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Long-term cytokine-free expansion of cord blood mononuclear cells in three-dimensional scaffolds

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ABSTRACT

Cord blood expansion *ex vivo* can be achieved in liquid suspension through the addition of cytokines at the expense of often undesirable cell differentiation. In order to derive a cytokine-free dynamic culture system, we hypothesised that a three-dimensional (3D) environment in the form of highly porous scaffolds made of poly (D,L-lactide-co-glycolide) (PLGA) or polyurethane (PU) for the biomimetic growth of cord blood mononuclear cells (CBMNCs), would facilitate expansion of hematopoietic cells without exogenous cytokines. Both scaffolds supported cellular expansion *ex vivo*. Cytokine-free, long-term culture was best in PU coated with collagen type I (54-fold expansion). In contrast, traditional 2D well-plate cultures collapsed within 4 days in the absence of cytokines. CBMNCs cultured in the scaffolds were visualised by scanning electron microscopy and immunophenotypic/immunostaining analysis and the studies validated the presence of a dynamic culture containing erythroid precursors ($CD45^-/CD71^+/CD235a^+$), hematopoietic stem/progenitor cells ($CD38^-CD34^+$, $CD117^+$), maturing myeloid cells ($CD38^+$, MPO^+), $CD4^+$ and $CD8^+$ T-lymphocytes and megakaryocytes ($FVIII^+$). Colony forming unit (CFU) assays indicated that BFU-E and CFU-GM increased ($p < 0.05$) whereas CFU-GEMM were maintained at week 4. In conclusion, this 3D culture system is capable of long-term, cytokine-free expansion of CBMNCs, enabling the study of hematopoiesis and providing a potential platform for drug discovery and therapeutic applications *ex vivo*.

1. Introduction

The availability of CB as a source of hematopoietic stem and progenitor cells (HSPCs) for transplantation and other cellular therapies is critical in expanding the potential donor pool, especially for minority populations [1]. In addition, CB contains mesenchymal stem cells that can form stromal elements required to sustain hematopoiesis [2] and can be expanded *ex vivo* in both two-dimensional (2D) and 3D culture systems using pre-selected and defined cell populations. The expansion of HSPCs, sometimes defined as $Lin^-/CD34^+$ cells, is more challenging. In 2D, liquid suspension [3] and automated perfusion [4] cultures of $CD34^+$ cells, cultures can be maintained for extended periods with the use of exogenous cytokines, such as stem cell factor (SCF), *fms*-like tyrosine kinase ligand (FLT3L), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-1, IL-3, IL-6 and others [5–8]. The addition of high concentrations of these cytokines (in the range of ng/ml) for *ex vivo* expansion is expensive and can mature HSPCs such that their self-renewal capacity becomes limited. These cytokine concentrations do not correspond to those found naturally in the BM, in the range of pg/ml [9], which act within a unique microenvironment in order to sustain normal hematopoiesis. This microenvironment incorporates 3D BM niches formed by stroma cells, which elaborate extracellular matrix proteins including fibronectin, collagen, vitronectin and tenascin [10,11].

In order to attempt the culture and expansion of HSPCs that can maintain self-renewal and repopulating capacity, *ex vivo* culture has evolved to 3D systems which may be able to recapitulate the *in vivo* microenvironment [12]. Hematopoietic cells have been

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encapsulated or cultured on hydrogel matrices [13], tantalum-coated biomaterial [14], porous biomatrix CellFoam™ [15], macroporous microspheres [16,17], or synthetic polymers [13]. Although these scaffolds may resemble some elements of the BM architecture and facilitate cell–cell, and cell–matrix interactions, these methods involve the addition of cytokine cocktails to the culture, resulting in unwanted differentiation, or co-culture with allogeneic or xenogeneic stromal feeder layers, potentially presenting foreign antigens into the culture.

We have previously shown that 3D scaffolds made of either poly (L-lactic-co-glycolic acid) (PLGA) or polyurethane (PU) were able to sustain long-term cultures of leukemic cell lines [18]. We have now extended our evaluation of these scaffolds to the long-term culture and expansion of unselected human CBMNCs. In order to enhance this BM biomimicry, we coated PU with an extracellular matrix (ECM) protein, collagen type I, that can provide strong mechanical contact between the scaffold material and CB cells [19].

