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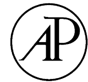

CYTOKINE-AUGMENTED CULTURE OF HAEMATOPOIETIC PROGENITOR CELLS IN A NOVEL THREE-DIMENSIONAL CELL GROWTH MATRIX

Naheed Banu, Michael Rosenzweig, Helen Kim, Jim Bagley, Mark Pykett

Studies aimed at the in vitro expansion of haematopoietic progenitor cells (HPCs) have suffered from the conflict of increasing cell numbers while maintaining long-term repopulating ability. We have developed a long-term bone marrow bioreactor culture system resembling the marrow-microenvironment that cultures HPCs in an inert, three-dimensional, porous biomatrix termed Cellfoam™. Previous studies have shown that the short-term culture of CD34$^+$ cells in Cellfoam™ improved the maintenance and multipotency of haematopoietic stem cells compared to cells cultured on plastic dishes. In this study, we examined the effects of low concentrations of cytokines including stem cell factor (SCF), IL-3, and Flk-2/Flt-3 ligand, on the maintenance, preservation and multipotency of CD34$^+$ cells cultured for 3 or 6 weeks in Cellfoam™. Analysis of cell yields using flow cytometry showed that in SCF and Flk-2/Flt-3 ligand-supplemented cultures as well as cytokine-free cultures, a higher number of CD45$^+$34$^+$ and CD45$^+$34$^+$38$^-$ cells is observed in Cellfoam™ cultures as compared to plastic cultures. The function of cultured cells was evaluated in colony-forming assays. The data demonstrate that Cellfoam™ cultures supplemented with SCF and Flk-2/Flt-3 ligand resulted in a higher output of colony activity compared to plastic cultures. Analysis of CAFC (29 days) activity also demonstrated that primitive progenitors were maintained to a greater extent in Cellfoam™ cultures containing either no cytokines or low concentrations of early-acting cytokines. These data suggest that culture of HPCs in three-dimensional bioreactors such as Cellfoam™ for extended periods may benefit from the addition of low levels of early-acting cytokines, including SCF and Flk-2/Flt-3 ligand, resulting in high yields of cells that are enriched for multipotent haematopoietic progenitors. These findings demonstrate that a three-dimensional matrix promotes the survival of primitive HPCs in culture and may modulate the in vitro effects of cytokines.

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Approaches to the ex vivo expansion of haematopoietic progenitor cells (HPCs) have generally involved culture techniques that include bone marrow stromal or cytokine support.[1–3] These approaches generally result in the expansion of CFU in vitro, but are complicated by a loss of more primitive cells. Studies performed in mice have shown that the ex vivo culture and expansion of marrow cells in the presence of a variety of growth factors may accelerate haematopoietic recovery.[4,5] However, vigorous assessments of these cells in secondary transplantation models have shown that they have diminished engraftment capacity,[6,7] suggesting that the choice and dose of cytokines used may negatively impact on the engraftment capacity of HPCs, including cells treated in transduction protocols.[8–13] Recently, serum has been shown to have a negative effect on engraftment.[14] Hirayama and Ogawa have noted that transduction of murine bone marrow cells in the presence of cytokines inhibited the capacity of the HPCs to differentiate into B cells.[10–12] Investigators demonstrated that an expansion protocol consisting of culture in the context of IL-3, IL-6, stem cell factor (SCF) and Flt-3 ligand with or without autologous stroma led to a diminished long-term repopulating ability of the graft.[14] Importantly, the effects of cytokines on HPC survival in vitro have not been separated from their effects on proliferation and differentiation.

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Recent evidence has indicated that the cytokine-driven proliferation and maturation of HPCs are associated with exit from G_0 quiescence and entrance into active cell cycling.[15–17] Such concerns are reinforced by recent data, which demonstrate a profound loss of engraftment capacity of primitive haematopoietic progenitors that have undergone ex vivo expansion via cytokine augmentation.[18–20] However, some recent data indicate that a proper balance between proliferation and quiescence of HPCs mediated through the use of certain cytokine cocktails and doses may enable the expansion of engraftable HPCs.[21] The basis for the differential effects of cytokines may be rooted in the heterogeneous cell cycle distribution of this collective cell population.[22] These observations reinforce the hypothesis that HPC immaturity and pluripotency are associated with a slowly proliferating, quiescent state.

Collectively, these data raise concerns about in vitro protocols that culture human progenitors in the presence of exogenous cytokines and suggest that certain types and doses of cytokines diminish the long-term repopulating ability of the graft. To improve the ability to provide cells with long-term engraftment potential, alternative culture systems must be developed that do not deplete the content of long-lived, immature, multipotent progenitors.

We have examined the utility of a three-dimensional tissue matrix termed Cellfoam™ to support HPCs for extended periods in vitro, as well as the effect of this tissue scaffold on modulating the in vitro effects of various cytokines. Human HPCs were cultured in the presence or absence of early-acting cytokines SCF or Flk-2/Flt-3 ligand, or the multilineage cytokine interleukin (IL)-3, using traditional liquid culture on fibronectin-coated plastic or fibronectin-coated Cellfoam™ devices for 3 or 6 weeks. Cytokines were used at doses 10–100 times lower than previously described. In general, Cellfoam™ cultures outperformed the controls, resulting in higher cell yields and greater number of CFU and CAFC. Our studies demonstrate that there may be a synergy between the uses of a three-dimensional scaffold together with low-dose cytokines to preserve and expand both more mature as well as immature HPCs.

