultures

We compared stroma-containing and stroma-free cultures of PB MNCs, obtained from patients following stem cell mobilization with G-CSF and cyclophosphamide, using serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. Stroma-free cultures were conducted in the grooved perfusion chamber and static petri dishes while stroma-containing cultures were conducted in the smooth perfusion chamber and static petri dishes. This was done for our first objective of evaluating the stromal layer requirements of perfusion and static cultures supplemented with IL-3, IL-6, G-CSF, and SCF. Cell results for the three separate paired experiments are shown in Figure 2 while the means are given in Table I. Cell expansion for all cultures was qualitatively similar over 15 days. Total cell numbers declined 20–60% from input on day 5 before proliferation was observed. Maximum cell numbers were observed on day 15 for all cultures. As expected, perfusion gave greater cell expansions than static cultures. Maximum average cell expansions were 5.4- and 9.9-fold for stroma-containing and stroma-free perfusion cultures, and 2.4- and 7.4-fold for stroma-containing and stroma-free static cultures, respectively. When 2×10^6 stromal cells (the number of irradiated stromal cells initially seeded) are subtracted from the initial and final values, average cell expansions increased to 9.9- and 3.4-fold for perfused and static stroma-containing cultures, respectively. Stroma-free static cultures gave greater maximum average cell expansion than the stroma-containing static cultures. In contrast, the stroma-containing and stroma-free perfusion cultures gave similar maximum average cell expansion. Perfusion

Table I. Summary statistics for cell numbers for the stroma-containing and stroma-free, perfusion and static PB MNC cultures shown in Figure 2.^a

Culture type	Stromal layer	Parameter	Cells per culture ($\times 10^6$)			
			Day 0	Day 5	Day 10	Day 15
Perfusion	no	mean	2.06	1.60	5.21 ^{b,c}	19.8 ^{b,c}
Static	no	mean	2.06	1.04 ^{b,d}	4.98 ^{b,c}	14.7 ^{b,c}
Perfusion	yes	mean	4.06	2.40	7.46 ^{b,c}	21.8 ^{b,d,e}
Static	yes	mean	4.06	1.49 ^{b,c}	6.30	9.68 ^{b,c,e}

^a Statistically significant differences are shown. No statistically significant differences were observed between the stroma-containing and stroma-free perfusion cultures; or between stroma-free perfusion and static cultures. No statistically significant differences were observed between the stroma-containing and stroma-free static cultures.

^b Significant difference between 0 and the indicated day using the paired Student's *t*-test.

^c $p < 0.05$.

^d $p < 0.01$.

^e Significant difference between perfusion and static cultures at the indicated day using the paired Student's *t*-test ($p < 0.05$).

culture enhanced maximum average cell expansion 2.6- and 1.3-fold for the stroma-containing and stroma-free cultures, respectively, over the corresponding static culture. Average viability after 5 days exceeded 78% and 86% for stroma-containing and stroma-free perfusion cultures, respectively, and 90% for stroma-containing and stroma-free static cultures (data not shown).

The CFU-GM results for the three separate paired experiments are shown in Figure 3; the means are given in Table II. CFU-GM expansion was obtained in all cultures. Average maximum CFU-GM expansion was obtained on day 10 for the stroma-free perfusion and static and stroma-containing static cultures, and days 10–15 in the stroma-containing perfusion culture. The maximum CFU-GM expansion from the individual experiments was obtained on day 10 for all culture types for two of the three PB samples; the third sample gave maximum expansions on day 15 for all culture types except the stroma-containing static culture which was obtained on day 10. Average maximum CFU-GM expansions were 16.6- and 19.4-fold in stroma-containing and stroma-free perfusion cultures, and 17.9- and 12.5-fold in stroma-containing and stroma-free static cultures, respectively. Perfusion enhanced maximum CFU-GM expansion by 56% in stroma-free cultures but surprisingly did not enhance CFU-GM expansion in stroma-containing cultures.