2. Materials and methods

2.1. Scaffold Preparation

Dimethylcarbonate (DMC, 99% pure; Sigma Aldrich, Dorset, UK) was used for the fabrication of PLGA (Purasorb® PDLG (53/47), PURAC Biochem, Gorinchem, The Netherlands). Dioxan (99.8% pure; Sigma-Aldrich) was used for the fabrication of PU (Noveon, Belgium). The solvents were used without further purification. The scaffolds (pore size 100–250 μm, porosity 90–95%) [20] were fabricated by thermally induced phase separation of polymer solutions (5 wt% in the appropriate solvent) and subsequent solvent sublimation as previously described (Table 1) [21]. Prior to cell seeding, the scaffolds were cut into cubes of 0.5 × 0.5 × 0.5 cm. Sterilisation was done using a combination of UV light (8 min exposure at 230 v, 50 Hz, 0.14 A, UV lamp, Kendro Laboratory Products, UK) and immersion for 2 h in Ethanol (70%); the scaffolds were then washed twice for 5 min in PBS before the addition of media and placement in a humidified incubator for 3 days at 37 °C and 5% CO_2 prior to use. PU scaffolds were coated with collagen type I from calf skin (Sigma-Aldrich) at a concentration of 62.5 μg/ml as previously described [18].

2.2. Cell culture

Human umbilical cord blood was obtained from the London Cord Blood Bank in cryopreserved form (London, UK; Harrow Research Ethics Committee 05/Q0405/20). The CB pack was rapidly thawed in a 37 °C water bath and MNCs were isolated using Ficoll–Paque (GE Healthcare, Sweden) density gradient centrifugation. CBMNCs were then seeded onto sterile scaffolds at a concentration of 2.5 × 10^6 cells/scaffold (100 μl of cell suspension), placed in 24-well tissue culture plates (Costar®, Corning® Inc, NY, USA) and incubated for 15 min at 37 °C and 5% CO_2 before adding 1.5 ml Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Invitrogen Ltd, UK) with 30% fetal bovine serum (FBS, heat-inactivated, Invitrogen Ltd, UK) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd, UK). Half-medium exchange was carried out every other day. Cytokines were not added at any stage of the cell culture. 2D control cell culture was performed by seeding CBMNCs onto sterile standard 24-well tissue culture plates (Costar®, Corning® Inc) at a concentration of 2.5 × 10^6 cells/well in 1.5 ml IMDM (Gibco, Invitrogen Ltd, UK) with 30% FBS heat-inactivated(Invitrogen Ltd) and 1% Penicillin/Streptomycin (Gibco, Invitrogen Ltd). Medium exchange was carried out every other day.

2.3. Seeding efficiency and Cell proliferation

Seeding efficiency was determined by subtracting the number of cells remaining in the media (i.e., did not seed into the scaffold) after the first 24 h of culture from the total number of cells seeded. Changes in the number of metabolically active cells within the scaffolds were quantitatively assessed at days 0, 2, 14, 21 and 28 with tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium], inner salt (MTS, Promega, Madison, WI, USA - CellTiter96® AQueous Solution Cell Proliferation Assay) [22]. In order to determine *in situ* cell proliferation, scaffolds seeded with the cells at days 0, 2, 14 and 28 were incubated with 200 μl of the tetrazolium compound for 3 h at 37 °C and 5% CO_2; absorbance was measured at 490 nm using a 96-well plate-reader (El_x 808, Ultra Microplate Reader, Bio-TEK Instruments Inc., Bath, UK) and graphed according to an internal standard. Empty scaffolds at the same culture time-points were used as controls. Viability of cells manually extracted from the scaffolds was also measured during the 28 day period using a standard hemocytometer and trypan blue dye exclusion at days 0, 2, 14, and 28; number of repetitions (n) = 2; number of experiments (N) = 6.

