British Journal of Haematology, 2003, **121**, 368–374

Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not

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Received 21 October 2002; accepted for publication 7 December 2002

Summary. In postnatal life, mesenchymal stem cells (MSC) self-replicate, proliferate and differentiate into mesenchymal tissues, including bone, fat, tendon, muscle and bone marrow (BM) stroma. Possible clinical applications for MSC in stem cell transplantation have been proposed. We have evaluated the frequency, phenotype and differentiation potential of MSC in adult BM, cord blood (CB) and peripheral blood stem cell collections (PBSC). During culture, BM MSC proliferated to confluence in 10–14 d, maintaining a stable non-haemopoietic phenotype, HLA class-1$^+$, CD29$^+$, CD44$^+$, CD90$^+$, CD45$^-$, CD34$^-$ and CD14 through subsequent passages. Using the colony forming unit fibroblasts assay, the estimated frequency of MSC in the BM nucleated cell population was 1 in $3{\cdot}4 \times 10^4$ cells. Both adipogenic and osteogenic differentiation of BM MSC was demonstrated. In contrast, CB and PBSC mononuclear cells cultured in MSC conditions for two passages produced a population of adherent, non-confluent fibroblast-like cells with a haemopoietic phenotype, CD45$^+$, CD14$^+$, CD34$^-$, CD44$^-$, CD90$^-$ and CD29$^-$. In paired experiments, cultured BM MSC and mature BM stroma were seeded with CB cells enriched for CD34$^+$. Similar numbers of colony-forming units of granulocytes–macrophages were produced by MSC-based and standard stroma cultures over 10 weeks. We conclude that adult BM is a reliable source of functional cultured MSC, but CB and PBSC are not.

Keywords: mesenchymal, stem cell, marrow, cord blood, culture.

Until recently, it had been widely assumed that embryonic stem cells were the only pluripotent stem cells capable of differentiating into cells of ectodermal, mesodermal and endodermal origin. In contrast, adult stem cells localized in different tissues were thought to be restricted to replenishing only one or two lineages specific to that tissue (Graf, 2002). Recently, it has been reported that adult haemopoietic stem cells are not limited to forming blood cells, but may also generate liver (Lagasse *et al,* 2000), heart (Martin *et al,* 2002) and skeletal muscle cells (Ferrari *et al*, 1998) in animal models. Conversely, non-haemopoietic stem cells such as neural- and muscle-derived stem cells have been reported to produce haemopoietic cells after transplantation into irradiated mice (Bjornson *et al,* 1999; Jackson *et al,* 1999). The validity of some of these studies requires confirmation. For example, it is possible that reports of muscle or neural stem cells producing haemopoietic cells might be due to the presence of haemopoietic cells in the vasculature of the organ examined. Despite the need for clarification of some experimental data reported in the literature, it is clear that adult bone marrow (BM) contains endothelial stem cells and primitive cells of mesenchymal origin first described by Friedenstein (Friedenstein *et al,* 1982). The latter is a primitive population of CD34$^-$, CD45$^-$ cells defined by an immunophenotype including positivity for CD44, CD105, CD166, CD28, CD33, CD13 and HLA class 1. Their reported ability to differentiate down multiple lineages includes osteogenic (Jaiswal *et al,* 1997), adipogenic (Pittenger *et al,* 1999), chondrogenic (Yoo *et al,* 1998) and neural differentiation (Sanchez-Ramos *et al,* 2000). Together with accessory cells of haemopoietic origin, principally lymphocytes and macrophages, and with the extra-cellular matrix, mesenchymal stem cells (MSC) generate the microenvironment essential for the maintenance, proliferation and differentiation of haemopoietic stem cells (Bianco *et al,* 2001).

We confirm that MSC are readily isolated from adult BM. However, cord blood (CB) and peripheral blood stem cell

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collections (PBSC) do not yield MSC in the culture conditions described. Thus adult BM is currently the best source of MSC for developing transplantation, tissue repair and tissue engineering strategies.