The LTC-IC results for the three separate paired experiments are shown in Figure 4 while the means are given in Table III. Perfusion cultures contained more LTC-IC than static cultures, but LTC-IC declined in all cultures. Static cultures exhibited a continual decline in LTC-IC, while perfusion cultures exhibited a recovery after day 5 or 10. Average LTC-IC numbers on day 15 were 34% and 64% of input in stroma-containing and stroma-free perfusion cultures, and 12% and 11% of input in stroma-containing and stroma-free static cultures, respectively. Thus, perfusion enhanced LTC-IC

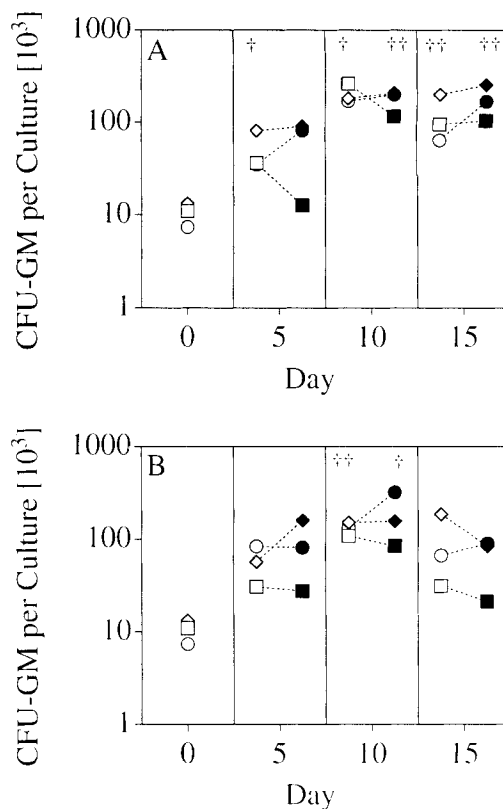


Figure 3. CFU-GM from stroma-containing and stroma-free PB MNC cultures in (A) perfusion culture system and (B) static petri dishes using serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. Symbols are as described in the legend to Figure 2. On day 0, we show three points (open symbols only) which represent the CFU-GM numbers for either the stroma-containing and stroma-free cultures, since stroma contains no CFU-GMs.

maintenance better in stroma-free cultures (5.8-fold, $p < 0.03$) than in stroma-containing cultures (2.8-fold). LTC-IC should not be removed from stroma-containing cultures during feeding or by perfusion because of their preferential location in the adherent layer.³⁰ Surpris-

Table II. Summary statistics for CFU-GM numbers for the stroma-containing and stroma-free, perfusion and static PB MNC cultures shown in Figure 3.^a

Culture type	Stromal layer	Parameter	CFU-GM per culture ($\times 10^3$)			
			Day 0	Day 5	Day 10	Day 15
Perfusion	no	mean	10.5	50.8 ^{b,c}	204 ^{b,c}	119 ^{b,d}
Static	no	mean	10.5	57.2	131 ^{b,d}	94.8
Perfusion	yes	mean	10.5	61.2	174 ^{b,d}	175 ^{b,d}
Static	yes	mean	10.5	89.4	188 ^{b,c}	65.3

^a Statistically significant differences are shown. No statistically significant differences were observed between the stroma-containing and stroma-free perfusion cultures, between stroma-containing perfusion and static cultures, or between stroma-free perfusion and static cultures. No statistically significant differences were observed between the stroma-containing or stroma-free static cultures.

^b Significant difference between day 0 and the indicated day using the paired Student's *t*-test.

^c $p < 0.05$.

^d $p < 0.01$.

