The International Journal of Biochemistry & Cell Biology 78 (2016) 43–51

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Low concentration of ethanol favors progenitor cell differentiation and neovascularization in high-fat diet-fed mice model

Luisa Vergori[a], Emilie Lauret[a], Raffaella Soleti[a], Maria Carmen Martinez[a], Ramaroson Andriantsitohaina[a,b,*]

[a] UMR INSERM 1063, Stress oxydant et pathologies métaboliques, Institut de Biologie en Santé, Université d'Angers, France
[b] CHU d'Angers, 4 rue Larrey, 49933 Angers cedex, France

ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 27 May 2016
Accepted 7 July 2016
Available online 10 July 2016

Keywords:
Angiogenesis
Bone marrow-derived cells
Differentiation
Ethanol
HFD diet

ABSTRACT

Endothelial progenitor cells (EPCs) and monocytic cells from bone marrow (BM) can be recruited to the injured endothelium and contribute to its regeneration. During metabolic diseases such as obesity and diabetes, progenitor cell function is impaired. Several studies have shown that moderate alcohol consumption prevents the development and progression of atherosclerosis in a variety of animal/mouse models and increases mobilization of progenitor cells. Along with these studies, we identify ethanol at low concentration as therapeutic tool to *in vitro* expand progenitor cells in order to obtain an adequate number of cells for their use in the treatment of cardiovascular diseases.

We evaluated the effects of ethanol on the phenotype of BM-derived cells from mice fed with high-fat diet (HFD). HFD did not induce changes in weight of mice but induced metabolic alterations. HFD feeding increased the differentiation of monocytic progenitors but not EPCs. Whereas ethanol at 0.6% is able to increase monocytic progenitor differentiation, 1% ethanol diminished it. Furthermore, ethanol at 0.6% increased the ability of progenitor cells to promote *in vivo* angiogenesis as well as secretome of BM-derived cells from mice fed with HFD, but not in mice fed normal diet. In conclusion, ethanol at low concentration is able to increase angiogenic abilities of progenitor cells from animals with early metabolic alterations.

1. Introduction

Endothelial progenitor cells (EPCs) represent one subset of progenitor cells derived from bone marrow (BM) multipotent adult progenitor cells. BM-derived cells also express CD133 and VEGFR-2 but lack CD34 or vascular endothelial cadherin expression (Khakoo and Finkel, 2005). *In vitro* experiments have shown that they differentiate into mature endothelial cells when grown in a serum-free media with vascular endothelial growth factor (VEGF). Lastly, the myelo/monocytic cells, also derived from the BM, can differentiate into EPCs (Khakoo and Finkel, 2005). The myelo/monocytic cells express CD14 on their surface and form mature endothelial cells positive for vWF, VEGFR-2, and CD45 (common leukocyte antigen) expression when cultured.

Regarding the function of these cells, BM-derived cells can participate not only in physiological repair but also in pathological neovascularization. Indeed, abnormal BM-derived EPC biology has been most characterized in diabetes mellitus (Fadini et al., 2006) and in metabolic syndrome (Fadini et al., 2009; Devaraj and Jialal, 2012) where the number and function of progenitor cells are reduced. Recently, potential therapeutic applications of BM-derived cells are extensively investigated in animal models as well as in patients with several types of diseases. In particular, isolated BM-derived cells may be used as therapeutic tools after *in vitro* treatment with soluble factors in order to enhance their weak ability to expand and differentiate in EPCs (Sen et al., 2011). For instance, BM-derived cells pretreated with estrogens, statins and GM-CSF display increased ability to repair endothelial denudation (Strehlow et al., 2003; Werner et al., 2002; Cho et al., 2003).

It is well known that moderate intake of ethanol possesses protective effect on survival among individuals at high cardiovascular risk (Vasdev et al., 2006). Several mechanisms have been proposed to explain the beneficial effects of alcohol intake on vascular

Abbreviations: BMB, one marrow; EtOH, ethanol; HFD, high fatdiet; EPCs, Endothelial progenitor cells.

* Corresponding author at: INSERM, U1063, Stress oxydant et pathologies métaboliques, Institut de Biologie en Santé Rue des Capucins, Angers F-49100, France.
E-mail address: ramaroson.andriantsitohaina@univ-angers.fr (R. Andriantsitohaina).

http://dx.doi.org/10.1016/j.biocel.2016.07.004
1357-2725/

events, including its inhibitory effects on hypercholesterolemia, insulin intolerance, oxidative stress and inflammation (da Luz and Coimbra, 2004). Ethanol has been shown to have direct effects on the endothelium to affect vascular functions in adults (Tawakol et al., 2004) and can modulate the expression of molecules such as VEGF in the context of angiogenesis (Gu et al., 2001).

Previous studies have shown that moderate doses of alcohol (10 mg/day) protect against the development of atherosclerosis and increase the level of growth factors that promote vascular remodeling (*i.e.* SDF-1, VEGF) with subsequent enhance mobilization of progenitor cells. In addition, *in vitro*, it is demonstrated that alcohol exerts an effect on SDF-1 secretion. Indeed, there is an inverse correlation between the increased SDF-1 secretion from fibroblasts and the reduced concentration of ethanol (from 1.6% to 0.1) (Gil-Bernabe et al., 2011). However, the effects of ethanol on the production and mobilization of EPCs and endothelial cells remain unclear.

The goal of the study is to identify an expansion technique that aim to facilitate significant increase differentiation capacities of BM-derived cells toward endothelial phenotype without negatively affecting their therapeutic potential. Therefore, we designed this study to test whether ethanol at low concentrations can reprogram BM-derived cells and increase angiogenic ability of these cells in high fat diet (HFD)-fed rodent model.

