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Vroling, L.

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CHAPTER 6

OUTGROWTH ARREST OF CIRCULATING ENDOTHELIAL COLONY FORMING CELLS BY HYPOXIA

Laura Vroling
Pieter Koolwijk
Richard R. de Haas
Michiel van Wijhe
Henk M.W. Verheul
Henk J. Broxterman
Victor W.M. van Hinsbergh

To be submitted

ABSTRACT

A role of endothelial progenitor cells (EPCs) in (tumor) angiogenesis and tissue repair has been proposed and recently debated. In circulation a very small subpopulation of EPCs, the endothelial colony forming cells (ECFCs) are present, which are capable to form colonies of true endothelial cells *in vitro*. These circulating ECFCs are thought to home into ischemic tissue, where they contribute to neovascularization. However, little is known about the initial response of ECFCs to hypoxia.

ECFC colonies were grown from cord-blood mononuclear cells (CB-MNCs). The effect of hypoxia was studied in special equipment, in which culture and handling of the cells occurred under 1% O₂, and compared with standard culture conditions (21% O₂). Colony formation was followed and quantified by phase contrast microscopy. Endothelial markers were determined by immunohistochemistry and flow cytometry.

Subcultured ECFCs proliferate both at 21% and 1% O₂. However, freshly obtained CB-MNCs formed ECFC colonies under 21% O₂, and not under 1% O₂. The inability to form ECFC colonies under hypoxia was overcome by subsequent transfer to 21% O₂, or by prior exposure to 21% O₂ for 2-3 days. A role of hypoxia inducible factors (HIFs) was observed after transfecting the initial CB-MNC fraction simultaneously with HIF-1 α and HIF-2 α double stranded small interfering RNAs. HIF-1/-2 down-regulation restored the ECFC colony outgrowth under 1% O₂, equal to 21% O₂. While subcultured ECFCs were able to proliferate under hypoxia, the presence of freshly isolated CB-MNC under hypoxia thereof prevented their proliferation.

The microenvironmental oxygen status determines outgrowth of circulating ECFCs to endothelial cells, either directly or via adjacent leukocytes. The data suggest that outgrowth of ECFCs would be most effective if ECFCs remain at circulation-hypoxic tissue borderzone and help sprouting of oxygenated vessels, rather than expanding in the core hypoxic area competing for the already scarce nutrient and oxygen supply.

INTRODUCTION

A role for endothelial progenitor cells (EPCs) in endothelial repair and angiogenesis has been proposed [1, 2] and in recent years debated [3, 4]. These cells have drawn a lot of attention as their number in the circulation is reduced in cardiovascular and diabetes patients, and are considered as a prognostic parameter for cardiovascular disease [5, 6]. Furthermore, EPCs were suggested to be required for ongoing tumor angiogenesis [4]. Participation of bone marrow-derived EPCs in the endothelial lining was reported early after vascular injury [7], but persistent incorporation of EPCs in the endothelial lining of tumors and tissues appeared hard to establish [8, 9].

Several theories about the origin and differentiation of EPCs have been put forward [10], but a major breakthrough has been the finding that two types of EPCs exist [11, 12], which can be separated on the basis of colony formation, i.e. "early" vs "late" outgrowth colonies with endothelial markers, and their hematopoietic or non-hematopoietic origin [13-15]. The large majority of EPCs represents myeloid cells which under specific conditions can acquire endothelial marker proteins. These myeloid cells, which grow out as early EPCs, are unable to form true endothelial colonies *in vitro* or endothelial tubules *in vivo*.

A second small fraction of cells, giving rise to the so-called late outgrowth EPCs, belong to the endothelial lineage. These cells are also indicated as blood outgrowth endothelial cells (BOECs) [16], endothelial outgrowth cells (EOCs) [10] or - as nowadays preferred - endothelial colony-forming cells (ECFCs) [17]. These cells, which we shall further indicate as ECFCs, have a high proliferative potential, are non-hematopoietic (CD34⁺/CD45⁻/CD133⁻), and express robust endothelial marker proteins [11, 13, 15]. They can be induced to form endothelial tubes *in vitro* [18-20] and vascular structures *in vivo*, the latter being stabilized in interaction with mural cells [21]. These ECFCs provide an interesting source of endothelial cells both for diagnostic means and for improvement of vascularization [18].

While the origin of ECFCs is still uncertain, it is generally believed that these cells home into ischemic tissue, where they can contribute to neovascularization. However, little is known about the behavior of ECFC in ischemic conditions, except for one study showing robust proliferation of subcultured ECFC in hypoxic conditions [22].

