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Priming Mononuclear Cells to Improve Outcomes of Regenerative Therapy

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In a seminal report in 1997, Asahara and colleagues[1] first described the existence of endothelial progenitor cells (EPCs) in human peripheral blood (PB) $CD34^+$ mononuclear cell (MNC) fraction. In a subsequent report,[2] they described the ability of bone marrow (BM)-derived circulating EPCs to induce neovascularization. The obvious therapeutic potential of EPCs for ischemic diseases has since driven intense research focused on vasculogenic cells from diverse sources with variable phenotypic attributes and biological functions. Although data from clinical trials have shown a modest positive impact of circulating progenitor therapy in cardiovascular and peripheral arterial diseases,[3–7] new and enhanced methods for cell isolation, expansion, and characterization are critically important to improve regenerative outcomes.

Indeed, the rarity of circulating EPCs remains a major hurdle toward successful and wider clinical application using autologous cells. The MNC fraction from PB contains primarily lineage-committed lymphoid and myeloid cells and a very small percentage of $CD34^+$ or $CD133^+$ stem/progenitor cells. Accordingly, attempts have been made to mobilize EPCs into PB with repeated administration of granulocyte colony stimulating factor (G-CSF) followed by apheresis, a common practice in the setting of BM transplantation. Although several clinical trials of tissue regeneration have been completed with progenitors harvested from PB following G-CSF injection, successful expansion of EPCs from PB would be a preferable approach to circumvent the need for G-CSF therapy. Moreover, although BM harvest is a minimally invasive procedure, phlebotomy for PB is less expensive and tolerated better by patients.

In this issue of *JAHA*, Masuda and colleagues[8] report successful enrichment of EPCs from human peripheral blood mononuclear cells (PBMNCs) using a quality and quantity culture (QQc) method and salvage of ischemic limbs in mice with injection of expanded cells. Culture of PBMNCs in QQc medium for 7 days resulted in a 19-fold increase in definitive EPC (dEPC) colony-forming cells, despite a ≈50% reduction in total number of cells. Moreover, these primed dEPCs showed a 2.7-fold greater endothelial differentiation potential. The frequency of dEPC colony-forming cells correlated positively with the primitive EPC (pEPC) colony-forming cells in PBMNCs, indicating that the QQc method effectively transitioned the pEPC colony-forming cells into dEPC colony-forming cells with increased potential for new vessel formation. Quality and quantity cultured mononuclear cells (QQMNCs) also expressed greater levels of mRNA for angiogenic molecules, including insulin-like growth factor-1 and interleukin (IL)-8, supporting the efficacy of QQc in inducing a vasculogenic phenotype in PBMNCs. Consistent with these favorable alterations in MNC phenotype, the injection of QQMNCs improved limb salvage following hindlimb ischemia in mice.[8] Multimodality assessments showed increased perfusion, angiogenesis, and myogenesis and reduced fibrosis in QQMNC-treated mice. Important from a therapeutic standpoint, compared with G-CSF–mobilized $CD34^+$ cell transplantation, QQMNC injection led to equal or greater improvement in outcomes in the setting of hindlimb ischemia.

With growing clinical need for EPCs in large numbers, various methods of enrichment and expansion have been developed by different laboratories for EPCs with diverse cellular phenotypes. Consistent with an endothelial or angiogenic theme, the culture medium for this purpose usually contains an endothelial medium (eg, endothelial basal medium-2[9] and endothelial growth medium-2[10,11]) with or without serum and angiogenic growth factors. These factors usually include varying combinations and concentrations of vascular endothelial growth factor, fibroblast growth factor-B, insulin-like growth factor-1, epidermal growth factor, hydrocortisone, ascorbic acid, and heparin.[10,12] Somewhat differently, the QQc medium used in this study by Masuda et al[8] contained Stemline® II (Sigma-Aldrich), a hematopoietic stem cell expansion medium, which did not contain any cytokine or

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serum. In addition to the angiogenic molecule vascular endothelial growth factor, the supplements in QQc medium included stem cell factor, thrombopoietin, IL-6, and Flt-3 ligand, molecules that are known to play important roles in the regulation of hematopoiesis and have been used extensively for expansion of cord blood– or peripheral blood–derived $CD34^+$ cells.[13–15] The success of this largely hematopoietic cocktail was documented by a nearly 6-fold increase in $CD34^+$ cells and a 3.5-fold increase in $CD133^+$ cells in QQMNCs after 7 days of culture.

