Introduction

Stem cells (SCs) are self-renewable and capable of differentiating into mature lineages [1, 2]. Conventionally, two main types of SCs are recognized. Those isolated from the embryo inner cell mass (embryonic SCs, or ESCs) or from foetal primordial germ cells (EG cells) are considered totipotent, since they also have the ability to entirely colonize an organism and give rise to all cell types. SCs found in adult or postnatal organisms, are referred to as adult, organ- or tissue-specific SCs, and are present in most, if not all, adult organs. They are considered pluripotent, since they can originate mature cell types of one or more lineages, but cannot reconstitute the organism as a whole. Human postnatal bone marrow stroma contains precursor cells that are capable of differentiating along multiple mesenchymal lineages [3]. These cells have been referred as mesenchymal stem cells (MSCs). MSCs are capable of differentiating into mesenchymal tissues such as bone, adipose, cartilage, myocardium and stroma [1, 4]. In addition, bone marrow is the major source of adult haematopoietic stem cells (HSCs), typically marked by the expression of surface CD34 antigen, that renew circulating blood elements as well as cellular components of vessel wall and myocardium [5]. Although adult bone marrow SCs (BMSCs) are arbitrarily separated into these two groups, MSCs also can differentiate into haematopoietic cells; and HSCs also can become non-haematopoietic tissues [1].

Abstract

Bone marrow stem cells (BMSCs) are mobilized in response to ischemic attacks, e.g. myocardial infarction, to repair the damage, or by cytokines, e.g. granulocyte colony-stimulating factor (G-CSF), which is used to harvest BMSCs for autologous transplantation. In order to optimize BMSC mobilization strategy for cardiovascular repair, we investigated whether BMSCs mobilized by G-CSF share the same subtype profile as that by ischemia in a non-human primate model. We subjected five baboons to subcutaneous G-CSF injection and five baboons to femoral artery ligation. Blood BMSCs were measured by surface antigens; functional differentiation to endothelial cells (ECs) was assessed by colony-forming capacity, expression of mature EC antigens and tube-like formation. The number of circulating CD34+/CD45RA- cells spiked on day 3 post-stimulation in both groups. While the number of CD34+ cells released by artery ligation was 2-fold lower by comparison with the number released by G-CSF administration, significantly more CD133+/KDR+/CXCR4+/CD31+ cells were detected in the baboons that underwent artery ligation. After culture in endothelial growth medium, mononuclear cells from baboons with artery ligation formed more EC colonies and more capillary-like tubes (P < 0.05), expressed higher vWF and phagocytosed more Dil-Ac-LDL (P < 0.05). While G-CSF and artery ligation can mobilize BMSCs capable of differentiating into ECs, BMSCs mobilized by the artery ligation simulating in vivo ischemic attacks have higher potential for vascular differentiation. Our findings demonstrate that different mobilization forces release different sets of BMSCs that may have different capacity for cardiovascular differentiation.

Keywords: bone marrow stem cells ● non-human primate ● endothelial progenitor cells

Differential bone marrow stem cell mobilization by G-CSF injection or arterial ligation in baboons

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While HSCs can be readily mobilized by chemotherapy or cytokines to become peripheral blood SCs, MSCs are generally difficult to mobilize. In adults, SCs with potency for further differentiation exist in bone marrow, circulating blood and organs with specialized functions. When there is an injury, such as ischemic attack in the myocardium, the locally reserved progenitor cells are clearly not sufficient to accomplish the functional repair. The stress signals released by injured myocardium, however, can mobilize BMSCs to circulating blood from which they aggregate to the injury site where they become terminally differentiated functional cells for tissue regeneration [4]. Endothelial progenitor cells (EPCs) are a subset of BMSCs that can readily differentiate to mature endothelial cells (ECs) under appropriate micro-environmental stimulations. These cells can be identified by surface antigens, e.g. CD34 or CD133 for the vascular lineage although the specificity is only a relative term [6]. The quantity of lineage-specific BMSCs is a reliable indicator of BMSC reserve. After an ischemic injury, such as myocardial infarction (MI) or unstable angina, or angioplastic balloon endothelial denudation, more EPCs are detected in the circulating blood [7]. The number of EPCs is associated with cardiovascular outcome [8]. Ischemia-initiated BMSC mobilization is a biological response towards stress, which improves the regeneration process of ischemic tissues [9, 10].

