Enriching Hematopoietic, Endothelial and Mesenchymal Functional Progenitors by Short-Term Culture of Steady-State Peripheral Blood Mononuclear Cells Obtained from Healthy Donors and Ischemic Patients

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ABSTRACT: **Background:** Non-mobilized peripheral blood contains mostly committed cells with limited numbers of early progenitors.

Objectives: To enrich functional progenitor cells from healthy donors and ischemic heart disease patients by short-term culture of mononuclear cells with defined culture conditions.

Methods: Mononuclear cells obtained from healthy donors and ischemic heart disease patients were cultured for 7 days in a cytokine cocktail. We tested the multilineage differentiation capacities and phenotype of cultured cells.

Results: The short-term culture (7 days) of all study groups with a defined cytokine cocktail resulted in two distinct cell populations (adherent and non-adherent) that differed in their differentiation capacities as well as their cell surface markers. Cultured adherent cells showed higher differentiation potential and expressed endothelial and mesenchymal fibroblast-like surface markers as compared to fresh non-cultured mononuclear cells. The non-adherent cell fraction demonstrated high numbers of colony-forming units, indicating a higher differentiation potential of hematopoietic lineage.

Conclusions: This study proved the feasibility of increasing limited numbers of multipotent progenitor cells obtained from the non-mobilized peripheral blood of healthy donors and ischemic patients. Moreover, we found that each of the two enriched subpopulations (adherent and non-adherent) has a different differentiation potential (mesenchymal, endothelial and hematopoietic).

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KEY WORDS: endothelial progenitor cells, hematopoietic cells, mesenchymal cells, non-mobilized peripheral blood mononuclear cells (NM-PBMNCs), cytokine supplement, short-term culture, multipotent cell enrichment

Bone marrow, granulocyte colony-stimulating factor-mobilized peripheral blood and umbilical cord blood are rich sources of adult pluripotent stem cells needed for hematopoietic reconstitution in autologous and allogeneic stem cell transplantation. These stem cells, which can differentiate into several hematopoietic and non-hematopoietic lineages [1], have been explored for clinical regenerative applications and damaged tissue repair, especially in ischemic heart disease. Collecting stem and progenitor cells from the peripheral blood is easier and safer than from any other tissue. However, progenitor cell mobilization to the circulating peripheral blood is subject to physiological or pathological stress [2]. Thus, infusing autologous non-mobilized peripheral blood mononuclear cells to patients with non-Hodgkin lymphoma or Hodgkin disease resulted in delayed engraftment as compared to cells from bone marrow [3]. Yet, in vitro and clinical studies have indicated that peripheral blood mononuclear cells (PBMNCs) possess a multilineage differentiation potential of non-hematopoietic lineages to induce neovasculature progression and erythroid progenitor differentiation, similar to bone marrow mononuclear cells and CD34+ selected cells [4,5]. Furthermore, studies have shown that fully differentiated circulating cells such as CD14^{neg} and CD14^{pos} have the ability to differentiate into bone, cartilage, fat, skeletal and cardiac muscle, neuron and endothelium [6-8]. However, expansion of these rare populations requires a long-term culture that is costly and unsafe due to the risk of acquiring chromosomal aberrations of the culture cells. Since steady-state peripheral blood is an unlimited source compared to bone marrow, cord blood and mobilized peripheral blood, increasing the number of their rare progenitor population using a simple modality is important as it might open the field to this source for regenerative therapy.

The present study tested the feasibility to increase the number of functional multilineage cell populations using a short-term simple modality for enriching non-mobilized (steady-state) PBMNCs obtained from healthy donors and ischemic patients. Mobilized PBMNCs obtained from healthy donors served as an enriched positive control. Results indicated that although enrichment potential differs according to the original

source (i.e., healthy donors vs. ischemic patients), the defined cytokine enrichment modality can enrich a mixture of endothelial, mesenchymal and hematopoietic populations in the same culture. This method might be used to expand mixed populations as well as separated adherent or non-adherent populations that specifically contain endothelial, mesenchymal-like and hematopoietic-enriched populations, respectively, according to different therapeutic needs.

MATERIALS AND METHODS

CELL SOURCES

Samples of non-mobilized peripheral blood were obtained from 33 healthy donors (mean age 50 years, range 40–59) and from 9 chronic ischemic heart disease patients (mean age 64 years, range 47–78) who underwent coronary artery bypass graft surgery. Granulocyte colony-stimulating factor mobilized PBMNCs were obtained from fresh hematopoietic stem cells (HSC) (harvested by apheresis) of 14 healthy donors (mean age 37 years).

The study protocol was approved by the Institutional Review Board Committee at Sheba Medical Center, Tel Hashomer, Israel. Written informed consent was obtained from all patients and healthy controls before enrolment to the study.

CELL CULTURE

Mononuclear cells were extracted from circulating blood samples using density gradient (1.077 g/dl) (Lymphoprep™, #1114547, Fresenius Kabi Norge AS, Norway) according to the manufacturer's instructions. Blood cells were loaded on the Lymphoprep™ density gradient in a 2:1 ratio (v:v). Cells were centrifuged for 20 minutes at 2000 rpm after washing with phosphate-buffered saline (PBS).

