Erythropoietin priming improves the vasculogenic potential of G-CSF mobilized human peripheral blood mononuclear cells

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Aims  
From our previous clinical trials, intracoronary infusion of granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (mobPBMCs) proved to be effective in improving myocardial contractility and reducing infarct volume in acute myocardial infarction. We tested the effect of priming mobPBMCs with erythropoietin (EPO) to augment its therapeutic efficacy.

Methods and results  
mobPBMCs were obtained from healthy volunteers after a 3-day subcutaneous injection of G-CSF (10 µg/kg). About 40% of mobPBMCs were EPO receptor (EPOR) (+) and responded to 6 h EPO-priming (10 IU/mL) by increasing the expression of vasculogenic factors (i.e. IL8, IL10, bFGF, PDGF, MMP9) and adhesion molecules (i.e. integrin αV, β1, β2, β8) through the JAK2 and Akt pathway. These responses were also observed in PBMCs from elderly patients with coronary disease. The conditioned media from EPO-primed mobPBMCs contained various cytokines such as IL8, IL10, TNFα, and PDGF, which enhanced the migration and tube formation capability of endothelial cells. EPO-primed mobPBMCs also showed increased adhesion on endothelial cells or fibronectin. Augmented vasculogenic potential of EPO-primed mobPBMCs was confirmed in a Matrigel plug assay, ischaemic hindlimb, and myocardial infarction models of athymic nude mice. There were two action mechanisms: (i) cellular effects confirmed by direct incorporation of human mobPBSCs into mouse vasculature and (ii) indirect humoral effects confirmed by the therapeutic effect of the supernatant of EPO-primed mobPBMCs.

Conclusion  
Brief ex vivo EPO-priming is a novel method to augment the vasculogenic potential of human mobPBMCs, which would help to achieve better results after intracoronary infusion in myocardial infarction patients.

Keywords  
Erythropoietin • G-CSF mobilized peripheral blood mononuclear cells • Priming • Vasculogenesis • Myocardial infarction

1. Introduction  
Stem-cell therapy in ischaemic diseases, such as myocardial infarction (MI) has been regarded as a new method to repair ischaemic tissue and promote neovascularization. Since the first in-human clinical trial using autologous bone marrow stem cells,1 numerous clinical trials of cell therapy have been carried out with various results. Recent several reports of meta-analysis2–4 demonstrated the promising results that stem-cell therapy improves cardiac function and reduces infarct after acute MI.5 Despite the proven efficacy of stem-cell therapy, there is still limitation in the therapeutic efficacy of current stem-cell therapy for patients with ischaemic heart disease.

As a source of stem cells for clinical trials, we have used mobilized peripheral blood mononuclear cells (mobPBMCs) that are enriched through
mobilization of bone marrow cells by granulocyte colony-stimulating factor (G-CSF) and apheresis process in MAGIC-CELL trials. \(^6\) The proportion of angiogenic progenitors was significantly increased and became comparable to bone marrow mononuclear cells. \(^7\) We have demonstrated that intracoronary infusion of the mobPBMCs was effective in improving myocardial contractility and reducing infarct volume in patients with acute MI. Furthermore, it improved long-term clinical outcomes. \(^8\) The promising effect of mobPBMCs may be due to the positive interaction in between various cells, and the minimal need of manipulation which is vulnerable to cell contamination. However, the therapeutic efficacy was limited mainly due to the poor retention rate after intracoronary infusion of the cells and the insufficient regenerative potency of the adult progenitors like mobPBMCs. \(^9,10\) As a practical breakthrough, we thought that priming of the mobPBMCs before infusion would be a feasible and reasonable strategy and that erythropoietin (EPO) would be a good candidate as a priming agent.

EPO, an erythropoietic growth factor that promotes survival, proliferation, and differentiation of erythroid progenitor cells, \(^11\) has long been used for cell-based therapy in ischemic diseases. The EPO receptor (EPOR) is a 59 kDa peptide found not only in erythroid cells, but also in endothelial cells, \(^12\) and mononuclear blood cells. \(^13\) Along with its erythropoietic effect, EPO is known to have various ischaemia-protective properties while the mechanisms were not yet fully elucidated. \(^14\) Previous studies have shown that EPO increased endothelial cell proliferation and neovascularization, \(^15\) induces the proliferation, differentiation, and adhesion of progenitor cells, \(^16\) and has anti-apoptotic effects. \(^17\)

Thus in this study, we checked the presence of EPOR on mobPBMCs and evaluated the effect of EPO-priming on mobPBMCs in terms of vasculogenic properties in vitro and in vivo.