MATERIALS AND METHODS

Sample collection and processing. All samples were obtained with written, informed consent in accordance with the local hospital ethics committee requirements. Cord blood was collected from the umbilical cord following full-term normal delivery and PBSC were obtained from washings of the bags used for normal donor PBSC collections. Femoral shaft marrow is a reliable source of primitive haemopoietic cells and mature stroma in that it functions as well as normal donor BM in haemopoietic long-term culture (LTC) (Wexler *et al*, 2002).

BM was obtained from the femoral shaft of patients undergoing total hip replacement at the time of surgery and was placed in a 50-ml Falcon tube (2070; Becton Dickinson, Cowley, Oxford, UK) containing 5000 units of preservative-free heparin.

Femoral tissue and BM were placed in a Petri dish, broken up with a scalpel and washed in Hank's balanced salt solution (H9269; Sigma, Poole, Dorset, UK). The washings were placed in a 50-ml Falcon tube and centrifuged at 200 ***g*** increasing to 700 ***g*** over 10 min. The plasma was removed and the buffy coat, with some of the fat layer, was placed in a 15-ml Falcon tube (Becton Dickinson). A white cell count was performed. The expected yield from a 20–40 ml sample was 1×10^8–1×10^9 cells. Cord blood and PBSC were processed in a similar way (Denning-Kendall *et al*, 1999).

Culture of MSC. Vented flasks (25 cm^2) with 10 ml MSC medium, consisting of Dulbecco's-modified Eagles medium (DMEM) (D5523; Sigma) with 10% fetal calf serum (FCS) selected for growth of MSC (06472; Stem Cell Technologies), were seeded with 1×10^7 cells for primary culture. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and were fed every week with MSC medium by half medium change until the fibroblast-like cells at the base of the flask reached confluence.

On reaching confluence, the adherent cells were re-suspended using 0·25% trypsin and re-seeded at 1×10^6 cells per flask (first passage). These were incubated again until confluence, and were once again trypsinized and re-seeded at 1×10^6 cells per flask (second passage). At the end of second passage when the cells reached confluence, they were trypsinized and either cryo-preserved or used immediately.

CFU-F frequency. Using the method of Castro-Malaspina with slight modifications, we measured the frequency of colony forming unit fibroblasts (CFU-F) as a surrogate marker for MSC frequency (Castro-Malaspina *et al*, 1980). Flasks were seeded with 5×10^5, 1×10^6 and 2×10^6 nucleated cells from CB, BM or PBSC collections.

Immunophenotyping. At the second passage, the morphologically homogeneous population of MSC were trypsinized and the immunophenotype examined by dual labelling with fluoroscein isothiocyanate (FITC)-conjugated anti-CD105 (Serotec, Oxford, UK), CD90 (BD Biosciences, Oxford, UK), CD13, CD45, CD29 (Dako, High Wycombe, UK) and human leucocyte antigen (HLA) class-I (Serotec), and with phycoerythrin (PE)-conjugated anti-CD14, CD44, CD34 and CD166 (Dako). Flow cytometric analysis was performed on a Coulter Epics® XLTM flow cytometer with EXPO32 software. Gates were set to exclude cellular debris.

Differentiation of MSC. MSCs were induced to differentiate into adipocytes by treating confluent second passage monolayer cultures with 0·5 μmol/l isobutyl methylxanthine (I5879; Sigma), 1 μmol/l dexamethesone (D-4902; Sigma), 60 μmol/l indomethacin (I-7378; Sigma) and 10 μg/ml insulin (I-6634; Sigma) in DMEM with 10% FCS. Adipogenic differentiation is apparent visually by the accumulation of lipid-containing vacuoles which stained red with oil red O (O-0625; Sigma) (Pittenger *et al*, 1999).

The osteogenic differentiation of MSCs was induced by culturing confluent monolayers in DMEM with 10% FCS, 0·1 μmol/l dexamethasone, 10 mmol/l β-glycerolphosphate (G-9891; Sigma) and 50 μmol/l ascorbate (A-8960; Sigma). Osteogenic differentiation was demonstrated by calcium deposition in culture over 1–2 weeks with Von Kossa's stain (05600; Fluka, from Sigma-Aldrich, Poole, Dorset, UK) (Jaiswal *et al*, 1997). CB and PBSC did not produce MSC and, therefore, differentiation experiments could not be performed.