RESULTS

Expansion of $CD34^+$ and $CD34^+CD38^-$ cells in long-term cultures

Primitive human bone marrow progenitor cells have been shown to express the CD34 antigen. Such $CD34^+$ cells comprise only 1–4% of bone marrow mononuclear cells. For the long-term cultures described, we isolated human bone marrow $CD34^+$ cells by immunomagnetic beads with greater than 90% purity. To assess the effects of cytokine augmentation on the viability and yield of $CD34^+$ haematopoietic cells in cultures in Cellfoam™ (CF) or plastic (PL), we performed a time course study that examined these parameters after 3 and 6 weeks in culture. After 3 and 6 weeks in culture, cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies to CD45. This enabled gating on $CD45^+$ haematopoietic cells to exclude non-haematopoietic cells.

To assess more directly the effects of cytokine-augmented cultures on haematopoietic progenitor cells, we next analyzed the expression patterns of CD34 and CD38 on cells isolated from the various cultures. Representative results are shown in Figure 1. Significantly higher numbers of $CD34^+$ cells were obtained in cytokine-free Cellfoam™ cultures at 6 weeks compared to cytokine-free plastic cultures. With the exception of IL-3 in ng/ml concentrations, all cytokine-supplemented cultures exhibited higher $CD34^+$ cell yields in CF cultures than in plastic (14 of 16, 88%). These findings are summarized in Figure 1B, which shows the relative fold increase in total $CD34^+$ cell numbers in Cellfoam™ compared to plastic. Again with the exception of cultures supplemented with IL-3, $CD34^+$ cell yields were generally two- to 16.6-fold higher in Cellfoam™. Analysis of the $CD34^+38^-$ content of the cultures demonstrated very similar findings, where again 14 of 16 conditions yielded higher numbers of these cells in Cellfoam™ cultures than in plastic controls (Fig. 2A and B).

Colony-forming activity of the haematopoietic progenitor cells in long-term cultures

Cells expressing the CD34 surface antigen constitute a heterogeneous population of haematopoietic cells that include primitive, uncommitted stem/progenitor cells capable of initiating long-term haematopoiesis in vitro as well as more mature progenitors committed to different lineages. To measure the colony-forming activity of the progenitor cells from 3- and 6-week cultures, standard methylcellulose assays were performed. Figure 3A shows the results from cultures supplemented with ng/ml levels of cytokines. With the exception of IL-3, total colony-forming unit (CFU) numbers were consistently higher in CF compared to plastic controls. This agrees with the functional ability of IL-3 to serve as a multi-lineage growth factor to stimulate HPCs to exit from the G_0 state and proliferate.[26] Further, with the exception of SCF and control culture in CF, CFU yields in both CF and PL were reduced over time from 3 to 6 weeks. Importantly, supplementation with IL-3 obliterated colony-forming activity. Figure 3B represents the fold differences in total colony-forming activity of cells isolated from CF cultures as compared to plastic. Of the various conditions assessed, the number of CFUs