Exposure of cells to hypoxia induces an extensive hypoxic response, which enables them to cope with the metabolic stress of oxygen limitation. While several pathways can be involved [23, 24], a central role has been attributed to the hypoxia inducible factors (HIFs). HIFs are transcription factors that consist of dimers between a HIF- α subunit, which is stabilized and transferred into the nucleus under hypoxia, and a stable HIF-1 β subunit [25]. All cells produce the HIF-1 α and HIF-1 β subunit, while a limited number of cells types including endothelial cells also synthesize HIF-2 α [26]. HIFs induce expression

of a large number of genes, including genes involved in glycolysis, angiogenesis, homing of progenitor cells and erythropoiesis [27]. Recent data indicate that while HIF-1 overexpression can induce uncontrolled endothelial sprouting, the additional activity of HIF-2 modulates the endothelial response into a more proper vessel formation [28].

Within the bone marrow stem cell niche hypoxia is critical for the maintenance of the undifferentiated stem cell or precursor phenotype. Circulating cells home via hypoxia-induced SDF-1:CXCR4 interaction, an interaction that is also involved in keeping progenitor cells within the hypoxic bone marrow [29]. Interestingly hypoxia also caused HIF-1-dependent differentiation arrest of adipose-tissue derived mesenchymal stem cells [30]. However, no information is available about the behavior of circulating and initially cultured ECFC in a hypoxic environment. As only few ECFC colonies are obtained from circulating blood, we wondered whether we could improve the recovery and function of ECFCs by seeding and culturing them in a hypoxic environment. Because at present there is still no unique marker combination to sort circulating ECFCs prospectively, we investigated how circulating ECFCs in a population of cord blood-derived mononuclear cells (CB-MNCs) expand into well defined endothelial colonies when seeded and cultured in a hypoxic (1% oxygen) environment. In contrast to our initial expectation, we observed a hypoxia-dependent inhibition of ECFC colony formation that did not occur after 2-3 days of priming of the cells to 21% O₂. Subsequently we investigated the involvement of HIF and a possible contribution of accessory cells.

MATERIALS AND METHODS

Fresh cord blood samples were collected after oral informed consent, and CB-MNCs were isolated within 24 hours. Endothelial outgrowth colonies of ECFCs were generated in culture according to established procedures [15, 17] and colonies were directly counted based on characteristic cobblestone morphology (Figure S1). In short, ECFCs were isolated from the mononuclear cell fraction that was obtained from the cord blood using Ficoll Paque density gradient separation medium (GE Healthcare Bio-sciences AB, Sweden) and Leucosep 50 mL tubes (Greiner bio-one, Melsungen, Germany). The CB-MNCs were cultured in a concentration of at least 2.5×10^6 cells per cm² in Endothelial cell basal medium-2 (EBM-2,) complete medium supplemented with 0.1% penicillin-streptomycin (Lonza, Verviers, Belgium), 10% Fetal calf serum (FCS) and EGM-2 SingleQuotes (Lonza, Walkersville, USA) but without hydrocortisone and gentamycin/amphotericin-B (Lonza, Walkersville, USA). When the colonies started to appear (after 8-16 days) ECFCs were trypsinized and subcultured into flasks. Colony outgrowth of ECFCs was performed at normoxic (21% O₂) and hypoxic (1% O₂) conditions (see Supplemental Methods, Table S1). ECFC colonies were stained for VE-cadherin (Figure S2) and the

endothelial phenotype was determined by flow cytometry analysis for endothelial markers (CD105, CD146, CD31, VE-cadherin, Tie-2, VEGFR1, uptake of AcLDL and UEA binding), monocyte and leukocyte markers (CD14 and CD45) and others (CD133, CXCR4, NP-1, EPO) (see Supplemental Methods, Figure S3 and Table S2). The proliferation and clonal growth of subcultured ECFCs was determined by staining the cells with crystal Violet and counted with the computer programme Image J. Clonal growth of subcultured ECFCs cultured with conditioned medium (CM) was determined by staining the cells with crystal Violet and ECFC colonies were counted (see Supplemental Methods). CB-MNCs lysates were dissolved in Laemmli buffer including β -mercaptoethanol, scraped, collected and protein levels of HIF-1 α and HIF-2 α were determined by Westernblot (see Supplemental Methods). Freshly isolated CB-MNCs were transfected with double stranded small interfering RNAs (dssiRNAs) for HIF-1 α and HIF-2 α using the AMAXA system (mononuclear factor kit) according to protocol of manufacturer (see Supplemental Methods).

