O₂ Level Controls Hematopoietic Circulating Progenitor Cells Differentiation into Endothelial or Smooth Muscle Cells

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Abstract

Background: Recent studies showed that progenitor cells could differentiate into mature vascular cells. The main physiological factors implicated in cell differentiation are specific growth factors. We hypothesized that simply by varying the oxygen content, progenitor cells can be differentiated either in mature endothelial cells (ECs) or contractile smooth muscle cells (SMCs) while keeping exactly the same culture medium.

Methodology/Principal Findings: Mononuclear cells were isolated by density gradient were cultivated under hypoxic (5% O₂) or normoxic (21% O₂) environment. Differentiated cells characterization was performed by confocal microscopy examination and flow cytometry analyses. The phenotype stability over a longer time period was also performed. The morphological examination of the confluent obtained cells after several weeks (between 2 and 4 weeks) showed two distinct morphologies: cobblestone shape in normoxia and a spindle like shape in hypoxia. The cell characterization showed that cobblestone cells were positive to ECs markers while spindle like shape cells were positive to contractile SMCs markers. Moreover, after several further amplification (until 3rd passage) in hypoxic or normoxic conditions of the previously differentiated SMC, immunofluorescence studies showed that more than 80% cells continued to express SMCs markers whatever the cell environmental culture conditions with a higher contractile markers expression compared to control (aorta SMCs) signature of phenotype stability.

Conclusion/Significance: We demonstrate in this paper that in vitro culture of peripheral blood mononuclear cells with specific angiogenic growth factors under hypoxic conditions leads to SMCs differentiation into a contractile phenotype, signature of their physiological state. Moreover after amplification, the differentiated SMC did not reverse and keep their contractile phenotype after the 3rd passage performed under hypoxic and normoxic conditions. These aspects are of the highest importance for tissue engineering strategies. These results highlight also the determinant role of the tissue environment in the differentiation process of vascular progenitor cells.

Introduction

During embryogenesis, vasculogenesis is one of the first initiated processes. Conversely in the adult, the new vessels formation is initiated from the existing blood vessel ramifications. Data accumulated in recent years indicate that the circulating mononuclear cell (MNCs) fractions contain a population of bone marrow derived cells called progenitor cells that contribute to the neovascularization of injured vessels. Different authors [1–5] suggested that these progenitor cells could differentiate in the presence of different specific cytokines and angiogenic growth factors (vascular endothelial growth factor (VEGF), platelet derived growth factor BB (PDGF-BB)...), into mature and functional endothelial (ECs) or vascular smooth muscle (SMCs) cells depending on the added specific growth factors. During wound healing, ischemia, vascular wall remodelling or tumour development, the formation of new blood vessels is preceded by the recruitment of MNCs at the injured sites which further promote vasculogenesis [6–9]. Various authors investigated also the role of the oxygen concentration on stem cells differentiation and it was shown that hypoxia increased the production of angiogenic growth factors such as transforming growth factor β1,
PDGF-BB and VEGF [10–12]. The main physiological factors implicated in cell differentiation are angiogenic growth factors (i.e., VEGF, bFGF and IGF) [2,3,13] and a decrease of the oxygen level in the tissue (hypoxia) [5]. Oxygen plays a main role in physiological and pathological states [14]; it is a potent biochemical signalling molecule with important regulation properties for cellular behaviour (migration, differentiation, proliferation...) [15–17]. However, the possible involvement of hypoxia in MNCs differentiation into SMCs has never been demonstrated and even mentioned up to now.

We hypothesized here that the only oxygen concentration tuning combined with growth factors favouring ECs differentiation (VEGF, FGF, EGF, IGF) [18] allow the differentiation of circulating progenitor cells into mature ECs or contractile SMCs, characteristic of mature vascular cells found in vivo.

We demonstrate that progenitor cells isolated from rabbit fraction cultivated onto specifically coated solid substrates (either by type I collagen: a compound of the arterial wall and known as an ideal substrate for adhesion and proliferation of vascular smooth muscle cells in vitro [2] or by a Polyelectrolyte Multilayered Film architecture which previously demonstrated an important speeding up of endothelial progenitor cells differentiation into mature and functional endothelial cells [19]) in normoxic conditions (21% O2 atmosphere or 151 mmHg) lead to mature ECs and to SMCs when cultivated in exactly the same medium but under moderate hypoxic conditions (3% O2 or 36 mmHg). Whereas it is well established that the culture of mature SMCs leads to a decrease of contractile markers associated with a pathological phenotype [20–22], we focused on SMCs-like cells obtained under hypoxia conditions and we checked the preservation of the contractile phenotype after further cell expansion (effect of passage number) and culture even under normoxic conditions.