2. Methods

Expanded methods are available in the Supplementary material online, Appendix.

2.1 Peripheral blood stem cells enriched by G-CSF mobilization (mobPBMCs) and EPO-priming

Peripheral blood (200 mL) was obtained from seven normal volunteers and three elderly patients with informed consent according to the Declaration of Helsinki and was approved by the Institutional Review Board of the Seoul National University Hospital. The normal volunteers underwent daily subcutaneous injections of G-CSF (Dong-A Pharmaceutical, Seoul, Korea) at 10 μg/kg body weight for 3 days. The elderly patients were those who visited our institute for treatment of coronary artery disease. Immediately after the blood was obtained, mononuclear cells were fractionated from other components of peripheral blood by centrifugation on Ficoll-Plaque (GE Healthcare) gradient. Mononuclear cells were immediately used for experiments with or without EPO-priming for 6 h. In experiments to test the cytokine release by EPO-primed cells, EPO-primed mobPBMCs were cultured for 36 h and the supernatant was used to evaluate secreted cytokines.

EPO-priming consisted of a 6 h incubation with human recombinant EPO (Espogen prefilled injection®, 10 000 IU/mL, LG Life Science) diluted in phosphate buffered saline (PBS), at a concentration of 10 IU/mL. A portion of mobPBMCs were used for analysis immediately after EPO-priming and others were washed and cultured in EGM2 MV (Lonza). After 36 h of cell culture, the supernatant was acquired by removing cellular debris via centrifuging. The concentration of secreted cytokines/chemokines was determined by using the Bio-Plex ProTM Array System (kits and equipment of Bio-Rad, USA based on Luminex xMAP technology) in the case of TNF-α, IL-8, IL-10, PDGF-BB, VEGF, and basic FGF.

To block the EPO pathway, EPO Rc blocking antibody (Santa Cruz), JAK inhibitor (AG490, Sigma), and AKT inhibitor (LY294002, Sigma) were used. Also for inhibiting integrin in the adhesion assay, integrin β1 and integrin β2 neutralizing antibodies (Santa Cruz) were used.

2.2 In vivo vasculogenesis in the ischemic limb of nude mouse

All procedures were approved according to the ‘Guide for the Care and Use of Laboratory Animals’ published by the United States National Institutes of Health, by the Experimental Animal Committee of Clinical Research Institute, Seoul National University Hospital, Seoul, Korea. Male athymic nude mice 7–9 weeks old, and 17–20 g in weight, were anaesthetized with 50 mg/kg intraperitoneal pentobarbital for Matrigel plug injection or unilateral femoral artery ligation. An adequate depth of anaesthesia was monitored by observing heart rate and the disappearance of pedal reflexes and briskness of corneal reflexes. For in vivo 3D Matrigel plug assay, four Matrigel \(^6\) plugs which contained four groups of 2 × 10⁵ cells in 350 μL Matrigel were subcutaneously injected into each quadrant of the dorsum of the mice. For evaluation of therapeutic neovasculogenesis using EPO-primed mobPBMCs, hindlimb ischemia was performed by ligation of the femoral artery, followed by injection of 2 × 10⁶ cells in 50 μL serum-free EBM medium, and to check the paracrine effect primed mobPBMCs only the cell supernatant was injected. Laser Doppler perfusion image analyser (LDPI, Moor Instrument, Wilmington, DE, USA) was used to record serial blood flow measurements over the course of 3 weeks after operation. Mice were sacrificed by cervical dislocation after anaesthesia as earlier, on Day 21 for histological evaluation and immunofluorescent staining.

For histological evaluation of arterioles, anti-a-smooth muscle actin (SMA, Sigma) antibody was used, and to evaluate the incorporated human cells in the ischemic muscles, the samples were co-stained with anti-human CD34 antibody (DAKO) followed by incubation with FITC-conjugated secondary antibodies with Cy3-conjugated anti-mouse SMA antibodies.