Support of haemopoiesis by MSC. To assess the capacity to support haemopoiesis, the ability of BM MSC and normal BM stroma were compared in LTCs by measuring the production of colony-forming units of granulocytes–macrophages (CFU-GM) in culture supernatants over 10 weeks (Denning-Kendall *et al*, 1999). Six paired experiments were carried out in which cells, enriched for $CD34^+$ from the same CB donor, were used to inoculate MSC-derived and standard BM stroma.

Statistics. All laboratory results are reported as means ± one or two standard deviations (SD). Statistical analysis of groups was performed using a two-tailed *t*-test. *P*-values of less than $P < 0{\cdot}05$ were considered significant.

RESULTS

The results of MSC culture

BM samples taken from the femoral shaft at the time of total hip replacement surgery produced a monomorphic confluent adherent layer of elongated fibroblast-like cells that survived multiple passages in mesenchymal culture conditions (Fig 1A). Primary culture took between 10 and 21 d (median 17 d for seven samples). CB samples produced a minimal, non-confluent adherent layer of heterogeneous cells, which did not proliferate beyond the first passage. The predominant cell after 2 weeks culture was fibroblast like (Fig 2A) and dislodged readily on trypsinization. PBSC cultured in the same way produced a scanty heterogeneous population similar to the CB cultures (Fig 2B). Cultured cells derived from CB or PBSC did not survive beyond the first passage.

The frequency of MSC measured by the CFU-F assay

The CFU-F assay was used as a surrogate assay for MSC. In the BM nucleated cell population, an estimated frequency of