These experiments demonstrate clearly the deterministic role of the oxygen content in vascular progenitor cells differentiation into mature functional cells constituting the vascular wall (media and intima).

Methods

1) Polyelectrolyte Multilayer Films (PEMs)

PEMs were built with cationic poly(allylamine hydrochloride) (PAH, MW = 70 kDa), and anionic polyanion (sodium-4-styrene sulfonate) (PSS, MW = 70 kDa) solutions (Sigma-Aldrich, France) as

![Figure 1. Morphological aspect of differentiated cell.](https://example.com/figure1.jpg)

Optical phase contrast microscopy visualization of differentiated cells seeded on type I collagen (A, B) and polyelectrolyte multilayer films (PEMs) (C, D) until confluence under normoxic (A, C) and hypoxic (B, D) environment. Objective ×20, scale bar 55 μm. The morphological examination of the confluent cells showed cobblestone shape (A, C) in normoxia and a spindle like (B, D) shape in hypoxia.

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previously described [19,23]. Briefly, PEMs were prepared on glass coverslips (CML, Nemours, France) pretreated with 0.01 M SDS and 0.12 M HCl for 15 min at 100°C and then extensively rinsed with deionized water. Glass coverslips were deposited in 24-well plates (Nunc, France). PAH-(PSS-PAH)₃ films were obtained by alternated immersion of the pretreated coverslips for 10 min in polyelectrolyte solutions (300 µL) at 5 mg/mL in the presence of 10 mM Tris-(hydroxymethyl) aminoethane (Tris) and 150 mM NaCl at pH 7.4. After each deposition, the coverslips were rinsed three times during 10 min with 10 mM Tris and 150 mM NaCl at pH 7.4. All the films were sterilized for 10 min by UV light (254 nm).

2) Isolation and culture of Mononuclear Cells from peripheral blood circulation

The experimental procedures were used in accordance with the “Principle of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals” (National Institute of Health publication No. 80–23, revised 1978). Blood (30 mL) was collected from white New Zealand rabbits (male, average weight 3–3.5 kg, CEGAV, France) carotid into heparinised plastic syringes. Peripheral Blood Mononuclear Cells (MNCs) were isolated using a density gradient as previously described [19]. The cells were then cultivated in endothelial basal medium (EBM-2: Lonza, Belgium) supplemented with angiogenic growth factors (EGM-2-single-

![Figure 2. Vascular cell phenotype characterization.](image)

The endothelial cell were characterized by the expression of specific markers: CD31 (A–D) and von Willebrand Factor (E–H) and the smooth muscle cells by the expression of contractile markers: α-Smooth Muscle Actin (α-SMA: E–H), Smooth Muscle Myosin Heavy Chain (SM-MHC: I–L) and Calponin (M–P). Images were obtained by confocal microscopy observation at cell confluence on both coated surfaces (type I collagen and Polyelectrolyte Multilayer films (PEMs)) and cultivated under normoxic and hypoxic conditions. Objective x40, NA = 0.8, scale bars 75 µm. The figure showed the positive expression of specific ECs markers for cell differentiated under normoxic environment and positive expression of specific contractile SMCs markers for cell differentiated under hypoxic environment.

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Cells were counted using Trypan Blue and were seeded at a density of $1 \times 10^5$ cells/cm$^2$ in 24-well plates containing glass coverslips coated either by Type I collagen 1% (BD Biosciences, France) or a PEMs films, made of PSS and PAH (Sigma, France) with a final PAH-(PSS-PAH)$_3$ architecture corresponding to 3.5 pairs of deposited PAH/PSS layers [19]. The cultures were placed in normal cell culture incubator at 37°C in an atmosphere with 5% CO$_2$ and 21% O$_2$ (O$_2$/CO$_2$ incubator, Sanyo, France). After three days, the medium was removed in order to discard unattached cells. The cells (CD34$^+$, CD133$^+$ were identified previously [19]) were then placed under hypoxia at 37°C, 5% CO$_2$ and 5% O$_2$ or under normoxia at 37°C, 5% CO$_2$ and 21% O$_2$ (control) and medium changed every two days. The differentiation and morphological evolution of the adherent cells were followed by Phase-contrast microscopy observations (Nikon DIAPHOT 300, Japan).

