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Expansion of engrafting human hematopoietic stem/progenitor cells in three-dimensional scaffolds with surface-immobilized fibronectin

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Abstract

An efficient and practical *ex vivo* expansion methodology for human hematopoietic stem/progenitor cells (HSPCs) is critical in realizing the potential of HSPC transplantation in treating a variety of hematologic disorders and as a supportive therapy for malignant diseases. We report here an expansion strategy using a three-dimensional (3D) scaffold conjugated with an extracellular matrix molecule, fibronectin (FN), to partially mimic the hematopoietic stem cell niche. FN-immobilized 3D polyethylene terephthalate (PET) scaffold was synthesized and evaluated for HSPC expansion efficiency, in comparison with a FN-immobilized 2D PET substrate and a 3D scaffold with FN supplemented in the medium. Covalent conjugation of FN produced substrate and scaffold with higher cell expansion efficiency than that on their unmodified counterparts. After 10 days of culture in serum-free medium, human umbilical cord blood CD34⁺ cells cultured in FN-conjugated scaffold yielded the highest expansion of CD34⁺ cells (~100 fold) and long-term culture initiating cells (~47-fold). The expanded human CD34⁺ cells successfully reconstituted hematopoiesis in NOD/SCID mice. This study demonstrated the synergistic effect between the three-dimensionality of the scaffold and surface-conjugated FN, and the potential of this FN-conjugated 3D scaffold for *ex vivo* expansion of HSPCs.

Keywords

hematopoietic stem cells; expansion; scaffold; fibronectin; immobilization

Introduction

Hematopoietic stem/progenitor cell (HSPC) transplantation is an established treatment for a variety of hematological disorders and as a supportive therapy for malignant diseases. However, insufficient number of donor HSPC cells, in many cases, limits its application.^{1,2} An efficient and practical *ex vivo* HSPC expansion methodology is critical in realizing its tremendous potential.³ The goal of *ex vivo* HSPC expansion is to produce sufficient number of cells that can engraft and sustain long-term hematopoiesis. HSPCs in bone marrow maintain the ability to balance self-renewal and commitment to differentiation, whereas in culture, they

tend to lose their self-renewal ability after extensive proliferation. The underlying mechanism that regulates the fate decision of HSC remains elusive, but it has increasingly been proposed that signals emanating from the HSC niche play a crucial role, apart from the autonomous signals.^{4,5} HSC niche is thought to be composed of a range of differentiated cell types (osteoblasts and stromal cells such as endothelial cells, fibroblasts, etc.), a rich milieu of extracellular matrix (ECM) molecules, and a variety of soluble and membrane/ECM bound hematopoietic growth factors or cytokines distributed in this three-dimensional (3D) niche.^{4,5}

Stromal cell-supported culture has been shown to preserve the ability of human HSPCs to sustain durable, long-term hematopoiesis in immune deficient mice.^{6–8} However, obtaining a sufficient quantity of autologous stromal cells for clinical applications can be problematic, laborious, and unpredictable. Allogeneic stromal cells or mesenchymal stem cell-derived cell lines, although feasible, suffer from potential contamination and immune response following transplantation.^{9,10} Therefore, alternate methods of HSPC expansion that are equally effective would be preferable.

ECM components are important elements in the HSC niche. They provide the adhesive interactions between HSPCs and cell adhesion molecules (CAMs), which are of critical importance in the regulation of hematopoiesis by lodging and retaining HSPCs in the bone marrow niche, in close proximity with osteoblasts, stromal cells, and signaling molecules they produce.^{11,12} For example, adhesion between fibronectin (FN) and the very late antigen-4 (VLA-4, $\alpha_4\beta_1$ integrin) and VLA-5 ($\alpha_5\beta_1$ integrin) on HSPCs, among a few other types of adhesion, have been shown to serve multiple functions in homing, lodging, and regulation of HSPCs in the niche.^{13–15} Bhatia *et al.* and Lundell *et al.* have shown an increased long-term culture initiating cell (LTC-IC) and extended LTC-IC preservation after expansion in contact with FN.^{16,17} Binding of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ -integrins to FN has also been shown to enhance LTC-IC preservation.¹⁶ Dao *et al.* have shown that HSPCs can traverse from M-phase back to quiescent state *ex vivo*, engraft, and sustain long term hematopoiesis in host animals, only if they have had the opportunity to engage their integrins to FN during the culture period.¹³ Recent studies have suggested a more direct role for HSPC adhesion with CAM molecules in regulating hematopoiesis by providing co-stimulatory signals to hematopoietic growth factors or cytokines.^{18,19}