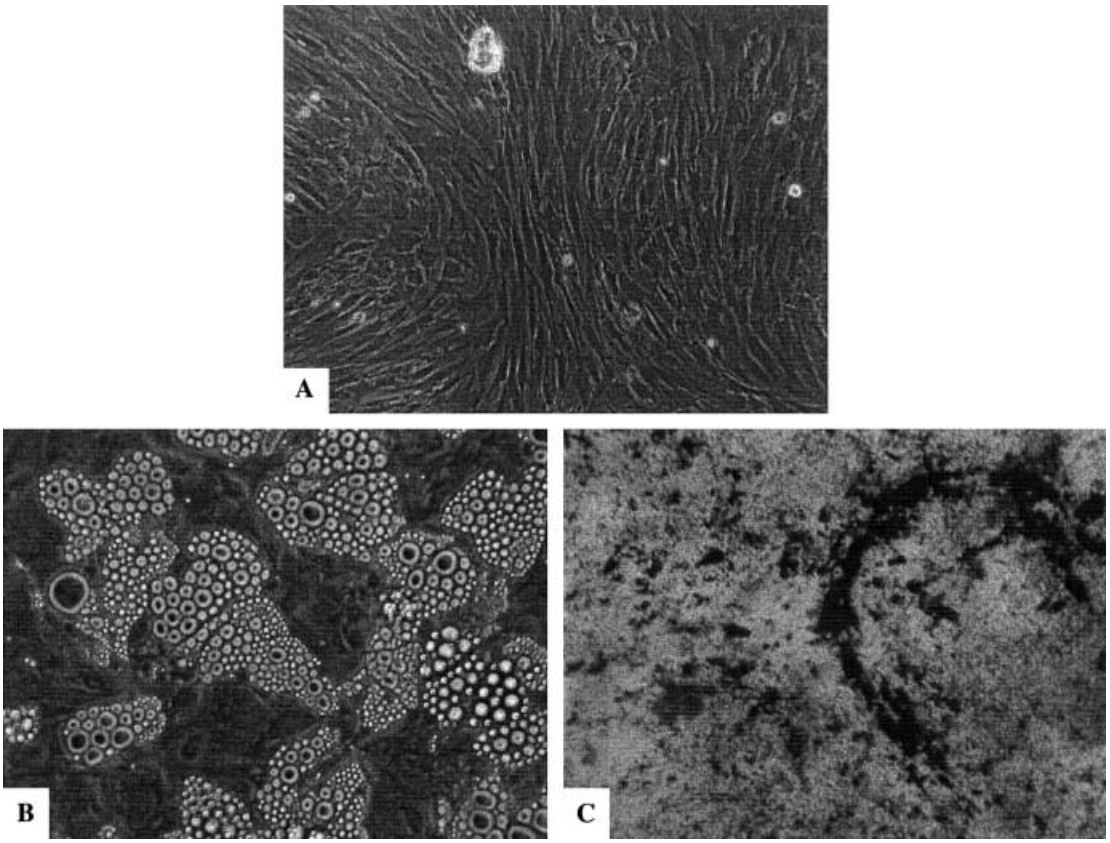

Fig 1. (A) Established, confluent BM MSCs in culture at the end of the second passage, displaying a typically homogeneous fibroblast-like pattern. Adipogenic differentiation is visually apparent by the accumulation of lipid containing vacuoles (B). Osteogenic differentiation was confirmed by calcium deposition in the culture over 1–2 weeks (C) (original magnification ×200).

one in $3{\cdot}4 \times 10^4$ cells was obtained [number of experiments (n) = 6; Table I]. Scanty macrophage-like cells were grown from nucleated cells derived from PBSC ($n = 4$) and CB ($n = 13$), but CFU-Fs were absent.

The immunophenotype of BM, CB and PBSC MSCs

BM-derived MSC were $CD34^-$, $CD45^-$ and expressed unique surface proteins, CD105 [endoglin/Src homology domain-2 (SH2)] and CD166 [activated leucocyte cell adhesion molecule-1 (ALCAM-1)], not found on haemopoietic precursors. They were uniformly positive for CD44 (the hyaluronate receptor), CD29 (β1 integrin), CD90, CD13 and HLA class-I (Fig 3). The lack of expression of CD45, CD34 and CD14 strongly suggested that by the end of the second passage, cultures were depleted of haemopoietic cells.

Immunophenotyping of weakly adherent cells from both CB and PBSC cultures showed a haemopoietic phenotype that was CD45 positive. The remaining population required further trypsinization and had a monocyte–macrophage phenotype, which was CD14 and CD45 positive.

The differentiation of BM MSC

At the end of the second passage, BM-derived MSC were successfully differentiated along adipogenic (Fig 1B) and osteogenic lineages (Fig 1C), using methods described earlier. Differentiation of cultured CB and PBSC adherent cells was not attempted because there was no phenotypic or morphological evidence that these were mesenchymal in origin.

The support of haemopoiesis in vitro by BM MSC

MSC-derived stroma was morphologically indistinguishable from stroma grown from the marrow buffy coat. Figure 4 shows the total number of CFU-GM colonies produced from culture supernatants over 10 weeks in LTC. There was no difference in the ability of BM stroma and MSC-derived stroma to support haemopoiesis when seeded with CB $CD34^+$-enriched cells in standard LTC conditions.

DISCUSSION

The ease with which BM MSC can be cultured and expanded *in vitro* has recently spurred a number of investigators to propose possible clinical applications for these cells. These include the enhancement of engraftment following HSC transplantation (Devine & Hoffman, 2000), stem cell strategies for the repair of damaged organs (Bruder *et al*, 1998) and gene therapy (Fukuda *et al*, 2000).

We have estimated the BM MSC frequency in adults to be one in $3{\cdot}4 \times 10^4$ cells, using the culture of CFU-F as a

