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Isolation of progenitor cells from cord blood using adhesion matrices

Sarah Maurice · Samer Srouji · Erella Livne

Abstract The aim of this study was to develop optimal conditions for selective adhesion and isolation of mesenchymal progenitor cells (MPCs) from cord blood and to determine their potential for osteogenic differentiation. Mononuclear cells (MNCs) were isolated by Ficoll-Paque gradient and plated onto 48-well culture plates precoated with: human or bovine collagen type I, human collagen type IV, fibronectin or matrigel. Cultures were incubated in αMEM containing fetal calf serum. Viability of the adherent cells was determined by alamarBlue® assay after 2, 3, and 4 weeks. After 4 weeks in culture, cells were typsinized and replated. Primary cultures were analyzed by histochemistry and third passage cells by FACS. Isolated fibroblast-like cells were cultured in the presence of osteogenic factors and differentiation determined by Alizarin Red S staining, RT-PCR and electron dispersive spectroscopy (EDS). MNCs adhered to all types of matrices with the greatest adhesion rates on fibronectin. These cells were CD45$^+$, CD105$^+$, CD14$^+$, CD49a$^+$, CD49f$^+$, CD44$^+$ and CD34$^-$. The highest incidence of progenitor cells (PC) was observed on fibronectin and polystyrene. Passages were CD45$^-$, CD14$^-$, CD34$^-$ and weakly CD105$^+$. Primary cultures expressed endothelial/macrophage RNA markers whether cultured on fibronectin or polystyrene and these markers decreased upon passage. The best osteogenic differentiation was observed in MPCs cultured in osteogenic medium containing vitamin D$_3$ and FGF9. These cells expressed the bone-related mRNA, collagen type I, core binding factor I (Cbfa I), osteocalcin and osteopontin. EDS of deposits produced by these cells demonstrated a calcium/phosphate ratio parallel to hydroxyapatite. It was concluded that fibronectin increased adhesion rates and isolation potential of cord blood mesenchymal progenitor cells.

Introduction

Mesenchymal progenitor cells are multipotential cells which are thought to act as long lasting precursors for connective tissues (Arinzeh 2005; Bruder et al. 1994; Bhagavati and Xu 2004; Dazzi et al. 2005; Dennis and Charbord 2002; Ge et al. 2005). To date, the most common source for harvesting of MSCs is bone marrow (Grove et al. 2004; Herzog et al. 2003) and fat tissue (Dicker et al. 2005; Zuk et al. 2001), but due to the invasive procedures necessary for the aspiration of sufficient numbers of target cells, alternative pools

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are being studied. It has been accepted that umbilical cord blood (UCB) and peripheral blood (PB) are resources for hematopoetic stem cells which can be utilized in place of bone marrow transplants (Cohen and Nagler 2004; Fibbe et al. 2001; Kogler et al. 2005; Rogers and Casper 2004; Muguruma et al. 2006) following myeloablation. In addition there have been several reports of the presence of circulating endothelial progenitors in CB and PB (Silva et al. 2005; Murohara et al. 2000; Hildbrand et al. 2004). On the contrary to bone marrow and PB-derived progenitors, cord blood progenitors are thought to possess a higher cell cycle rate. This property should favor the growth of cord blood progenitors compared with adult PB. In spite of reports of in vitro skeletal myogenic (Gang et al. 2004), hepatic (Hong et al. 2005), neural (Jeong et al. 2004), osteogenic, chondrogenic and adipogenic differentiation (Chang et al. 2006) of UCB-derived MNCs, controversy still exists as to the identity of these cells and the reproducibility of the various isolation and culture protocols.

Efforts to isolate pure cultures of these multipotential cells from samples of fresh cord blood while employing the plastic adhesion method with variations of supplemented cell media have demonstrated levels of success ranging from 7.4% (Kang et al. 2004), 24% (Erices et al. 2000) to 40.3% (Kogler et al. 2004). Bieback et al. (2004) developed optimized isolation and culture conditions and were able to reach a 63% rate of isolation. Their study determined 4 criteria which significantly affected the success rate of MPC isolation; (1) time of culture (not >15 h from collection), (2) volume of sample (not <33 ml), (3) number of MNCs/ml (not $<10^8$) and (4) minimal state of hemolysis of sample. The goals of the present study were to determine if the addition of protein adhesion molecules to the surface of plastic culture plates would assist in increasing the incidence of MPC isolation from fresh samples of cord blood, to identify the cells which bind to the plate surface and to evaluate the osteogenic potential of the resultant mesenchymal isolates.

Materials and methods

Coating of plates

Forty-eight-well (1.1 cm^2) polystyrene culture plates (Nunc) were coated prior to initiation of cell culture with either human collagen types I and III, bovine collagen types I and III (both Gibco-Invitrogen, USA), collagen type IV (Sigma, USA), fibronectin (Biological Industries, Israel) or Cultrex Basement Membrane Extract (Trevigen, USA). Coating was accomplished in the following manner: (1) Working solutions of human collagen type IV (Col IV), bovine collagen (b-Col) and human collagen (h-Col) (97% type I and 3% type II) were prepared by diluting to a concentration of 100 μg ml^{-1} with 0.25% acetic acid. 0.5 ml of collagen were spread evenly over the surface, followed by incubation overnight (o/n) at 4°C. Excess matrix was removed, the plates dried under UV for 20 min followed by two washes with phosphate buffered saline (PBS). (2) Fibronection was diluted (5 μg ml^{-1}) in Minimal Essential Medium-Alpha with 1,000 mg/ml D-glucose (αMEM—Biological Industries, Israel), added to culture dishes and incubated (60 min, 37°C, 5% CO$_2$) prior to seeding of the cord blood cells. (3) Cultrex was diluted with cold serum-free medium (0.1 mg ml^{-1}) and a sufficient volume necessary to cover the surface area of each well was applied prior to incubation for 60 min at 37°C. Each trial consisted of six replicates/plate of each coating and three plates/sample i.e. a total of 18 replicate wells of each coating/experiment. Cells were also seeded onto non-coated polystyrene culture plates as well as 4-well polystyrene chamber slides (Lab-Tek, USA). The chamber slides were employed for histochemical staining. A total of six cord blood samples from different donors were studied in this manner.