Various clinical trials have reported the results of transplanting BMSCs for the treatment of cardiovascular diseases [11]. In these studies, BMSCs were either harvested by direct bone marrow aspiration or from the peripheral blood after granulocyte colony-stimulating factor (G-CSF)-mediated mobilization [12]. One of the underlying assumptions is that BMSCs artificially mobilized by administration of G-CSF have similar functional repair capacities as those naturally mobilized after ischemic injuries. However, there are no data reporting potential differences in functional subtypes of BMSCs mobilized by the cytokine and ischemia. Understanding the difference is of great importance not only to the SC biology with regard to factors responsible for mobilizable subtypes of BMSCs, but also to the development of therapeutic strategies applied to the harvest of BMSCs for autologous transplantation in treating various diseases including MI. It should be noted that the therapeutic effects of the G-CSF are pleiotropic and non-SC associated benefits of the G-CSF are pleiotropic and non-SC associated benefits that they were healthy and had blood parameters in the normal ranges. The mean age was 10.8 (7–15) years and the mean weight was 21.5 (14.4–31.0) kg. The baboons were moved into single cages 2 weeks before the experiments. Peripheral blood samples were collected in heparin tubes on days 0, 3, 5 and 10. Mononuclear cells were isolated from the blood before flow cytometry analysis. EDTA plasma also was collected to determine cytokine levels. The study was approved by the Institutional Animal Care and Use Committee of Southwest Foundation for Biomedical Research, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Animal treatments

A group of five baboons were injected subcutaneously with G-CSF (NEUPHOCEN, Amgen Inc., Thousand Oaks, CA, USA) 100 μg/kg/day daily for 2 days—regimen based on previous studies in human beings and animals [13–15]. The other five baboons were subjected to femoral artery ligation as described previously [16]. In brief, baboons were immobilized with ketamine (10 mg/kg, IM) and valium (5 mg, IV) and anaesthetized with isofluorane (1.5% V/V, inhalation). The left leg was prepared for aseptic surgery. A skin incision was made over the femoral region, midway between the pubis and knee. One inch of the artery was isolated and ligated; the branches were ligated and the segment was removed. Muscle and skin were closed with simple continuous sutures. After baboons recovered from the anaesthesia, they were returned to their normal living conditions. Bone marrow samples were taken via needle aspirate from the head of the humerus by veterinarians following standard procedures.

Mononuclear cell isolation from peripheral blood

To isolate peripheral blood mononuclear cells (PBMCs), baboon peripheral blood was diluted 1:1 by PBS. The blood/PBS mixture was then slowly layered on Ficoll-Hypaque at a density of 1.077 (Sigma, #H8889 HISTOPAQUE). We used 3 ml Ficoll-Hypaque per 10 ml blood/PBS mixture and 5 ml Ficoll-Hypaque per 10 ml bone marrow/PBS mixture. The samples were centrifuged for 30 min at 2000 rpm (900g) at 18°C. Using a sterile pipette, plasma and most of the platelets in the upper layer were removed. Cells were washed with excess HBSS (Gibco BRL, #14080-055) and centrifuged for additional 10 min at 1300 rpm (400g) at 18°C. The cell wash was repeated before resuspended in complete F–12K medium. The number of cells was counted and cell viability was determined by trypan blue exclusion.

Flow cytometry analysis

Freshly isolated PBMCs were used for the study. All cells were washed twice with cold PBS containing 2% human AB serum (Invitrogen, Cat#34005100, Carlsbad, CA, USA) before they were used. Majority of antibodies used in this study were raised against human antigens. Flow cytometry was carried out in a Becton-Dickinson FACSCalibur using BD CellQuest software (BD Immunocytometry Systems, CA). Positive cells...
were selected by gating on a discrete population of brightly stained cells that had fluorescence intensity greater than 99% of isotype controls.