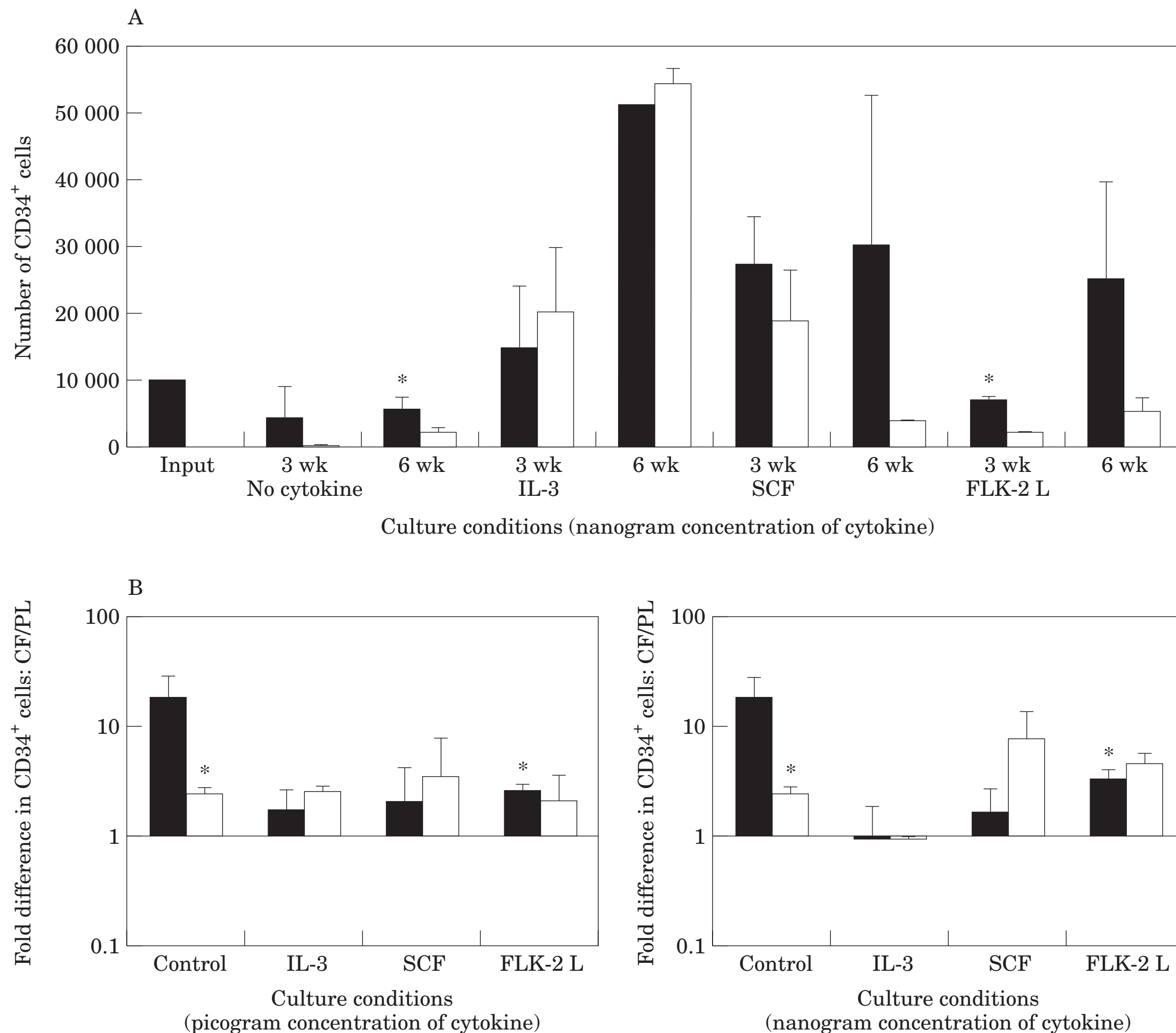

Figure 1.

A: Yield of $CD34^+$ cells in Cellfoam™ (CF, ■) and plastic (PL, □) cultures without exogenous cytokines (control) or supplemented with single cytokines in nanogram/ml concentrations at 3 and 6 weeks. $CD34^+$ cell numbers in CF and PL cultures were determined by gating $CD45^+$ haematopoietic cells and then sub-gating on $CD34^+$ haematopoietic progenitors in the flow cytometry analysis. The input $CD34^+$ cell number is presented by the bar at left hand side of the graph. Statistically significant ($P<0.05$) $CD34^+$ cell yields between CF and PL are shown by asterisks. B: Comparison of $CD34^+$ cell yields in Cellfoam™ (CF) plastic (PL) cultures without exogenous cytokines or supplemented with single cytokines for 3 (■) and 6 (□) weeks. The fold difference of $CD45^+CD34^+$ cell yields in CF cultures as compared to PL is shown for both picogram/ml concentrations (left) and nanogram/ml concentrations (right). Bars above the 1.00 line indicate the number of fold higher cell yields were in CF as compared to PL; bars below the 1.00 line indicate the number of fold higher cell yields were in PL as compared to CF. Scale=log base. Asterisks indicate statistically significant values ($P<0.05$, two-tailed Student's *t*-test) between Cellfoam™ and plastic culture cell yields.

recovered was observed to be significantly higher in CF cultures than in PL cultures (91%, $P=0.001$, two-tailed Student's *t*-test). With the exception of SCF, the colony-forming activity of cultures with higher (i.e. ng/ml) concentration of cytokines decreased from 3 weeks to 6 weeks. These data support the proliferative role of IL-3 on progenitors. The results also confirm the previous findings that SCF plays a greater role than in sustaining more primitive HPC, namely pre-CFC.[27]

Endogenous secretion of cytokines

To date, extended in vitro maintenance and differentiation of haematopoietic stem cells have required growth factors in the culture system. Interestingly, Cellfoam™ cultures without any exogenous cytokines showed significantly higher numbers of $CD34^+$ cells and colony-forming cell yields at 6 week of cultures compared to plastic cultures. To examine the nature and level of any endogenously secreted cytokine in the long-term Cellfoam™ cultures, ELISAs were done. Supernatants of the cultures were collected over a 6 week time period and the concentrations of secreted cytokines were determined by ELISA assays. Figure 4 indicates that the cytokines examined were secreted at picogram concentrations. The early acting cytokine SCF was secreted at highest concentration.