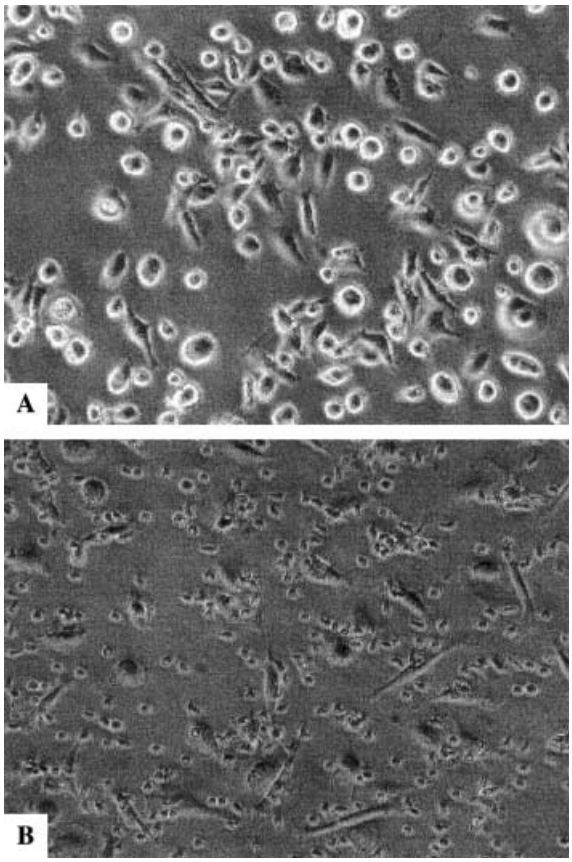

Fig 2. After 2 weeks culture of CB-nucleated cells in MSC conditions, sparse fibroblast-like cells were observed (A), which dislodged readily on trypsinization. PBSC MNC cultured in the same way initially produced a similar scanty heterogeneous population (B). We were not able to culture these cells beyond the first passage. Immunophenotyping of cultured CB and PBSC showed that they were positive for CD45 and CD14, confirming that these cells were monocytes and macrophages (original magnification ×200).

surrogate assay (Table I), and have shown that CB and PBSC do not produce CFU-F in identical conditions. Morphologically, the adherent cells in the MSC culture of BM produced a homogeneous confluent monolayer (Fig 1), whereas CB and PBSC culture produced a heterogeneous mixture of non-confluent cells, which cannot be passaged (Fig 2). BM MSC expressed a unique combination of surface proteins, not found on haemopoietic cells, but in our laboratory cultured CB and PBSC adherent cells expressed a monocyte and macrophage phenotype, which was $CD45^+$ and $CD14^+$. We have also shown that irradiated BM MSCs are capable of supporting haemopoiesis as efficiently as normal irradiated BM stroma in LTC (Fig 4). Fresh CB does not form stroma in LTC conditions and CB mononuclear cells seeded into flasks without supportive stromal layers do not produce CFU-GM after 4 weeks (Hows *et al*, 1992). Our observation that indicated that MSC are sparse or absent from CB is consistent with results obtained by Hows *et al* (1992). Interestingly, using different culture conditions, we have previously shown that von Willebrand factor $(VWF)^+$, $CD54^+$, $CD62E^+$ and $CD31^+$ endothelial cells derived from the $CD34^+$ fraction of CB form a confluent adherent monolayer layer over several weeks that was capable of supporting haemopoiesis after irradiation. These fibroblast-like endothelial cells were $CD45^-$, $CD34^-$ and $CD14^-$ (Nieda *et al*, 1997), and were ,therefore, very unlike the sparse $CD45^+$, $CD14^+$ adherent cells grown from CB-nucleated cells in our current investigation.

Table I. The frequency of MSCs in the total nucleated BM cell population measured by the CFU-F assay.*

BM sample (number)	CFU-F		
	$0{\cdot}5 \times 10^6$	1×10^6	2×10^6
1	22	64	90
2	14	16	42
3	4	16	14
4	17	50	79
5	10	32	65
6	9	39	51

*Table 1 indicates the frequencies of CFU-Fs when doubling concentrations of BM buffy coat cells ($0{\cdot}5 \times 10^6$, 1×10^6 and 2×10^6 cells) were seeded into MSC medium. The estimated CFU-F frequency is one in $3{\cdot}4 \times 10^4$ cells in the BM-nucleated cell population. No CFU-Fs were grown from PBSC ($n = 6$) and CB ($n = 13$) under the same conditions.

