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Forearm ischemia decreases endothelial colony-forming cell angiogenic potential

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Abstract

Background aims. Circulating endothelial progenitor cells and especially endothelial colony-forming cells (ECFCs) are promising candidate cells for endothelial regenerative medicine of ischemic diseases, but the conditions for an optimal collection from adult blood must be improved. *Methods.* On the basis of a recently reported vascular niche of ECFCs, we hypothesized that a local ischemia could trigger ECFC mobilization from the vascular wall into peripheral blood to optimize their collection for autologous implantation in critical leg ischemia. Because the target population with critical leg ischemia is composed of elderly patients in whom a vascular impairment has been documented, we also analyzed the impact of aging on ECFC mobilization and vascular integrity. *Results.* After having defined optimized ECFC culture conditions, we studied the effect of forearm ischemia on ECFC numbers and functions in 26 healthy volunteers (13 volunteers ages 20–30-years old versus 13 volunteers ages 60–70 years old). The results show that forearm ischemia induced an efficient local ischemia and a normal endothelial response but did not mobilize ECFCs regardless of the age group. Moreover, we report an alteration of angiogenic properties of ECFCs obtained after forearm ischemia, *in vitro* as well as *in vivo* in a hindlimb ischemia murine model. This impaired ECFC angiogenic potential was not associated with a quantitative modification of the circulating endothelial compartment. *Conclusions.* The procedure of local ischemia, although reulting in a preserved endothelial reactivity, did not mobilize ECFCs but altered their angiogenic potential.

Key Words: *aging, endothelial colony-forming cells, endothelial progenitor cells, forearm ischemia, mobilization*

Introduction

The description of circulating endothelial progenitor cells (EPCs) originating from bone marrow has revolutionized the concept of postnatal vasculogenesis (1–3) and paved the way for the development of a cell therapy approach for tissue ischemia. On the other side, both EPC level and an altered function were associated with cardiovascular risk factors and have been shown to be predictive of cardiovascular outcomes (4,5).

Several types of cells are referred to as EPCs according to their phenotype, the method used or their origin (6). Cells expressing CD34 and KDR (VEGF-R2) antigens when analyzed by flow cytometry are commonly called EPCs (7). In culture, two types of EPCs have been isolated. Colony-forming unit–endothelial cells (CFU-ECs), also called CFU-Hill or early EPCs, are spindle-shaped cells that express leukocyte markers and a very low level of endothelial markers. They do not give rise to pseudotubes *in vitro* and do not form human vessels in preclinical models of vascularization (8). Late EPCs, or endothelial colony-forming cells (ECFCs) according to Yoder's group classification, are rare, circulating viable cells obtained within 1 to 3 weeks of culture that show a robust clonal proliferative potential (8–10). ECFCs express endothelial markers as well as the embryonic marker BMP4 (11). By contrast to

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early EPCs, they give rise to true endothelial progenitors of endothelial cells and merge into neovessels *in vivo* (8). ECFCs are thus promising candidate cells for an autologous cell therapy product to treat ischemic diseases such as critical limb ischemia. Two main limiting factors hinder such a therapy in adults: first, ECFCs obtained in culture from adults have impaired proliferative and angiogenic properties (9,12,13). Second, huge amounts of blood are required to obtain a sufficient cell number, and thus an efficient mobilization procedure is needed. Several procedures have been considered, such as administration of growth factors (3,14), *ex vivo* expansion (15) and gene therapy (16). Friedrich *et al.* (17) observed a mobilization of a very immature progenitor cell population $CD34^{-}CD133^{+}$ triggered by local ischemia. This finding, together with the study of Ingram *et al.* (18) suggesting that ECFCs have a vascular origin, led us to hypothesize that ECFCs could be efficiently mobilized in peripheral blood on vascular stimulation induced by local ischemia. Because impaired vascular function has been documented in elderly patients, we also investigated the differences between age groups in terms of vascular response, which comprised vascular reactivity and exploration of the whole circulating endothelial compartment. This compartment reflects both lesion/activation (endothelial microparticles and circulating endothelial cells) and regeneration (endothelial progenitor cells) and has indeed recently been proposed to define the vascular competence (19).

The aims of the study were first to optimize ECFC culture from adult blood and second, to determine whether a local forearm ischemia could recruit ECFCs in two groups of healthy volunteers ages 20–30 years old and 60–70 years old. Finally, we investigated the effect of forearm ischemia on ECFC angiogenic potential and vascular response.

Methods

Optimization of ECFC culture

Experiments are described in detail in the Supplementary material.

Vascular assessment in healthy volunteers and response to forearm ischemia

Study population. Twenty-six male healthy volunteers were included on the basis of the following criteria: absence of any known medical disease, no treatment by any drug known to affect EPC mobilization, normal physical examination and laboratory results within reference ranges. Two groups of 13 volunteers of 20–30 and 60–70 years old, respectively, were recruited. This study was approved by the Paris-Ile de France II ethics committee and is registered at http://clinicaltrials.gov (NCT01038700). The Center of Clinical Investigation was in charge of the recruitment and the follow-up of the study population.

Design of the study

The schematic presented in Figure 1 summarizes the design of the study. Pharmacological parameters and flow-mediated endothelial dilatation were measured 10 days after each volunteer's inclusion. Volunteers were subjected to a second procedure of forearm ischemia (FI) 1 week apart, this time dedicated to the quantification of circulating biological markers in whole blood, at rest and at different time points after ischemia. The total blood volume taken was equivalent to a blood donation. The first blood sample of 100 mL was collected from the brachial vein draining the ischemic territory the day before the induction of ischemia (baseline sample). The same volume was collected immediately after deflating the cuff (after FI sample), 2 h later (2 h after FI sample), and 24 h after (24 h after FI sample). The volunteers fasted for the past 12 h each morning along the 3 days of the trial. They stayed at rest for 30 min before blood sampling and did not practice exercise during these 3 days. The morning of the ischemia procedure, they did not eat till the sample 2 h after ischemia was realized. This part was managed by the center of clinical investigation. Of note, no lipemic plasma was observed in any samples of the volunteers. At each time point, the whole circulating endothelial compartment was quantified. This included markers of endothelial lesions, namely microparticles and circulating endothelial cells, and markers of endothelial repair, the progenitor cells. Because of EPC heterogeneity in terms of ontogeny and the lack of correlations between the different EPC-like definitions, we quantified three types of EPCs and other progenitor cells. The EPCs are $CD34^{+}KDR^{+}$ cells, ECFC and CFU-EC. Progenitor cells are $CD34^{+}CD45^{dim}$ cells or hematopoietic progenitor cells (HPCs) and $CD34^{+}CD133^{+}$ cells or circulating progenitor cells (CPCs). Functional studies with EPCs obtained in culture, ECFCs and CFU-ECs were also performed.

Pharmacological and biological measurements realized in this study have been described elsewhere and in detail in the Supplementary material file.

Statistical analysis

The results of ECFC culture optimization and functional studies were analyzed by means of paired