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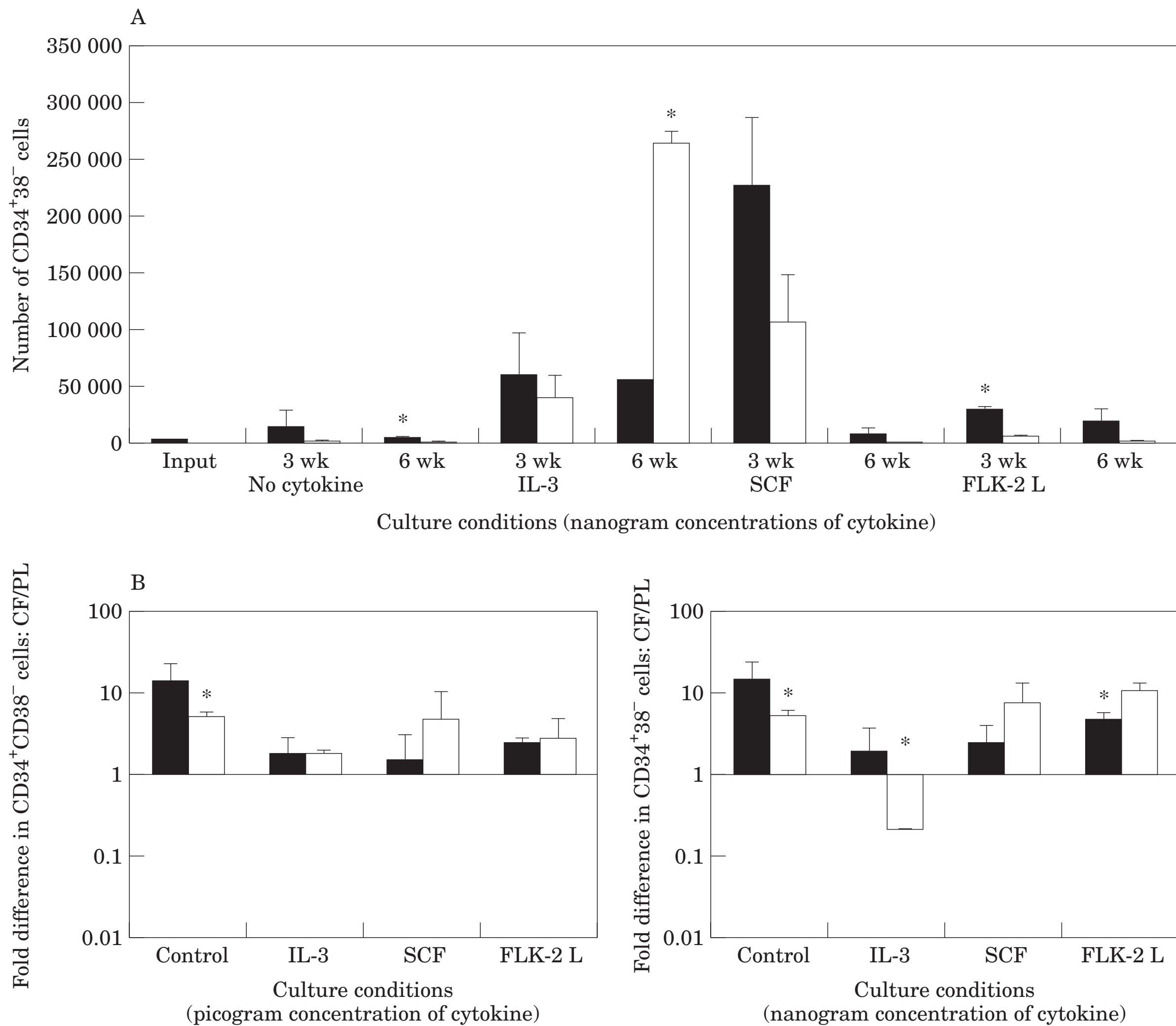

Figure 2.

A: Yield of $CD45^+CD34^+CD38^-$ cells in Cellfoam™ (CF, ■) and plastic (PL, □) cultures without exogenous cytokines or supplemented with single cytokines in nanogram/ml concentrations at 3 and 6 weeks. $CD34^+CD38^-$ cell numbers in CF and PL cultures were determined first by gating on $CD45^+$ cells and then sub-gating on CD34 and CD38. The bar at left hand side of the graph presents the input $CD45^+CD34^+CD38^-$ cell number. Statistically significant ($P<0.05$) results between Cellfoam™ and plastic cultures are shown by asterisks. B: Comparison of $CD34^+CD38^-$ cells yield in Cellfoam™ (CF) and plastic (PL) cultures without exogenous cytokines or supplemented with single cytokines in picogram/ml (top) or nanogram/ml (bottom) concentrations for 3 (■) and 6 (□) weeks. The fold difference of $CD45^+CD34^+CD38^-$ cell yields in CF cultures as compared to PL is shown for both picogram/ml concentration (left) and nanogram/ml (right) concentrations. Bars above the 1.00 line indicate the number of fold higher that cell yields were in CF as compared to PL; bars below the 1.00 line indicate the fold higher numbers obtained in PL as compared with CF cultures. Scale=log base. Statistically significant values ($P<0.05$, two-tailed Student's *t*-test) between Cellfoam™ and plastic cultures are denoted by asterisks.

Secretion of IL-3 and Flk-2/Flt-3 ligand was very low compared to SCF secretion. Cytokine production in Cellfoam™ was greatest after 3 weeks. At this point, there was no change in SCF secretion in plastic control cultures and viability of the cells in the cultures started to decline and so no comparison could be made.

Maintenance of long-term precursor cells

To investigate the "primitiveness" of the haematopoietic cells isolated from CF and PL cultures, cobblestone area-forming cell (CAFC) assays were done. CAFC assays, which relate the primitiveness of the stem cell to the onset and duration of cobblestone area formation beneath a stromal layer, were performed on cells isolated from 6-week cultures in CF and PL supplemented with ng/ml levels of cytokine. Figure 5 shows that CAFC readouts were greater in all cytokine-augmented CF and PL cultures compared to parallel controls receiving no cytokines. With the exception of 15-day CAFC readouts from IL-3-supplemented cultures, 15- and 29-day CAFC readouts for cultures grown in the presence or absence of cytokines were greater in CF than in PL. This suggests