Recent developments in characterizing cell adhesions occurred in 3D matrixes have underscored the importance of topographical features of the ECM in regulating cell growth, migration, differentiation, survival, tissue reorganization, and matrix remodeling.^{20,21} More relevant to HSPC expansion, several studies have suggested that a 3D culture microenvironment is conducive to regulating HSPC proliferation and differentiation.^{22–25} Bagley *et al.* have showed that a 3D tantalum porous scaffold supported the maintenance of primitive CD34⁺CD38[−] cells for up to 6 weeks and yielded a 6.7-fold increase in colony-forming cells (CFCs) without supplementing cytokines in serum-free medium.²³ In the presence of low concentration of stem cell factor (SCF) and Flk-2/flt-3 ligand (FL), culture of HSPCs in the scaffold resulted in higher yield of cells that are enriched for hematopoietic progenitors.²² Li *et al.* cultured human cord blood (CB) cells in a nonwoven polyethylene terephthalate (PET) mesh and obtained significantly higher numbers of CD34⁺ cells and CFCs after 7–9 weeks of culture in the 3D scaffold compared with that cultured on a 2D substrate.²⁴

To investigate the effect of CAM in a 3D scaffold on HSPC expansion, we have synthesized 3D PET scaffolds that are surface-immobilized with FN or collagen, in part to mimic the characteristics of HSC niche. FN and collagen were immobilized separately on the scaffold by covalent conjugation or by physical adsorption. These functionalized scaffolds were compared

for their efficiency in expanding HSPCs with their corresponding 2D substrates (FN-immobilized PET films) and tissue-culture polystyrene surface. Cells expanded in these scaffolds were characterized by FACS analysis, CFU assay, LTC-IC assay, as well as engraftment capability assayed in NOD/SCID mice.

Materials and Methods

Human cells, recombinant cytokines, and culture medium

Frozen human CB CD34⁺ cells were purchased from All-Cells (San Mateo, CA), which were obtained from normal volunteers participating in an Institute Reviewing Board (IRB) approved donor program (AllCells). Cells (92% CD34⁺, 95% viable) were thawed according to the reference.²⁶ The recombinant human stem cell factor (SCF), Flt-3 ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3), StemSpan SFEM Medium, MethoCult GF+ H4435, and MyeloCult H5100 were all from StemCell Technologies (Vancouver, BC, Canada).

Immobilization of FN and collagen on nonwoven PET membranes and PET scaffolds

Nonwoven 3D polyethylene terephthalate (PET) mesh (diameter of PET fibers, 18 μ m; porosity of mesh, 82%; Fibra-Cel®, New Brunswick Scientific, Edison, NJ) and PET film (Goodfellow, Huntingdon, Cambridgeshire, UK) were used. For covalent conjugation of FN and collagen, Fibra-Cel scaffold and PET film were first aminated in ethylenediamine/ethanol mixture (1:1, v/v) at 50°C for 40 min and then activated by 2% glutaraldehyde in 0.25M pH 11.25 carbonate buffer for 20 h. Human FN (Roche, Singapore) or collagen (95–98% type I collagen with 2–5% type III collagen, Stem-Cell Technologies) was conjugated onto the activated PET substrates in 0.25M sodium cyanoborohydride (pH 8.5) for 12 h at a concentration of 50 μ g/mL. For physical adsorption of FN and collagen, PET films and scaffolds were first treated with 1% NaOH solution at boiling temperature for 1 h followed by extensive washing with distilled water, and incubating with 50 μ g/mL FN or collagen solution for 12 h at 4°C.

The amount of protein adsorbed or conjugated on PET film or scaffold was determined by the micro-BCA protein assay using a microplate protocol adapted from Pierce product manual (Pierce, Rockford, IL). Briefly, FN- or collagen-immobilized PET films or scaffolds were immersed in 150 μ L of PBS in a microplate well, followed by addition of 150 μ L of BCA working reagent and incubation at 37°C for 2 h. The absorbance at 562 nm for the solution was used to quantify the concentration of FN or collagen against a standard curve obtained using the same protocol.

CD34⁺ cell culture

One piece of modified or unmodified Fibra-Cel mesh or PET film was placed at the bottom of the well in a 96-well plate (Nalge Nunc International, Rochester, NY). The Fibracel disc (mesh dimension, 6 mm in diameter and 1.5 mm in thickness; average surface area, 6.34 cm²) and PET film (discs with a diameter of 6 mm) fit exactly into each well of a 96-well plate. Tissue culture polystyrene (TCPS) dish/well was used as a control substrate. One hundred thawed CB CD34⁺ cells were seeded in 100 μ L StemSpan serum-free medium (1% BSA, 10 μ g/mL recombinant human insulin, 200 μ g/mL human transferrin, 10⁻⁴M 2-mercaptoethanol and 2 mM L-glutamine in Iscove's MDM) at 37°C for 10 days supplemented with 40 μ g/mL low density lipoprotein (Academy Biomedical, Houston, TX) and a combination of cytokines: 100 ng/mL SCF, 100 ng/mL FL, 50 ng/mL TPO, and 20 ng/mL IL-3. Cells were seeded to Fibracel scaffold by pipetting cell suspension directly onto the top of the scaffold slowly. On day 10, cells were harvested by repeated pipetting and counted on a hemocytometer. Antibodies for CD34 and surface markers for other progenitor and progeny cells (CD38, CD13, CD33, CD14, CD15, VLA-4, VLA-5, CD3, CD19, and α GpA, from Becton-Dickinson, Franklin Lakes, NJ; and CD41, from DakoCytomation, Carpinteria, CA) were incubated with cells at 4°C for 30

min and analyzed using a FACSVantage SE (Becton-Dickinson). At least 10,000 events were acquired for each analysis.