**Haematopoietic stem cells (HSCs) colony-forming assay**

The colony-forming cell assay is based on the ability of HSCs to proliferate into colonies in a semi-solid medium in response to cytokine stimulation [17]. A total of $1 \times 10^5$ PBMCs were mixed with 2.8% methylcellulose (R&D Systems, Cat# HSC001, Minneapolis, MN, USA) containing 30% FCS in 10 ng/ml interleukin 3 (Cat# 203-IL), 10 ng/ml GM-CSF (Cat# 215-GM) and 50 ng/ml SCF (Cat# 255-SC), and plated in three wells of a 6-well plate. After 14 days at 37°C, 5% CO$_2$, the numbers of erythroid burst colony-forming units (EB-CFU) and granulocyte macrophage colony-forming units (GM-CFU) were counted using standard criteria according to their unique morphologies [17, 18].

**Endothelial progenitor colony colony-forming assay**

Samples were processed within 4 hrs after collection. Recovered cells were washed twice with PBS and once in growth medium consisting of 20% FCS F-12K Medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Isolated PBMCs were subsequently resuspended in endothelial growth medium (EndoCult™, StemCell Technologies Inc, Vancouver, Canada, #5900) and plated on fibronectin-coated 6-well plate using $5 \times 10^5$ PBMCs per well (VWR, International, West Chester, PA, USA, Cat# 62405–662) for 48 hrs. In order to remove the mature circulating ECs and macrophages, the non-adherent cells were collected and $1 \times 10^5$ cells were replated onto fibronectin-coated 24-well plates (VWR, Cat# 62405-666) for colony-forming analysis. Growth medium was changed every three days, and the numbers of colonies were counted 7 days after plating. A colony of EPCs consisted of multiple thin and flat cells emanating from a central cluster of rounded cells. Colonies were counted manually in at least three wells.

**Differentiated functionalities of endothelial progenitor cell**

We used two methods to evaluate the differentiation capacity of EPCs derived from mobilized PBMCs into functional ECs, including Dil-Ac-LDL uptake and Ulex Europaeus Agglutinin (UEA-1) binding ability. Our preliminary experiments showed that no typical capillary or tube structure was formed from the relatively small number of EPC lineage cells in the PBMCs within 4 weeks. We therefore used bone marrow mononuclear cells (BMMNCs) to evaluate their tube-forming capacity on Matrigel-coated plates. Confirmation of EPC lineage was performed in samples of all baboons. Fluorescence-stained cells were analysed with a Becton-Dickinson FACSCalibur flow cytometer. Percentages of Dil-Ac-LDL or UEA-1 positive cells were defined as the percentage of the cells within the mononuclear cell subpopulation with fluorescence intensity exceeding that of the maximum level of autofluorescence of unlabelled cells in the same population. Mean fluorescence intensity (MFI) levels of Dil-Ac-LDL as well as UEA-1 were used to identify the tube-formation ability. An aliquot of $1 \times 10^5$ EPCs was seeded either on Matrigel-coated plates (ECM, Sigma, #E1270) supplemented with EPC growth medium; or fibronectin-coated plates (BD Biosciences, CA, USA). The tube-like structure was examined using an inverted Nikon microscope after culture for up to 4 weeks; and photographs were taken at various time-points.

**Immunofluorescence staining**

In order to define the origin of the cells that formed endothelial colonies, we carried out immunofluorescence staining of the collected EPCs before and after in vitro culture.

**Results**

**Effects of femoral artery ligation and G-CSF administration on blood leucocyte and platelet counts**

Table 1 shows the total counts of leucocytes and platelets in peripheral blood after femoral artery ligation and subcutaneous administration of G-CSF. Circulating blood counts were determined before and after femoral ligation or G-CSF administration. No elevation of WBCs was seen in baboons with femoral artery ligation. However, significant leucytosis was observed, peaked at day 5, and remained at a high level until day 10, in baboons that received the G-CSF injection. The major component of the increased WBC counts was monocytes, whereas neutrophil counts were not changed. Baboons in both groups were shown to have significantly decreased platelets at both day 5 and day 10 compared to baseline levels.