STATISTICAL ANALYSIS

The data are expressed as the mean \pm SEM. Unpaired t-test (SPSS for windows 14.0, SPSS, Inc., Chicago, IL) was used to compare the number of colonies under 21% and 1% O₂, and between the counted ECFC colonies determined by different transfection conditions. Values of P \leq 0.05 (two-sided) were considered statistically significant.

RESULTS

In accordance with published data, cobblestone ECFC colonies grew under ambient oxygen levels (21% O₂) [13, 15] with a mean of 14 (range 3-28) colonies after 6-18 days (n=14) when seeded with median of 4.49×10^7 cells/ 10 cm² (range 1.27×10^7 - 2.88×10^8 cells/ 10 cm²) Table S1. These ECFC-colonies had classical cobblestone morphology and expressed VE-cadherin antigen (Figures S1 and S2), together indicative for endothelial cells (EC). Subcultured ECFCs showed indeed characteristics of *bona fide* ECs, including CD105, CD146, CD31, VE-cadherin, Tie-2, VEGFR1, uptake of acLDL and UEA binding, while CD34 expression decreased in culture, and monocyte markers (CD14 and CD45), and progenitor marker CD133 were absent (Figure S3, Table S2). Once ECFCs were expanded and further passaged under 21% O₂, the subcultured ECFCs adapted and grew well under 1% O₂. The division rate under hypoxia was slightly less ($82 \pm 5\%$) than that of subcultured ECFCs under 21% O₂, as determined after 4 or 7 days in 7 independent ECFC cultures of different donors.

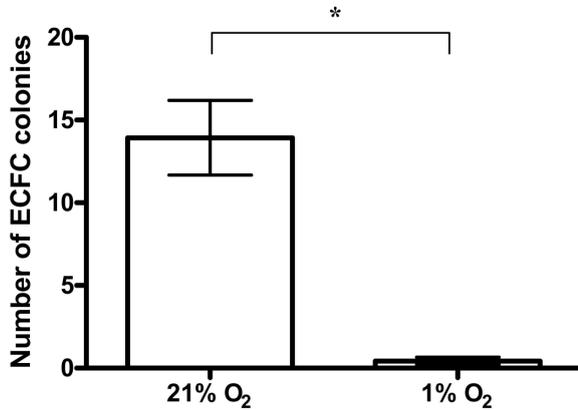


Figure 1 - ECFC colony formation under 21% and 1% O₂.

The mean number of colonies obtained under 21% and 1% O₂ culture conditions. Freshly isolated mononuclear cells were cultured in identical conditions except for the oxygen tension as described in the supplemental methods. A mean of 14 (range 3-28) ECFC colonies at 21% O₂ and virtually no (mean; 0, range 0-2) colony formation under 1% O₂ concentration. Bars reflect mean \pm SEM in ECFC colonies of 14 different cord blood samples. See supplemental table S1 for individual ECFC colony counts. * $P < 0.001$.

ARREST OF ECFC COLONY FORMATION BY HYPOXIA

As 21% O₂ is supra-physiological and 1% O₂ reflects more the condition of tissue (or tumor) where active neo-angiogenesis is needed, we anticipated that the formation of colonies of ECFCs might proceed more efficiently in 1% O₂ than at 21% O₂. However, virtually no outgrowth of ECFC colonies occurred at 1% O₂, in contrast to the 21% O₂ condition (Figure 1, Table S1). Even after 4 weeks no colonies became visible excluding a contribution of a slightly delayed ECFC growth rate at 1% O₂. Subsequently, the possibility was considered that a minimal number of ECFCs might be required to enable them to grow in 1% O₂. However, at a density of 1 cell/cm² the outgrowth of subcultured ECFCs still proceeded under 1% O₂, and resulted without delay in secondary colonies (data not shown).

NO LOSS OF VIABILITY OR ADHERENCE DUE TO HYPOXIA

To identify possible causes for loss of colony formation ability under 1% O₂ we tested whether the ECFCs lost their viability under hypoxic conditions. Therefore, CB-MNCs plated initially under 1% O₂ were transferred after 2 weeks to 21% O₂; then recovery of

Oxygen concentration during ECFC colony-culture procedure

| | |
|--------------------|--|
| 21% O ₂ | |
| 1% O ₂ | |
| ■ | D1: 21% → 1% O ₂ after 1 day |
| ■ ■ | D2: 21% → 1% O ₂ after 2 days |
| ■ ■ ■ | D3: 21% → 1% O ₂ after 3 days |
| ■ ■ ■ ■ | D4: 21% → 1% O ₂ after 4 days |