Colony-forming unit assay

After 10 days of expansion, 10,000 of the expanded cells were transferred to 3 mL of H4435 medium and plated in methylcellulose medium into two dishes (1% methylcellulose in Iscove's MDM, 30% fetal bovine serum, 1% bovine serum albumin, 3 U/mL EPO, $10^{-4}M$ 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL SCF, 20 ng/mL GM-CSF, 20 ng/mL IL-3, 20 ng/mL IL-6, and 20 ng/mL G-CSF). After incubation for 14 days at 37°C, granulopoietic, erythroid, and multilineage (granulocyte-erythroid-macrophage-megakaryocyte) colony-forming units (CFU-GM, BFU-E/CFU-E, CFU-GEMM, respectively) were counted under an inverted microscope (Axiovert 25, Carl Zeiss Pte Ltd, Singapore). CFU numbers were then normalized against 100 initial CD34⁺ CB cells.

Long-term culture-initiating cell (LTC-IC) assay

All cells expanded from 100 CD34⁺ CB cells under different conditions were harvested and seeded onto irradiated (8000 cGy) M2-10B4 stromal cells (StemCell Technologies) in H5100 medium (12.5% horse serum, 12.5% FBS, 0.2 mM inositol, 20 mM folic acid, $10^{-4}M$ 2-mercaptoethanol, 2 mM L-glutamine in MEM- α medium) and maintained at 37°C for 5 weeks with half medium change weekly. At the end of the 5th week, cells were trypsinized and fractions of cells were plated for CFU assay in H4435 medium as described above. Aliquots of the trypsinized cells were cytospun on a glass slide at 500 rpm and stained with Wright-Giemsa to visualize the morphology difference of cells that were differentiated from expanded cells under various conditions.

Engraftment of expanded cells in NOD/SCID mice

NOD/LtSz scid/scid (NOD/SCID) mice were supplied by Jackson Laboratories (Bar Harbor, ME) and maintained at animal holding unit of National University of Singapore (NUS). All animals were handled according to protocol approved by NUS Animal Care and Use Committee, under sterile conditions and maintained in microisolator cages. Mice to be transplanted were given total body irradiation at 6–8 weeks of age with 350–375 cGy from a ⁶⁰Co source. Within 24 h, expanded cells were harvested and injected into NOD/SCID mice through tail vein. A dose of 2×10^5 human BM CD34⁺ cells were irradiated with 1500 cGy and injected together as carrier cells.

Flow cytometric detection of human cells in murine BM

To assess the human engraftment efficiency, mice were sacrificed 6 to 8 weeks after transplantation. BM cells were flushed from both sides of femurs and tibias in Hanks' Balanced Salt Solution (HBSS) with 2% FBS and 5% human serum using a syringe and a 26-gauge needle. Red blood cells were lysed by 0.16M ammonium chloride and the remaining cells were washed with HBSS supplemented with 2% FBS. To facilitate blocking of Fc receptors and prevent nonspecific binding of antibodies, cells were suspended in HBSS with 2% FBS and 5% human serum and incubated with mouse 2.4G2 MAb (Becton-Dickinson) for 10 min at 4°C. Cells were then incubated with monoclonal antibodies specific for human CD45-PerCP-Cy5.5, CD71-FITC, and α -Glycophorin A-PE for 30 min at 4°C. Analysis was performed using a FACSVantage SE analyzer (Becton-Dickinson) and more than 20,000 events were acquired. Successful engraftment of human HSPC was defined by the presence of at least 0.1% of human CD45⁺, CD71⁺, and α -GpA⁺ cells. To verify the multilineage reconstitution of human cells, in some mice, additional aliquots were stained with anti-human CD34-FITC, CD19-PE, CD41-FITC, and CD33-FITC together with anti-human CD45-PerCP-Cy5.5, and scanned on the FACSVantage SE analyzer.

Analysis of human engraftment by real time PCR

Genomic DNA from mice BM cells was extracted by a DNeasy® Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A standard curve was constructed based on levels of the human chromosome 17 specific α -satellite p17H8 fragment in DNA samples extracted from 10 to 10^7 healthy human mono-nuclear cells (Blood and Cell Culture DNA Midi Kit, Qiagen). The primer sequences are as follows: forward, 5'-CTCTCCTTTTGAGAGCGCAGTTTGTG and reverse, 5'-GCATCTGTGTAGTTTTTATGTGAAGATGATTC. DNA samples were mixed with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), $10\ \mu\text{M}$ of sense, and antisense primers in $50\ \mu\text{L}$ reaction buffer. Real-time PCR amplification was carried out for 55 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec on an iCycler iQ Real-Time PCR Detection System from Bio-Rad Laboratories (Hercules, CA). The number of cycles was converted to the number of human cells according to the standard curve (correlation coefficient was 0.997). The engraftment percentage was calculated from the ratio of numbers of human cells and total BM cells harvested.²⁷

Statistical analysis

We used Microsoft Excel software for calculation of mean and standard deviation and for running Student's t tests (two-sided, unequal variance). For LTC-IC assay and engraftment results, we used the nonparametric Mann-Whitney test (Wilcoxon rank sum test), which was run unsupervised with the SAS 9.0 program (SAS Institute, Cary, NC). We judged findings to be significant if p value was 0.05 or less by both tests.