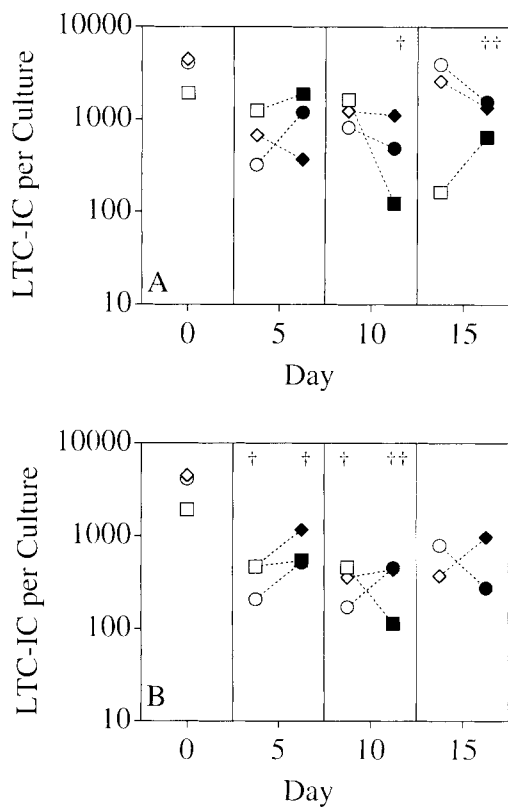


Figure 4. LTC-IC from stroma-containing and stroma-free PB MNC cultures in (A) perfusion cultures and (B) static Petri dishes using serum-containing medium supplemented with IL-3, IL-6, G-CSF, and SCF. Symbols are as described in the legend to Figure 2. The day 15 LTC-IC numbers for one of the experiments were zero and are not shown in B. On day 0, we show three points (open symbols only) which represent the LTC-IC numbers for either the stroma-containing and stroma-free cultures, since stroma contains no LTC-ICs.

ingly then, stroma-free static cultures maintained LTC-IC at identical levels as stroma-containing static cultures and stroma-free perfusion cultures maintained LTC-ICs better than stroma-containing perfusion cultures. These differences were not statistically significant and did not appear to be due to losses incurred during inoculation because LTC-IC were maintained better on day 5 in the stroma-containing cultures.

Perfusion enhanced CFU-GM expansion and LTC-IC maintenance more for the stroma-free cultures than for the stroma-containing cultures. This was surprising because analysis of media supernatant samples indicated that the stroma-containing cultures were more metabolically active than the stroma-free cultures. Perfusion is assumed to improve culture performance by maintaining a time-invariant environment, and thus improve the performance of the most metabolically active cultures the most. None of the cultures appeared to rapidly deplete glutamine, and only the stroma-containing cultures depleted glucose and accumulated lactate in appreciable amounts. At day zero, all cultures contained 12.8 mM glucose and 2.5 mM lactate. In perfusion cultures at day 15, the stroma-containing cultures had an average of 10.5 mM glucose and 11.8 mM lactate, while the stroma-free cultures had 11.4 mM glucose and 4.4 mM lactate. No data are available for day 15 static cultures. At day 10, the stroma-containing static cultures had an average of 6.5 mM glucose and 16.4 mM lactate, while the static stroma-free cultures had 11.1 mM glucose and 6.7 mM lactate. Following the lactate buildup, the medium pH in the stroma-containing static cultures declined from 7.35 to 6.95 by day 10, while the pH in stroma-free static cultures only declined to 7.23 over the same period. It is not known if these levels of lactate accumulation and

Table III. Summary statistics for LTC-IC numbers for the stroma-containing and stroma-free, perfusion and static PB MNC cultures shown in Figure 4.^a

Culture type	Stromal layer	Parameter	LTC-IC per culture			
			Day 0	Day 5	Day 10	Day 15
Perfusion	no	mean	3500	750	1200 ^{b,c}	2300 ^{b,d}
Static	no	mean	3500	380 ^{e,f}	330 ^{b,c,e,f}	390 ^{b,d}
Perfusion	yes	mean	3500	1200	580 ^{e,f}	1200 ^{e,g}
Static	yes	mean	3500	750 ^{e,f}	340 ^{e,g}	420

^a Statistically significant differences are shown. No statistically significant differences were observed between the stroma-containing and stroma-free perfusion cultures or between stroma-containing perfusion and static cultures. No statistically significant differences were observed between the stroma-containing or stroma-free static cultures.

^b Significant difference between perfusion and static cultures at the indicated day using the paired Student's *t*-test.