2.3 Myocardial infarction models of nude mouse

The mice were anaesthetized with 50 mg/kg intraperitoneal pentobarbital and then fixed in the supine position under anaesthesia as earlier. Then, the skin was dissected by a lateral 1.0 cm cut along the left side of the sternum, and the main trunk of the left coronary artery was ligated. Immediately after ligation, 2 × 10⁵ cells from the respective groups in 30 μL serum-free EBM medium, or PBS were injected into the apex of the mouse. Mice were sacrificed by cervical dislocation and harvest was done 2 weeks after surgery.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the mean ± standard error of the mean. Comparisons between groups were performed using analysis of variance, with post hoc testing performed with Bonferroni analysis or unpaired t-tests, as appropriate. Values of P < 0.05 were considered statistically significant.

3. Results

3.1 EPO-priming induced the angiogenic factors and integrins on EPOR(+)

mobPBMCs

The volunteer demographics and the complete blood count before and after G-CSF injection are shown in Supplementary material online, Table S1. The effect of G-CSF mobilization was proved by the Complete
Blood Count, which was done immediately before peripheral blood collection. G-CSF injection significantly increased the number of total leukocytes, segmented neutrophils, and monocytes (see Supplementary material online, Table S2). After Ficoll-plaque separation of $^{\text{mob}}$PBMCs, we checked the cell population by Wright-Giemsa staining (see Supplementary material online, Figure S1). We analysed the effect of EPO-priming on $^{\text{mob}}$PBMCs in two experimental steps; with or without FACS sorting by EPOR (see Supplementary material online, Figure S2).

EPO was expressed on $41.2 \pm 12.3\%$ of $^{\text{mob}}$PBMCs. We sorted the cells by the presence of EPORs, into EPOR(+) $^{\text{mob}}$PBMCs and EPOR(-) $^{\text{mob}}$PBMCs. The purity of the sorted cells was confirmed by FACS (Figure 1A) and immunofluorescence staining (Figure 1B). Before and after EPO-priming of the FACS sorted cells, we screened the mRNA level of several cytokines. The expression of genes before EPO-priming, varied according to the presence of EPOR. The expression of several genes such as IL8, TNFα, and MMP9 was higher in EPOR(+) cells than in EPOR(-) ones even before priming. However, after EPO-priming, the gene expression difference was significantly augmented. EPO-priming induced specifically EPOR(+) cells to markedly express IL8, IL10, bFGF, PDGF, MMP2, integrin αV, β1, β2, and β8, which is assumed as the effect of EPO-priming (Figure 1C). Considering that IL-8, IL-10, bFGF, and TNFα are well-known cytokines having vasculo-genic potentials and that MMP9 and integrins are essential in vasculo-genic cell recruitment, we focused on the vasculo-genic effect of ex vivo EPO-priming.

3.2 The effect of EPO-priming in the whole $^{\text{mob}}$PBMCs and its signalling pathway

In the future clinical application, FACS sorting of $^{\text{mob}}$PBMCs by EPOR would be a certain burden in terms of practicability of the protocol. Moreover, $^{\text{mob}}$PBMCs are currently used cells for stem-cell therapy and have proved to be effective in our prior trials.\(^8\) – \(^{10}\) Thus, using the whole cell group (without EPOR sorting), we assessed the effect of EPO-priming. EPO-priming of whole $^{\text{mob}}$PBMCs also significantly induced the expression of IL8, IL10, basic FGF, MMP9, Integrin αV, and β8 compared with veh-priming (Figure 2A). Although the significant increase in some molecules disappeared in the whole cell group (i.e. PDGF, integrin β1, β2), EPO-priming was still proved to increase the expression of vasculo-genic molecules in whole $^{\text{mob}}$PBMCs that are used in MAGIC-CELL clinical trial.\(^8\) We then evaluated the surface molecules of EPO-primed $^{\text{mob}}$PBMCs (Figure 2B). The integrin expression profile was altered by EPO-priming. The expression of integrin β1, β2 increased in EPO-primed $^{\text{mob}}$PBMCs, whereas integrin α4 and α5 were similar to the veh-primed $^{\text{mob}}$PBMCs. This was concordant with the quantitative real time PCR results of EPOR (+) $^{\text{mob}}$PBMCs.