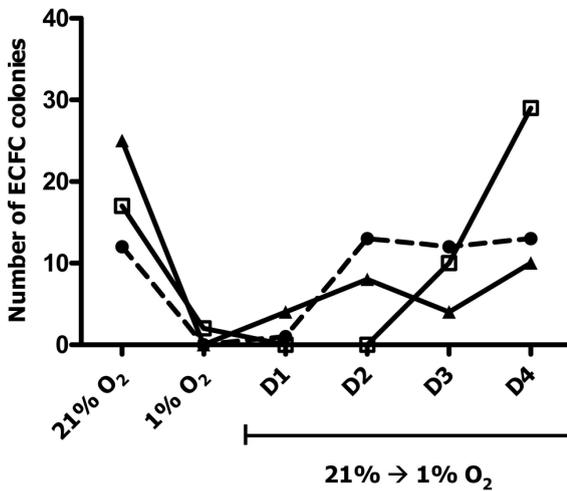


Figure 2 - Effect of prior exposure to 21% O₂ on the induction of ECFC colony formation in hypoxia.

To determine the minimally exposure time to 21% O₂ needed for overcoming the lack of colony outgrowth under hypoxia, three different CB-MNCs were isolated and refreshed as described in the ECFC culture section of the supplemental material and methods, and individual 6-well plates were transferred after 1, 2, 3 or 4 days (D1, D2, D3 and D4) from 21% O₂ to 1% O₂. Cells cultured only under 21% O₂ or 1% O₂ served as controls. Each condition contained an equal amount of CB-MNCs; ECFC colonies were quantified when the colonies had become visible in the culture under 21% O₂. Three different CB-MNC isolations showed that at least 2 days exposure to 21% O₂ is required before ECFC colony outgrowth occurred under 1% O₂. After 4 days of 21% O₂ incubation outgrowth of ECFCs under 1% O₂ was comparable to ECFC colony formation under normoxic circumstances.

ECFC colony formation occurred in 4 of 5 experiments. The colonies formed after 1% to 21% O₂ transfer were present in lower numbers compared to the initially normoxic cultures but were larger in size, and their structure was more compact. These cells had full endothelial properties. In addition, the non-adherent cells of the hypoxic cultures were collected after 3 days, re-plated on gelatin-coated wells, and colony outgrowth of ECFCs was evaluated at 21% O₂. No ECFC colonies were obtained after culture of the non-adherent cells up to 3 weeks, which excludes lack of adherence of ECFCs under 1% O₂ conditions as major cause for the hypoxic outgrowth arrest.

PRIOR EXPOSURE TO OXYGEN OVERCOMES HYPOXIA-INDUCED ECFC ARREST

To evaluate whether exposure to oxygen was essential to initiate the process of ECFC colony formation by freshly collected CB-MNC, seeding and culturing of the entire CB-MNC fraction was started at ambient oxygen (21% O₂); and after 0, 1, 2, 3, or 4 days the adherent cells were transferred to 1% O₂ atmosphere for additional culture for several weeks. Figure 2 shows that 2-3 days of 21% O₂ exposure was sufficient to initialize later outgrowth of ECFC colonies, an ability that also continued to act at 1% O₂ at a nearly similar success rate as in 21% O₂ (mean of 18 colonies under normoxia, and mean of 17 colonies after 4 days of priming at 21% O₂). Importantly, these experiments show that ECFCs are still present and viable, and that they can proliferate with full colony forming ability during hypoxia.

ROLE OF HIF-1 α AND HIF-2 α IN HYPOXIA-INDUCED ECFC ARREST

Subsequently we studied the possibility that exposure to hypoxia kept the circulating ECFCs in a quiescent condition via a HIF-dependent program. As expected in established subcultured ECFCs both HIF-1 α and HIF-2 α were highly expressed after exposure to 1% O₂. Both HIF-1 α and HIF-2 α accumulated after 6-24 hrs hypoxia and subsequently decreased slowly (Figure 3). Since circulating ECFCs represent a very rare population of cells (< 10 cells/mL of blood) in a large number of other mononuclear cells and no fully specific markers are known, it is not possible to measure HIF expression levels in the initial ECFCs. To obtain information on the effect of HIF-expression, the whole CB-MNC fraction was transfected with dssiRNAs for HIF-1 α and HIF-2 α (Figure S4 shows their efficacy). Both dssiRNAs were used, because the subcultured ECFCs contained both HIF-1 α and HIF-2 α under hypoxia and in the first experiments we observed only recovery of outgrowth with the combination of both. In three different isolates obtained from independent donors we consistently observed that HIF-1 α and HIF-2 α dssiRNAs together reversed the colony outgrowth arrest in 1% O₂ (Figure 4).