Isolation and culture of mononuclear cells

Fresh cord blood was collected after full-term deliveries in accordance with local ethical standards and after informed consent from the donors. MNCs were isolated in Uni-Sep$_{MAXI}$ tubes (NovaMed, Israel) using Lymphoprep^{TM} (density 1.007 g ml^{-1}—osmolality 290 mOSm) (Axis Sheild, Norway) according to the manufacturer's protocol. Briefly, 10 ml of Lymphoprep solution were overlaid onto the separation membrane followed by centrifugation (400 × g, 1 min, RT), after which undiluted cord blood (15 ml) was added above the membrane and centrifuged (1,000 × g, 30 min, RT, no brake). The resultant mononuclear layer was then removed with the aid of a Pasteur pipette, washed twice with PBS and resuspended at a concentration of

25×10^6 cells ml^{-1} in αMEM supplemented with 20% FCS, glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 µg ml^{-1}), amphotericin (0.25 µg ml^{-1}) and 1% non-essential amino acids (all from Biological Industries, Israel). The cells were then seeded onto tissue culture plates in the following volumes: 1 ml for 48-well (1.1 cm^2 area) polystyrene dishes and 0.6 ml for 4-well chamber slides. Following two days incubation, the cultures were washed twice with PBS and fresh medium added. Subsequent to 2 weeks in culture the concentration of FCS was reduced to 10% (basic medium). Unless otherwise noted all cultures were maintained at 37°C, 5%CO$_2$ in a moist atmosphere and medium changed twice weekly. Adherent cells were detached from the primary (P$_0$) culture plates by trypsinization (0.05% trypsin/0.25% EDTA- Biological Industries, Israel) after 4 weeks and replated onto freshly coated plates. These cells were termed P$_1$. The number of wells in which putative PCs developed following the first passage was determined from each experiment and the average number from all experiments calculated. P$_1$ cells were collected and recultured (P$_2$) for use in the differentiation experiments and for FACS.

Cell proliferation assay

Cell attachment, survival and proliferation were monitored employing the alamarBlue® assay (Serotec, Oxford UK) 2, 3 and 4 weeks following initial culture. The medium was removed from the culture wells, replaced with 10% (v/v) alamarBlue® in αMEM without FCS and incubated for 2 h (37°C, 5% CO$_2$). Redox levels of the alamarBlue® were determined fluorometrically employing a spectrofluorometer microtiter well-plate reader (Fluostar Galaxy, USA) at excitation 530 nm/emission 590 nm with an internal gain of 10. Results were noted as fluorescence emission intensity units (FEIUs) and normalized relative to the level of adhesion to uncoated polystyrene culture wells.

Osteogenic differentiation

P$_1$ PCs were replated onto 6-well polystyrene plates at a concentration of 4×10^4/well in αMEM (10% FCS). Cultures were allowed to reach ∼60% confluence before differentiation was initiated. Three different types of osteogenic differentiation medium, based on αMEM supplemented with 0.1 µM dexamethasone, 10 mM β-glycerolphosphate, 50µM ascorbic acid (all from Sigma-Aldrich USA) (basic osteogenic medium), were employed in order to induce differentiation. PCs were incubated with either basic osteogenic medium alone or enriched with 10 ng/ml recombinant FGF9 , 10^{-7}M 1,25-(OH)$_2$ vitamin D$_3$ (Sigma-Aldrich, USA) or FGF-9 together with vitamin D$_3$ at the same concentrations as mentioned previously. Cultures were maintained for 18 days for RT-PCR and 21 days for calcium deposit analysis.

Calcium analysis

The presence of calcium deposits was determined in 3-week cultures by Alizarin Red S and electron dispersive spectroscopy (EDS). For Alizarin Red S staining, cultures were prefixed with 95% ethanol (4°C, 10 min) and stained (30 min) with a 2% solution of Alizarin Red S (pH 4.3) followed by intense washing with double distilled water (ddw).

Prior to EDS analysis the same cultures were fixed with 4% glutaraldehyde, washed intensively with ddw, dehydrated in graded alcohols (70, 95 and 100%, three changes, 10 min each) and vacuum dried. Charcoal adhesive tape was used to lift small samples of each culture which were subsequently examined utilizing a Jeol JSM-35 C scanning electron microscope at 15 kV with attached Kevex energy dispersive spectrophotometer.

Histochemistry

Histochemical staining was carried out on 2-week P$_0$ cord blood cultured in 4-well chamber slides. General cell morphology was observed by light microscopy (100×) following staining with Modified Giemsa Stain (Sigma, USA) employing the manufacturers instructions.

Naphthol AS-D chloroacetate (NCAE) and α-naphthyl acetate esterase (NAE) activity was determined employing a Sigma Diagnostics kit using the manufacturer's instructions (procedure 91). Cells were scaled as positive, weakly positive or negative according to the intensity of red (NCAE) or black (NAE) granular staining apparent in the cytoplasm.