^c $p < 0.01$.

^d $p < 0.05$.

^e Significant difference between day 0 and the indicated day using the paired Student's *t*-test.

^f $p < 0.05$.

^g $p < 0.01$.

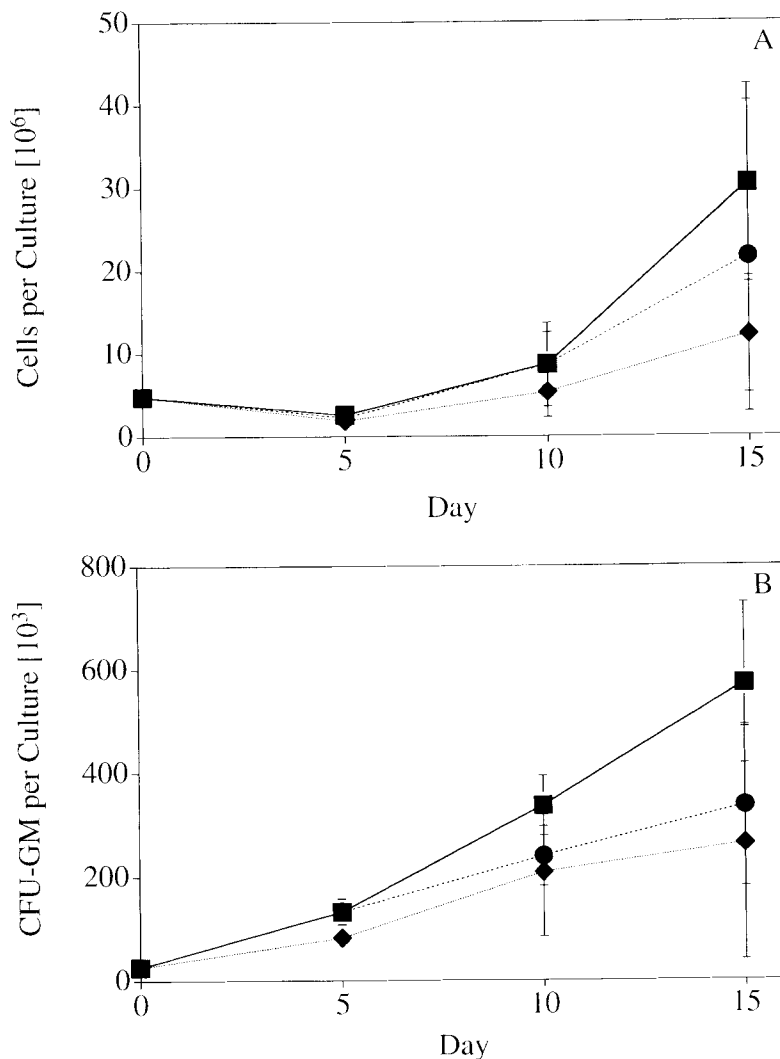


Figure 5. Total cells (A) and CFU-GM (B) from 15-day CB MNC cultures supplemented with IL-3, IL-6, G-CSF, and SCF. Cultures were inoculated with $5.1 \pm 0.1 \times 10^6$ MNCs containing $26 \pm 5 \times 10^3$ CFU-GM. Results are shown for the average of three (A) or two (B) separate experiments, and error bars represent the standard deviation. Symbols represent perfusion cultures in the grooved chambers (■) and smooth chambers (◆) and static cultures in petri dishes (●).

lower pH affect culture proliferation and differentiation; experiments are currently being carried out in our laboratories to address this issue. We also examined if the levels of some key cytokines were affected by the culture type. The different culture types did not have appreciably different IL-6, GM-CSF, or TNF- α concentrations,²² but other stimulatory or inhibitory factors might have been affected.