Results

Immobilization of FN and collagen on PET film and scaffolds

PET film is among a small group of substrates that could support HPC expansion in serum free culture, and it is as efficient as TCPS in supporting HSPC expansion.²⁸ In this study, FN and collagen were immobilized on PET surface to promote specific adhesion with HSPCs. The immobilization was achieved through physical adsorption or covalent conjugation to compare the potency of the two different formats of the immobilized adhesion molecules. To minimize confusion in the following presentation, the term conjugation and conjugated are reserved for covalent attachment.

For adsorption of FN, PET film, or scaffold was pretreated with sodium hydroxide to improve the surface wettability.^{29–31} The surface density of FN and collagen was dependent on the concentration of the incubating proteins, and reached an average of $610\ \text{ng}/\text{cm}^2$ and $630\ \text{ng}/\text{cm}^2$, respectively, when $50\ \mu\text{g}/\text{mL}$ FN or collagen solution was used. These densities corresponded to the coating level for full coverage of substrate surface.³² For FN or collagen conjugation, PET was first aminated by ethylenediamine as detailed in the Method section, without significantly altering the surface morphology of the PET film (data not shown). As the reaction time extended beyond 1 h, PET surface became roughened and the film turned opaque. The aminated PET film was incubated in 2% glutaraldehyde aqueous solution for 20 h before washing and reacting with FN or collagen solution ($50\ \mu\text{g}/\text{mL}$). After extensive wash with 5M NaCl solution to remove nonspecifically adsorbed proteins, the densities of FN and collagen on FN- and collagen-conjugated PET films were $629\ \text{ng}/\text{cm}^2$ and $646\ \text{ng}/\text{cm}^2$, respectively.

Effects of functional surface and topography of the scaffold/substrate on HSPC expansion

After 10 days of serum-free expansion culture, $\text{CD}34^+$ CB cells cultured in TCPS wells showed 75-fold total cell expansion and twofold $\text{CD}34^+$ cell expansion (Fig. 1). FN-conjugated PET

film resulted in significant improvement in CD34⁺ cell expansion with 116-fold total cell expansion and 19-fold CD34⁺ cell expansion ($p < 0.001$). Culturing CD34⁺ cells in unmodified 3D PET scaffold yielded 153-fold of total cell expansion and 11-fold of CD34⁺ cell expansion (50 $\mu\text{g/mL}$ of FN was supplemented in the medium). The highest CD34⁺ expansion was obtained using FN-conjugated PET scaffold, leading to about 100-fold CD34⁺ expansion and 185-fold total cell expansion (50.8% CD34⁺ in expanded cells). Interestingly, when FN was physically adsorbed onto the PET scaffold surface at similar surface FN density, the total cell expansion fold was the lowest among all surfaces tested (56-fold), whereas CD34⁺ cell expansion fold was similar to that in TCPS wells. It is worth noting that the expansion efficiency of CB CD34⁺ cells cultured in TCPS wells was significantly lower than that reported in the literature.^{33–36} This might be related to the progenitor content of input cells—the percentages of CD34⁺CD38[−] ($16.4 \pm 2.3\%$) or more primitive cells,^{37,38} culture medium (cytokine) composition,^{34,39} and the inherently high degree of heterogeneity between CB samples in terms of proliferative capacity in culture. Nevertheless, results collected using this batch of cells were consistent. Data shown in Figure 1 were collected from three separate experiments performed in quintuplicate.

To examine whether CD34⁺ cell expansion obtained in FN-conjugated scaffold is related to the interaction between immobilized FN and CD34⁺ cells, we studied the effect of increasing concentrations of FN supplemented in the culture medium as a blocking reagent⁴⁰ on the expansion outcome (Fig. 2). Following 10 days of culture, CD34⁺ percentage in the expanded cells decreased sharply from an average of 51% to 16% ($p < 0.001$) when 10 $\mu\text{g/mL}$ of soluble FN was present, whereas total cell expansion only decreased slightly (by about 8%, $p > 0.05$). Increasing concentration of free FN in the medium from 10 to 100 $\mu\text{g/mL}$ did not further decrease the CD34⁺ cell expansion efficiency (23- to 28-fold expansion, $p > 0.05$). The low saturation concentration of blocking FN (10 $\mu\text{g/mL}$) was probably due to the fact that the initial cell seeding number per scaffold was low (100 cells/scaffold) and 10 $\mu\text{g/mL}$ of soluble FN was sufficient to saturate the receptors. These data showed that CD34⁺ expression is maintained by surface-immobilized FN.

This general trend of CD34⁺ cell expansion was observed with another series of scaffolds with conjugated collagen instead of FN (Fig. 1). The expansion efficiency for CD34⁺ cells in/on collagen-immobilized scaffold or substrate was slightly lower than that of the FN counterparts ($p > 0.05$). For instance, collagen-conjugated scaffold yielded 40.3% of CD34⁺ cells, compared to 50.8% for FN-conjugated scaffold ($p > 0.05$). This corresponded to about 73-fold CD34⁺ cell expansion.