Tartrate-resistant acid phosphatase (TRAP) staining was carried out employing the protocol described

Table 1 List of primer names, sequence and predicted size

Primer name	Sequence (5'–3')	Predicted size (bp)
Glyceraldehyde-3-p dehydrogenase		
Sense	tgaacgggaagctcactgg	307
Antisense	tccaccaccctgttgctgta	
Collagen type I		
Sense	tgacgagaccaagaactg	600
Antisense	ccatccaaaccatgaaacc	
Bone sialoprotein		
Sense	atttccagttcagggcagtag	447
Antisense	acactttcttcttccccttct	
Core binding factorα1		
Sense	taccagaccgagaccaacagag	310
Antisense	caccaccgggtcacgtcgc	
Osteocalcin		
Sense	tcacactcctcgccctattgg	371
Antisense	tcacactcctcgccctattgg	
Osteopontin		
Sense	agaccccaaaagtaaggaagaag	541
Antisense	gacaaccgtggaaacaaataag	
VEGFR 1		
Sense	gtcacagaagaggatgaaggtgtcta	414
Antisense	cacagtccggcacgtaggtgatt	
VEGFR 2		
Sense	ctggcatggtcttctgtgaagca	795
Antisense	aataccagtggatgtgatgccgg	
TIE-2		
Sense	tctgtgctgttccttcttgc	376
Antisense	cttgagtaacttccagcgga	
eNOS		
Sense	caccgtttgcccacccttcg	434
Antisense	gcccactgggagccgacact	

in the Beckton Dickinson technical bulletin 445. The presence of a deep red sediment within the cells was considered a positive reaction.

RT-PCR

RNA was prepared from cells (P_0, P_1 or P_3 cells in differentiation) collected from cultures using a Qiagene mini-kit and according to the manufacturer's instructions. Reverse transcription was carried out employing a final concentration of 0.5 µg random primers (Promega, USA)/1µg RNA, 500 µM dNTP mix, 10 U RNase inhibitor, 40 U M-MuLV reverse transcriptase (RT) and its accompanying buffer (25 µl final volume). The RT program consisted of heating the mRNA and random primers (final volume 15 µl) for 5 min at 70°C followed by 5 min 0°C. A mixture of buffer, enzymes and dNTPs were added and heated to 39°C (1 h) followed by 90°C (10 min). Each PCR reaction was accomplished using ∼100 ng cDNA, 400 nM each of sense and antisense primers (Table 1), 200 µM dNTP mix, 0.4 U/reaction of Taq polymerase and its accompanying buffer (final volume 25 µl). The PCR program consisted of 5 min 94°C denaturation followed by 29 cycles of 94°C (3 min), annealing (45 s), elongation (1 min) and terminating with 5 min elongation. Both RT and PCR were carried out in PTC-100™ Thermal Control (MJ Research, Waltham, MA/

US). PCR products were resolved on 1.5% agarose gels containing ethidium bromide.

Flow cytometry

For flow cytometry analysis, P_0 and P_1 cultures were detached from their culture plates with trypsin/ EDTA (on ice) and labeled with the following monoclonal antibodies: CD49a-FITC (P1H5-Santa-Cruz), CD49f-FITC (MCA1457F, Serotec), CD105 (266; BD PharMingen), CD44Std. (SFF-304; Bender MedSystems), CD34-FITC (Class III 581; Serotec), CD45-FITC (2B11; Serotec), CD14 (MEM-18, Abcam, USA). Acquisition and analysis was performed on a FACSCalibur flow cytometer (Becton Dickenso, USA). All antibodies were used at a concentration of 0.5 µg/10^6 cells in a volume of 100 µl unless otherwise recommended by the manufacturer.

Statistical analysis

Intra and inter plate assay differences were determined prior to interpretation of the data. The effect of adhesion matrices on adhesion and isolation of PCs was examined using a one-way analysis of variance (ANOVA) with the aid of the program, GraphPad Prism, Version 4 (GraphPad Software, USA). The Dunnet post-test was employed for comparison of the experimental groups. In all cases, $P < 0.05$ with a Cl of 95% was considered significant.

Results

Isolation and proliferation of PCs on different matrices

The relative numbers of cells which adhered to the various matrices was determined by the alamarBlue Assay 2, 3, and 4 weeks after initial culture (Fig. 1a). The average intra plate disparity was 5.2% and the inter plate 8.3%. The lowest number of residual cells recorded following 2 weeks in culture was observed on the collagen type IV surface. The numbers decreased significantly after 3 and 4 weeks incubation (29.8 and 18.0% respectively, $P < 0.05$). A similar profile was observed

with the matrigel coating. Human collagen and fibronectin films initiated adhesion levels which were equivalent to those observed in uncoated polystyrene plates (1.08 ± 1.78 and 0.99 ± 0.315 FEIUs relative to the polystyrene plates). A slight but insignificant decrease in the cell numbers occurred during the next two weeks with these two matrices while the number of cells in the fibronectin coated plates remained constant.

Long-term adherent cells were recultured onto freshly coated culture wells. Most of the P_0 cells did not adhere and were lost upon passage. The average number of P_0 wells (18 replications per experiment) in which PCs were observed microscopically in all six experiments were: fibronectin 13.17 ± 2.93, polystyrene 7.0 ± 1.29, collagen type IV 3.83 ± 1.6, human collagen 0.83 ± 0.75, bovine collagen 1.0 ± 1.26 and matrigel 0.67 ± 1.21 coated wells (Fig. 1b).