2.4. Scanning electron microscopy (SEM)

Following cultivation of the CBMNCs in scaffolds for 48 h, 14 days and 28 days, the scaffolds were removed from the media, fixed with 2.5% PBS-buffered glutaraldehyde solution (Fluka BioChemika, Switzerland) for 40 min at 4 °C and washed twice with PBS. They were then dehydrated in a graded series of ethanol (50, 70, 90, 95 and 100%), each for 5 min and the samples were dried in an aseptic environment for 4 h. The specimens were sectioned and then sputter-coated with gold in an argon atmosphere for 2 min prior to SEM (JEOL JSM-840A, JEOL Ltd., Welwyn Garden City, U.K.) evaluation at an acceleration voltage of 20 kV.

2.5. Immunophenotype by flow cytometry

At defined time-points (days 0, 14 and 28), cultured cells were extracted from the scaffolds by gentle aspiration using a 5 ml syringe (BD Plastipak™, Biosciences, NJ, USA) and 18G needle (Terumo®, Somerset, NJ, USA). Cells were exposed to directly-conjugated mouse anti-human monoclonal antibodies (mAbs) to assess hematopoietic populations with CD45-fluorescein isothiocyanate (FITC; clone HI30, BD Biosciences), erythrocyte precursors with CD71-phycoerythrin (PE; clone M-A712, BD Biosciences), early myeloid precursors with CD33-FITC (clone MOPC-21, BD Biosciences) and CD33-PE (clone P67.6, BD Biosciences), mature erythrocytes with CD235a-phycoerythrin-cyanine 5 (PE-Cy5; clone 27-35, BD Biosciences), T-lymphocytes with CD3-PE-Cy5 (clone G155-178, BD Biosciences), CD4-FITC (clone SK3, BD Biosciences), CD8-PE (clone HIT8a, BD Biosciences), hematopoietic stem and progenitor cells with CD34-PE (clone 8G12, BD Biosciences) and maturing cells with CD38-PE-Cy5 (clone HIT2, BD Biosciences), all with their respective isotype controls (all from BD Biosciences). The cells were incubated with directly-labelled antibody clones at 4 °C in the dark for 30 min, washed and fixed with 2% (v/v) Paraformaldehyde (BDH Industries Ltd, India) then acquired on an EPICS ALTRA flow cytometer (Beckman Coulter Inc, CA, USA) within 24 h. Thirty thousand events were acquired for each sample and WinList 5.0 software (Verity software house, Maine, USA) was used for data analysis.

2.6. Immunostaining

Samples were fixed with 4% paraformaldehyde for 30 min. They were then processed overnight, as for routine histopathological biopsies and by using vacuum impregnated processing (VIP) which involves removal of tissue water through successive alcohol gradients ending with the final step in absolute methanol. The tissue was thereafter embedded in paraffin wax. Paraffin sections (6 microns thickness) were cut for immunostaining and heat pressure cooker antigen retrieval was employed. The following antibodies were used: mouse anti-human CD45 (dilution 1:200), mouse anti-human CD3 (dilution 1:25), mouse anti-human CD20 (dilution 1:200), mouse anti-human glycophorin/Ret 40f (dilution 1:25), rabbit anti-human CD117 (dilution 1:100), rabbit anti-human MPO (dilution 1:4000), rabbit anti-human Factor VIII (dilution 1:600) and mouse anti-human CD34 (Nova Castra, dilution 1:100). A dextran polymer labelled with HRP (Dako; EnVision™) was applied, using Dako Autostainer Link 48. Haematoxylin was used as nuclear stain. Images were taken using an Olympus BX51 microscope with a Nikon (DS-2Mv) camera and analysed using the software program AnalySIS[B and D] (Soft Imaging System, Munster, Germany). Images were not manipulated.

Table 1
Summary of the physical properties of PLGA and PU scaffolds prior to cell seeding.

Property	3D Scaffolds	
	PLGA	PU
Scaffold diameter (mm)	5	5
Density ρ (g/ml)	1.53 ± 0.035	1.25 ± 0.023
Specific pore volume V_p (ml/g)	11.76 ± 1.31	12.31 ± 1.42
Surface area A_s(m^2/g)	0.6	1.03
Porosity (%)	94.74 ± 1.30	93.90 ± 0.04
Pore size range (μm)	micropores 50–100 macropores 100–300	≈100