Phenotypic characteristics of the expanded cells

The unexpanded cells started with $16.4 \pm 2.3\%$ CD34⁺CD38[−], which was reduced to $0.6 \pm 0.4\%$ after 10 days of culture in TCPS wells ($p < 0.001$, Table I). Cells expanded in FN-conjugated scaffold and collagen-conjugated scaffold could maintain the percentages of CD34⁺CD38[−] cells at $(12.6 \pm 5.9)\%$ and $(8.2 \pm 3.7)\%$, respectively ($p < 0.01$ between these two groups).

HSPC binding to FN through β_1 integrin in VLA-4 and VLA-5 has been shown to be involved in the regulation of HSPCs and their homing/lodging to HSC niche.^{41–44} For all three groups of expanded cells in our study, the total percentages of positively stained cells for VLA-4⁺ and VLA-5⁺ remained similar to that of unexpanded cells. Nevertheless, when gating was limited to only CD34⁺ cell population, VLA-4⁺ percentage among cells expanded in FN- and collagen-conjugated scaffolds remained essentially unchanged (98.2%) from that of unexpanded cells (98.9%, $p > 0.05$), whereas this percentage decreased in cells expanded in TCPS wells (82.7%, $p < 0.001$).

Compared with cells expanded in TCPS wells, cells expanded in FN-conjugated scaffold exhibited lower levels of surface markers for committed granulocyte-monocyte progenitors (CD33⁺) and mature granulocytes/monocytes (CD14⁺/CD15⁺) ($p < 0.05$). Cells expanded in collagen-immobilized scaffold showed no significant difference in the expression levels of CD33, CD13, and CD15 compared to cells expanded in TCPS wells ($p > 0.05$). Interestingly, more cells expanded on FN-scaffold ($p < 0.05$) (but not collagen-conjugated scaffold, $p > 0.05$) expressed erythrocyte marker α -GpA (Table I). Markers for mature megakaryocytes (CD41⁺) and lymphocytes (CD19⁺ for B cells and CD3⁺ for T cell) did not change significantly for all groups compared to unexpanded cells ($p > 0.05$).

CFU and LTC-IC assays on expanded cells

To quantitate the clonogenic hematopoietic progenitors among the expanded cells, CFU assays were conducted on cells expanded under different conditions [Fig. 3(a)]. Compared with that cultured in TCPS wells, cells expanded in FN-conjugated scaffold showed 10.5-fold increase of CFU-GEMM number, 6.7-fold increase of BFU-E/CFU-E number, and 3.8-fold increase of total CFU number ($p < 0.001$). The total CFU number of cells expanded on collagen-conjugated scaffold was about twofold lower than that on FN-conjugated scaffold.

The proportions of CFU-GM, BFU-E/CFU-E, and CFU-GEMM in total CFU number are plotted in Figure 3(b). Compared with unexpanded CD34⁺ cells, FN-conjugated scaffold maintained a similar percentage of CFU-GEMM ((25.7 $\pm$ 3.8)% vs. (27.6 $\pm$ 3.9)% for unexpanded cells, $p > 0.05$), a 45% increase of BFU-E/CFU-E ((53.4 $\pm$ 7.4)% vs. (36.9 $\pm$ 5.9)%, $p < 0.001$) and a 41% decrease of CFU-GM ((20.9 $\pm$ 3.2)% vs. (35.6 $\pm$ 4.4)%, $p < 0.001$). Cells expanded in collagen-conjugated scaffold showed maintenance of CFU-GM ($p > 0.05$), increased BFU-E/CFU-E ((45.6 $\pm$ 6.4)%, $p < 0.05$), and slightly decreased CFU-GEMM ((20.0 $\pm$ 2.5)%, $p < 0.001$), compared with unexpanded cells. For cells expanded in TCPS wells, CFU-GEMM number decreased significantly ((9.3 $\pm$ 2.1)%, $p < 0.001$) with a substantial increase in CFU-GM ((60.5 $\pm$ 8.5)%, $p < 0.001$). These CFU data are consistent with the results on surface marker expression profile (Table I) and further confirmed by a bulk LTC-IC assay.

Previous experiments have established that under these culture conditions, an average of 8 CFU output in the bulk culture corresponds to one LTC-IC.^{45,46} LTC-IC frequencies are calculated accordingly and listed in Table II. Among all substrates/scaffolds tested, FN-conjugated scaffold produced the highest number of LTC-IC-derived CFUs. Table II listed the results from three separate experiments showing that LTC-IC expansion correlated with the LTC-IC frequency of the input cells: an average of 34.3-fold expansion was obtained for input cells with 2.3% LTC-IC, 65.4-fold expansion for input cells with 5.5% LTC-IC, and 40.5-fold expansion for input cells with 3.0% LTC-IC. The overall average of LTC-IC expansion from these three different batches of cells is 46.7 $\pm$ 16.5-fold ($p < 0.001$). For cells expanded in collagen-conjugated scaffold, the LTC-IC expansion fold was 4.4 $\pm$ 3.4, substantially lower than that expanded in FN-conjugated scaffold. For cells expanded in TCPS wells, colonies can only be observed in one experiment in low numbers.