Cell Retention in the Grooved Chamber Using CB MNC Cultures

We compared stroma-free cultures in the grooved and smooth perfusion chambers and static petri dishes using CB MNCs in order to further evaluate the performance of the grooved chamber (Fig. 1). CB MNCs were chosen for these experiments because of their ready availability

and also because of their consistent expansion capability (as compared to PB MNCs). Cell retention in the grooved chamber was demonstrated by the 41% greater cell expansion in the grooved chamber compared to the static culture (Fig. 5A). In contrast to the grooved chamber, perfusion cultures in the smooth chamber (stroma free) gave only 56% of the cell expansion obtained in the static culture. Maximum cell expansions were observed on day 15 for all cultures. Average maximum cell expansions of 6.4-, 2.5-, and 4.5-fold were obtained in cultures using the grooved chamber, smooth chamber, and petri dish, respectively.

Further demonstration of efficient cell retention in the grooved perfusion chamber is the superior CFU-GM expansion obtained in the grooved chamber compared to the static cultures (Fig. 5B). Again, in contrast to the grooved chamber, perfusion cultures in the

smooth chamber contained fewer CFU-GM than the static cultures. Maximum CFU-GM expansions were observed on day 15 for all cultures. Average maximum CFU-GM expansions were 22-, 10-, and 13-fold in cultures using the grooved chamber, smooth chamber, and petri dish, respectively.

Cells are lost from the smooth chamber by being slowly swept toward the chamber outlet. Conversely, cells are retained in the grooved chamber by being isolated in the grooves from the bulk medium flow and therefore evenly distributed throughout the chamber.

An experiment was conducted in specially designed grooved and smooth perfusion chambers (Fig. 6) to allow visual inspection in order to observe the cell distribution in the chambers. Initially, the CB MNCs uniformly settle throughout both the grooved and smooth chambers (Figs. 6A and 6B), including the top of the groove walls in the grooved chamber (data not shown). After 14 days of culture, there were markedly fewer cells in the front two-thirds of the smooth chamber than the last one-third (Figs. 7.A–C), which corresponds with how we anticipated cell loss to occur. In contrast to the uneven cell distribution in the smooth chamber, the cell distribution in the grooved chamber near the inlet, middle, and outlet were very similar (Figs. 7.D–F).

In other experiments, although on day 0 there was an even distribution of cells in the grooved chamber both in the grooves and on top of the groove walls, local groove-to-groove variations were observed on day 14 (data not shown). On several instances, many cells could be observed in individual grooves while the grooves immediately next to it on the downstream side contained few cells (data not shown). This is most likely due to the initial distribution of cells with high replicative capacity and provides additional evidence that cells are not lost from the grooved chamber. If cells were swept out of an individual groove, they would most likely settle into a groove downstream. It would be anticipated therefore that grooves toward the outlet would contain more cells than grooves near the inlet, which was not observed. Finally, no cells were observed on top of the groove walls on day 14 (data not shown).

DISCUSSION

Perfusion cultures of PB MNCs obtained from patients following stem cell mobilization did not require a stromal layer for CFU-GM expansion or LTC-IC maintenance, while static cultures benefited from a stromal layer as demonstrated by enhanced CFU-GM expansion.