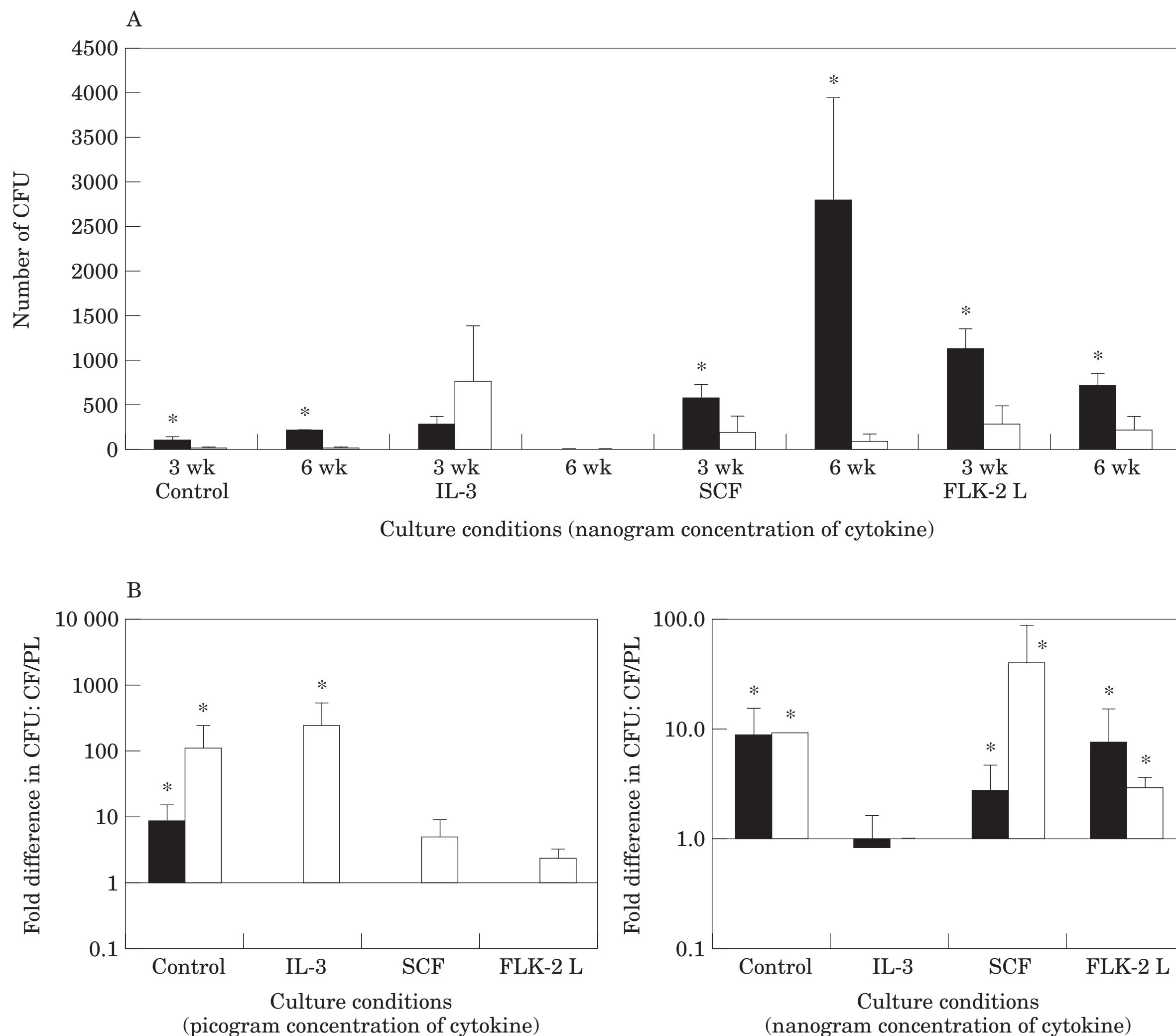

Figure 3.

A: Total colony activity of the cells isolated from Cellfoam™ (CF, ■) and plastic (PL, □) cultures at the respective time points in the absence or presence of single cytokines. Colony-forming activity in CF cultures statistically significant ($P<0.05$, two-tailed Student's *t*-test) from plastic cultures is shown by asterisks. B: Determination of colony-forming unit (CFU) activity of cells cultured in Cellfoam™ (CF) and plastic (PL) in the absence or presence of single cytokines for 3 (■) and 6 (□) weeks. The graphs represent the fold difference in total colony activity of cells obtained from CF cultures as compared to PL cultures. Cytokines were used in picogram/ml concentration (left) and nanogram/ml concentration (right) alone or in combination. Bars above the 1.00 line indicate the fold higher CFU activity obtained in CF compared to PL; bars below the 1.00 line indicate the fold higher CFU activity in PL compared to CF. Scale=log base. Asterisks indicate statistically significant values ($P<0.05$, two-tailed Student's *t*-test) between Cellfoam™ and plastic culture CFU yields.

that primitive stem cells may benefit from the three-dimensional structure and a low concentration of early-acting cytokine (SCF) to survive for longer periods of time in in vitro cultures.