**Time-dependent changes in mobilized CD34+**

We used FACS to follow up the dynamic changes of CD34+ population after two treatments. At baseline, the CD34+ counts were not different between males ($0.11 \pm 0.01\%$, $n = 4$) and females ($0.18 \pm 0.11\%$, $n = 6$, $P = 0.14$). Three days after treatment, male baboons with femoral arterial ligation had significantly higher elevation in circulating CD34+ cells ($8.83 \pm 3.36\%$, $n = 2$) than the female baboons ($1.22 \pm 0.81\%$, $n = 3$, $P = 0.01$). On the other hand, G-CSF mobilized CD34+ cells were similar between male ($6.11 \pm 1.61\%$, $n = 2$) and female baboons ($7.17 \pm 3.23\%$, $n = 3$, $P = 0.35$). It should be noted that the numbers of baboons in the subgroup analyses were too small to be properly statistically powered. The ages were not statistically different between baboons treated with G-CSF ($7.4 \pm 1.5$ years, 5, 7, 8, 8, 9 years) or with artery ligation ($8.2 \pm 1.1$ years, 7, 8, 8, 8, 10 years, $P = 0.367$). The body weights were also not differed between the two groups (G-CSF group: $23.2 \pm 7.2$, ligation group: $19.6 \pm 6.5$, $P = 0.429$). Using Pearson correlation coefficient analysis, there were no significant correlations between ages, body weights and numbers of CD34+ cells.

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Subsets of progenitor cells mobilized by two treatments

To elucidate more detailed antigen molecules on mobilized cells, we determined their EPC marker expression profiles using monoclonal or polyclonal antibodies (CD34, CD31, CD133, CXCR4, KDR and c-kit) in PBMCNs at day 3 by FACS. Although both mobilizations resulted in common immunophenotypic profile changes, artery ligation appeared to be more effective in mobilizing CD31+/KDR+/CXCR4+/CD133+ among the mobilized progenitor cells (online supplement Figure I). At the same time, neither G-CSF nor artery ligation mobilized the c-kit+ cells, which were highly expressed in BMSCs collected from bone marrow of the same baboons (data not shown). G-CSF mobilized more CD45+ PBMCNs than artery ligation as detected by monoclonal antibody (data not shown). These findings suggest that artery ligation may be more effective in mobilizing cells of the vascular lineage, e.g. EPCs. Progenitor cells mobilized by G-CSF may be more predominantly in the haematopoietic lineage, i.e. CD34+/CD45+, as indicated in Figure 1.

Colony-forming capacity of the mobilized PBMCNs

In order to assess the colony-forming capacities of PBMCNs mobilized by these two methods, we cultured PBMCNs collected at baseline (8 days before the surgery), day 3 and day 10 in EPC culture medium or HSC culture medium. We seeded 2 × 10^5 PBMCNs onto 100-mm Petri dish coated with fibronectin cultured for 14 days to form visible and distinct colonies. The EPC colonies have typical cobblestone shape under phase-contrast inverted microscope; and randomly selected samples were confirmed by immunostaining by antibody against vWF to confirm the exact count of EPCs. Morphological features of typical colonies were shown in Figures 2A–C. The numbers of endothelial colonies, expressed as per 10^6 PBMCNs, from baboons with femoral artery ligation at baseline (1.26 ± 0.72) were not different from the baboons treated with G-CSF (1.72 ± 0.36). However, colonies form from PBMCNs collected 3 days post-treatments were significantly higher in arterial ligation group (8.12 ± 1.76) than the CSF group (2.52 ± 0.73, P < 0.01). PBMCNs harvested on day 10 formed more EPC colonies than those harvested on day 3 or at baseline. On the other hand, PBMCNs from G-CSF mobilization formed more HSC colonies.