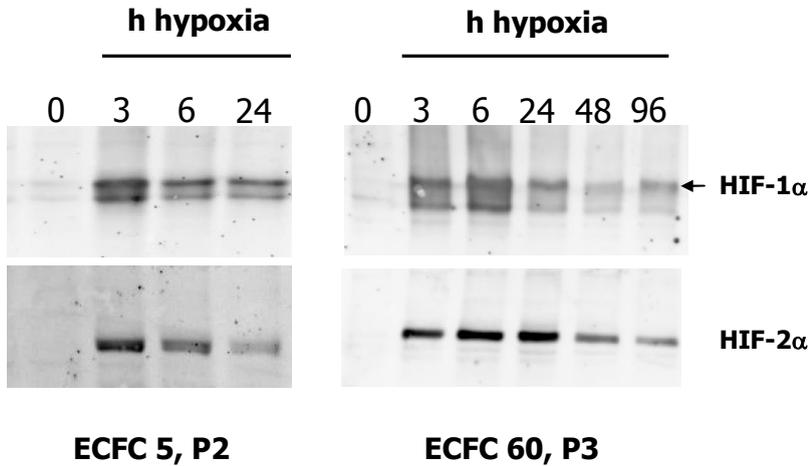


Figure 3 - Induction of hypoxia inducible factors (HIF) in subcultured ECFCs by 1% oxygen.

Expression of HIF-1 α and HIF-2 α protein in subcultured ECFCs after several hours of 1% O₂ exposure. Confluent ECFCs were detached and collected at (0), 3, 6, 24, 48 and 96 hours after the onset of exposure to 1% O₂ and proteins of the cell extracts were analyzed by Western blotting. Panels show the antigen expression of HIF-1 α (upper panels) and HIF-2 α (lower panels) for two representative ECFC cultures exposed to 1% O₂. The data are representative for 4 independent cord-blood-derived ECFCs obtained from 4 different donors.

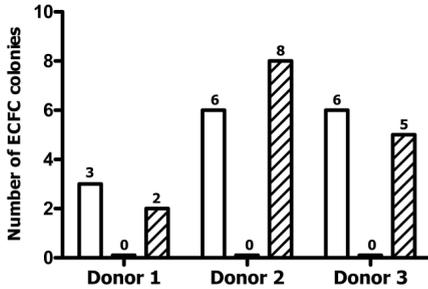
UPREGULATION OF HIF-1 α IN CB-MNCs EXPOSED TO 1% O₂

Since the whole CB-MNC population is present in the wells during the first 3 days of colony outgrowth, and is conditioning the medium during the first 3 days, any effects of hypoxia or of HIF-1 α /2 α dsiRNAs on the primary ECFCs, and thereby on ECFC colony outgrowth may also involve indirect effects of other cells present in the CB-MNC population. First, HIF-1 α and HIF-2 α protein expressions were assessed in the adherent and non-adherent CB-MNC fractions 24 hours after seeding and culture under 1% O₂. The results show that upregulation of HIF-1 α was detectable both in the adherent and non-adherent cells, while HIF-2 α protein remained undetectable (data not shown).

ROLE OF ACCESSORY CELLS IN ECFC COLONY FORMATION ARREST

Because it is not yet possible to perform direct cloning experiments on single sorted primary ECFCs, subcultured ECFCs in low density (10 cells/10 cm²) were incubated in conditioned medium of freshly isolated CB-MNCs that were cultured for 3 days either

A



B

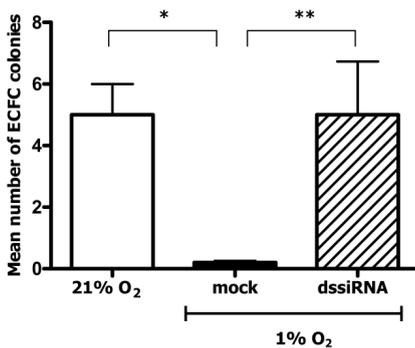


Figure 4 - Transfection with dssiRNAs for HIF-1 α and HIF-2 α induces recovery of ECFC colony outgrowth at 1% O₂.