DISCUSSION

The expansion of haematopoietic progenitor cells (HPCs) in in vitro cultures is an area of active interest in the field of stem cell transplantation, haematopoietic stem cell gene therapy and transfusion medicine. Many investigators have already shown that it is possible to expand sub-populations of haematopoietic cells, including colony-forming cells, by the addition of cytokines. However, expansion of the cells responsible for long-term reconstitution has not met with similar success.[26,28–30] The addition of high doses of cytokines to HPC cultures promotes the proliferation and differentiation of early pre-progenitors by terminal differentiation, thereby amplifying the progenitor and precursor pools. Unfortunately, as this happens the number of $CD34^+$ cells and primitive long-term culture initiating cells (LTC-IC) in these cultures decreased. As a result of longer incubation periods with cytokines, the cultures become depleted in pre-progenitors

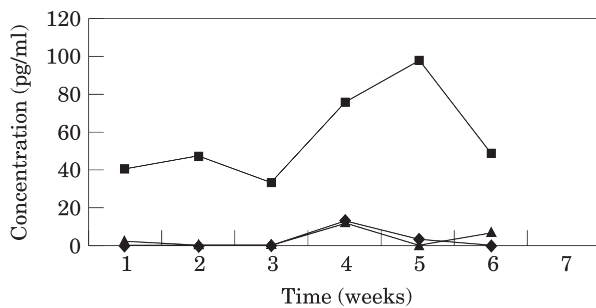

Figure 4. A representative ELISA profile of the endogenous production of cytokines over 6 weeks of Cellfoam™ cultures.

Supernatant of cytokine-free Cellfoam™ cultures seeded with CD34$^+$ cells in 10% FBS containing RPMI-1640 media was collected for 6 weeks. Following the manufacturer's instruction, the levels of secretion of SCF (—■—), Flk-2/Flt-3 (—▲—) ligand, IL-3 (—◆—) in the supernatant were examined. The graph shows the secretion of the cytokines in Cellfoam™ cultures for 6 weeks above the control media which contains 10% FBS.

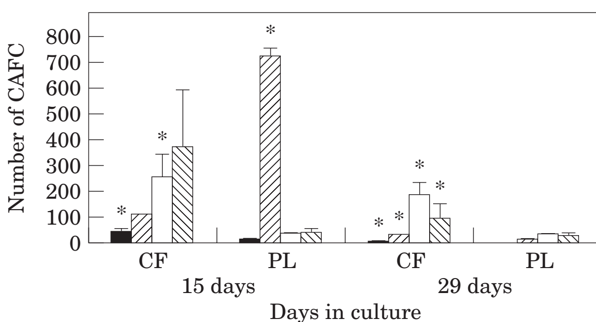

Figure 5. Limit dilution analysis of CAFC numbers in cells cultured in the Cellfoam™ (CF) and plastic (PL) systems.

CAFC readouts at 15 and 29 days are shown for CF or PL cultures maintained for 6 weeks in the presence of nanogram/ml concentrations of the indicated cytokines or in cytokine-free media (control). Statistically significant values (P<0.05, two-tailed Student's t-test) between Cellfoam™ and plastic CAFC culture yields are denoted by asterisks. Data are from two independent experiments. No cytokine, ■; IL-3, ▨; SCF, □; FLK-2L, ▧.

cells.[31–34] Thus, such cultures may not show any evidence of true stem cell expansion, but rather of in vitro differentiation.

In this study, we examined the effects of low concentrations of cytokines (picogram/ml and nanogram/ml), on the maintenance, preservation, and colony activity of CD34$^+$ cells cultured for 3 to 6 weeks in a novel, three-dimensional porous cell-growth matrix (Cellfoam™) compared to conventional monolayer, control cultures in plastic. We chose low concentrations of cytokines in order to achieve an environment similar to steady state in vivo haematopoiesis levels as deduced from stromal supernatants.[35–37] It should be noted here that the local concentration of SCF and Flk-2/Flt-3 ligand might be higher in the extracellular matrix than in the supernatant as such cytokines are also expressed as transmembrane, cell-associated forms which may make important contributions to the control of haematopoiesis by the stromal microenvironment.

Previously, we have reported that Cellfoam™ supports CD34$^+$ cell viability, multipotency and maintenance of long-term progenitor cells in the absence of exogenous cytokines at time points beyond other systems, including fibronectin-coated plastic dishes and bone marrow stroma as well as other three-dimensional devices.[38] It was shown that after initial cultures in Cellfoam™ (3 or 6 weeks) with no exogenous cytokines, significantly greater expansion in colony-forming ability and ELTCIC activity were observed in Cellfoam™ compared to bone marrow stroma cultures, fibronectin-coated plastic cultures and three-dimensional ceramic cultures. Furthermore, we have observed that the endogenous production of cytokines in otherwise unsupplemented CD34$^+$ cultures in CF is in the pg/ml range. The present experiments demonstrate that cytokines, when used at concentrations that are lower than generally applied in the field, can expand at least certain components of the heterogeneous CD34$^+$ haematopoietic progenitor cell population in cultures performed in the context of a three-dimensional growth support. In particular, Cellfoam™ cultures were observed to support higher levels of cell expansion than plastic cultures, yielding higher numbers of CD45$^+$CD34$^+$ and CD45$^+$CD34$^+$CD38$^-$ cells at both 3 and 6 weeks. This is also true for both colony-forming and primitive long-term progenitor cells present in 3 and 6 week cultures in Cellfoam™. It appears that the porous three-dimensional Cellfoam™ matrix may mimic the in vivo spatial arrangements of cytokine and extracellular matrix distribution which haematopoietic progenitor cells encounter and which may enhance cytokine–receptor interactions. Although CD38 negativity can be used to assess HPC immaturity, caution should be used in such interpretations of long-term cultures such as examined in the present study, as the level of CD38 on HPC may be highly variable.