3) Immunostaining for smooth muscle cells (SMCs) and endothelial cells (ECs) specific markers

At confluence and after the third passage, cells were also immunolabelled against SMCs and ECs specific markers. Three antibodies were used to characterize the contractile SMCs phenotype: i) Alpha Smooth Muscle Actin (α-SMA), ii) Smooth Muscle Myosin Heavy Chain (SM-MHC) and iii) Calponin. Two other antibodies were used for the ECs phenotype: i) CD31 ii) von Willebrand factor (vWF) (all from Dako, France). Prior to the immunolabelling with the intracellular antibodies (α-SMA, SM-MHC, Calponin and vWF), the cells were fixed with paraformaldehyde (PAF) 4% (w/v in phosphate buffer saline) for 10 min and permeabilized with Triton X-100 0.5% (w/v in distilled water) for 15 min. For CD31 labelling the second step (permeabilization) was not performed. The cells were incubated for 45 min at 37°C with the primary monoclonal antibodies, diluted at 1/50 in RPMI 1640 without phenol red, containing bovine serum albumin (BSA 0.5%, w/v). After two washes with RPMI 1640, the secondary antibody labelled with Alexa-Fluor 488 diluted at 1/100 was incubated for 30 min at 37°C. The cells were observed by fluorescence confocal microscopy (LEICA DMIRE2 HC Fluo TCS 1-B, Germany) using the 488 nm spectral line.

4) Immunostaining for extracellular matrix (ECM) proteins

At confluence, hypoxia differentiated cells were immunostained for ECM proteins characterization via two specific proteins such as i) laminin and ii) type IV collagen. The differentiated cells were fixed with PAF 4% for 10 min and incubated for 45 min at 37°C with the primary monoclonal antibodies, diluted at 1/50 in RPMI 1640 without phenol red, containing 0.5% BSA. After two washes with RPMI 1640, the secondary antibody labelled with Alexa-Fluor 488 diluted at 1/100 was incubated for 30 min at 37°C. The cells were observed using fluorescence confocal microscopy (LEICA DMIRE2 HC Fluo TCS 1-B, Germany).

5) Evaluation of the maintenance of the SMCs phenotype

In order to check that after a first step of culture under hypoxia, the differentiation into SMCs was stable versus time, cells were further cultivated either under hypoxia or normoxia. After differentiation the
confluent cells cultivated on type I collagen and PEMs were amplified and separated in two batches. The first batch was kept under hypoxic condition (37°C, 5% CO2 and 5% O2) whereas the second batch was placed in normoxic conditions (37°C, 5% CO2 and 21% O2). Cells were then cultivated in these different conditions until the third passage (P3) and mature SMCs from rabbit aorta cultivated under the same conditions were used as control.

6) Fluorescence Activated Cell Sorting (FACS)

FACS analyses (EPICS XL, Beckman Coulter, France) were performed to quantify the percentage of positive cells and the fluorescence intensity of the specific contractile markers expressed by the differentiated SMCs. After P3, FACS was performed to identify intracellular antigens in cells. For that, trypsinized differentiated cells were labelled as previously described. The non-specific binding was evaluated by the incubation of cells only with the second antibody. Within the differentiated cell area, as determined by forward and sideward scattering, 10,000 events were collected and the percentage of positive cells and the mean fluorescence intensity (MFI) were determined.

7) Statistics

The data were expressed as mean±standard error of the mean (s.e.m.) for each condition. Each experiment was repeated in triplicate independently three times. Mean values were compared with the unpaired t-test (Statview IV, Abacus Concepts Inc, Berkley, CA, USA), in which p represents the rejection level of the null-hypothesis of equal means.

Results and Discussion

Peripheral blood mononuclear cells (MNCs) fraction containing progenitor cells was isolated and seeded in 24-well plates containing glass coverslips coated with type I collagen or a Polyelectrolyte Multilayer Film (PEMs) at 1×10⁶ cells/cm². We used type I collagen known as an ideal substrate for vascular progenitor cells culture [2] and PEMs for their high potentialities to boost progenitor cell differentiation [19]. After 4 days of culture in normoxic conditions, unattached cells were removed and the adherent cells (CD34+, CD133+) were divided in two batches and placed under hypoxia (5% CO2 and 5% O2) or normoxia (5% CO2 and 21% O2) until confluence (between 2 and 4 weeks). At confluence and for both surface types, the phase-contrast microscopy cell observation showed cobblestone morphology in normoxic conditions (Figure 1A, 1C) and a spindle like morphology in hypoxic conditions (Figure 1B, 1D).