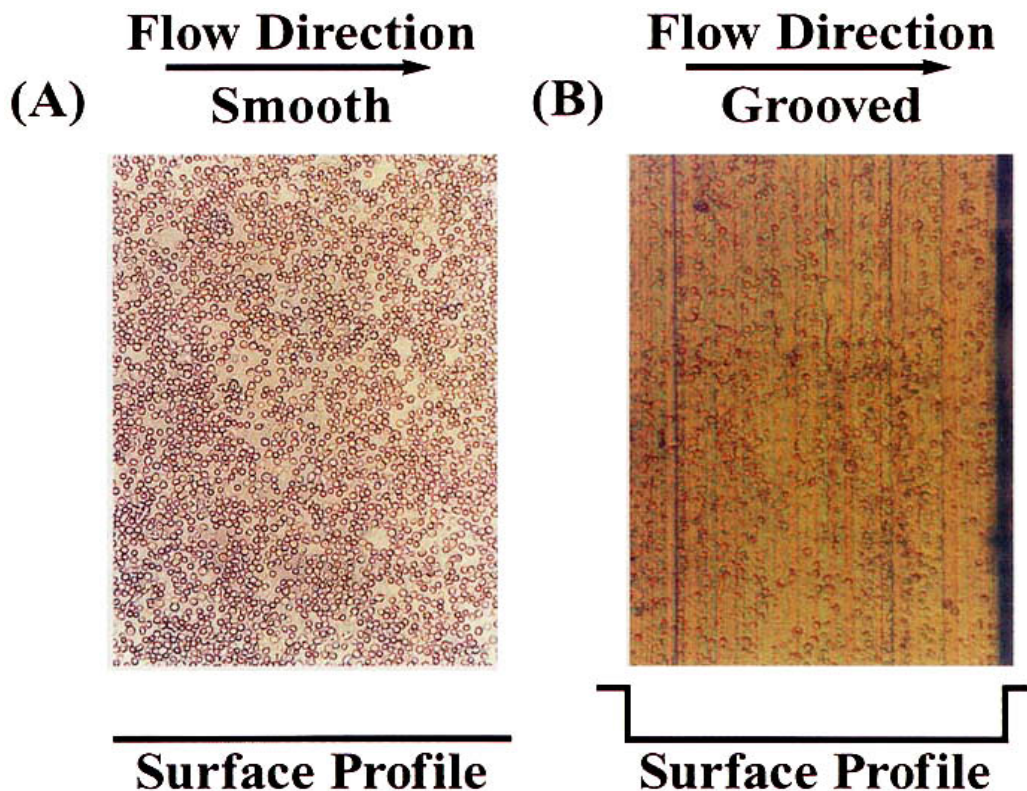


Figure 6. Pictures of day 0 cultures in the smooth (A) and grooved (B) chambers inoculated at 3.40×10^5 CB MNCs per cm^2 . The pictures were taken at $200\times$ magnification approximately 2 h after the cultures were inoculated in order to allow the cells to settle to the lower surface. Cells appeared uniformly distributed in both the smooth and grooved chambers. The direction of bulk medium flow and the relative position of the grooves are shown above and beneath the pictures, respectively. The parallel lines in the grooved chamber are scratches which resulted from the groove manufacture and the thick dark line is the groove wall. For the experiment of this figure and Fig. 7 we used a modified grooved perfusion chamber with grooves $400 \mu\text{m}$ across.