Wright-Giemsa staining, at the end of 5 weeks of coculture with M2-10B4 cells, also showed that the majority of expanded cells in TCPS wells differentiated to mature blood cells, whereas those expanded in FN-conjugated scaffold retained a large number of cells exhibiting typical primitive/stem cell morphology with large nuclei and uniformly dispersed chromatin (data not shown).

Engraftment assay of cells expanded in FN-conjugated scaffold

NOD/SCID mouse repopulating assay was used to evaluate the expansion of SCID-repopulating cells (SRCs). The efficiency of engraftment was analyzed by FACS and RT-PCR

analyses.³⁵ As established in the literature,^{35,47} the minimum number of CD34⁺ CB cells (cryopreserved) required for successful engraftment in NOD/SCID mouse (detectable by FACS) is about $2-4 \times 10^4$ cells. We have shown that the FN-conjugated scaffold could generate 47-fold expansion of LTC-ICs and 100-fold expansion of CD34⁺ cells after 10 days of culture. On these bases, we estimated that cells expanded from 200–400 initial CD34⁺ cells should have the engraftment capability to reconstitute hematopoiesis. Therefore, we tested the engraftment efficiencies of cells expanded from 100 to 900 initially seeded CD34⁺ CB cells. To keep the culture conditions consistent, a cell seeding density of 100 CD34⁺ cells per FN-conjugated scaffold was maintained. After 10 days of culture, cells expanded from 1, 3, 6, or 9 scaffolds were pooled and injected intravenously into sublethally irradiated NOD/SCID mice. Unexpanded CD34⁺ CB cells (40,000 per mouse) from the same cell source were administered as a control. We detected human cells in 2 out of 6 mice (four mice died between weeks 4 and 6, likely due to irradiation-induced pancytopenia). This result is consistent with literature reports.^{35,47} All 5 control-mice receiving only the irradiated carrier cells died within 4 weeks. For cells expanded in FN-conjugated scaffold, human cells were detectable in mice receiving cells expanded from as low as 100 CD34⁺ CB cells (Fig. 4). Increasing the number of transplanted cells (expanded from higher numbers of CD34⁺ cells) clearly increased the overall survival rate and mean engraftment percentage: 1.15% to 9.65% according to the presence of human CD45⁺, CD71⁺, and α -GpA⁺ cells detected by FACS ($p < 0.005$); 1.04%–28.9% detected by RT-PCR assay²⁷ ($p < 0.008$). The engraftment efficiencies were higher by RT-PCR assay than that by FACS for some of the samples (Fig. 4), due to the higher sensitivity of the RT-PCR assay. As a control, DNA extracted from 10⁷ BM cells of untransplanted NOD/SCID mice had no detectable level of human genes following the same amplification protocol. Cells expanded from 100 to 300 initial CD34⁺ cells for 10 days in FN-conjugated scaffold reconstituted hematopoiesis in NOD/SCID mice, similar to the engraftment efficacy of 40,000 unexpanded CD34⁺ cells. Figure 5 confirmed that multiple human hematopoietic lineages were present in the BM of the transplanted NOD/SCID mice 6–8 weeks after infusion: human platelets/megakaryocytes represented by CD41⁺, B cells by CD19⁺, myeloid lineages by CD33⁺, and primitive CD34⁺ cells within CD45⁺ cells.

Discussion

Self-renewal and proliferation of HSPCs is controlled by a combination of the intrinsic factors of HSPCs and HSC niche signals.^{4,42,48–50} This study examines the effects of two of the features of the HSC niche, three-dimensionality and adhesion signals, on HSPC expansion. Effect of FN on HSPC expansion was investigated here in the context of 3D scaffold and in three different formats: covalently conjugated, physically adsorbed, and soluble FN in the medium. We have shown that in PET nonwoven scaffold, adsorbed FN yielded similar CD34⁺ cell-expansion fold as TCPS group. In contrast, when FN was covalently conjugated to the scaffold, the percentage of CD34⁺ cells was retained at a much higher level, corresponding to about 30-fold higher CD34⁺ cell expansion efficiency compared to FN-adsorbed scaffold, even though the difference in total cell expansion was much less. These data suggest that the engagement of the integrin receptors, likely β_1 integrins, probably enhances the maintenance of CD34⁺ progenitors, thus corroborating the findings of Bhatia *et al.*¹⁶

One possible reason for the enhanced expansion of HSPCs on FN-conjugated PET scaffold compared to FN-adsorbed scaffold is that covalently conjugating FN to the matrix prevents it from detaching during culture. It has been widely recognized that protein adsorbed on polymer substrates gradually detaches during culture, particularly for proteins adsorbed on a weakly charged surface. For example, Ito *et al.* reported that FN adsorbed onto partially hydrolyzed poly(methyl methacrylate) membrane (similar to our hydrolyzed PET membrane) lost more than 50% in 1 week, whereas chemically conjugated FN showed only 5% loss.³² A report by Noiset *et al.* also showed a similar trend.⁵¹ Based on these observations, it is expected that on

PET surface, chemically conjugated FN will have a higher stability compared with physically adsorbed FN.