Three independent Cord Blood-MNC (CB-MNC) fractions were each transfected with double stranded small interfering RNA (dssiRNAs) for HIF-1 α and HIF-2 α simultaneously. (A) data of 3 individual donors, (B) mean number of colonies from 3 independent experiments. Open bars represent the outgrowth of ECFC colonies under 21% O₂ (mock transfected), closed bars represent outgrowth of ECFC colony under 1% O₂ (mock transfected), and hatched bars demonstrate the CB-MNCs transfected with dssiRNAs for HIF-1 α and HIF-2 α under 1% O₂. All experiments showed a recovery of ECFC colony outgrowth after treating the total CB-MNC fractions with dssiRNAs for HIF-1 α and HIF-2 α . No colonies appeared in mock transfected cultures exposed to 1% O₂ but when the CB-MNCs were treated with dssiRNA HIF-1 α and HIF-2 α the number of ECFC colonies were equal to the number of ECFC colonies cultured under normoxia. * $P < 0.01$, $P \leq 0.05$.

under hypoxia (CM-hyp) or normoxia (CM-norm), and subsequent ECFC colony formation was evaluated. The cellular response to CM-hyp and CM-norm was identical, but varied

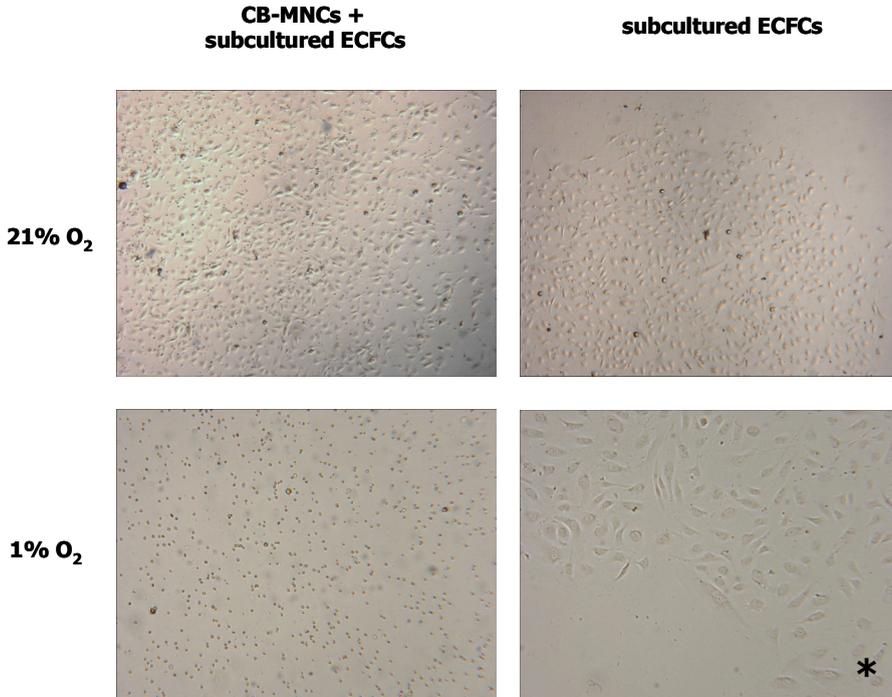


Figure 5 - Evaluation of cellular interaction between subcultured ECFCs and freshly isolated cord blood mononuclear cells (CB-MNCs) under 1% and 21% O₂.

Pictures show clonal growth of subcultured ECFCs with or without freshly isolated CB-MNCs under 1% or 21% O₂. Subcultured ECFCs in combination with CB-MNCs could only clonally grow out under 21% O₂ as compared with 1% O₂ where clonal growth was impaired. Single seeded ECFCs under 1% and 21% O₂ served as a control and showed comparable clonal growth. Pictures were taken with 2.5x magnification. * magnification of 10x.

depending on the incubation time. CM-hyp but also CM-norm collected during the first 3 days (both 50% mixed with fresh EGM culture medium) completely inhibited colony formation from subcultured ECFCs, while ECFC colony outgrowth occurred to the same extent in CM-hyp and CM-norm produced during 5-7 days of incubation (3 independent cultures; Table 1). While initially the CB-MNCs may produce growth inhibitory factors, these data do not point to the production of a secreted factor that hypoxia-specifically prevents or retards ECFC colony formation.

Subsequently, a direct cellular interaction was evaluated. To that end, small numbers of subcultured ECFCs were mixed with freshly isolated CB-MNCs and cultured under 1% or

| | Donor 5 [^] | | Donor 7 ^{^^} | | Donor 10 ^{^^} | |
|------------------|----------------------|--------------------|-----------------------|--------------------|------------------------|--------------------|
| | 1 % O ₂ | 21% O ₂ | 1 % O ₂ | 21% O ₂ | 1 % O ₂ | 21% O ₂ |
| EGM * | 1 | 8 | 42 | 48 | 30 | 23 |
| CM# day 3 | none | none | none | none | none | 3 |
| CM# day 7 | 5 | 5 | 27 | 35 | 25 | 22 |

Table 1 - Conditioned medium influences clonal endothelial colony forming cells (ECFC) growth.