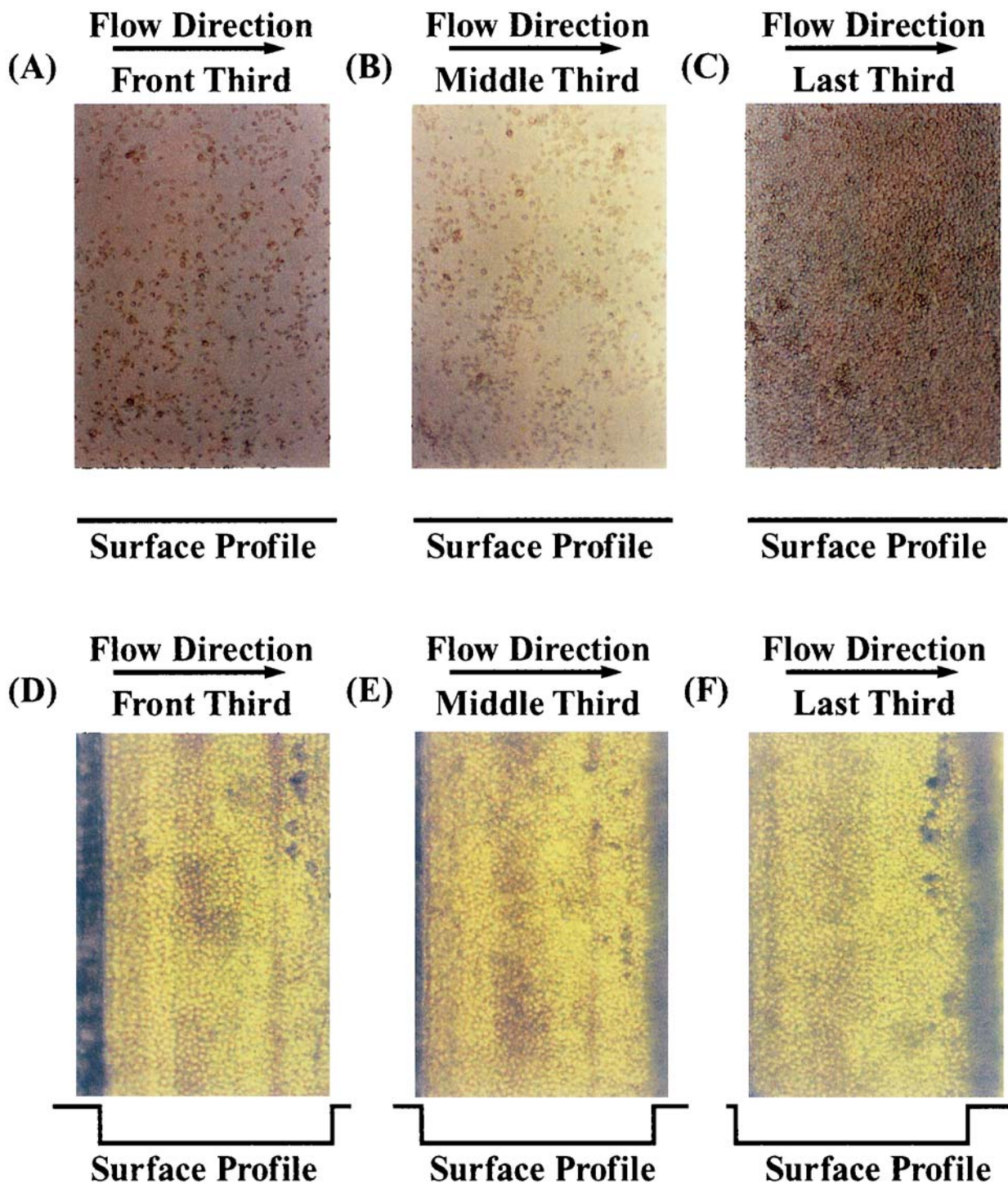


Figure 7. Pictures of day 14 cultures in the smooth and grooved chambers inoculated at 3.40×10^5 CB MNCs per cm^2 . These cultures were conducted at 37°C in HLTM supplemented with 0.50 ng/mL IL-3, 10 ng/mL SCF, and 3.0 ng/mL G-CSF. The flow rate in these perfusion cultures was 10 mL/min/chamber and the medium pH was controlled at 7.35 ± 0.05 . Perfusion cultures were fed 3 times by replacing one-third of the medium with fresh HLTM and HGFs. The pictures were taken at $200\times$ magnification of the front one-third (A; closest to the inlet), middle one-third (B), and last one-third (C; closest to the exit) of the smooth chamber and the front one-third (D), middle one-third (E), and last one-third (F) of the grooved chamber. The average final cell densities were 0.6×10^6 and 3.30×10^6 cell per cm^2 in the smooth and grooved chambers, respectively. The direction of bulk medium flow and the relative position of the grooves are shown above and beneath the pictures, respectively. The parallel lines in the grooved chamber are scratches which resulted from the groove manufacture and the thick dark lines on the edges of the pictures are the groove walls.

sion. Stroma-free perfusion cultures supported greater maximum CFU-GM expansion (19.4- and 16.6-fold for stroma-free and stroma-containing cultures on day 10, respectively) and LTC-IC maintenance (64% and 34% of input on day 15, respectively) than the stroma-containing perfusion cultures. In contrast, stroma-containing static cultures supported greater maximum CFU-GM expansion (17.9- and 12.5-fold for stroma-containing and stroma-free cultures on day 10, respectively) and equivalent LTC-IC maintenance (12% and 11% of input on day 15, respectively) when compared to the stroma-free static cultures.