Mononuclear cells 4 x 10^6/ml (total number of cells 100 x 10^6 cells/T75 culture flask) were cultured for 7 days. All culture media were based on low glucose DMEM supplemented with 10% fetal bovine serum, 2 mM/L of L-glutamin, 100 U/ml penicillin-streptomycin solution (all produced by Biological Industries, Beit HaEmek, Israel). The basic growing medium was supplemented with the following cytokines: macrophage colony-stimulating factor (25 ng/ml), interleukin-6 (IL-6) (20 ng/ml), leukemia inhibitory factor 1000 U/ml, basic fibroblast growth factor (FGF) (10 ng/ml), stem cell factor (SCF) (25 ng/ml), FLT3 ligand (25 ng/ml) and thrombopoietin (25 ng/ml) – all produced by Peprotech, Rocky Hill, NJ, USA. Three control groups for cytokine supplements were used only for cells obtained from healthy donors:

- Basic medium – without cytokine supplement, that contains low glucose DMEM supplemented with 10% fetal bovine serum, 2 mM/ml L-glutamin, and 100 U/ml penicillin-streptomycin solution
- Basic medium supplemented with a combination of four cytokines: M-CSF (25 ng/ml), IL-6 (20 ng/ml), leukemia inhibitory factor 1000 U/ml, basic FGF (10 ng/ml) (named MILF combination to denote the initials of the cytokines, designed to support the adherent cell subpopulation, as described previously) [9]
- Basic medium supplemented with a combination of the following three cytokines: SCF (25 ng/ml), FLT3 ligand (25 ng/ml), and thrombopoietin (25 ng/ml), named SCF combination for their function as SCF cytokines as previously described [10].

On day 3 of culture, the medium was collected and changed in the original seeded culture flasks. The medium was centrifuged and cells were seeded at a concentration of 0.5 x 10^6 cells/ml (total number of cells 7.5 x 10^6 cells in T75 culture flask). The cell cultures were continued for another 4 days, after which adherent and non-adherent cells were collected. On day 7 non-adherent cells were obtained by centrifuging (1250 rpm for 10 minutes) the collected growth medium; adherent cells were obtained by washing cells with PBS, followed by the addition of trypsin-EDTA (0.25%) solution (Beit HaEmek, Israel) for 5 minutes and 10 ml of growth medium, then spinning the cells at 1250 rpm for 10 minutes.

Human umbilical vein endothelial cells (HUVEC) (Promo Cell GmbH, Heidelberg, Germany, # C12200), serving as a positive control for tube formation assay, were cultured in endothelial cell basal medium-2 (Clonetics # CC-3156) supplemented with EGM-2 (SingleQuots # 4176). Upon confluence, cells were collected using trypsin, and tube formation assay was performed as described [11].

Bone marrow mesenchymal stromal cells (BM-MNCs) were obtained from consenting healthy bone marrow donors for allogeneic transplantation. These cells were used as a positive control for cultured non-mobilized peripheral blood mesenchymal characterization tests. Mesenchymal cells were obtained from passage-1 of fresh healthy bone marrow aspirates cultured for 2–3 weeks in mesenchymal growing medium (MesenCult Stem Cell Technologies, Vancouver, Canada).

DIFFERENTIATION ASSAYS

The collected adherent and non-adherent cells were tested for their ability to differentiate into various cell lineages. Fresh non-cultured MNCs were used as internal controls, mobilized PBMNCs were used as positive controls for hematopoietic stem cells differentiation, HUVEC served as a positive control for endothelial cell function, and bone marrow mesenchymal stromal cells as positive controls for mesenchymal cell function.

HEMATOPOIETIC DIFFERENTIATION

Hematopoietic differentiation was tested by using a colony-forming unit (CFU) assay to detect and quantify hematopoietic progenitor cells. CFUs were tested according to the manufacturer's instructions with slight modifications. Briefly, fresh

and cultured cells were re-suspended in culture medium to a final concentration of 0.5 x 10^6cells/ml. A mixture of 1:10 (v:v) of diluted cells and methylcellulose (Methocult kit - H4034, Stem Cells Technologies) was produced. Fifty percent of the cell mixture (2.5 x 10^4 cells) was seeded in each well (24-well plate) and cultured in a humidified CO_2 incubator for 10–14 days. Hematopoietic colonies (blast-forming unit-erythroid, CFU-myeloid, and CFU-granulocyte, erythroid, monocyte and myeloid) were counted, and numbers were extrapolated as per 10^6 cells.

ENDOTHELIAL CELL FUNCTIONAL ABILITIES

Endothelial cell (EC) differentiation was determined by short-term CFU-EC and long-term growth endothelial progenitor cell-derived colonies. Fresh (non-cultured) MNCs, adherent and non-adherent collected cultured cells were tested. To assess the short-term EC (CFU-EC) potential, EndoCult Liquid Medium Kit # 05900/05950 (Stem Cell Technologies, Canada) was used according to the manufacturer's instructions. In brief, cells were mixed with EndoCult Liquid Medium to a final concentration of 1 x 10^6 cells/ml and seeded on a fibronectin (# F-2006, Sigma) pre-coated 24-well plate. Forty-eight hours later, the non-adherent cells were collected and re-seeded at a concentration of 0.5 x 10^6/ml on a fibronectin pre-coated 24-well dish for an additional 72 hours. The early outgrowth colony-forming unit (CFU-EC) was defined as consisting of two cell types: a central cluster of round cells and sprouting elongated cells at the periphery, as described previously [12]. The number of colonies was calculated per 10^6 cells. Late outgrowth EC potential was tested by culturing cells for 2–4 weeks on 0.1% gelatin in endothelial cell basal medium-2 (Clonetics # CC-3156, USA) supplemented with EGM-2 (SingleQuots # 4176) as previously described [13]. The ability of cells to form capillary tube networks was tested using Matrigel matrix (BD Bioscience # FAL354234, USA) as previously described [11].