In contrast to our current report, Erices and colleagues were able to isolate mesenchymal-like cells (MLC) from CB (Erices *et al,* 2000). However, they used different culture conditions [alpha minimal essential medium (α-MEM) and 20% FCS] and five of the seven samples producing MLC were from preterm deliveries. MSC have been isolated from first-trimester fetal blood (Campagnoli *et al,* 2000) and from second-trimester fetal lung samples (Noort *et al,* 2002). All our CB samples were collected after full-term normal deliveries, so the presence of MSC in CB might be related to gestational age. Of note, there has been a single publication reporting the establishment of confluent adherent cell layers from human CB that were capable of supporting haemopoiesis for many weeks (Ye *et al*, 1994). The authors do not state the gestational age of the infants they studied. Previous reports (Gutierrez-Rodriguez *et al,* 2000) and our own observations confirm the inability of CB MNC to produce CFU-F. We agree with these authors' observations that the adherent cells, grown from CB cultured in conditions that primarily support MSC, are of haemopoietic origin and were predominantly monocytes and macrophages.

The evidence for the isolation of fibroblast-like cells with a mesenchymal phenotype from PBSC collections is conflicting. Fernandez and colleagues reported the detection of MSC from the PBSC collections of patients with breast cancer (Fernandez *et al*, 1997), whereas Lazarus' group reported no evidence of MSC in PBSC from patients undergoing autologous haemopoietic stem cell transplants and normal