Classification of adherent cells

Within 24 h MNCs adhered in large numbers to all surfaces regardless of the coating. Morphology and staining properties were similar with all of the adhesion proteins with the exception of the matrigel. A representation of the cells found on the polystyrene surface is shown in Fig. 2. The h-Col and b-Col appeared unstable and small areas of the matrix became partially detached with time. All cultures, excluding the matrigel, developed into a field of flattened single or multinucleated cells containing distinct cytoplasm (Fig. 2f). Cells cultured on the matrigel tended to clump together forming small mounds (Fig. 2g).

Cells which adhered to the various coated and noncoated surfaces for a period of two weeks were identified by histochemical and enzymatic stains. Giemsa staining revealed a mixture of cell morphologies with two major types; elongated thin cells carrying a funnel-like cytoplasmic extension containing a small but distinct nucleus and larger cells containing cytoplasm surrounding a central nucleus (Fig. 2a). The rounded population was composed of a substantial number of larger multinucleated cells. These cells were weakly alkaline phosphatase positive (Fig. 2b), TRAP (Fig. 2c) reactive as well as NCAE (Fig. 2d) and NAE (Fig. 2e) positive. The fibroblast-like elongated cells were mostly alkaline
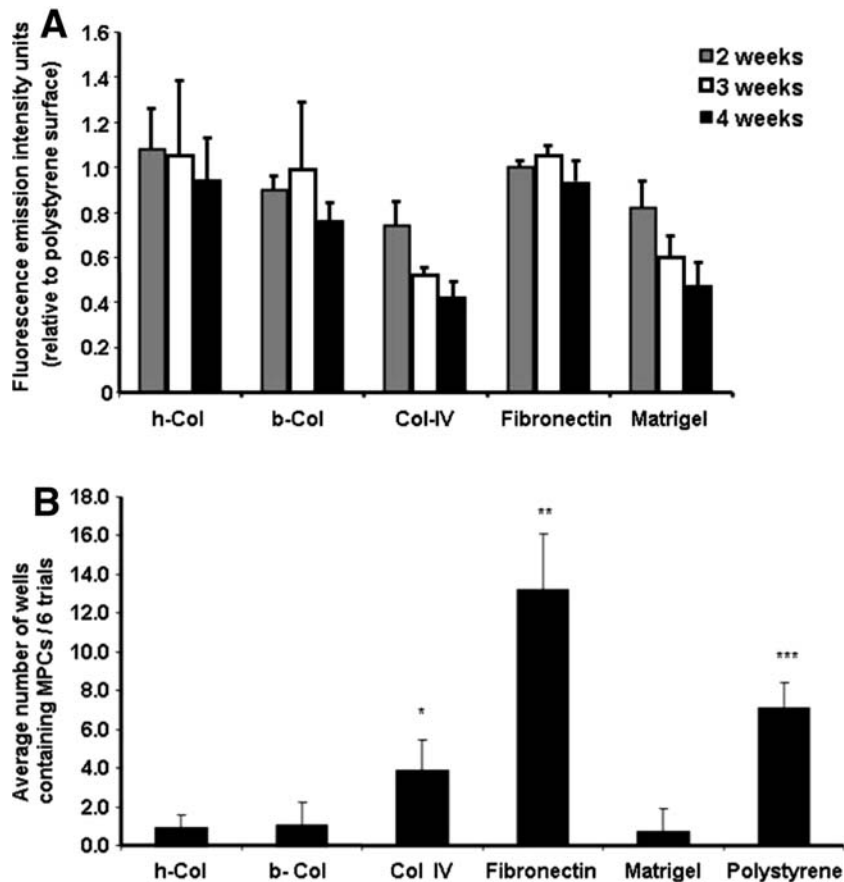
Fig. 1 (**a**) Relative percentage of cord blood cells which adhered to coated plates. Mononuclear cord blood cells were isolated by Ficoll-Paque gradient and seeded onto 48-well polystyrene culture plates or the same plates pre coated with either h-Col I, b-Col I, human Col IV, fibronectin or matrigel. The presence of live adherent cells was determined 2, 3 and 4 weeks post seeding employing the alamarBlue™ assay and results normalized relative to the level of cell adhesion on uncoated polystyrene culture wells. Redox levels were determined fluorometrically at excitation 530 nm and emission 590 nm with an internal gain of 10. Results are noted as fluorescence emission intensity units and represent the average

numbers from six separate experiments with 18 replicates/ experiment ± standard deviation. (**b**) Percentage of PC isolations on different adhesion proteins. Adherent cells were trypsinized from P0 cultures and replated onto matrix coated plates. The number of wells in which PCs developed were counted and displayed on a *bar graph*. Each *bar* represents the average number of wells in which PCs developed in all six trials ± standard deviation. Significant differences in PC isolation were observed between protein coatings with fibronectin > polystyrene > collagen type IV > human collagen type I ($P < 0.05$, CI 95)

and TRAP negative and weakly NAE and NCAE positive. Cells cultured on matrigel produced clusters with elongated cells extending from the periphery (Fig. 2g). Staining was poor and not definitive (Results not shown).

P_1 positive cultures on all of the plate surfaces revealed elongated fibroblast cells which developed randomly and which replicated very slowly (Fig. 2h). P_2 cultures replicated more rapidly and formed of loosely woven clusters (Fig. 2i).