2. Materials and methods

2.1. Animal model

All animal studies were carried out using approved institutional protocols (CEEA.2012.125) and were conformed the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male C57BL/6N (20 week-old) are used. They were housed at an ambient temperature of $23 \pm 2\,^{\circ}C$ under a 12 h light/12 h dark cycle (lights on, 8:00 AM). The animals were allowed free access to water and standard diet or high-fat diet (HFD, 37% Kcal from fat, 47.5% from carbohydrates, SAFE, Augy, France) for 12 weeks.

2.2. Biochemical analyses

Mice were anesthetized by isoflurane for all procedures. At sacrifice, after an overnight fast (18 h), blood samples were gathered by cardiac exsanguinations and plasma was collected and stored at $-80\,^{\circ}C$ until analysis. Serum glucose, triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol profiles were determined by spectrophotometry UV/visible Roche/Hitachi Modular P (Roche Diagnostics, Mannheim, Germany) using enzyme-linked immunosorbent assay and Konelab™/T Series according to the protocol of the manufacturer. Serum insulin levels were measured by ELISA kit (Mouse insulin Elisa kit, Millipore, Billerica, MA; ref: EZRMI-13K) following the manufacturer's instructions.

2.3. Isolation, culture and characterization of EPCs

Femurs and tibiae were removed from each mouse, cleaned of muscle and connective tissue, and ~1 mm of the end of each bone was removed. BM-derived cells were obtained by isolating mononuclear cells from BM using Histopaque H1083 (Sigma-Aldrich) density-gradient centrifugation. Briefly, immediately after isolation, total mononuclear cells (10^6 cells/cm^2) were plated on culture dishes coated with fibronectin (BD Biosciences, San Jose, CA; 10 μg/mL) and maintained in EGM-2 endothelial medium Bullet Kit system (Lonza, Walkersville, MD) consisting of endothelial basal medium supplemented with 5% foetal bovine serum, VEGF, fibroblast growth factor 2 basic (bFGF-2), epidermal growth factor, insulin-like growth factor, ascorbic acid, gentamicin sulphate amphotericin, hydrocortisone and heparin. After 24 h, non-adherent cells were removed by washing with PBS, and adherent cells were cultured in EGM-2 endothelial medium in the absence or in the presence of varying concentrations of ethanol.

The concentrations of ethanol used in the present study, *i.e.* 0.6% (20 mM) and 1% (34 mM), are levels that do not cause significant intoxication *ex vivo*. As previously performed by Chen et al. (1999), brief exposure of isolated adult rat cardiomyocytes to 10–50 mM ethanol induced cardioprotective effects.

To analyse cell phenotype, characterization of isolated cells after 7 days of culture was performed. Labelled cells were analysed by flow cytometry using the following antibodies: anti-CD14-FITC, anti-Ly-6A/E (Sca1)-PE/Cy7, anti-Flk1 (VEGFR2)-PE, (BioLegend, San Diego, CA). Irrelevant mouse IgGs were used as isotype-matched negative controls. Samples were analysed in a flow cytometer 500 MPL system (Beckman Coulter, Villepinte, France).

2.4. Proliferation assay

Effects of ethanol on proliferation of BM-derived cells were analysed by using CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Briefly, 5×10^3 cells per well were seeded into 96-well plates and allowed to attach overnight and then cells were treated with ethanol (0.6% or 1%) for 24 h and 48 h. After growth medium removal, dye-binding solution was added into each microplate well and cells were incubated at 37C for 30 min. The fluorescence levels were read on a fluorescent microplate reader (Synergy HT, Biotek) with filters for 485 nm excitation and 530 nm emission.

2.5. Cell cycle and apoptosis analysis

10^6 cells/well were seeded and treated with ethanol (0.6% or 1%) for 7 days. Cells were then trypsinized, washed in PBS, fixed with 70% ethanol at $4\,^{\circ}C$ overnight. The cells were first incubated with RNAse A (50 μg/mL, Sigma Co. St. Louis, MO) at $37\,^{\circ}C$ for 10 min and then labelled with propidium iodide (Sigma-Aldrich, 0.1 mg/mL) and incubated at room temperature in the dark for 30 min. DNA content was then analysed by flow cytometry.

2.6. In vivo *matrigel plug assay*

For *in vivo* studies of angiogenesis, BM cells were isolated from mice fed with normal chow. Then, they were treated either with solvent, 0.6% or 1% ethanol for 7 days. After treatment, cells were detached and were mixed with 500 μL of Matrigel (ECMgel® Sigma-Aldrich) with recombinant bFGF-2 (300 ng/mL, Peprotech, Rocky Hill, NJ). This mixture was injected subcutaneously on the back of mice fed with normal chow. The same experimental protocol was conducted using BM cells isolated from mice fed with HFD injected in HFD mice. At day 7, Matrigel plugs were removed and divided into two blocks, one homogenized in lysis buffer and incubated for 24 h at $4\,^{\circ}C$ and then, disrupted with a Polytron (PRO250, Monroe, CT), and another directly frozen in OCT embedding compound (Tissue-Tek) for immunohistochemistry.

Hemoglobin levels were determined using Drabkin's reagent (Sigma-Aldrich) according to manufacturer's instructions.

2.7. Immunohistochemistry of matrigel plugs

Frozen Matrigel plugs sections (30 μm thickness) were prepared using a Leica CM3050 S cryostat (Leica, Rueil-Malmaison, France) and then briefly fixed with 4% PFA. Slides were incubated with rat anti-mouse CD31 antibody (BD Biosciences, dilution 1:100) followed by incubation with goat anti-rat IgG-FITC (Southern