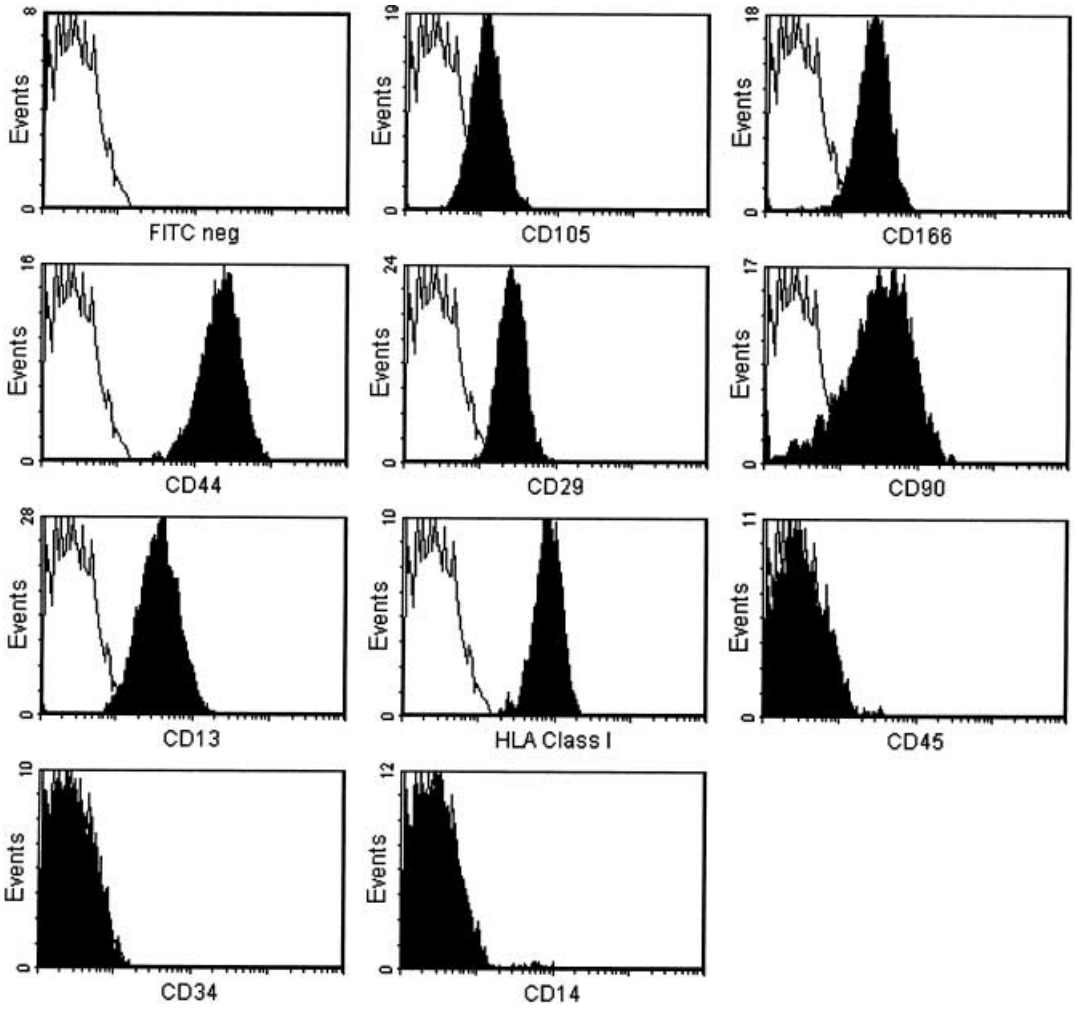

Fig 3. Flow cytometric plots showing the immunophenotype of BM MSCs that were obtained from the homogeneous confluent monolayer at the end of the second passage. The cells expressed CD105 (endoglin/SH2) and CD166 (ALCAM-1), CD44 (the hyaluronate receptor), CD29 (β1 integrin), CD90, CD13 and HLA class-I, and lacked the expression of CD45, CD34 and CD14.

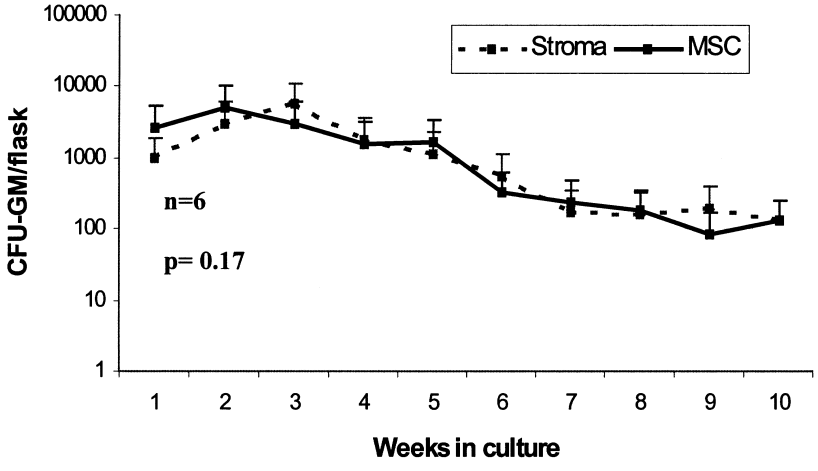

Fig 4. The haemopoietic potential of third-party $CD34^+$-enriched CB cells, inoculated onto preirradiated standard stroma and MSC-derived stroma, and grown in LTC in six paired experiments. The graph shows the number of CFU-GM colonies produced per flask (± SD) over 10 weeks of culture. There was no difference in the ability of BM stroma and MSC-derived stroma to support haemopoiesis ($P = 0{\cdot}17$).

sibling donor transplants (Lazarus *et al*, 1997). Again, the culture conditions in these reports differ; the former cultured cells in α-MEM and the latter in DMEM. A further report by Zvaifler and colleagues describes the detection of MSC in the peripheral blood of normal individuals following elutriation (Zvaifler *et al*, 2000).

We cannot exclude the possibility that MSC or their precursors could be present in mobilized adult peripheral blood and CB at a low frequency, however, they did not proliferate in our culture system. Further investigation is warranted to assess different culture conditions and CB samples from preterm deliveries, to confirm the absence or very low frequency of MSC in both CB and PBSC. From the results of this study, we recommend that adult BM is the best source of human-marrow-derived MSCs for both research and cellular therapies.

ACKNOWLEDGMENTS

We thank the patients, medical and nursing staff in the Avon Orthopaedic Centre, Southmead Hospital for their co-operation with this project, and the mothers and midwives on the Central Delivery Suite, Southmead Hospital for donation and collection of cord blood. The staff of the Stem Cell Laboratories, National Blood Service, Southmead kindly provided the adult PBSC. We acknowledge support from the Leukaemia Research Fund. We express our gratitude to the Bunzl group who supported Dr Sarah Wexler's laboratory research through Children with Leukaemia, and Children with Leukaemia who funded the Paul O'Gorman Lifeline Centre.

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