FACS and RT-PCR analysis of P_0 and P_1 cultures

P_0 (Fig. 2f) and P_3 cultures (Fig. 2i) were typsinized from polystyrene plates and analyzed by FACS and RT-PCR. A large portion of the primary population strongly expressed CD45 and CD44-standard, weakly CD14, CD105, CD49a, CD49f and negative for CD34 (Fig. 3a). PCs which developed after passage did not display the markers for CD45, CD14 or CD34 while both CD105 and CD49f remained stable and
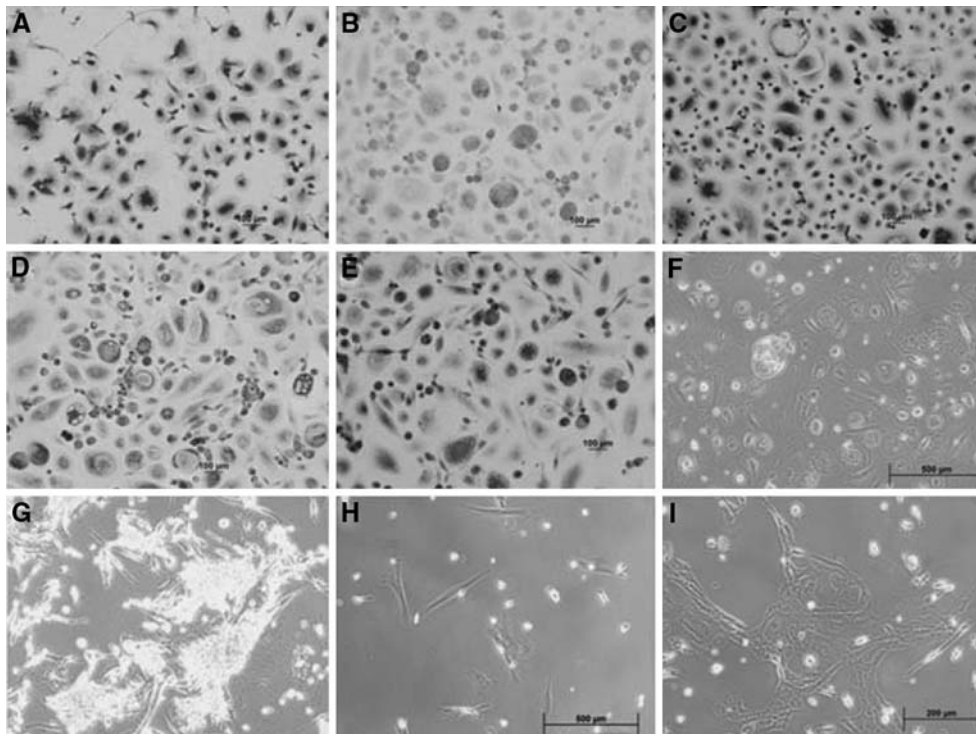
Fig. 2 Morphology and histochemistry of P0 adherent cells. Cord blood cells which adhered to polystyrene surfaces for 2 weeks were stained with (**A**) Giemsa; (**B**) Alkaline phosphatase; (**C**) Tartrate-resistant acid phosphatase; (**D**) Napthol AS-D chloroacetate; (**E**) α-napthyl acetate esterase; (**F**) P0 culture on polystyrene (unstained); (**G**) Morphology of clusters which developed on matrigel coated plates; (**H**) P1 culture; (**I**) P2 culture. (Magnification 100×) Two major types; elongated thin cells carrying a funnel-like cytoplasmic extension containing a small but distinct nucleus and larger cells containing cytoplasm surrounding a central nucleus were observed. The rounded population was weakly alkaline phosphatase positive, TRAP reactive as well as NCAE and NAE positive. The fibroblast-like elongated cells were mostly alkaline and TRAP negative and weakly NAE and NCAE positive. (Bar sizes indicated in each slide are as follows:- Figs A–E.100 μm; F–H 500 μm; Fig I. 200 μm)

expression of CD49a and CD44 increased (Fig. 3b). RT-PCR of the primary cord blood cultures expressed RNA sequences for VEGFR1, VEGFR2, TIE2 and E-nos (Fig. 4a) while only the endothelial marker eNOS was weakly observed in the P_1 passage of the same cells (Fig. 4b).

Osteogenic differentiation of isolated MPCs

Isolated fibroblast-like cord blood cells (P_2) were cultured in 6-well polystyrene plates and exposed to ''osteogenic medium'' with or without β-glycerophosphate, FGF9 and/or vitamin D_3. Subsequent to 18 days in culture, control cultures, incubated in basic medium only, express RNA for the housekeeping gene GAPDH. The addition of ascorbic acid and dexamethasone to the growth medium resulted in the appearance of RNA associated with collagen type I and CBFAI. Adding β-glycerophosphate to the osteogenic media did not alter this profile. The enrichment with vitamin D_3 induced the production of mRNA for osteopontin but eliminated the presence of CBFA1, while addition of FGF9 in place of vitamin D_3 resulted in strong CBFAI expression together with osteopontin and collagen type I. Only when both vitmin D_3 and FGF9 were added to the culture media was osteocalcin observed (Fig. 5). BSP expression was not observed in any of the above mentioned culture conditions although it did occur in human bone marrow osteoprogenitors cultured in our lab in osteogenic basic medium (results not shown).