Results shown represent total counted ECFC colonies per 20 cm². ECFC colonies were counted either on day 9 (donor 7 and 10) or on day 12 (donor 5). CM; conditioned medium, EGM; endothelial growth medium. * EGM; EGM complete medium as described in materials and methods section. # CM; consisted of 50% EGM + 50% CM. ^ < 20 subcultured ECFCs seeded. ^^ mean of 20 subcultured ECFCs/ 10 cm² seeded.

21% O₂ atmosphere. In the presence of CB-MNCs, clonal growth of subcultured ECFCs was only seen under 21% O₂, while secondary ECFC colony growth did not develop under 1% O₂ (Figure 5). This suggests that direct cellular interaction plays a role in keeping ECFCs quiescent.

Collectively, our data show that a very small number of circulating cells from human CB, by definition ECFCs, can form true endothelial cell colonies, as shown before by others. The outgrowth of ECFCs is sensitive to O₂ levels and is severely or completely inhibited under hypoxia (1% O₂). This growth arrest can be overcome by 2-3 days normoxic priming, showing that the ECFCs are present, viable and proliferation-competent. In addition blocking upregulation of HIF-1 α and HIF-2 α protein expression by dsiRNA overcomes the growth arrest under 1% O₂. Furthermore, accessory cells may contribute to the arrest of colony formation by subcultured ECFCs.

DISCUSSION

Here we show that primary circulating ECFCs are unable to exert their capacity to form endothelial cell colonies when the initially seeded CB-MNCs are exposed to a hypoxic condition. This unexpected phenomenon was not due to a loss of viability or impairment of the adhesion of the freshly isolated ECFCs. Since the growth arrest was overcome by combined HIF-1 α /HIF-2 α dsiRNAs transfection of the cells before initial seeding, our data suggest an important role of HIF-1 α /HIF-2 α in the growth arrest of the initial outgrowth of ECFC colonies. Furthermore, our data point to a paracrine inhibiting

interaction between CB-MNCs and ECFCs in hypoxic conditions, which was overcome after 2-3 days exposure of the cells to 21% O₂.

The observation of a hypoxia-mediated arrest of colony formation that was eliminated by 2-3 days *in vitro* exposure to a 21% O₂ conditioning is surprising, because the cells were freshly isolated from cord blood, which should provide sufficient oxygen. Because the adherent ECFCs required several days before the hypoxia-dependent inhibition was overcome, it might be reasoned that the period during which the ECFCs were in the blood was not long enough for them to switch into a proliferation-prone colony forming phenotype. This delay may also explain why these cells are "late-outgrowth" despite of their high division rate once they proliferate.

The most interesting question raised by our results is certainly why is there no outgrowth of circulating ECFCs under hypoxia, a property which would be essential for vessel repair or new vessel forming abilities under hypoxic conditions? Three possibilities might explain the outgrowth arrest of ECFC colonies under hypoxia: (1) direct retardation of ECFC proliferation by oxygen deprivation; (2) differentiation arrest of ECFCs; and (3) paracrine regulation of the initial ECFC growth by accessory CB-MNCs.

First, a considerable delay in proliferation rate of initial colonies would mask colony outgrowth of ECFCs within the time-frame of the experiments. However, if we would assume that the primary ECFCs are in fact mature endothelial cells shedded from vessels into the circulation [31], a direct growth inhibition of ECFCs by hypoxia can be excluded, since the doubling time of already established colonies was only slightly smaller under 1% O₂ than under 21% O₂. Furthermore, no primary ECFC colonies were observed after considerable extension of the evaluation period of the hypoxic wells up to 4 weeks. This strongly indicates that we could not have "missed" the colonies under hypoxia because of "simple" direct growth delay.