The reduced (relative to stroma-free) culture enhancement of CFU-GM and LTC-IC numbers by perfusion in stroma-containing cultures may be due to a combination of two possible effects. First, stroma cultures allow direct contact between hematopoietic progenitor cells and stromal cells, which has been reported to inhibit progenitor cell expansion.³¹⁻³³ Second, perfusion may dilute some soluble stimulatory compounds produced by the stromal layer, which would offset the benefits of perfusion. Thus, the time-invariant environment approximated by perfusion has not benefited stroma cultures as much as stroma-free cultures in terms of colony-forming cells. Nevertheless, perfusion has substantially increased total PB MNC expansion in stroma-containing cultures. In contrast to the present PB MNC data, the data of Figure 5 and our earlier¹³ data show that perfusion dramatically improves both CFU-GM and LTC-IC cell expansion of CB MNCs on stroma.

The grooved perfusion chamber met or exceeded all of our original design requirements. Efficient cell retention in the grooved chamber was demonstrated with stroma-free CB MNC cultures by the greater cell and CFU-GM expansions than corresponding static cultures or perfusion cultures in the smooth chamber. In addition, the cell distribution in the smooth chamber correlates well with how cells are anticipated to be washed out (cells pile up near the chamber exit), while the cell distribution in the grooved chamber indicates that cells are not washed out. In excess of 97% of the cells in the grooved culture chamber could be routinely harvested without trypsinization. The harvesting procedure for the grooved chamber was very simple and only involved gentle agitation and a series of rinsing steps. This compares very favorably with the labor-intensive procedure required to harvest the stroma-containing cultures. (Stroma-containing cultures were harvested by trypsinizing the stroma-containing slide after it was aseptically removed from the chamber.) The inoculation procedure was equally simple and only involved injecting the cells into the chamber and letting them settle for a short period (1.5 h) before beginning perfusion. This compares very favorably to the labor-intensive procedure required for the smooth chamber design in which an irradiated stromal layer must be preformed and subsequently transferred to the smooth chamber before the

cultures can be inoculated. Finally, the grooved chamber design was relatively simple, did not employ any type of membrane, and allowed easy construction. The maximum cell density observed in the grooved chamber in these studies was 3×10^6 cells/cm², which would result in a maximum drop between the top and bottom of the groove in O₂ concentration of 4% of air saturation.

A therapeutic dose of 20×10^4 CFU-GM per kilogram of body weight has been suggested for rapid engraftment of neutrophils.² A perfusion system would require a surface area of approximately 670 cm² to produce therapeutic CFU-GM numbers from PB MNCs from patients following stem cell mobilizations with G-CSF and cyclophosphamide.²³ The grooved chamber design allows several possible scale-up strategies based on one or more of the following: (a) an increase of the gross surface area, and thus of the area occupied by the grooves, where the cells grow; (b) allowing multiple layers of cells to be cultured while still maintaining minimal concentration gradients (by increasing the medium flow rate); and (c) an increase of the width of the grooves, or a decrease in the space between the grooves, and thus an increase in the fraction of the gross surface area which is available to the cultured cells.

We thank Baxter employees Steve Smith for assistance with the preparation of the stromal layers, Daniel Boggs for assistance in designing the grooved perfusion chamber, and Garry Tierney for assistance with the computer data acquisition and control system. We thank Yoon Berm Kim at the University of Health Sciences, The Chicago Medical School (Department of Microbiology and Immunology, North Chicago, IL), for allowing access to the Mark I irradiator. We are grateful for the Fenwal bone marrow collection container filter units made available after bone marrow harvests by the University of Chicago Medical School (Chicago, IL), for the umbilical cord blood samples by St. Theresa Hospital (Waukegan, IL), and for the peripheral blood samples provided by Response Technologies (Memphis, TN). We are grateful for the donation of SCF by Amgen. Supported in part by National Institutes of Health grant HL48276 (to E. T. P. and W. M. M.) and Baxter Healthcare (Immunotherapy Division, Round Lake, IL).

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