In brief, Matrigel complex was diluted 1:1 (v:v) with endothelial cell basal medium-2 (Clonetics # CC-3156) on ice after plating the mixture for 3 hours to enable the mixture to solidify. Cells (5 x 10^4) were suspended in 500 μl EGM-2 and seeded on a 24-well plate pre-coated with matrigel matrix (BD Bioscience # FAL354234). Seeded cells were incubated in humidified 5% CO_2 incubator for 24 hours. Tubes were counted under an inverted light microscope after 8 hours, while early tubes started to form at 4 hours and no progress was detected at 24 hours of incubation.

MESENCHYMAL DIFFERENTIATION POTENTIAL

In order to test the CFU-fibroblast frequencies of fresh and cultured adherent and non-adherent cells, limited dilutions were performed as previously described by Castro-Malaspina et al. [14] with slight modifications. Cells (2 x 10^4, 2 x 10^5, 2 x 10^6 in 6-well plates) were re-suspended with cytokine-supplemented culture medium MS (x7 cytokines medium supplement) and seeded in 6-well dishes for 10 days. The culture medium was changed twice a week. Colonies were counted after 10 days under light microscopy. After 10 days, the colonies mixture was collected and cultured for expansion with MesenCult medium (Stem Cell Technologies) until confluence. These cells were then used for phenotype characterizations and differentiation capacity tests. The differentiation abilities of osteoblasts and adipocytes were tested using Miltenyi kits (# 130-091-677 and 130-091-678, respectively), according to the manufacturer's instructions. For detection of osteocytes, we used Alizarin red staining (Sigma) that stains the mineralized matrix formed by the osteocytes. Adipocyte differentiation was tested 3 weeks after cell induction for differentiation. For detection of oil droplets, cultures were stained in saturated Oil-Red O solution (Sigma) in 60% isopropanol. BM-MSCs were used as positive controls for colony-forming unit-fibroblast (CFU-F) formation and differentiation assays.

SURFACE MARKER ANALYSIS

Surface marker expression of fresh and cultured cells used in this study was analyzed by using a FACSCalibur instrument (Becton Dickinson, USA). Cells were blocked with PBS containing 10% human serum albumin, washed with PBS that contains 0.5% fetal calf serum, re-suspended as a single cell suspension (10^5 cells/100 μl reaction) and labeled for 30 minutes at 2–8°C.

To characterize the cultured cell phenotype, adherent and non-adherent cells were single stained with the following panel: CD14(FITC), CD73(PE), CD90(PE), CD117(APC) produced by Becton Dickinson; CD31(PECAM-1-FITC), CD45(APC), CD105(APC), CD144(FITC) CD146(PE) produced by EBioscience, CD133(PE) (BD Bioscience), vimentin(FITC), fibronectin(FITC) and CD34-FITC (IQProduct) monoclonal antibodies. Appropriate isotype-matched antibodies were used as negative controls (Becton Dickinson). Fresh (not cultured) PBMNCs were stained with the same panel of markers and used as untreated controls. Single staining measurements were performed from viable cells (7AAD negative staining) and a minimum of 10,000 events were stored. Data were collected from gated live cells, and the percentages of positive cells were calculated using isotype control. In order to specifically characterize cells obtained after differentiation assays, cells (obtained from non-mobilized healthy donors only, n=4) – CFU hematopoietic, CFU-EC and CFU-F – were stained with different specific cell surface panels as follows:

- Hematopoietic CFUs were collected by dissolving the soft agar methylcellulose with basal culture at room temperature. The following cell surface markers were used in two different sets of four color staining (n=4): a) CD11b(FITC), CD13(PE), CD117(APC), and b) CD38(FITC), CD33(PE)

and CD34(APC) and CD45(PerCP). Gates were performed on viable cells and on CD45PerCP-positive cells
- CFU-ECs (n=4) were collected using trypsin-EDTA and stained with CD14(FITC), CD31(FITC), CD34(FITC), CD105(APC), CD144(FITC), CD146(PE), KDR(APC) and uptake of acetylated low density lipoprotein (acLDL)
- Cells collected from expanded (n=4) CFU-F cultures were stained with CD3(PE), CD14(FITC), CD34(FITC), CD73(PE), CD90(PE), CD105(APC), CD45(APC), HLA-DR(APC), vimentin(FITC) and fibronectin(FITC).

STATISTICAL ANALYSIS

Data were analyzed using the FlowJo software. Results were expressed as mean standard error of the mean from at least three experiments. Statistical significance was performed by paired *t*-test analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Short-term culture of non-mobilized peripheral blood mononuclear cells (NM-PBMNCs) in a defined cytokine cocktail resulted in two different functional subpopulations – adherent and non-adherent cells – which showed endothelial, mesenchymal and hematopoietic progenitor differentiation capacities proven by specific cell surface marker expression and functional analysis.