We further tested the surface antigens of the EPCs before and after in vitro culture. PBMCNs that formed the endothelial colonies expressed high levels of mature endothelial markers including antigen CD31, vWF, lectin-binding molecules (UEA-1) and CD146 (online supplement Figure II). On the other hand, markers for haematopoietic cells and monocytes/macrophage (CD45 and CD14), or markers for precursor erythrocyte cells (CD235a) were negative (online supplement Figure II). On the other hand, the expression levels of mature endothelial markers, e.g. vWF, were scarce in PBMCNs before in vitro endothelial culture induction. These observations suggest that directional differentiation may also occur in vivo after exposure to mobilization factors.
Differentiation potential towards ECs

We then determined whether the two mobilization methods differentially impacted the capacities of the mobilized cells to transform into mature ECs. We used three methods to evaluate EC differentiation capacity. We firstly examined the Dil-Ac-LDL uptake, which is a marker of mature ECs. PBMNCs collected on day 3 from both groups were cultured for 14 days before being labeled by incubating with Dil-Ac-LDL (10 nM) for 4 hrs. As shown in Figure 3A, the average Dil-Ac-LDL uptake as measured by the MFI in both groups was significantly higher than for cells incubated with vehicles (dashed line). Baboons with artery ligation (gray line) exhibited greater uptake than those collected from baboons by G-CSF administration (black line). We further examined the UEA-1 binding affinity using FITC-conjugated UEA-1 to detect endothelial-specific lectin binding proteins. By using mature ECs as control (95.6% positivity), we found that a proportion of EPCs from both groups could positively bind the FITC labels. A representative result of flow cytometry is shown in Figure 3B. In five baboons with artery ligation treatment, 2.15 ± 1.85% (n = 5) EPCs were able to bind FITC-conjugated UEA-1, whereas 1.92 ± 1.15% (n = 5) of EPCs treated with G-CSF were able to do so.

To further characterize the types of cells formed the colonies, we used immunocytochemical method to detect the surface antigens of the colony-forming cells. We hand-picked representative colonies from both groups and spun down by Cytofuge (StatSpin) onto slides. Immunocytochemical staining with various antibodies were carried out. Images revealed that most colonies (73.3%, 11 out of 15 colonies) in G-CSF treatment samples expressed a wide range of haematopoietic-related proteins (CD45, CD14 and CD235a) as well as EC markers like CD31 and CD146, but not vWF (online supplemental Figure III). Only 4 of 15 colonies (26.7%)...
Table 2 Levels of cytokines in baboons either treated with femoral artery ligation or G-CSF injection