Regarding cell viability, EPO-priming reduced the number of apoptotic cells double-positive for PI and Annexin V. Regarding cell sub-populations, EPO-priming significantly increased CD14(+) monocytes resulting in the reduced proportion of CD66(+) neutrophils. Among the monocyte population, we observed a polarization shift like induction of highly vasculo-genic CD14(++)/CD16(+) cells.

To confirm the signalling pathway down to EPOR, we performed western blot analysis for total and phosphorylated JAK2 and Akt protein. After 5 min of EPO-priming, these signalling molecules are phosphorylated and activated, which was blocked by the EPOR antibody (Figure 3A). Next, we examined the effect of JAK or Akt inhibitor on the release of pro-angiogenic growth factors from these cells. EPO-priming significantly increased mRNA expression of IL8, IL10, bFGF, and PDGF, which was effectively prevented by EPOR blocking antibody, JAK inhibitor, or Akt inhibitor (Figure 3B). ELISA of supernatant from $^{\text{mob}}$PBMCs demonstrated that secretion of these factors was remarkably induced by EPO-priming, which was obliterated by EPOR blocking antibody, JAK inhibitor, or Akt inhibitor. ELISA results were corrected by the viable cell count after priming agent without/with blockers (Figure 3C, Supplementary material online, Figure S3).

3.3 Two faceted effects of EPO-priming on $^{\text{mob}}$PBMCs: paracrine action and adhesion on endothelial cells

$^{\text{mob}}$PBMCs can directly take part in angiogenesis, whereas the paracrine effect may also be important. Especially, monocytes are known to differentiate into various cells and secrete cytokines which are pro-vasculo-genic.\(^16\) We analysed the culture supernatant of EPO-primed $^{\text{mob}}$PBMCs cultured for 36 h. Compared with the veh-primed $^{\text{mob}}$PBMCs, EPO-primed $^{\text{mob}}$PBMCs had a higher amount of secreted IL8, IL10, TNFα, and PDGF (Figure 4A) in their culture-supernatant. A Matrigel tube formation assay was used to check whether the culture supernatant of EPO-primed $^{\text{mob}}$PBMCs can induce vasculo-genesis. Formation of capillary-like structures was significantly enhanced when human umbilical vein endothelial cells (HUVECs) were cultured in the supernatant of EPO-primed $^{\text{mob}}$PBMCs rather than in the supernatant from veh-primed $^{\text{mob}}$PBMCs (Figure 4B). Also in a random migration assay, the supernatant of EPO-primed $^{\text{mob}}$PBMCs induced a significantly large amount of migration of HUVECs (Figure 4C). Both tube formation and migration of endothelial cells were significantly disturbed by pre-treatment with EPOR blocking antibody, JAK inhibitor, or Akt inhibitor (Figure 4B and C).

Also, adhesion of $^{\text{mob}}$PBMCs to either endothelial cells or extracellular matrix is known to be the initial step during formation of new blood vessels.\(^17\) Therefore, we assessed the consequence of the induced integrins on the surface of EPO-primed $^{\text{mob}}$PBMCs through in-vitro adhesion assay on fibronectin (50 μg/mL) or on HUVECs (1 x 10^5 cells per plate). EPO-priming increased $^{\text{mob}}$PBMCs adhesion to both fibronectin and HUVECs, which were reduced by EPOR blocking antibodies, integrin β1 and β2 neutralizing antibodies (Figure 5A – C). The increased adhesion could have originated from the improved survival by EPO-priming. However, integrin neutralizing antibodies had no effect on cell viability but reduced adhesion, whereas EPOR blocking antibody pre-treatment reduced viability along with adhesion (Figure 5D and E). Therefore, we concluded that the improved adhesion is integrin-mediated.

3.4 EPO-primed $^{\text{mob}}$PBMCs enhance neovascularization in ischaemic limb and repair myocardium after infarction through cellular and humoral mechanisms

Based on the prior results of increased vasculo-genic potential by EPO-priming, we investigated the vasculo-genic effect of EPO-primed $^{\text{mob}}$PBMCs using a 3D Matrigel plug model. We injected into the dorsal subcutaneous tissue of nude mice Matrigel plugs mixed with PBS and veh-primed $^{\text{mob}}$PBMCs or EPO-primed $^{\text{mob}}$PBMCs (see Supplementary material online, Figure S4). Two weeks later, histological evaluation revealed more vessels in Matrigel plugs injected with the EPO-primed $^{\text{mob}}$PBMCs than in the other plugs (see Supplementary material online, Figure S5).