One other possible reason that leads to the difference in expansion outcome is the physical status of FN on the substrate. Katz *et al.*²⁹ and Garcia *et al.*³⁰ have shown that physically adsorbed FN and chemically conjugated FN mediated different “focal adhesion” structures in fibroblasts. These two distinct types of adhesion patterns have different focal adhesion contact molecules²⁹ that lead to different cell motility, cytoskeleton reorganization, and signaling activity. However, there remain to be investigated whether HSPCs cultured in the two types of substrate operate under a similar mechanism, and whether FN adsorbed or conjugated on substrates with different surface chemistries will lead to different HSPC expansion outcomes.

The collagen-immobilized scaffold (adsorption vs. covalent conjugation), by and large, reproduce the same trend, but the total cell expansion and CD34⁺ expansion are less efficient than the corresponding FN-immobilized scaffold. These results are in good agreement with the report by Yokota *et al.* showing that FN is more efficient than other ECM molecules in enhancing HSC expansion.¹⁴

Substrate dimensionality also has significant impact on HSC expansion. Our functionalized nonwoven PET mesh creates a 3D-like microenvironment for HSC culture.^{24,52,53} The 3D matrix with FN conjugated apparently promoted higher level of HSPC expansion than FN-conjugated PET film. This echoes a number of other studies demonstrating the higher expansion efficiency in 3D matrixes than on 2D substrates in maintaining the multipotency and improving the expansion of HPCs.^{24,25}

A more accurate estimation of SRC frequency should be analyzed by the limiting dilution analysis.^{54–56} Nevertheless, this preliminary engraftment assay demonstrated that SRCs and progenitor cells could be significantly expanded in FN-conjugated scaffold. The successful engraftment is believed to be a result of SRC expansion and probably related to improved homing and lodging property of the expanded cells. This is supported by the high levels of maintenance of VLA-4 and VLA-5 expression among CD34⁺ cells expanded in the FN-conjugated scaffolds. It has been documented that the interaction between CAMs receptors on HSPC and ECM in the bone marrow microenvironment plays an important role in stem cell homing to the extravascular bone marrow niche.^{41–44} Papayannopoulou *et al.* have showed that anti-integrin $\alpha_4\beta_1$ (VLA-4) antibody treatment selectively induced up to 200-fold increase of mobilization of hematopoietic progenitors in primates.^{41,57,58} In NOD/SCID mouse model, pretreatment of donor cells with anti-VLA-4 antibody also significantly reduced the homing of HSPCs to the bone marrow, as indicated by an increase of circulating HSPCs.⁵⁷

The underlying mechanism by which immobilized FN and three-dimensionality influence HSPC self-renewal vs. differentiation remains to be uncovered. The covalently conjugated FN on PET scaffold provides a functioning substrate that might mediate several crucial cellular events at different levels. Increasing evidence has suggested the interdependence and functional overlap between growth factors and CAM. This indicates that medium composition, particularly growth factor concentrations, could likely influence the expansion outcome and engraftment capability of HPCs expanded in these scaffolds.^{18,59,60} Given the significant expansion of engrafting HSPCs in the FN-conjugated scaffold and recent findings on the involvement of Delta/Notch ligands and Wnt in regulating HSPC self-renewal,^{49,61} it is conceivable that incorporation of these signals into our 3D PET mesh could further enhance the function of the scaffold as an artificial HSC niche.

In summary, the three-dimensionality of PET scaffold and surface-conjugated FN act synergistically to promote the expansion of human HSPCs in frozen CB cells. Frozen CB CD34⁺ cells cultured in FN-conjugated scaffold for 10 days yielded over 100-fold-expansion

of SCID repopulating cells. This study demonstrated the potential of this functional 3D scaffold for *ex vivo* expansion of HSPCs.

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Figure 1.

Effects of conjugated or adsorbed FN and collagen and topography of the substrate (2D vs. 3D) on HSPC expansion. One hundred CB CD34⁺ cells (92%) were seeded on each substrate/scaffold and cultured for 10 days with serum free medium before harvested for analysis. Each bar in the graphs represents mean $\pm$ SD from three separate experiments performed in quintuplicate.

Figure 2. Blocking surface-conjugated FN in PET scaffold with different concentrations of soluble FN. Each data point represents mean $\pm$ SD from three separate experiments performed in duplicate.

Figure 3.

CFU numbers (a) and progenitor distribution (CFU-GM, BFU-E/CFU-E, CFU-GEMM) based on total CFU (b) analyzed for cells expanded in different scaffolds for 10 days. CFU numbers were normalized against 100 initial CD34⁺ CB cells. Each bar in the graphs represents mean $\pm$ SD from three separate experiments performed in quintuplicate.

Figure 4.