In order to evaluate the cell phenotype of differentiated cells, we checked the expression of specific markers of vascular cells (SMCs and ECs) i.e. alpha-Smooth Muscle Actin (α-SMA), Smooth Muscle Myosin Heavy Chain (SM-MHC) and Calponin known to assess vascular SMCs differentiation and their contractile function [2,24,25] and CD31 and von Willebrand Factor (vWF) for the ECs phenotype evaluation [26,27]. As expected under normoxic conditions, the confocal microscopy observations showed the presence of positive cells for ECs markers [Figure 2A and 2E (for type I collagen coating), 2C and 2G (for PEMs coating)] and negative cells for SMCs markers [Figure 2I, 2M and 2O (for type I collagen), 2K, 2O and 2S (for PEMs)]. Under hypoxia a surprising positive expression of SMCs markers was observed [Figure 2J, 2N and 2R (for type I collagen), 2L, 2P and 2T (for PEMs)]. No expression of ECs markers was noticed under this condition whatever the surface coating (Figure 2B and 2F for Type I collagen), Figure 2D and 2H (for PEMs) indicating thus a total absence of cellular differentiation into ECs at a low concentration of O2. All these observations constitute a signature for the progenitor cells switching into SMCs phenotype. These results suggest first the potentiality of MNCs cells to differentiate into a SMCs phenotype under a hypoxic environment and second the expression of the specific markers confirmed the contractile phenotype of these cells [28] (similar to SMCs in vivo). In the literature the hematopoietic stem cells differentiation into mature and functional SMCs requires the culture medium supplementation with specific growth factors, especially PDGF-BB [2,3]. Our results demonstrate that the oxygen concentration tuning alone allows phenotype switch either to endothelial cells or smooth muscle cells.

The extracellular matrix (ECM) contributes to the control of the cellular function and is involved in maintaining the cells in a differentiated state [29,30]. During blood vessel formation the SMCs are responsible for extracellular matrix formation via protein (fibronectin, laminin, collagens...) secretion [31]. The ECM deposition contributes in vivo and in vitro (tissue engineering approach) to arterial wall constitution and cell function via different signalling pathways (kinase pathways activation) [31,32]. We investigated the capacity of the differentiated cells under hypoxic conditions to synthesize their own ECM, and we evaluated the secretion of two extracellular proteins (Laminin and type IV collagen), which play a major role in ECM synthesis and contribute to maintain the contractile phenotype of the differentiated cells [31]. Confocal microscopy observations showed the deposition of both of these proteins. The comparison between both surfaces showed moreover a stronger synthesis of ECM by the cells cultivated on PEMs (Figure 3). These data obtained under hypoxic conditions confirmed the capacity of MNCs to differentiate into SMCs, exhibiting a contractile phenotype, sign of a correct physiological state and integrity of the ECM. This integrity plays a key role to maintain this state and suggests stability over longer time periods.

The phenotype stability over a longer time period of the SMCs derived from MNCs cultivated under hypoxia is a major issue to use this route in tissue engineering for example. The SMCs phenotype stability was investigated at low or high oxygen concentration. After the first passage of hypoxic differentiated cells (cells positive to SMCs markers), the obtained cells were expanded under two conditions. For the first assay we maintained cells under hypoxic condition and for the second assay we placed cells in normoxic condition. In order to check the stability of the SMCs phenotype under these conditions, several passages (P3) were performed. Whatever the experimental condition (hypoxic...
Figure 5. Phenotype stability under normoxia. After the third passage, the smooth muscle cells phenotype stability of differentiated cell cultivated under normoxic conditions was investigated by confocal microscopy observation (A) and flow cytometry analyses (B, C). A: Microscopical observations show positive cells for contractile markers: \( \alpha \)- Smooth Muscle Actin (\( \alpha \)-SMA), Smooth Muscle Myosin Heavy Chain (SM-MHC) and Calponin.

B: Flow cytometry analysis showing the percentage of cells positive for Type I collagen and isotypic control.

C: Bar graph showing mean fluorescence intensity of \( \alpha \)-SMA, SM-MHC, and Calponin for control, Type I collagen, and PEM conditions.
vascular tissue engineering. We observed effectively a quite present differentiation approach allowed us to obtain a "healthy" proliferative (pathological) phenotype [34,35]. This switch constituted their phenotype from a contractile (healthy) to a

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