Also, we injected PBS, veh-primed $^{\text{mob}}$PBMCs, or EPO-primed $^{\text{mob}}$PBMCs into the ischaemic hindlimb of athymic nude mice. By serial
**Figure 1** Expression of EPOR on CottonPBMCs and Effect of EPO-priming. (A) CottonPBMCs were sorted by the presence of EPOR. By FACS analysis, the purity of EPOR-sorted CottonPBMCs was proved. (B) Immunofluorescence showed again the purity of sorting that anti-EPOR antibody-stained cells were only present in the EPOR(+) CottonPBMCs group. (C) Gene expression of EPOR(+) and (-) cells before EPO-priming and in response to EPO-priming. Results are shown as the relative amount of mRNA expression compared with GAPDH, and fold-expression compared with EPOR(-) CottonPBMCs (thick black bars indicate difference between EPOR(+) CottonPBMCs, before and after EPO-priming (n = 3 for all results).
Figure 2  Effect of EPO-priming on the mobilized PBMCs. (A) Gene expression of veh-primed mobilized PBMCs and EPO-primed mobilized PBMCs. EPO-priming significantly increased IL8, IL10, bFGF, MMP9, Integrin αV, and Integrin β8. Results are shown in fold expression compared with veh-primed mobilized PBMC. (B) FACS analysis, EPO-priming showed an induction of integrins on cell surface, decreased of apoptotic cells and a phenotypic shift from mobilized PBMCs towards angiogenic CD14/16-double positive cells. Representative figures are shown and proportion of cells in each box is shown in numbers (n = 3 for all results).
Figure 3  Signal pathway of EPO-priming. (A) Signalling pathway underlying EPO-priming of \( \text{mob} \) PBMCs. Western blot for EPO-primed \( \text{mob} \) PBMCs cultured with additional veh or EPOR blocking antibodies. EPOR blocking antibodies reduced the phospho-form of Akt and JAK2, without effect on the total amount. (B and C) The vasculogenic secretome primed by EPO was checked at the mRNA level (B) and at the protein level (C). IL8, IL10, bFGF, PDGF levels were augmented by EPO-priming, which were abolished by EPOR blocking antibody, JAK inhibitor, and Akt inhibitor. Results in (B) are shown as the relative amount of mRNA expression compared to GAPDH and fold-expression compared with veh-primed \( \text{mob} \) PBMCs. Cytokine levels were shown in pg/mL (\( n = 3 \) for all results).
Figure 4  Vasculogenic potential of the EPO-primed \( ^{\text{mob}} \)PBMCs. (A) Veh- or EPO-primed \( ^{\text{mob}} \)PBMCs were cultured for 36 h. ELISA assay of the supernatants showed a significant increase in IL8, IL10, TNF\( \alpha \), and PDGF. Y axis represents relative amount of concentration compared with veh-primed \( ^{\text{mob}} \)PBMCs culture supernatant. (B) HUVECs were cultured in each cell-culture supernatant. Tube formation assay showed an increase in tube like structures, resulting in a significant increase in branching points and tube length. (C) Random migration assay showed a significant increase in wound healing in the HUVECs cultured in EPO-primed \( ^{\text{mob}} \)PBMCs culture supernatant. Both tube formation and random migration activity of HUVEC were decreased by EPOR blocking antibody, JAK inhibitor, and Akt inhibitor (\( n = 3 \) for all results).
Figure 5 Adhesion of the EPO-primed \textsuperscript{mob}PBMCs. (A) Adhesion assays of primed \textsuperscript{mob}PBMCs were done on HUVECs and (B) Fibronectin. (C) EPO-priming increased the adhesion ability of \textsuperscript{mob}PBMCs on HUVECs and fibronectin, which were abolished by EPOR blocking antibody, integrin \(\beta_1\) and \(\beta_2\) neutralizing antibodies. (D) Cell viability was improved by EPO-priming and reduced by EPOR blocking antibody, whereas this was not influenced by integrin neutralizing antibodies. Therefore, increased adhesion was not due to increased viability, but was an integrin-mediated effect (\(n = 3\) for all results).
measurement of the blood flow by the LDPI analyser on Day 0, 3, 7, 14, and 21, the blood flow recovery to the ischaemic hindlimb was significantly facilitated by EPO-primed mbPBMCs, compared with PBS or veh-primed mbPBMCs (Figure 6A). We confirmed that sufficient degree of ischaemia was induced in this model by histological examination of the ischaemic tissue with regenerating muscles at 21 days (see Supplementary material online, Figure S6). For quantification of angiogenesis, we counted the number and area of newly formed mature vessels in the ischaemic limb. EPO-primed mbPBMCs significantly increased the number and area of SMA(+) vessels (Figure 6B). Furthermore, we confirmed the direct incorporation of human mbPBMCs into mouse vessels through staining with human specific endothelial marker anti-CD34 antibody (Figure 6C and Supplementary material online, Video S1).