Engraftment percentage of expanded human CB cells in bone marrow of the transplanted NOD/SCID mice. (a) Percent of engraftment of total human CD45⁺, CD71⁺, and α -GpA⁺ cells in the harvested BM by FACS analysis. (b) Engraftment percentage of human cells by real time PCR amplification of human chromosome 17 specific satellite p17H8 fragment. Each mouse in the negative control group received 2×10^5 carrier cells [irradiated (1500 cGy) human bone marrow CD34⁺ cells].

Figure 5.

Representative FACS plots of marrow cells from NOD/SCID mice transplanted 8 weeks previously with human CB cells expanded from 900 CD34⁺ cells in FN-immobilized scaffold. (a) Isotype control for PE vs. FITC, (b) αGpA vs. CD71, (c) Isotype control for FITC-PE vs. PerCp-Cy5.5, (d). CD34 vs. CD45, (e). CD33 vs. CD45, (f). CD19 vs. CD45, and (g). CD41 vs. CD45 expressions.

Table I**Expression of Surface Markers on Cells Before and After Expansion Under Different Conditions**

Surface Marker	Unexpanded Cells (%)	FN-Conjugated Scaffold (3D) (%)	Collagen-Conjugated Scaffold (3D) (%)	TCPS (%)
CD34 ⁺	92.0 ± 4.2	50.8 ± 12.7	40.3 ± 9.9	2.5 ± 1.1
CD34 ⁺ CD38 ⁻	16.4 ± 2.3	12.6 ± 5.9	8.2 ± 3.7	0.6 ± 0.4
CD34 ⁺ VLA4 ⁺	91.0 ± 2.2	49.9 ± 13.1	40.2 ± 8.7	2.1 ± 0.8
CD34 ⁺ VLA5 ⁺	23.4 ± 3.5	40.8 ± 6.3	31.7 ± 5.6	1.5 ± 0.9
VLA4 ⁺	93.1 ± 2.1	92.8 ± 7.9	91.2 ± 6.3	91.8 ± 5.7
VLA5 ⁺	53.1 ± 2.9	54.1 ± 3.1	53.1 ± 2.7	52.6 ± 3.4
CD33 ⁺	0.4 ± 0.1	29.8 ± 12.3	49.0 ± 11.9	55.3 ± 0.8
CD13 ⁺	19.1 ± 5.9	41.8 ± 12.4	53.4 ± 3.4	56.4 ± 10.0
CD14 ⁺	6.6 ± 2.1	15.1 ± 3.3	19.2 ± 5.0	31.6 ± 5.3
CD15 ⁺	5.5 ± 1.8	41.4 ± 8.9	56.7 ± 7.3	65.9 ± 3.1
αGpA ⁺	3.4 ± 1.5	13.7 ± 2.6	9.5 ± 4.2	5.6 ± 2.3
CD41 ⁺	1.3 ± 0.7	1.9 ± 0.5	2.1 ± 0.4	1.6 ± 0.6
CD19 ⁺	0.9 ± 0.4	2.3 ± 0.7	2.1 ± 0.5	1.9 ± 0.4
CD3 ⁺	0.8 ± 0.6	1.8 ± 0.4	1.4 ± 0.5	1.7 ± 0.6

Table II

LTC-IC Derived CFUs Normalized Against 100 CD34⁺ CB Cells Seeded in Each Scaffold/Substrate Before and After Expansion

Experiment	Culture Conditions	LTC-IC Derived CFUs Before Expansion ^a	LTC-IC Derived CFUs After Expansion ^a	Expansion Fold
Exp 1 (<i>n</i> = 3)	FN-conjugated scaffold	18.4 ± 3.5 (2.30 ± 0.44%) ^b	630 ± 79 (0.44 ± 0.05%) ^b	34.2
	Collagen-conjugated scaffold	18.4 ± 3.5 (2.30 ± 0.44%) ^b	25 ± 4 (0.02 ± 0.00%) ^b	1.4
	TCPS	18.4 ± 3.5 (2.30 ± 0.44%) ^b	0	0
Exp 2 (<i>n</i> = 5)	FN-conjugated scaffold	44.0 ± 6.8 (5.5 ± 0.9%) ^b	2875 ± 234 (1.94 ± 0.16%) ^b	65.3
	Collagen-conjugated scaffold	44.0 ± 6.8 (5.5 ± 0.9%) ^b	351 ± 43 (0.26 ± 0.03%) ^b	8.0
	TCPS	44.0 ± 6.8 (5.5 ± 0.9%) ^b	11 ± 4 (0.02 ± 0.01%) ^b	0.3
Exp 3 (<i>n</i> = 3)	FN-conjugated scaffold	23.6 ± 11.7 (3.0 ± 1.5%) ^b	955 ± 94 (0.65 ± 0.06%) ^b	40.5
	Collagen-conjugated scaffold	23.6 ± 11.7 (3.0 ± 1.5%) ^b	90 ± 23 (0.07 ± 0.02%) ^b	3.8
	TCPS	23.6 ± 11.7 (3.0 ± 1.5%) ^b	0	0

^aThe number of LTC-IC derived CFUs is normalized against 100 initially seeded CD34⁺ cells.

^bThe number in parenthesis is the estimated LTC-IC frequency.^{23,39}