| Cytokines | Day 8 (Artery ligation) | Day 1 (Artery ligation) | Day 3 (Artery ligation) | Day 3 (G-CSF) | Day 5 (Artery ligation) | Day 5 (G-CSF) |
|-----------|-------------------------|-------------------------|-------------------------|---------------|-------------------------|---------------|
| IL-1B     | 3.9 ± 1.5               | 3.2 ± 0                 | 4.3 ± 2.5               | 3.2 ± 0       | 3.6 ± 1                 | 3.2 ± 1.0     |
| IL-2      | 5.5 ± 4.6               | 3.2 ± 0                 | 7.1 ± 8.7               | 3.2 ± 0       | 5.5 ± 5.2               | 3.2 ± 5.2     |
| IL-1ra    | 405.3 ± 373.0           | 248.8 ± 117.7           | 1,694.1 ± 1161.5        | 279.5 ± 162.6| 1,144.6 ± 818.3         | 261.0 ± 225.9 |
| IL-4      | 3.2 ± 3.2               | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| IL-5      | 3.2 ± 3.2               | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| EGF       | 21.6 ± 23.3             | 28.7 ± 13.6             | 14.4 ± 7.4              | 22.1 ± 21.7   | 17.9 ± 9.3              | 10.4 ± 14.7   |
| IL-6      | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| IL-7      | 3.2 ± 3.2               | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| TGF-α     | 9.8 ± 6.2               | 13.0 ± 6.1              | 10.6 ± 2.9              | 7.9 ± 4.6     | 13.0 ± 4.6              | 4.0 ± 1.1     |
| Fractalkin| 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| IL-8      | 883.0 ± 330.4           | 1351.6 ± 528.1          | 531.2 ± 130.2           | 544.6 ± 412.7| 803.0 ± 188.7           | 276.9 ± 254.0 |
| IL-10     | 31.4 ± 60.2             | 7.0 ± 5.1               | 3.2 ± 0                 | 26.5 ± 52.1   | 3.2 ± 0                 | 3.2 ± 0       |
| IL-12p70  | 5.4 ± 2.2               | 7.1 ± 4.7               | 3.2 ± 0                 | 4.0 ± 1.1     | 3.5 ± 0.7               | 3.2 ± 0       |
| IL-13     | 9.3 ± 6.4               | 13.9 ± 9.7              | 3.3 ± 0.2               | 6.7 ± 2.8     | 5.1 ± 2.4               | 3.4 ± 0.5     |
| IL-15     | 5.8 ± 5.0               | 8.3 ± 2.3               | 4.4 ± 1.7               | 6.2 ± 4.0     | 4.7 ± 2.0               | 3.2 ± 0.5     |
| IL-17     | 6.0 ± 4.9               | 5.4 ± 2.4               | 3.9 ± 1.5               | 3.9 ± 0.8     | 3.8 ± 1.3               | 3.3 ± 0.3     |
| IL-1α     | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| INF-γ     | 4.0 ± 1.7               | 3.2 ± 0                 | 3.2 ± 0                 | 4.1 ± 1.9     | 3.2 ± 0                 | 3.2 ± 0       |
| G-CSF     | 3.2 ± 0                 | 3.2 ± 0                 | >10,000 ± 0 †           | 8.4 ± 11.6    | 463.4 ± 181.7 †         | 3.2 ± 0       |
| GM-CSF    | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| TNF-α     | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| Entaxin   | 46.6 ± 86.7             | 22.6 ± 23.4             | 6.0 ± 6.2               | 41.8 ± 71.0   | 5.3 ± 4.8               | 24.4 ± 39.5   |
| MCP-1     | 123.6 ± 30.1            | 177.7 ± 58.5            | 97.1 ± 15.5             | 125.8 ± 38.6  | 158.9 ± 37.8            | 104.7 ± 31.4  |
| CD40L     | 2181.7 ± 1300.8         | 2752 ± 1495.6           | 1160.6 ± 859.8           | 1070.1 ± 660.5| 1633.7 ± 1324.1         | 1218.9 ± 538.8 |
| IL-12p40  | 3.2 ± 0                 | 14.5 ± 25.3             | 6.7 ± 7.8               | 5.2 ± 4.4     | 9.1 ± 13.1              | 3.2 ± 0       |
| MIP-1α    | 3.8 ± 1.3               | 8.6 ± 11.6              | 5.1 ± 2.7               | 3.3 ± 0.2     | 7.9 ± 9.8               | 3.2 ± 0       |
| MIP-1β    | 5.4 ± 4.9               | 3.2 ± 0                 | 3.2 ± 0                 | 9.7 ± 14.5    | 3.2 ± 0                 | 6.4 ± 0       |
| IP-10     | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0                 | 3.2 ± 0       | 3.2 ± 0                 | 3.2 ± 0       |
| VEGF      | 8.6 ± 5.7               | 9.8 ± 6.9               | 3.2 ± 0                 | 8.0 ± 5.9     | 5.0 ± 3.5               | 3.2 ± 0       |

*P < 0.05 between basal levels and day(s) after G-CSF administration.
**P < 0.05 between basal levels and day(s) after artery ligation.
†P < 0.05 between artery ligation and G-CSF at day 3.
‡G-CSF treatment was given in these subjects by subcutaneous injection.
had positive anti-vWF staining in this group. In contrary, most colonies (93.3%) from the arterial ligation group (14 out of 15 colonies) were strongly positive for CD31, CD146 and vWF, moderately for CD45 positivity, but negative CD14 and CD235a (online supplemental Figure III). These results suggest that G-CSF administration releases more haematopoietic progenitor cells, whereas arterial ligation results in more release of vascular progenitor cells.