A second explanation for the inhibition of ECFC outgrowth might be a hypoxia-induced differentiation arrest. Several studies pointed to an important effect of oxygen tension on cell differentiation [30, 32-34]. Hypoxia reversibly arrested pre-adipocytes in an undifferentiated state [30]. HIF-1 constituted an important mechanism for the inhibition of adipogenic differentiation by hypoxia [30]. Similarly, pancreatic beta-cell differentiation is controlled by pO₂ through HIF-1 α [35]. The fact that exposure of the freshly obtained CB-MNCs to 21% O₂ for 2-3 days did overcome the inhibition of ECFC colony formation in 1% O₂ condition is in support of a putative differentiation step. Furthermore, the growth of such colonies in 21% O₂ after a 2 week period of growth arrest under 1% O₂ also indicates that the cells remained in a quiescent but viable state. Finally, the combined inhibition of HIF-1 α /HIF-2 α indicates that HIF activation plays a crucial role in prohibiting ECFCs from forming colonies. However, a more definitive conclusion on a differentiation arrest wait for procedures to prospective purify ECFCs from blood, to allow profiling the

gene expression program of primary ECFCs and comparison with ECFCs grown in culture under 1% and 21% O₂.

Thirdly, one may consider that not only ECFCs are affected by hypoxia, but also the other mononuclear cells in the initial inoculate. Therefore, ECFC colony outgrowth might be inhibited indirectly by the action of accessory cells, an inhibition that is relieved when these cells have been exposed to oxygen for several days. Although no paracrine inhibition was observed that specifically contributed to the observed arrest of ECFC colony formation in hypoxia, our experiments indicate that cell-cell interaction may play a role. Interestingly, when the cells are exposed to 21% O₂ this inhibitory effect disappears within several days, a loss of inhibition that remains if the cells are replaced to hypoxia. This may be explained by a differentiation of the accompanying cells, by which they lose their capability to inhibit ECFCs in hypoxia.

While the data are in favor of a differentiation step at 21% O₂ that overcomes the hypoxia-induced arrest of colony formation of primary ECFCs, it is at present not possible to decide whether the ECFCs themselves or accessory cells within the CB-MNC population or both are the subject of such a differentiation step. Notwithstanding, it is safe to say that the action of HIF1/HIF2 is required for the hypoxia-induced arrest of ECFC colony growth. Subcultured ECFCs produce both HIF-1 α and HIF-2 α when exposed to hypoxia. This suggests that both HIF1 and HIF2 have important roles in ECFCs outgrowth. In adherent CB-MNCs only HIF-1 α was expressed under hypoxia. Furthermore, adherent 7-day old monocyte-derived macrophages did accumulate both HIF-1 α and HIF-2 α after exposure to hypoxia [36]. Therefore, at present the HIF-1 α /HIF-2 α -dependency does not exclude paracrine inhibition of ECFC colony formation by other mononuclear cells in favor of a hypoxia-induced differentiation arrest of ECFCs.

A limiting factor for rapid progress in this area of research is the inability to obtain sufficient numbers of pure, sorted starting cells, primary circulating ECFCs. Recent studies have been able to better define on the ECFCs and shown based on marker expression that these cells can be distinguished from hematopoietic progenitor cells (HPCs) by the lack of the hematopoietic surface marker CD133 and the leukocyte marker CD45 [11-13, 15]. However, to the best of our knowledge no reproducible method is available yet to form endothelial colonies from circulating single ECFCs after purification by cell sorting. Moreover, it may be that cellular cooperation is not only involved in inhibition of colony formation under hypoxic conditions, but is also required to initiate proliferation and/or colony formation of ECFCs under 21% oxygen. If that would be the case, the circulating ECFCs differ in this respect from subcultured ECFCs which are able to propagate at clonal density, similar as mature endothelial cells.

The here presented data demonstrate that hypoxia is able to maintain circulating ECFCs in a quiescent state without decreasing their outgrowth potential. The occurrence of a

hypoxia-induced arrest of ECFC colony formation may seem counterintuitive as hypoxia is a strong angiogenesis stimulator. However, while an hypoxic tissue or tumor indeed needs neo-angiogenesis, the mere proliferation and in tissue accumulation of new endothelial cells without proper connection to the flowing blood would not be helpful and even may put demand on the limited oxygen available. In this respect one should note that neovascularization proceeds at the borderzone between the perfused vasculature and the hypoxic tissue. Therefore, one may envisage that outgrowth of ECFCs would be the most effective if the ECFCs remain in the vicinity of the circulation and help to speed up the growth of vascular sprouts from an oxygenated vessel into the hypoxic tissue. The hypoxia that we introduced artificially in our experiments may keep the ECFCs in a quiescent state until a condition occurs in which proliferation would indeed help to improve vascularization. In this respect it is of interest that down-regulation of hypoxia-induced HIF-1 α /HIF-2 α activation, either in ECFCs or in adjacent mononuclear leukocytes, overcomes the quiescent circulating ECFC phenotype under hypoxic conditions.

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