Another important indication of EPO-primed cell is the myocardial repair after infarction. Thus we made myocardial infarction in nude mice and compared therapeutic efficacy of EPO-primed mbPBMCs vs. veh-primed mbPBMCs vs. PBS control. EPO-primed mbPBMC showed a significantly better therapeutic efficacy than veh-primed cell or PBS control in terms of infarct size and infarct wall thickness. Veh-primed cell was better than PBS control (Figure 6D). Furthermore, injection of EPO-primed mbPBMC resulted in the least amount of cardiac fibrosis compared with the other two treatments (see Supplementary material online, Figure S7).

Next, to show the paracrine vasculogenic properties of mbPBMCs, we created mouse ischaemic hindlimb models and injected cell supernatant from primed mbPBMCs. As a result, injection of supernatant from EPO-primed mbPBMC showed a significantly better therapeutic efficacy than veh-primed cell or PBS control in terms of blood flow recovery to ischaemic limb (Figure 6E), as well as neovascularization with mature vessels (see Supplementary material online, Figure S8).

3.5 EPO-priming of PBMCs from the elderly patients with coronary artery disease

Those who would benefit from EPO-priming are patients with myocardial infarction. Therefore, we performed EPO-priming of PBMCs from three elderly patients with coronary artery diseases. Patients were 66.7 ± 5.0 years old and the clinical diagnosis was angiographically confirmed. EPO-priming significantly induced the expression of IL10, PDGF, MMP2, integrin α4, β1, β2, and β8, which was reduced by EPOR blocking antibody. Furthermore, EPO-priming induced M2 polarization, reduced apoptosis, and stimulated integrin surface expression, which was prevented by blocking antibody against EPOR. Such responses of PBMCs from elderly patients to EPO-priming were consistent with responses of mbPBMCs from healthy young volunteers (see Supplementary material online, Figure S9).

4. Discussion

Through this study, we have shown the beneficial effect of ex vivo EPO-priming to increase the vasculogenic potential of human mobilized peripheral blood stem cells after G-CSF administration. By EPO-priming, mbPBMCs increased the synthesis of cytokines and integrins that involve in vasculogenesis. Furthermore, EPO-priming changed the cell viability or phenotype of mbPBMCs by reducing apoptosis and inducing shift of monocyte polarity toward vasculogenic CD14(++)/CD16(+) mononuclear cells. In vivo models also demonstrated that injection of EPO-primed mbPBMCs achieved a greater vasculogenesis in the ischaemic tissue than that of veh-primed mbPBMCs did.

These findings strongly suggest that ex vivo EPO-priming of mbPBMCs can be a feasible and effective method to augment the efficacy of cytokine-base peripheral blood stem-cell therapy for patients with MI.