DEFINING CULTURE CYTOKINE SUPPLEMENT

The mixture of seven different cytokines (named MS) used to supplement the culture growth medium was selected after testing of three different cytokine mixtures. MS cytokine supplement contained a combination of seven cytokines, while MILF (a name given for the four cytokine supplement mixture) and SCF (three cytokines) supplements were partial combinations of the MS (seven) cytokine mixture (as described in Materials and Methods). Results (data not shown) indicated that the average number of non-adherent cells collected from MILF, SCF and MS cytokine combination cultures were similar (31 ± 3.0, 30.75 ± 9.6, 29.5 ± 6.9 x 10^6 cells, respectively). The average number of adherent cells collected from MILF and MS cells were similar and significantly higher (21.5 ± 3.5, 19.4 ± 3.9 x 10^6 cells, respectively) than the average number of cells collected from SCF supplement cultures (8.2 ± 2.6 x10^6 cells) ($P < 0.0001$). Moreover, differentiation tests showed that adherent cells collected from MILF, SCF and MS cytokine mixtures produced higher numbers of endothelial cell colonies (28.4 ± 13.1, 34.3 ± 13.5, 46.4 ± 17.7/10^6 cells, respectively) compared to non-adherent cell fraction (8.6 ± 3.9, 8.1 ± 4.9, 4.9± 1.7/10^6 cells, respectively) ($P < 0.0001$). Yet, only an adherent fraction of cells cultured with MS supplement mixture was able to produce substantial tube networks compared to adherent cells cultured with MILF or SCF. Cultured non-adherent cells collected from MILF, SCF and MS cytokine mixtures produced significantly higher numbers of hematopoietic colonies (74 ± 42.9, 474 ± 97, 686.2 ± 91.6/10^6 cells, respectively), compared to the adherent cells (1.4 ± 2.5, 65.5 ± 36, 41.2 ± 24.8/10^6 cells, respectively) ($P < 0.0001$). Moreover, adherent and non-adherent cells collected from MS cytokine mixture cultures produced higher numbers of endothelial and hematopoietic colonies compared to MILF and SCF cytokine supplement.

Cells collected after short-term culture with basic culture media (no cytokines) (n=3) resulted in very low numbers of adherent cell fraction (0.8 ± 0.5 x 10^6 cells) and viable non-adherent cell fraction (10 ± 1.8 x 10^6 cells) that failed to produce hematopoietic and endothelial colonies (data not shown). The results described above prompted us to use the MS cytokine mixture to culture cells from ischemic heart disease patients and to test hematopoietic differentiation abilities from non-adherent cell fraction and the endothelial differentiation abilities from adherent cell fraction.

ANALYSIS OF CELL SURFACE MARKERS

Cell surface markers expressed by cultured adherent and non-adherent cell populations were analyzed and compared to the expression on fresh non-cultured cells. Results (data not shown) indicated that the cell marker expression profile differed between sources (i.e., non-mobilized healthy donor, non-mobilized ischemic patient, and mobilized healthy donor), fresh (pre-cultured) or cultured, and fractions collected from cultured cells (adherent and non-adherent).

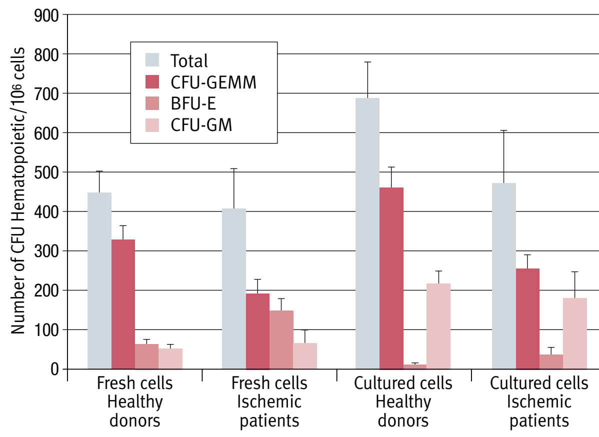
A significantly higher percentage of fresh mobilized peripheral blood cells expressed CD31, CD105, KDR, CD14 and CD34 compared to fresh healthy and ischemic patient non-mobilized cells. The percentage of positive adherent cells that expressed CD14, CD31, CD90, CD105 and CD117 increased significantly compared to that obtained from non-cultured fresh PBMNCs. Cultured non-adherent cells obtained from peripheral blood of healthy donors (non-mobilized) expressed more KDR (2.5 ± 1.7) and CD117 (2.6 ± 1.6) compared to the significantly low percentage expressed on fresh cells (0.35 ± 0.1 and 0.09 ± 0.04, respectively, $P < 0.001$). Cultured non-adherent cells obtained from healthy donor (mobilized) peripheral blood expressed slightly more CD14 (34.4 ± 7.8 vs. 25.9 ± 8.1) and CD45 (81.4 ± 21.1 vs. 73 ± 11.8), but significantly more CD90 (5.5 ± 0.8 vs. 2.5 ± 0.1), CD105 (40.9 ± 7.53 vs. 11.1 ± 1.3) and CD117 (11.1 ± 1.3 vs. 3.6 ± 0.8), compared to expression by fresh cells, respectively, $P < 0.001$. The percentage of cells that were positive for CD133 and CD34 on adherent and non-adherent cell fractions was low or undetectable in all study groups.