Fig. 3 FACS analysis of P0 and P2 cultures employing markers (**a**) CDs 14, 105, 45, 34; (**b**) 49a, 49f, and 44. FACS analysis determined that the majority of P0 cells were of the macrophage/ monocyte lineage while P3 cultures demonstrated typical mesenchymal markers
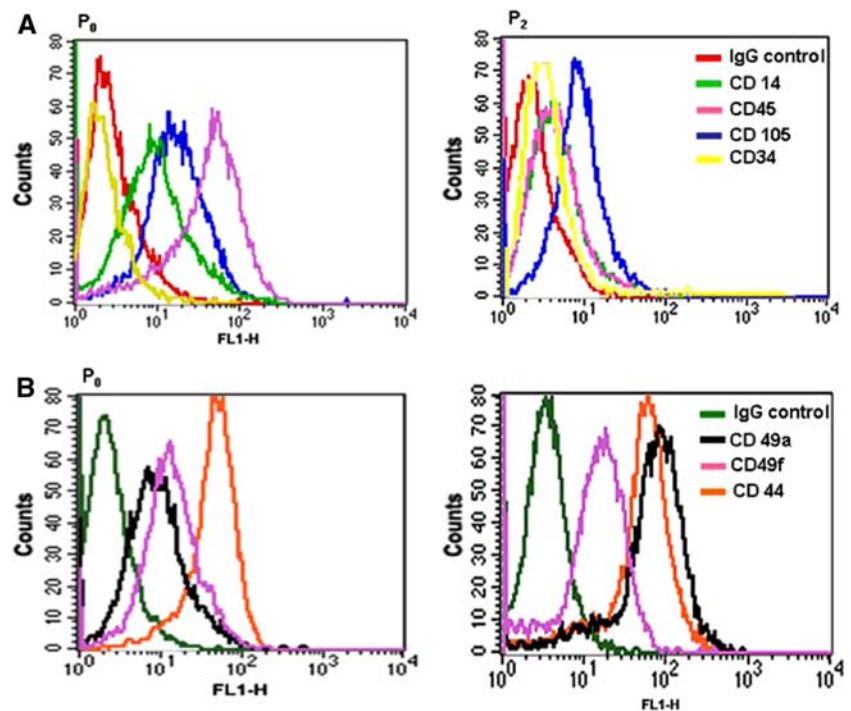

Compared to PCs cultured in basic medium (Fig. 6a-1), only cells exposed to osteogenic medium containing both vitamin D_3 and FGF9 produced large deposits of calcium (Fig. 6a-2) as seen by Alizarin staining (Fig. 6a-3). Cells which had been induced with each vehicle alone produced small and scattered mineralized matrix deposits. EDS analysis of the control culture (Fig. 6b-1) and the osteogenic cultures containing sediment (Fig. 6b-2) revealed a calcium to phosphate ratio of 1.68 which is parallel to that of hydroxyapatite.

Discussion

The interaction of cell receptors with extra cellular matrix (ECM) adhesion proteins provides signals
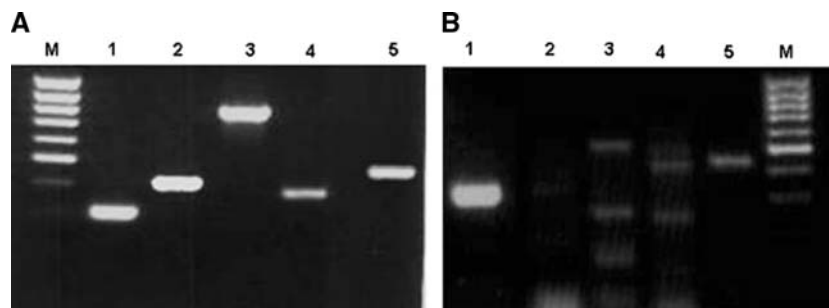
Fig. 4 RT-PCR of cord blood cells cultured on polystyrene plates. 5A P0 cord blood culture after 2 weeks adhesion: *left to right*: M 100 bp marker (*top to bottom* 1,031, 900, 800, 700, 600, 500, 400, 300, 200 bp); *1* GAPDH; *2* VEGFR1; *3* VEGFR2; *4* TIE2; *5* eNOS. 5B P1 cord blood culture (PCs) after 2 weeks in culture: *1* GAPDH; *2* VEGFR1; *3* VEGRF2; *4* TIE2; *5* eNOS; *M* marker (*top to bottom* 1,031, 900, 800, 700,

600, 500, 400, 300,200 bp). Primary cord blood cultures expressed RNA sequences for VEGFR1, VEGFR2, TIE2 and E-nos (**A**) while only eNOS was weakly observed in the P1 passage of the same cells (**B**) The full length markers for VEGFR1, VEGFR2 and TIE2 were not expressed but appeared as small misprimed fractions ofDNA (Fig. 4B lanes 2–4)
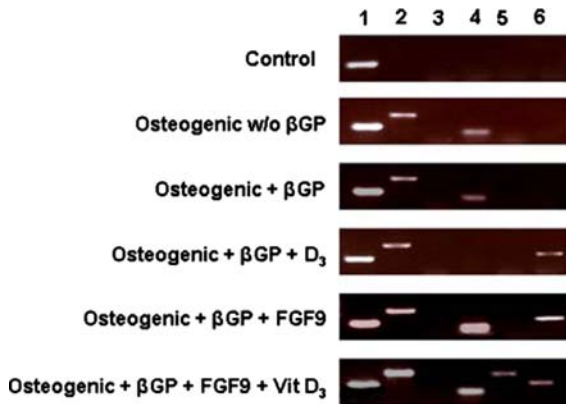
Fig. 5 RT-PCR of PCs (P2) cultured for 18 days in standard medium and in medium designed for osteogenic differentiation. *1* GAPDH; *2* collagen type I; *3* BSP; *4* CBFAα1; *5* osteocalcin; *6* osteopontin. (*w/o* without; *βGP* β-glycerophosphate)

which can affect the survival of adherent cells. Salasznyk et al. (2004) assessed the contribution of extra cellular matrix proteins on the ability of MSCs to adhere to ECM proteins found in bone marrow.