4.1 New strategy to overcome the limited efficacy of adult stem-cell therapy

During the past decade, a vast body of researches has been done on stem-cell therapy for patients with MI and reported significant efficacy in improving contractility and reducing infarct scar. But the problem was the limited efficacy: improvement of left-ventricular ejection fraction just by 3% and reduction of infarct size just by 5 mL. Several methods to overcome such a limitation have been proposed, such as, increasing cell numbers, genetic manipulation of the adult progenitor cells, or adoption of potent stem cells rather than adult progenitor cells. Increasing cell numbers may not be feasible because of the technical limitation of intracoronary or trans-catheter endomyocardial injection. Genetic manipulation or adoption of more potent cells derived from embryonic stem cells may encounter the safety issues before clinical application. Under these situations, priming of the adult progenitor cells before implantation would be a very feasible and safe way to augment the therapeutic efficacy, which has been well demonstrated by our previous report. In this previous paper, we found that most of mbPBMCs expressed Tie-2 receptor and that priming of mbPBMCs with angiopoietin-1 significantly enhanced the transdifferentiation of mbPBMCs to endothelial lineage by turning on Ets-1 factor and the expression of integrins on the surface of mbPBMCs, leading to significant improvement of retention efficiency and therapeutic efficacy of mbPBMCs after intra-arterial delivery. But we encountered the unexpected difficulty to realize this concept in clinics because it is very difficult to make the potent angiopoietin-1 as human- or clinical-grade to meet the economical criteria during mass production. This is the reason why we searched for another cell-primer that is feasible in clinical application and paid attention to EPO because it is currently used in clinics and has several beneficial effects on cardiovascular system in the situation of MI.

4.2 EPO as a priming agent

EPO, being a well-known cytokine controlling erythropoiesis, has been tried to patients with MI with disappointing results, probably because of the insufficient local concentration at the target infarcted myocardium or because of the unwanted systemic effects. With adoption of ex vivo cell priming strategy, we expect several benefits; to maximize the effects of EPO on target cells, i.e. enhanced proliferative, vasculogenic, and anti-apoptotic properties, while to avoid the systemic side effects of EPO. With this ex vivo cell-specific priming strategy, we could use the highest dose of 10 IU/mL, which is known to have maximal proliferative effect in prior studies.

In a previous study, EPO was once used as a priming agent for late endothelial progenitor cells (EPCs) that already have good vasculogenic potentials with high expression of VEGFR-2, CD31, and Tie2. However, late EPCs are not actively used in the current clinical cell therapy, due to the burden of ex vivo cell culture (requires at least 10 days for culture), limited replicative capacity, and the possibility of contamination in the process of ex vivo manipulation. In contrast, mbPBMCs have been used for 10 years’ MAGIC-CELL program.
Figure 6  Transplantation of EPO-primed $\text{m}^{\text{ol}}$PBMCs or their supernatant improved blood flow recovery to ischaemic limb and repaired the damaged heart after myocardial infarction in nude mice. (A) Representative figure of LDPI measurements and the perfusion ratio of the ischaemic limb compared with the non-ischaemic limb. After 3 weeks of femoral artery ligation and $\text{m}^{\text{ol}}$PBMCs injection, the perfusion ratio was $30.8 \pm 1.8\%$ in the PBS injected group and $41.7 \pm 2.7\%$ in the veh-primed $\text{m}^{\text{ol}}$PBMCs injected group, whereas it was $50.6 \pm 1.3\%$ in the EPO-primed $\text{m}^{\text{ol}}$PBMCs injected group. (*$P < 0.01$ for EPO vs. PBS, †$P$ of trend $< 0.05$ for veh vs. PBS, ‡$P < 0.01$ for veh vs. PBS, §§$P$ of trend $< 0.01$, $n = 21$; $n = 7$ in each group). (B) The mature vessel count was analysed by SMA(+)- circular structures in low power fields ($\times 40$). EPO-primed $\text{m}^{\text{ol}}$PBMCs induced a larger number and broader area of mature vessels than did veh-primed cells or PBS. ($n = 21$; $n = 7$ in each group). (C) Immunofluorescence staining showed the evidence that human specific CD34(+) cells (white arrowheads) incorporated into the mouse vascular structures. (D) In the myocardial infarction model of nude mice, transplantation of EPO-primed $\text{m}^{\text{ol}}$PBMC showed the better therapeutic efficacy than veh-primed cells or PBS control in terms of infarct size and wall thickness. MT staining in the first row and H&E staining in the second row. ($n = 9$; $n = 3$ in each group). (E) Paracrine humoral effects of EPO-primed cells were tested in the ischaemic hindlimb model. Injection of supernatants from EPO-primed $\text{m}^{\text{ol}}$PBMC showed the better efficacy than supernatants from veh-primed cells or PBS control in terms of blood flow recovery to ischaemic limb (perfusion ratio; $36.9 \pm 10.0\%$ vs. $25.7 \pm 8.6\%$ vs. $17.7 \pm 9.3\%$ (*$P < 0.01$ for EPO vs. PBS, **$P < 0.05$ for EPO vs. veh, †$P < 0.01$ for EPO vs. PBS, $n = 21$; $n = 7$ in each group).
and our strategy to prime these cells ex vivo requires just several hours’ incubation without ex vivo cell culture.