HEMATOPOIETIC LINEAGE DIFFERENTIATION

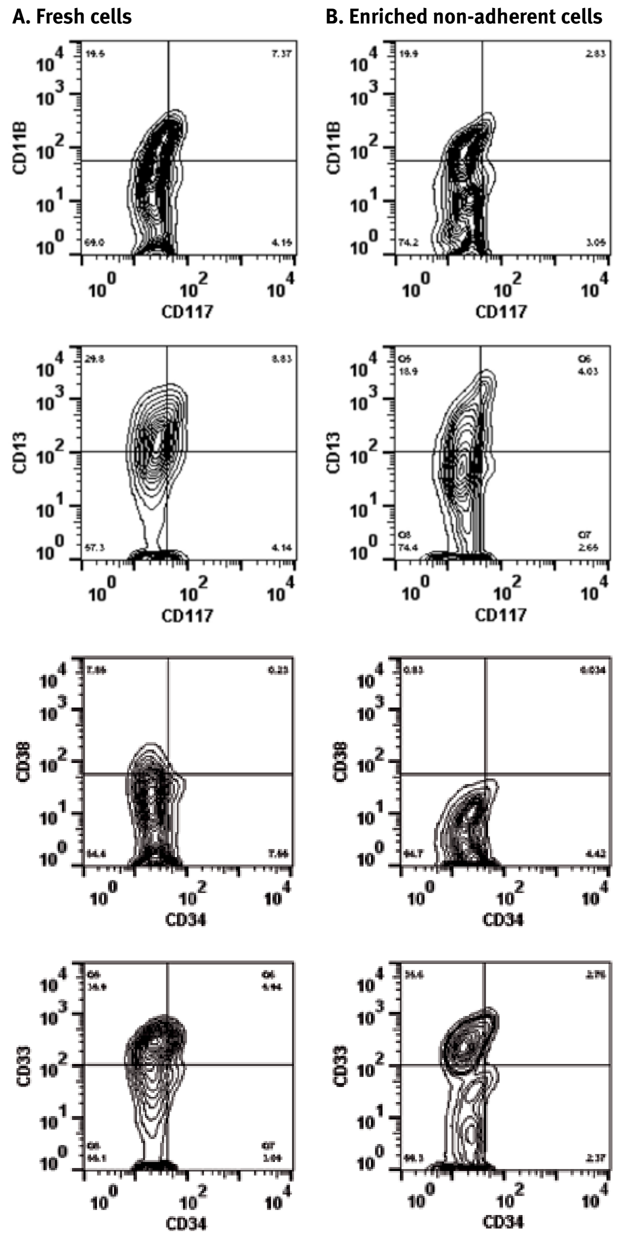
Hematopoietic differentiation was tested by evaluating the cultured cells' ability to produce CFUs. Cultured non-adherent cells

Figure 1. Hematopoietic differentiation potential of cultured non-mobilized peripheral blood cells (NM-PBMNCs)

Panel 1: Mean numbers of all hematopoietic lineages obtained from fresh and cultured non-adherent cells originated from healthy donors (n=33) and ischemic patients (n=9). Total CFUs, CFU-GEMM, BFU-E and CFU-GM hematopoietic colonies are presented. While cultured ischemic patients' non-adherent cells produced 15% more total CFUs than fresh cells ($P < 0.01$), healthy donor (non-mobilized) cultures yielded 54% more total hematopoietic colonies compared to CFU produced by fresh non-cultured cells ($P < 0.0001$)



Panel 2: Cell surface markers analysis of all hematopoietic colonies. Mixed CFUs produced by fresh cells **[A]** and non-adherent cells [B] from healthy donors (n=4) were collected and stained with hematopoietic lineage markers such as CD11b, CD13, CD33, CD34, CD38 and CD117, gated on CD45pos cells



from ischemic patients produced 15% more total CFUs (468.6 ± 137.1/10^6 cells, $P < 0.025$) than fresh cells (405.9 ± 102.7 mixed CFUs/10^6 cells). The numbers of CFU-GEMM increased to 253.7 ± 36.9/10^6 cells (30% increment from fresh), CFU-GM increased to 179.2 ± 69.1/10^6 cells (2.6-fold increment from fresh), while BFU-E decreased to 36 ± 21.1/10^6 cells, $P < 0.0001$ [Figure 1, Panel 1].

Cultured cells obtained from healthy non-mobilized donors yielded 54% more total hematopoietic colonies (686.2 ± 91.6) than fresh (444.2 ± 59.7), 40% more CFU-GEMM (459.7 ± 52.4) compared to 328.9 ± 35, 216.5 ± 32.7 CFU-GM (four times more) compared to 52.8 ± 11.0 produced by fresh cells, and reduced numbers of burst-forming unit-erythroids (BFU-E) (10.0 ± 6.5/10^6) compared to 62.5 ± 13.7 produced by fresh non-cultured cells ($P < 0.0001$) [Figure 1, panel 1]. Fresh cells obtained from healthy donor mobilized mononuclear cells (n=6) yielded total numbers of 2338 ± 752/10^6 cells, CFUs that contained 250 ± 102.9 CFU-GEMM, 308 ± 116.4 CFU-GM, and 1780 ± 674 BFU-E ($P < 0.0001$).

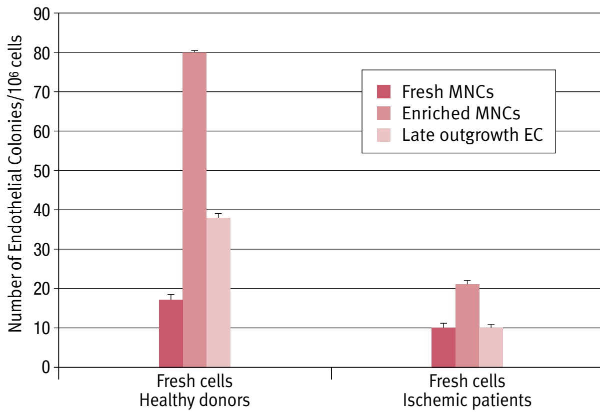
To characterize the hematopoietic phenotype of the hematopoietic colonies, CFU single cell suspensions were stained with two different marker mixtures, each with four color combinations, as described in Materials and Methods.