While employing 30-min adhesion assays they determined that fibronectin, Col I, Col IV and vitronectin supported approximately 6-to 8-fold greater static adhesion compared to the negative control (culture well blocked with dairy creamer) or laminin. We examined the long-term adhesion and possible replication potential of mononuclear cord blood cells 2, 3 and 4 weeks following seeding onto five different pro-adhesive proteins. Over time, the numbers of adherent cells changed only slightly on the standard polystyrene surface, as well as on the h-Col, b-Col and fibronectin coated plates; indicating absence of cell proliferation and perhaps characteristic of the state of differentiation of the adherent cells. In comparison, cells attached poorly and decreasing metabolic activity and/or numbers was determined in plates coated with matrigel and Col IV (Fig. 1a). No obvious changes in cell morphology were perceptible in any of the plates and PC colonies did not develop during the 4 week incubation period. Only following trypsinization and reculture did the MPCs become visible (Fig. 1b). A significant differ-
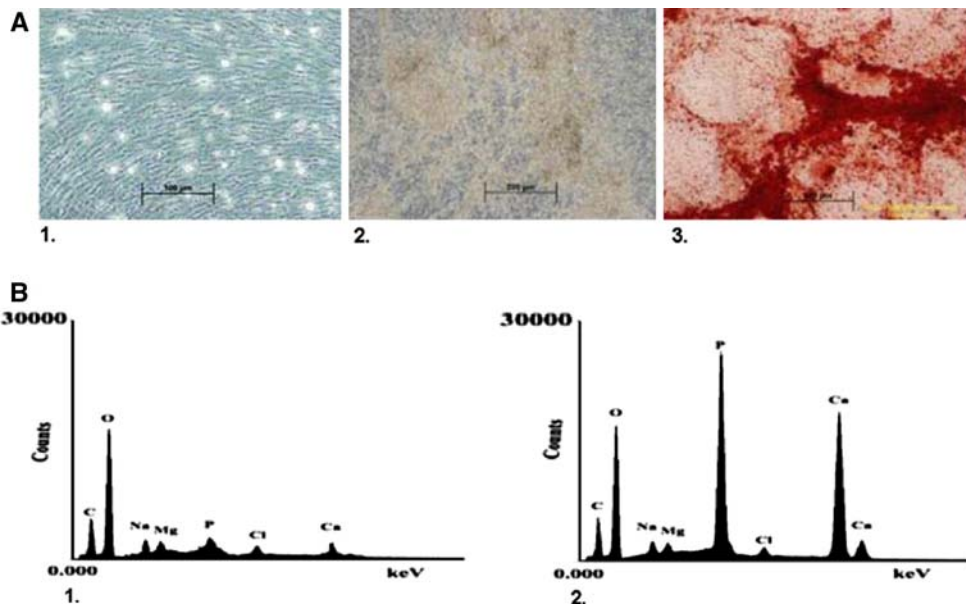
Fig. 6 PCs were cultured in standard medium or osteogenic medium containing both FGF9 and vitamin D$_3$ for a period of 21 days. The presence of calcium deposits was determined by Alizarin Red S and Electron Dispersive Spectroscopy. (**a***1*) PCs cultured in standard medium; (**a***2*) PCs cultured in osteogenic medium containing FGF9 and vitamin D$_3$ demonstrating calcium deposits; (**a***3*) Alizarin Red staining culture observed in (**a***2*). *Red* staining indicates presence of calcium deposits. (**b**) EDS analysis of culture (**a***1*) and (**a***2*). (**b***1*) EDS of control culture (**a***1*) with very minimal presence of calcium and phosphate; (**b***2*) EDS of osteogenic culture (**a***2*) with a strong signal for phosphate and calcium demonstrating a ratio similar to hydroxyapatite

ence ($P < 0.05$, CI 95%) was observed in the percentage of positive culture wells between the various matrices. The largest number of clones was isolated on fibronectin (73.2%) while 1.9-fold fewer isolations were observed with uncoated plates (38.89%). Although collagen type IV appeared to be a poor candidate for adhesion, it proved to be a more efficient matrix (21.3%) for the isolation of PCs than type I human (4.63%) and bovine (4.63%) collagens. Matrigel coating, which produced a very different initial adhesion pattern, also demonstrated very limited potential for isolation of the target cells. Fibronectin is a large multidomain glycoprotein found in connective tissue, on cell surfaces, and in plasma. It is a potential ligand for most cells involved in cellular processes, including tissue repair, cell migration and adhesion. It is known to interact with cell-surface receptors on a variety of cells including fibroblasts, neurons and phagocytes. The RGD motive found in fibronectin is the most important recognition site for about half of all known integrin cellular receptors and these receptors have a dominating role in the anchoring of cells to the ECM. A striking feature of many integrins is their ability to bind multiple ligands such as fibronectin, vitronectin, collagens and laminin (Jiang et al. 2006; Keselowsky et al. (2005). When examined by FACS, P_0 and P_3 cultures consistently expressed high levels of integrins CD49a and CD49f (Fig. 3b). This would explain the superior adhesion observed with the fibronectin, h-Col and b-Col. Although $\alpha 2$ integrin is a known receptor for Col IV (Keselowsky et al. 2005), isolated MNCs did not bind well or remain attached to this matrix.