We further investigated the capacity of tube formation, which marks the angiogenesis capacity of EPCs from circulating blood. We isolated BMMNCs from both groups and seeded them in medium containing VEGF, bFGF, EGF and IGF on fibronectin-coated plates. In agreement with previous observations [19], we found a cord-like structure at day 7 of the culture (Fig. 4A). We continued to maintain the cultures under angiogenic conditions. After 4 weeks of culture, two tube-like structures were observed. As shown in Figure 4, a cord-like structure grew predominantly in PBMNCs from arterial ligation mobilized samples. The structure became complex, with either two layers of cells (Fig. 4B) or multiple layers (Figs. 4C and D). Branching points could also be found, indicating an important characteristic of EPCs (Fig. 4E). These cells at this stage formed a capillary-like structure, apparent at a higher magnification with the lumen (Fig. 4F). We counted both cord-like and capillary-like structures in these two groups. While there was no difference in the numbers of tubes formed from the BMMNCs collected before the challenges, the number of tubes after the challenge was significantly higher in the artery ligation group (6.6 ± 1.5) than the G-CSF group (3.4 ± 1.1, P < 0.01). We further found that by seeding the same numbers of cells in Matrigel, BMMNCs from artery ligation differentiated into a more complex three-dimensional capillary-tube structure (Fig. 4G), while BMMNCs from G-CSF remained as cell aggregates or a cluster structure (Fig. 4H) for the same culture period (up to 6 weeks). By immunocytochemistry, we found that these cells were stained positive for vWF with varied intensities among samples (online supplement Figure IV). The percentage of cells with positive vWF stains was significantly more in cells from baboons with artery ligation (online supplement Figure IV.A) than those from baboons with G-CSF treatment (online supplement Figure IVB).

Cytokine measurement

We also measured the levels of 29 plasma cytokines in these baboons receiving different treatments using Lincoplex immunobeads. As shown in Table 2, only IL-1α levels were elevated in correspondence with the changes in CD34+ by the G-CSF administration, which was not seen in baboons with artery ligation. Artery ligation appeared to reduce the plasma levels of IL-8 (P < 0.05) and IL-10 (P = NS). In addition, G-CSF administration introduced a wide range of changes in cytokine profiles including levels of IL-1, IL-8, IL-10, EGF, MCP-1, CD40L, TNFα and others.
Discussion

The promise of SC-based therapy is built on both theoretical prediction and limited experimental evidence from human trials and animal experiments. While much of the controversy remains about whether ESCs should be developed for therapeutic purposes, development and validation of therapeutic uses of adult BMSCs have been extensive. The commonly used G-CSF-based BMSC mobilization as a convenient way to harvest BMSCs for autologous transplantation was mainly derived from previous experience of treating haematological diseases. Whether the G-CSF-mobilized SC profiles are indeed suitable in treating cardiovascular diseases have never been properly investigated. The main findings of our current report indicate that G-CSF-mobilized BMSCs are somewhat less potent to differentiate into cardiovascular cells by comparison with those mobilized by the ischemic attacks, e.g. artery ligation. However, the difference is more quantitative rather than qualitative, and should be interpreted with caution since our study used a single dose G-CSF even though the selected dose was based on previous studies [13, 14]. Our findings nevertheless suggest a fact that natural biological response to ischemic attack mobilizes BMSCs that are more destined to become cardiovascular cells. On the other hand, the G-CSF-released BMSCs are more destined towards haematological differentiation. G-CSF-associated beneficial effects may not be mediated by the mobilized BMSCs.

As one would expect, there is a surge of inflammatory responses to G-CSF injection as marked by the elevated number of WBCs at 5 days post-injection. Whether elevated WBCs affect the treatment efficacy in some clinical trials in which MI patients who received G-CSF injection had improved myocardial function remains to be investigated [13, 14]. This elevation is accompanied by decreased platelet counts [20], which could be the result of direct G-CSF...
effects. On the other hand, WBCs were not elevated in baboons with artery ligation although platelet counts were also decreased.