Results (n=4) indicated that although the percentage of CD34 expressed on both fresh and cultured non-adherent CFUs is low, cells obtained from non-adherent CFUs lost their CD38+ expression compared to fresh cells (2.0 ± 0.2% and 12.8 ± 2.7%, respectively).

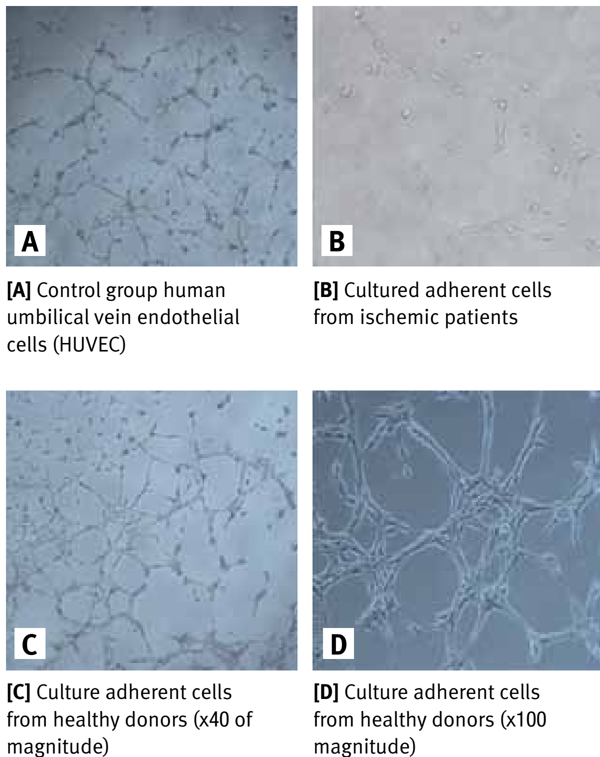
The percentage of CD33pos CFUs cultured from non-adherent cells was doubled compared to the expression on CFUs obtained from fresh cells (60 ± 6.9%, 29.7 ± 6.0%, respectively). Moreover, the percentage of CD11b expressed on CFU cells

Figure 2. Endothelial differentiation capacities of adherent and non-adherent cultured cells

Panel 1: Total numbers of short-term (early) endothelial colonies and late outgrowth endothelial colonies produced by fresh PBMNCs and enriched adherent cells obtained from healthy donors (n=33, n=15 for long-term culture) and ischemic heart disease patients (n=9)



Panel 2: In vitro tube-forming networks by cultured cells. Representative pictures of capillary tube networks formed 4 hours after seeding cells on matrigel matrix



[A] Control group human umbilical vein endothelial cells (HUVEC)

[B] Cultured adherent cells from ischemic patients

[C] Culture adherent cells from healthy donors (x40 of magnitude)

[D] Culture adherent cells from healthy donors (x100 magnitude)

Panel 3: Endothelial cell surface marker analysis of mixed colony-forming units-endothelial cells (CFU-EC). CFU-EC were collected and cells were stained for specific endothelial surface markers such as CD31, CD34, CD45, KDR, CD105 and CD146

produced by non-adherent cells was 2.5 times higher than on cells produced from fresh CFUs (21 ± 5.5, 8.5 ± 3.0 respectively, $P < 0.001$) [Figure 1, panel 1B].

ENDOTHELIAL LINEAGE DIFFERENTIATION

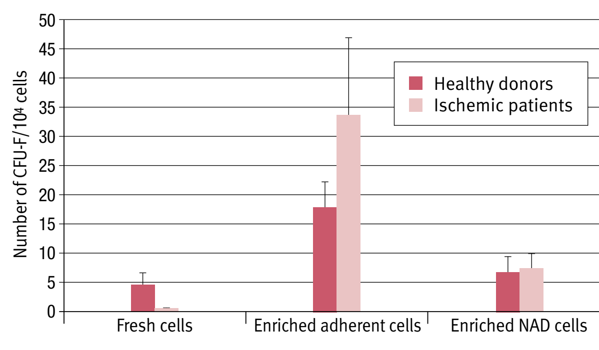
Endothelial differentiation capacities were tested by cells' ability to produce short- and long-term outgrowth colonies and their ability to form tube networks. Results indicated that cultured cells obtained from healthy (non-mobilized) donors produced significantly more endothelial colonies (79.3 ± 21.3/10^6) compared to fresh non-cultured seeded cells (17.6 ± 3.7/10^6) ($P < 0.0001$) [Figure 2, panel 1].

The mean number of CFU-ECs produced by PBMNCs originated from ischemic patients was doubled in cultured adherent cells (21.0 ± 6.7/10^6 cells) compared to the number of colonies produced by fresh non-cultured mononuclear cells (10.3 ± 3.4/10^6 cells) ($P < 0.001$) [Figure 2, panel 1]. The vasculogenesis potential of cultured cells was evaluated by the cells' ability to produce late outgrowth EC colonies and tube networks. Adherent cultured cells of both healthy donors and ischemic patients produced significantly high numbers of long-term endothelial colonies compared to no colonies produced by fresh cells obtained from ischemic patients and healthy donors (10.0 ± 3.5/10^6 cells, 38 ± 10.0/10^6 cells respectively, $P < 0.0001$) [Figure 2, panel 1]. The effect of short-term culture on in vitro angiogenesis potential was studied by testing cells' ability to produce a tube network. The assays were performed from fresh and from cultured adherent cells. Human umbilical vein endothelial cells were used as a positive control. Cultured adherent cells of non-mobilized or mobilized PBMNCs from healthy donors resulted in production of complete tube branches (4.3 ± 0.7, 5.2 ± 1.1/5 x 10^4 cells seeded, respectively), compared to complete tube networks produced by HUVEC (2.3 ± 0.5/5 x 10^4 cells) ($P < 0.001$) in 15 fields of x400 magnification [Figure 2, panel 2 A-D]. Fresh cells obtained from healthy donors and ischemic patients, and cultured adherent cells originated from ischemic patients failed to produce a tube network.