Interestingly, careful phenotypic characterization of cultured cells in the current study also revealed that QQc could promote an antiinflammatory phenotype in MNCs. Specifically, gene expression of the proinflammatory cytokine IL-1β was lower and expression of the antiinflammatory molecule IL-10 was higher in QQMNCs. Further, QQc increased the proportion of $CD206^+$ alternatively activated M2 macrophages with antiinflammatory properties by 5-fold and reduced the $CCR2^+$ classical M1 population known to exert proinflammatory effects. Importantly, QQc also induced a decrease in $CD19^+$ lymphoid B cells and $CD56^+$ natural killer cells, while inducing a nearly 6-fold increase in both $CD4^+$/interferon-γ^-/$IL\text{-}4^+$ T helper 2 (Th2) type T cells and regulatory T cells in response to phorbol 12-myristate 13-acetate and ionomycin. Considering the prominent roles played by regulatory T cells, natural killer cells, and other immune cell subsets in transplant tolerance,[16,17] these changes in immunological composition of MNCs have profound implications for survival of transplanted EPCs, especially in the context of allogeneic off-the-shelf products.

While the molecular regulation of immune cells and inflammation in vivo is extremely complex, the ingredients of this QQc medium may potentially exert antiinflammatory and immune-tolerant actions by altering the T helper 1 (Th1)/Th2 cell balance. In this regard, IL-6 has been shown to promote the generation of the Th2 subset,[18] and human PBMNCs produce Th2 cytokines in response to vascular endothelial growth factor.[19] In humans, Flt-3 ligand increases both $CD11c^+$ and $CD11c^-$ dendritic cells,[20] which promote the generation of Th1 and Th2 cytokines, respectively. Moreover, recent evidence indicates that Th1 cytokines induce classical M1 macrophage activation, while Th2 cytokines favor the M2 characteristics. Thus, IL-6 and stem cell factor, components of the QQc medium, may favorably modulate the activation of macrophages,[21,22] which may serve to quench inflammation and promote tissue repair in an ischemic milieu in vivo. Nonetheless, to further improve this culture technology, the precise molecular signaling that produces these phenotypic shifts in QQMNCs should be elucidated in greater detail in future studies. Whether adding other agents known to augment antiinflammatory attributes of EPCs[23] to QQc medium would further enhance its efficacy should also be explored. Finally, there remains a possibility that the current in vivo data with human cells in athymic mice that are unable to produce T cells may not faithfully predict the results of QQMNC transplantation in immunocompetent human recipients. Additional in vitro characterization of immunogenicity of QQMNCs and in vivo testing in relevant preclinical models will be prudent before clinical translation.

The formulation of a serum-free culture process that transforms human PBMNCs into vasculogenic cells has major translational relevance. In conjunction with the authors' previous report with umbilical cord blood–derived $CD133^+$ cells,[24] the current observations support the efficacy of the QQc method to produce vasculogenic cells from multiple sources with a significant increase in dEPC colonies and the induction of an antiinflammatory and immune-tolerant phenotype in expanded cells. These results also indicate that after in vivo transplantation, QQc products are able to improve outcomes in the setting of myocardial infarction[24] as well as limb ischemia.[8] If replicated successfully in clinical trials, the QQc method may lead to a cellular product that will effectively alleviate ischemic diseases in humans, fulfilling the primary goal of regenerative research.

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