BMSCs released into the peripheral blood after G-CSF injection contained significantly higher percentages of CD34+ with a clear peak on day 3 for CD34+/CD45RA− and day 1 for CD34+/CD45RA+. While exact mechanisms of G-CSF mediated BMSC mobilization are unclear, proteolysis may have a critical role. Metalloproteinase enzymes released by activated granulocytes have been implicated in G-CSF-induced BMSC mobilization [21], which sets in motion an intra-marrow proteolytic machinery with participation of diverse cell-bound or free proteases implicating multiple target molecules on haematopoietic cells or stromal cells and their matrix (adhesion receptors, chemokines and their receptors, or signalling molecules, etc). Depending on the stimulus applied, several distinct pathways can initiate mobilization. However, there is a broad interdependency between the pathways initiating and/or amplifying mobilization, underscoring the complexities involved. In comparing to G-CSF initiated mobilization, the number of CD34+ cells released by arterial ligation was significantly lower (1.3- to 2.0-fold) than the number mobilized by the G-CSF injection. However, subsets of the artery ligation-released BMSCs were more predominant of the EPC lineage as judged by their surface antigens and capacity to form tubes. This is demonstrated by the findings that the number of CD34+ cells released by arterial ligation was 2-fold lower than the number released by G-CSF administration; the ligation group had more CD133+/KDR+/CXCR4+/CD31+ cells than the G-CSF group. Our findings suggest that different mobilization forces as mediated

Fig. 4 Direct observation of tube-forming process by BMMNCs cultured on fibronectin-coated plates. BMMNCs were initially suspended in angiogenic culture conditions and plated in fibronectin-coated wells. After 7 days of culture, some cells with oval or spindle shapes aggregated together, as indicated by arrow (A, 100× magnification). After 4 weeks of culture, cells were differentiated further and two tube-like structures were seen: a complex cord-like structure with either two layers of cells (B, 200× magnification) or multiple layers (C and D, 100× magnification). Some of the tubes also formed a branching point, which represents a capillary-like structure (E, 100× magnification). Panel (F) shows the capillary-forming structures at higher magnification (400×). Panel (G) shows the capillary-like structure formed by BMMNCs grown in Matrigel. A wide range of sizes were consistently seen in cultures from baboons with artery ligation by comparison with the G-CSF treated group. Three circular capillary-like structures are indicated by arrows (400×). In contrast, more cell aggregates or clusters (H) were presented in PBMNCs collected from baboons treated with G-CSF than with artery ligation.
by different factors release different subsets of BMSCs. Subsets of BMSCs released by vascular injury may be more destined towards vascular repair than those released by the G-CSF activation, which releases more BMSCs in the haematopoietic lineage. Our findings may explain some of the inconsistent reports in relation to G-CSF-mediated therapeutic effects on MI. While Zohlnhöfer et al. reported no therapeutic effects of G-CSF based BMSC mobilization for the treatment of MI [22], protective effects were reported in other studies [13, 14]. These protective effects could be independent of BMSC mobilization, such as via direct binding between the G-CSF and G-CSF receptors on cardiomyocytes, thereby promoting cell survival [13, 14].

Comparison of the endothelial differentiation capacities of the circulating BMSCs collected at different days after the challenge reveals an interesting phenomenon. Peripheral BMSCs harvested on day 10 are farther developed along the direction of functional EC maturation than those collected at baseline or day 3. This result suggests that the differentiation process may occur in vivo post-injury or post-stimulation. This in vivo process appears to be more efficient in promoting cell differentiation, which is more consistent with an adequate growth environment in vivo that cannot be duplicated in vitro. This in vivo differentiation further indicates that the BMSCs mobilized by the ischemic injury are indeed fulfilling their role to repair vascular damage.

In conclusion, these experiments with the baboon model provide valuable evidence to guide the autologous transplantation of adult BMSC in treating various diseases. Our findings suggest that different mobilization stimulations will result in release of different BMSC subsets that are programmed to specific terminal cell types in response to the biological or pathological requirements, e.g. MI, diabetes or neuron injury. When BMSC harvest protocols are established to treat certain types of diseases, this tissue- or injury-specific difference should be considered. If no stimulators are known to mobilize subsets of BMSCs destined to specific functional cells, direct bone marrow aspiration to harvest BMSC should be utilized in order to obtain the pool of BMSCs containing all subtypes with desired terminal differentiation capacity.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
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