In order to characterize the endothelial colonies phenotype (CFU-EC), a mixture of 7 days short-term colonies produced by fresh (not cultured) or cultured adherent cells (both obtained from healthy donors) were collected and stained with the following markers: KDR, CD31, CD34, CD105, CD45, CD146 and acetylated LDL (n=4). Results [Figure 2, panel 3] showed that cells extracted from CFU-EC (n=4) highly expressed endothelial cell markers such as KDR, CD31, CD105 and CD146 in both fresh and cultured adherent CFU-EC. Yet, a higher percentage of cells collected from CFU-EC produced by cultured adherent cells expressed CD146 (57.5 ± 0.98%) and acLDL uptake (80.6 ± 10.0%) compared to CFU-EC produced by fresh non-cultured cells (18.6 ± 2.1% and 62.2 ± 1.57%, respectively).

Figure 3. Mesenchymal differentiation capacities

Panel 1: Mean numbers of fibroblast-like mesenchymal colonies (CFU-F) produced by fresh, cultured adherent and non-adherent (NAD) cells obtained from peripheral blood of healthy donors and ischemic patients



MESENCHYMAL LINEAGE DIFFERENTIATION

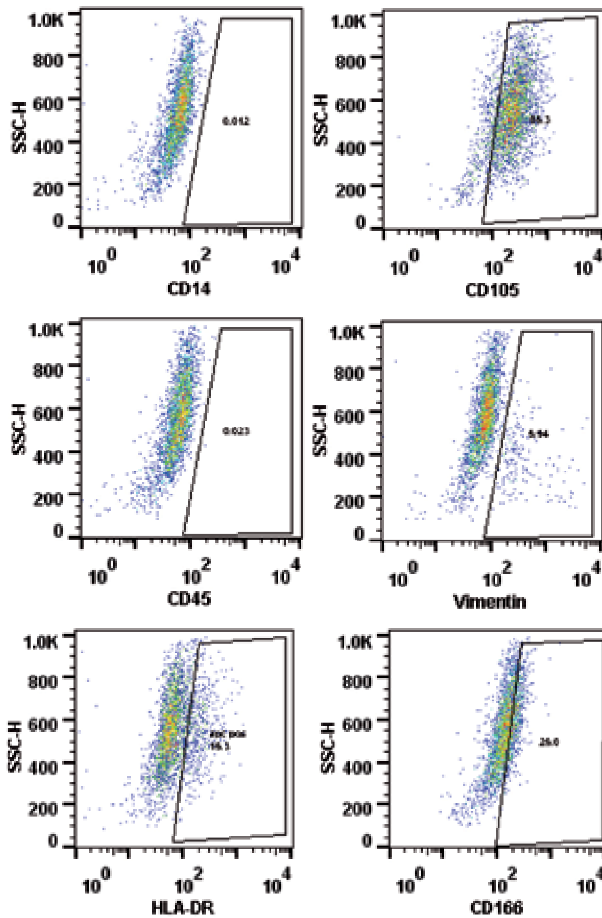
Mesenchymal differentiation capacities of cultured cells were tested by cells' abilities to produce CFU-F and to differentiate into osteoblasts and adipocytes.

High CFU-Fs were produced by adherent cells from ischemic patients (33.6 ± 13.4/2 x 10^4 seeded cells), compared to zero fibroblast colony produced by fresh PBMNCs. Significantly higher numbers of CFU-Fs were produced by adherent cells originated from healthy donors (17.8 ± 4.5/2 x 10^4 seeded cells) compared to low numbers produced by fresh PBMNCs (4.6 ± 2.05/2 x 10^4 seeded cells) [Figure 3, panel 1]. Adherent cells cultured for 21 days with adipocyte differentiation medium produced small lipid vacuoles stained by Oil-Red O compared to large vacuoles produced by BM-MSCs (cultured in the same conditions). Adherent cells cultured with osteoblast differentiation medium for 10 days produced less mineralized matrix (stained by Alizarin red) compared to more mineralized matrix produced by BM-MSCs. All cells failed to differentiate into chondrocytes. In order to characterize the mesenchymal lineage of differentiated cells, mixed CFU-Fs were collected (n=4) using trypsin-EDTA and stained with CD14(FITC), CD3(FITC), CD34(FITC), CD44(PE), CD166(PE), CD45(APC/FITC), CD90(PE), CD105(APC) and HLA-DR. Results shown in Figure 3, panel 2 indicate that cells collected from expanded CFU-Fs lost their CD14, CD3 and CD45 expression, yet gained expression of CD90 (57.4%), CD73 (38.4%) and CD105 (85%). Moreover, these cells were found to express HLA-DR class I (15.3%) and vimentin (5.94%) [Figure 3, panel 2].