The collective data indicate that selective use of particular cytokines can lead to expansion of colony-forming cells and that greater increases in colony-forming activity are achieved in Cellfoam™ cultures under most conditions. It is interesting to note that the colony-forming activity of cells cultured in nanogram concentrations of cytokines was found to decrease from 3 to 6 weeks (except with SCF). These results agree with previous findings of other investigators showing that exposure of haematopoietic progenitor cells to cytokines results in initial increases in total cell numbers and CFU content through proliferative mechanisms that trigger terminal differentiation. Our studies also show that the colony-forming abilities of the harvested cells from cultures supplemented with

IL-3 or combinations of cytokines were lower than control cultures with no cytokines. These data suggest that supplementation with IL-3, even at low doses, depletes CFU activity. Cultures of HPCs in Cellfoam™ without the addition of exogenous cytokines, or with SCF alone, can lead to increases in CFU content over 6 weeks of culture. These data indicate that SCF and FLK-2 ligand alone may have the potential to promote CFU increases over prolonged periods in the context of a three-dimensional cell-growth scaffold such as Cellfoam™. These results appear to differ from previous reports on in vitro cultures showing that these two early-acting growth factors alone have little or no effect on primitive stem cells, but exhibit synergy with other early-acting growth factors.[39–44] However, our data agree with an in vivo study by Tong *et al*., which showed that administration of SCF to human subjects increased the absolute number of $CD34^+$ cells and primitive and myeloid progenitor cells in bone marrow.[45] Recent publications, however, have observed that the addition of thrombopoietin to HPC cultures results in the expansion of CFU and LTCIC.[23] We are currently investigating the effects of this cytokine in our system.

In the current study, the survival and proliferation of the more primitive haematopoietic stem cells was assessed by CAFC assays. Interestingly, CAFC assays indicated that over 6 weeks of Cellfoam™ cultures, supplementation with only a single, early-acting cytokine, particularly SCF, can maintain and preserve long-term progenitor cells, whereas the multilineage cytokine IL-3 depleted primitive precursor cell content after just 3 weeks. This corroborates previous findings of the role of SCF in survival of primitive stem cells.[27] Zandstra *et al*.[24] showed that 10 days of incubation with high concentrations of cytokines significantly amplified LTC-IC content of the haematopoietic progenitor cells. In contrast, our data suggest that culturing haematopoietic progenitor cells with low concentrations of cytokines for extended periods of time (3 or 6 weeks) in the three-dimensional Cellfoam™ matrix maintains LTC-IC content. Our data showed that LTC-IC amplification is reduced with decreases in the concentration of SCF and FLK-2 ligand or with increased concentrations of IL-3, which is consistent with previous observations.[24] Overall, our study indicates that three-dimensional structures such as that embodied by Cellfoam™ are useful for stem cell expansion.

MATERIALS AND METHODS

Culture materials

The culture devices used in these studies consisted of the following: (1) Cellfoam™, a biocompatible, three dimensional matrix arranged as a series of continuous channels and interconnected pores with a porosity of approximately 90% (Cytomatrix, Woburn, MA, USA) coated with full-length fibronectin (Boehringer Mannheim, Germany); (2) multiwell plastic dishes (Corning-Costar, Acton, MA, USA) coated with full-length fibronectin (Boehringer Mannheim, Germany). The Cellfoam™ biomatrix is fabricated by a vapor deposition/infiltration process in which solid tantalum metal is vaporized at high temperature and precipitated as a thin layer onto a carbon lattice, which comprises the strut or ligament skeleton of the matrix. The lattice is arranged in interconnected pores, each roughly approximating a dodecahedron, which create a series of continuous microniches. This geometry and the surface texturing arising from the metal vapor deposition process produce high surface area:volume ratios. The matrix is readily coated with substrate molecules such as fibronectin and collagens through standard incubation procedures.

Long-term cultures

$CD34^+$ haematopoietic progenitor cells derived from commercially purchased human bone marrow samples (Poietic Technologies, Gaithersburg, MD, USA) were isolated by magnetic cell separation column (Miltenyi Biotech, Auburn, CA, USA). To establish the long-term cultures, $66–160 \times 10^5$ $CD34^+$ cells were seeded onto plastic 48-well dishes coated with fibronectin (plastic), or into fibronectin-coated Cellfoam™ (Cytomatrix) also in 48-well dishes. Cultures utilized 1 ml of RPMI (Sigma, St. Louis, MO, USA) supplemented with 10% Fetal Bovine Serum (Sigma), penicillin (10 IU/ml), streptomycin (10 ug/ml), L-glutamine (1 mM), Hepes (10 mM), Fungizone 2.5 ug/ml (Life Technologies, Gaithersburg, MD, USA). In cytokine-augmented cultures, human recombinant cytokines were added at two different concentrations ("ng/ml" and "pg/ml" concentrations) as follows: IL-3 (Genzyme, Cambridge, MA, USA) at 10 ng/ml or 100 pg/ml; IL-6 (Genzyme) at 10 ng/ml or 100 pg/ml; and SCF and FLK-2 ligand both at 25 ng/ml or 250 pg/ml (R & D system, Minneapolis, MN, USA). The cytokines were titrated and the optimal concentration of each cytokine was determined. The concentrations of the cytokines used in this study were 1- to 200-fold below concentrations described by others, but were still within the active range.[23,24] In control cultures, no exogenous cytokines were added. Half-volume media changes were performed twice a week. Cultures were performed in duplicate.