Positive NAE, NCAE and general morphology indicated that the majority of the adherent primary cells are related to the monocyte/macrophage lineage (Fig. 2a, d, e). Within this complex, cells presenting staining properties (TRAP, Fig. 2c) and morphology (multiple nuclei) of osteoclasts were also evident in fairly large numbers. TRAP positive osteoclasts, in their precursor stage are found in the PB among the monocytic population (Shalhoub et al. 2000) but, although the expression of TRAP is regarded as an osteoclast marker, under certain conditions, cells of the macrophage/dendritic cell family also express TRAP (Tsuboi et al. 2003). FACS analysis further fortified the monocyte/macrophage identity of the primary adherent cells

which demonstrated 90% positive for the leukocyte-common antigen CD45 and negative for CD34 (Fig. 3a). During the 4 weeks of culture, the population of adherent cells observed on all coatings, excluding the matrigel, consisted of a heterogenic mixture of hematopoietic cells, monocytes/macrophages and pre-steoclasts.

Paradoxically, a greater isolation rate of PCs was observed on Col IV plates than with collagen type I. PC colonies were not observed in P_0 cultures, but became apparent only after transfer to fresh plates (Fig. 2h). The enhanced adhesion to fibronectin in the primary cultures resulted in an increased incidence of PC isolation (Fig. 1b) when compared to the other coatings and the standard polystyrene culture plates. Protein adhesion molecules in the ECM trigger changes in the cytoplasmic domains which eventually alter their interaction with cytoskeletal and/or other proteins that regulate cell adhesion, growth and migration (Damsky and Ilic 2002). A 1.9-fold increase in PC colony numbers compared to the polystryrene plates would represent a significant effect of the fibronectin coating on these cells. The large and varied cell population which adhered to the culture plates in P_0 could be analogous to the micro environment present in the native cord blood state. Similar to the niche which has been observed in bone marrow (Diaz-Flores et al. 2006; Yin and Li 2006), replication of the non differentiated cells did not occur until appropriate signals have been initiated. It would seem that separation of the PCs from the hematopoetic population by the process of trypsinization induced a modification in these cells which initiated division. After passage, adherent cells were constituted by a homogeneous population of PCs which exhibited a fibroblast-like morphology (Fig. 2h) and typical mesenchymal-like phenotypes.

RT-PCR analysis of P_0 produced products for the Receptor Tyrosine kinases VEGFR 1, VEGFR 2 and TIE 2 as well as the endothelial eNOS (Fig. 4a). Buhring et al. (1999) described these genes in normal hematopoietic tissue and define them as bipotent hematopoietic/endothelial precursors. Following trysinization and reculture, with the exception of the declining eNOS signal, expression of these RNA sequences was progressively lost after cell proliferation, demonstrating the dominance of a unique type of cells.

To examine whether the P_2 generation of PCs could be induced to differentiate into mature osteoblasts and produce a mineralized matrix in culture we exposed these cells to various soluble osteogenic supplements and assayed for the presence of RNA differentiation markers (Fig. 5). During 18 days in culture, no morphological transition from elongated to cuboidal shaped cells was noted. Cells that were cultured in standard medium expressed only GAPDH while the addition of ascorbic acid and dexamethasone stimulated the upregulation of the early osteogenic gene, collagen type I and Cbfa I. These markers remained stable regardless of the additives employed. Glucocorticoids such as dexamethasone promote osteoblastic differentiation in *in vitro* models employing bone marrow derived mesenchymal cells. This is thought to take place through the coordinated action of dexamethasone and the transcription factor Runx2/Cbfa 1 which in turn induces osteocalcin expression and terminal mineral deposition (Ducy et al. 1997; Yang et al. 2003; Kundu et al. 2002). β-glycerophosphate is added to bone marrow-derived osteogenic cultures in order to provide a source of phosphate necessary for construction of the hydroxyapatite configuration (Bellows et al. 1992). Calcium deposits were not apparent in cord blood PCs cultured in the presence of β-glycerophosphate, nor was the genetic expression pattern altered. Osteopontin, an intermediate marker expressed during osteoblast maturation, appeared subsequent to the addition of vitamin D_3, but at the same time Cbfa 1 receded. When cells were cultured with 10 ng/ml FGF9, Cbfa I and osteopontin were present. Only when osteogenic medium was enriched with both vitamin D_3 and FGF9 was the late marker, osteocalcin, expressed and considerable amounts of hydroxyapatite crystals (Fig. 6b-1 and 2) deposited (Fig. 6a-2 and 3).

Bsp 1 was not detected in any of the osteogenic cultures. Temporal sampling of these cultures would have been advantageous for better defining the window of expression of each marker and may have increased the possibility of observing Bsp 1.

The genetic markers of differentiation expressed by the PCs, and the phenotypic display of matrix production, is comparable to intramembranous ossification. Variation in the projected appearance of each marker, as affected by the osteogenic supple-

ments when compared to osteoblasts, could be due to a large degree on differenced in osteogenic cell type, culture conditions and the ECM environment.

Conclusions

Various preparative protocols have been proposed using surface antigen markers for the acquisition of mesenchymal progenitor cells from cord blood. Since MPCs have been defined as adherent cells we proposed a simple method of adherence to a specific adhesion matrix for the isolation of these cells. PCs derived from different donors using the same culture procedure yielded consistent and reproducible surface marker and gene expression profiles when cultured under standard or osteogenic conditions. Isolated cells could be induced to differentiate into osteoblasts and to produce a hydroxyapatite mineralized matrix. Strong alkaline phosphatase activity is a well documented marker of early osteoblast differentiation. Within the adherent cell population (P_0), positive alkaline phosphatase cells were not observed indicating that the cells which later developed into osteoprogenitors were not present in the primary cell population as osteoblasts.

Whether the model of differentiation observed with cord blood PCs is truly osteogenisis or just an artifact of in vitro culture can only be determined with in vivo models involving bone defects which are greater than the critical size necessary for natural healing.

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