DISCUSSION

The current study demonstrates the feasibility of increasing limited numbers of progenitor cells with multilineage differentiation potential from NM-PBMNCs, using a short-term culture with a defined cytokine cocktail. Ex vivo expansion

Panel 2: Cell surface markers of mesenchymal lineage expressed on CFU-F. Mesenchymal lineage cell surface markers staining was performed on mixed CFU-F collected (n=1) with CD14(FITC), CD3(FITC), CD34(FITC), CD44(PE), CD166(PE), CD45(APC/FITC), CD90(PE), CD105(APC) and HLA-DR



of hematopoietic stem cells, mesenchymal stromal cells, erythroid and endothelial progenitor cells from different sources were previously described [10,15-17]. With our modality, however, it is possible to collect two distinct subpopulations that are able to differentiate into hematopoietic, endothelial and mesenchymal cells. Indeed, the MILF supplement induced endothelial cell lineage and the SC supplement induced hematopoietic lineage (data not shown). Combining both mixture contents (in the MS supplement) improved cultured cells' ability to produce hematopoietic, endothelial and mesenchymal-like progenitors from the same original culture with no extra manipulations (such as specific subpopulation isolation or enrichment). Moreover, in view of the simplicity and low cost of this method, non-mobilized peripheral blood, which is almost an unlimited source, might be an attractive modality for clinical use.

The use of the MILF and SCF cytokines, each combined from a partial mixture of the MS supplement, induced the culture to favor either endothelial or hematopoietic lineage characterization (respectively). Cells cultured with no cytokine supplement failed to enrich any of the tested lineages (n=4, data not shown). The numbers of hematopoietic, endothelial and mesenchymal colonies were found to be dependent on the cell source (i.e., healthy donor or ischemic patient). Results indicated that cell multilineage potential capacities of healthy donor cultured cells were enhanced compared to cells originated from ischemic patients. Factors such as age, diabetes mellitus, hypertension and hyperlipidemia, which were among the disease complications, may explain the difference between patients and healthy controls [18]. Yet, despite the poor starting point, the overall enrichment process of cells from chronic ischemic patients was feasible. The adjustment between the patient's pathophysiology (i.e., acute vs. chronic heart disease, age-related) and culture conditions (cytokine concentrations) needs to be further evaluated.

The ability of adherent and non-adherent cells to expand was tested by using a Cell Proliferation Kit (XTT). Results (data not shown) indicated that adherent cells doubled their population number in the short-term culture (7 days), compared to non-adherent cells. This ability to expand and enrich the multilineage capacities of cell populations may result from the dynamic interactions that the heterogeneous (adherent and non-adherent) cultured cells provide as a micro-environment, which is needed for the enrichment of primitive cells, as suggested by Jing et al. [19].

To better characterize cultured cells, various cell surface markers were used to stain adherent and non-adherent subpopulations and to compare the percentage of expressing cells to fresh (non-cultured) cells. The results indicated that cultured cells are composed of both mature myeloid and endothelial cells (CD14^{pos}, CD34^{low} , CD45^{pos} and CD31^{pos}) and with immature cells which express KDR^{pos}, CD90^{pos}, CD105^{pos}, and CD117^{pos}, and CD146^{pos} (data described in results section). These cells were able to produce significantly higher numbers of specific lineage differentiation colonies (i.e., CFU-HSC, CFU-EC and CFU-F) compared to those produced by fresh non-cultured cells (data described in results section). To better characterize the colonies obtained when differentiation assays were performed, specific cell surface markers for endothelial, hematopoietic and mesenchymal markers were employed.

Cells obtained from endothelial and hematopoietic colonies expressed more specific lineage markers such as CD31^{pos}, CD105P^{pos}, CD146^{pos} and acLDL uptake, and immature myeloid markers such as CD13^{pos}, CD33^{pos}, CD11b^{pos} and CD38^{neg} [Figure 1 panel 2 and Figure 2 panel 3], respectively. These results proved that cultured cells (adherent and non-adherent) gained a multilineage potential that is absent in fresh PBMNCs. It became apparent that "lineage-committed" cells

that are cultured under specific growth conditions display a high plasticity and can differentiate into cells of another lineage with distinct functional properties [5]. Moreover, our finding of low CD34 expression of the cultured cells is in line with previous findings of Dimmeler [5], who showed that CD34lowCD14pos cells in peripheral blood are a major source of endothelial progenitor cells. Surprisingly, although adherent cells expressed mesenchymal cell surface markers [Figure 3, panel 2], they failed to differentiate into osteoblasts, adipocytes and chondrocytes as BM-MSCs. This might be due to the need for more stringent differentiation conditions than those appropriate to BM-MSC differentiation abilities.

The phenomenon of different lineage activities with or without acquiring a specific lineage surface marker in the same culture might result from the supportive culture microenvironment introduced to non-mobilized peripheral blood first by the cytokine supplement provided in the culture medium and next by the interaction between adherent and non-adherent cells. A wide variety of ex vivo expansion methodologies use the co-culture modality to expand hematopoietic (CD34pos) stem cells by mesenchymal stromal cells and cytokine-enriched culture medium that provide an optimal environmental niche [20-22].

In summary, our challenge to enrich limited numbers of non-mobilized PBMNCs from healthy donors or ischemic patients was proven feasible. This low cost, effective and safe modality of enriching an unlimited source of cells simplifies their use as a source for multilineage cells, especially for autologous use. This tool may also be employed for future clinical use where mesenchymal and endothelial progenitor cells are needed for ex vivo expansion [16,17] of hematopoietic progenitors or as accessory cells to improve hematopoietic engraftment, as recently described in a pre-clinical study [23,24].

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"Foreign aid might be defined as a transfer of money from poor people in rich countries to rich people in poor countries"

Douglas Casey, classmate of Bill Clinton at Georgetown University