4.3 Specific effects of EPO priming through EPO-EPOR pathway

About 40% of mobilized PBMCs were positive for EPOR and these cells were used to evaluate the specific effects of EPO-priming through its receptor. EPO-primed mobilized PBMCs showed dramatic increases of various angiogenic molecules. Especially, IL8 increased in a 38.8-fold, IL10 in a 33.3-fold, and bFGF in a 96.8-fold. EPO-priming also induced integrins which are essential for adhesion on the vascular lumen, trans-endothelial migration, further extra-vascular migration of the circulating or intra-arterially delivered mobilized PBMCs. These effects of EPO-priming were all reversed by EPOR blocking antibodies. This proved the rationale of using EPO to augment the vasculogenic potential of mobilized PBMCs. Previous studies have shown that EPO can affect endothelial cells, vascular smooth muscle cells, neuronal cells, myeloid cells, etc. and can provide cytoprotection to ischaemia through the JAK2, AKT, and ERK pathway in a parallel order. This pathway can maintain the mitochondrial membrane potential, prevent the cellular release of cytochrome c, and modulate caspase activity.

Furthermore, we also checked the effect of EPO-priming on whole population of mobilized PBMCs without EPOR sorting. In the practical point of view, ex vivo cell sorting or purification process by FACS would be a limiting step that may be associated with damage to cell viability, risk of contamination, deprivation of possible cell-to-cell synergistic interactions, or economic burden. That is the rationale for us to use whole mobilized PBMCs without sorting in MAGIC-CELL trials.6,8,10,25 We were able to reproduce the beneficial effect of EPO-priming also in the whole mobilized PBMCs population in terms of in vitro angiogenic gene expression as well as in vivo vasculogenic effect in the two different models, proving this strategy readily applicable in the clinic. The effect of EPO-priming could be classified into two levels; the paracrine effect to form a vasculogenic niche and the direct cell-modulating effect.

4.4 Paracrine action of EPO-primed mobilized PBMCs: formation of a vasculogenic niche

Secretion of cytokines such as IL8 and IL10 from EPO-primed mobilized PBMCs may be helpful to make the vasculogenic niche in the target ischaemic tissue when these cells delivered. IL8 is a well-known cytokine that enhance proliferation and survival of endothelial cells by activation of MMP2 and MMP9.28,29 IL10 is also a well-known vasculogenic cytokine that enhances proliferation and survival of endothelial cells by activation of MMP2 and MMP9.28,29 IL10 is also a well-known vasculogenic cytokine that may be helpful to make the vasculogenic niche in the target ischaemic tissue. IL8 is a well-known cytokine that enhances proliferation and survival of endothelial cells by activation of MMP2 and MMP9.28,29 IL10 is also a well-known vasculogenic cytokine that may be helpful to make the vasculogenic niche in the target ischaemic tissue. IL8 is a well-known cytokine that enhances proliferation and survival of endothelial cells by activation of MMP2 and MMP9.28,29 IL10 is also a well-known vasculogenic cytokine that may be helpful to make the vasculogenic niche in the target ischaemic tissue.

5. Conclusion

EPO-priming can induce vasculogenic niche by promoting vasculogenic potential of mobilized PBMCs. EPO-priming of mobilized PBMCs can provide vasculogenic niche to the target ischaemic tissue by expression of vasculogenic cytokines and more easily incorporate into the target ischaemic tissue through up-regulated integrins as well as they differentiate into vasculogenic monocytes. Such mechanisms are well supported by the enhanced angiogenesis, limb, and myocardial salvage, and evidence of incorporation of human cell in mice limb after transplantation of EPO-primed mobilized PBMCs in nude mice. EPO-priming can be a promising and practical method to augment the therapeutic efficacy of mobilized PBMCs in the cytokine-based cell therapy for patients with MI.

Supplementary material

Supplementary Material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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