After each incubation period, all cells (adherent and non-adherent) were harvested from all culture conditions. Non-adherent cells were harvested from Cellfoam™ devices by centrifugation for 10 min at approximately $250 \times \boldsymbol{g}$. Adherent cells from three-dimensional culture devices were collected by incubating cellfoam with Cell Dissociation Solution (CDS; Sigma) for 30 min at 37°C and then centrifuging at approximately $250 \times \boldsymbol{g}$ for 10 min. Non-adherent cells from fibronectin-coated plastic cultures were collected by gentle washing; adherent cells were collected by incubation in CDS for 30 min at 37°C. Both adherent and non-adherent cells were pooled and viable cells were counted.

Flow cytometry analysis

After 3 and 6 weeks of culture, all cells (adherent and non-adherent) were harvested from all culture conditions, counted, and stained with fluorochrome-conjugated antibodies to surface antigens. Antibodies for phenotype determination included anti-CD34 (Qbend10, Immunotech, Westbrook, Maine, USA), anti-CD38 (Becton Dickinson, San Jose, CA, USA) and anti-CD45 (Becton Dickinson) antibodies to evaluate progenitor cell distributions. Cells were stained in the presence of staining buffer (PBS with 2% fetal bovine serum). After staining, the cells were fixed with 2% paraformaldehyde. Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson). Appropriate controls included matched isotype antibodies to establish positive and negative quadrants, as well as appropriate single color stains for compensation. For each sample, at least 20 000 list mode events were collected.

Colony-formation assays

To assess the colony-forming ability of cells isolated from cultures in Cellfoam™ or plastic, traditional methylcellulose assays were performed. Equal numbers of cells isolated from Cellfoam™ or plastic cultures, as described above, were added at 6.67×10^4 cells/ml to 3.0 ml of methylcellulose medium with cytokines (IL-3 20 ng/ml: erythropoietin 3 IU/ml; stem cell factor 50 ng/ml; all Stem Cell Technologies, Vancouver, Canada) plus 0.5 ml of DMEM (2% FCS, 10 IU/ml penicillin, 10 μg/ml streptomycin, 1 mM L-glutamine). 1.5 ml of this mixture was added to a 35 mm gridded petri dish. Duplicate assays were performed for each condition. Duplicate petri dishes were placed in a humidified incubator (37°C temperature and 5% CO_2) for 10–21 days. Following the incubation period, the number of colonies was determined by light microscopy. Positive colonies were scored on the basis of an accumulation of 40 or more cells. Counts were done in duplicate.

Enzyme-linked immunosorbent assays (ELISA) for cytokines

Supernatants were collected from cytokine-free Cellfoam™ cultures during bi-weekly media changes. After centrifugation to remove cell debris, supernatants were aliquoted and frozen at −80°C until use. After the collection of all supernatants over the 6-week period, samples were thawed on ice, mixed well and the concentrations of secreted cytokines were measured with commercially available ELISA kits (R&D Quantikine Immunoassay Kits from R&D Systems) for human IL-3, human SCF and human Flk-2/Flt-3 ligand. Following the manufacturer's instructions, reagents and working standards were prepared and assays were done. Briefly, diluents and 50–200 ul of supernatant or standard (depending on particular assay) were added in duplicate into each well of the pre-coated micro-titer plate and allowed to incubate at room temperature for 2 h. Control wells contained RPMI-1640 media with 10% serum without any cytokine. The wells were then washed with wash buffer and the conjugate was added and allowed to incubate for 1.5–2 h. After washing, the substrate solution was added and allowed to incubate for 20–30 min. Finally, 50 ul of stop solution was added and the optical density was read immediately using the Titertek Multiskan Plus set at 450 nm. A standard curve was established and cytokine concentrations (pg/ml) were derived from the standard curve.

CAFC assays

Confluent layers of FBMD-1, a mouse stroma cell line, (Genetics Institute, Cambridge, MA, USA) in flat 96-well plates were overlaid with cells harvested from various culture conditions in limiting dilution.[25] Input numbers of cells ranged from 6 to 6000 per well. Four dilutions ten-fold apart were used for each sample with six replicate wells per dilution. The cells were cultured in DMEM (Life Technologies) supplemented with penicillin (10 IU/ml), streptomycin (10 ug/ml), L-glutamine (1 mM), Fungizone (2.5 ug/ml) (Life Technologies), 20% Horse serum (Sigma), 10^{-5} M Hydrocortisone (Sigma), 10^{-5} M 2-mercaptoethanol (Sigma), 10 ng/ml IL-3 (Genzyme) and 20 ng/ml G-CSF (R & D Systems) at 34°C in 5% CO_2. Fresh media with growth factors was added weekly. The percentage of wells with at least one phase-dark haematopoietic clone of at least five cells (Cobblestone area) beneath the stroma layer was determined weekly for 2–6 weeks. CAFC frequencies were calculated using Poisson statistics.

Statistical analysis

The results were expressed as the mean ± SD of data obtained from three different experiments performed in duplicate. Statistical significance was determined using the Student's *t*-test.

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