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Endothelial colony-forming cells: Biological and functional abnormalities in patients with recurrent, unprovoked venous thromboembolic disease

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ABSTRACT

Introduction: Endothelial cells (ECs) are an important component of the blood coagulation system because it maintains blood fluid. Because in patients with venous thromboembolic disease (VTD) a thrombophilic condition is not found sometimes, we investigated if endothelial colony-forming cells (ECFCs) from these patients have biological and functional abnormalities.
Patients and methods: Human mononuclear cells (MNCs) were obtained from peripheral blood from patients with VTD and controls to obtain ECFCs. These cells were assayed for their immunophenotype and electron microscopy characteristics and their ability to form capillary-like structures and to produce pro-inflammatory and pro-angiogenic cytokines and reactive oxygen species (ROS).
Results: ECFCs appeared at 7 and 21 days of culture in VTD patients and controls, respectively. ECFCs increased 8-fold in patients and emerged 1 week earlier. No differences in the size of the colonies of ECFCs were found. Numbers and time of appearance of ECFCs was different between groups. ECFC-derived ECs (ECFC-ECs) of both groups expressed CD31, CD34, CD146, and CD-309 but none expressed CD45, CD14, or CD90. Interest CD34 was highly expressed in ECFC-ECs from patients. In both groups, ECFC-ECs showed similar capacity to form capillary-like structures but ECFC-ECs from patients had significant abnormalities in the mitochondrial membrane. We found a significant increase in ROS production in ECFC-ECs from patients. There were significant differences in cytokine profiles between VTD patients and controls.
Conclusions: We found a dysfunctional state in ECFC from VTD patients resembling some characteristics of dysfunctional ECs. These findings may help to understand some pathophysiological aspects of VTD.
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1. Introduction

The term "venous thromboembolic disease" (VTD) encompasses two previously separate clinical entities, deep venous thrombosis and pulmonary embolism. VTD is currently considered a public health problem. It is estimated that VTD occurs in 350,000 to 600,000 persons per year in the U.S. and that >250,000 of them experience a first thrombotic episode [1]. The number of incident and recurrent VTD events is estimated at >1 million/year [2]. Beyond the initial risk of death estimated to be >30% within the first 30 days after the event, a significant

Abbreviations: ECs, endothelial cells; VTD, venous thromboembolic disease; ECFCs, endothelial colony-forming cells; ROS, reactive oxygen species; ECFC-ECs, ECFC-derived endothelial cells; MNCs, mononuclear cells; PBS, phosphate-buffered saline; FBS, fetal bovine serum; SCF, stem cell factor; FLT-3, free light ligand-3; GM-CSF, granulocyte-macrophage colony stimulating factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; VEGF, vascular-endothelial growth factor; IL-1β, interleukin 1β; MIP-1, monocyte inhibitor protein 1; TNF-α, tumor necrosis factor-α; TPO, thrombopoietin; IL-3, interleukin 3; IL-6, interleukin 6; IFN-γ, interferon gamma; HUVECs, human umbilical vein endothelial cells; EPCs, endothelial progenitor cell; HPP-ECFCs, high proliferative potential ECFC; ICAM-1, intercellular adhesion molecule; VCAM-1, vascular cell adhesion molecule-1; VWF, von Willebrand factor; DVT LL, deep venous thrombosis of the left leg; DVT RL, deep venous thrombosis of the right leg; PE, pulmonary embolism; MFI, mean fluorescence intensity.

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percentage of surviving patients develop recurrent thrombosis or long-term morbidity associated with the post-thrombotic syndrome [3,4].

Although it has recently been suggested that susceptibility for VTD may be governed by a complex interplay of gene expression, inflammation, and other processes [5], major risk factors associated with VTD are always related to hypercoagulability, blood flow stasis, and vascular injury [6]. Significant advances have been achieved in terms of recognizing new VTD risk factors; however, in a high number of patients, laboratory abnormalities or a thrombophilic pattern are never found. Therefore, the search for new thrombophilic conditions is highly desirable.

Endothelial cells (ECs) represent an important component of the blood coagulation system because they are mainly responsible for maintaining the blood in a fluid state. This function is achieved due to the production of antiplatelet, anticoagulant, and fibrinolytic substances. Therefore, if ECs dysfunction is present, the antithrombotic potential of ECs significantly falls while the risk of thrombosis increases.

Because the role of biological and functional abnormalities of ECs in the pathogenesis of VTD remains incompletely characterized [7], we investigated if endothelial colony-forming cells (ECFCs) [8] from patients with VTD displayed biological and functional abnormalities as compared with cells from healthy individuals.

2. Patients and methods

2.1. Cell collection and sample processing

All patients had a history of recurrent (>3 VTD episodes), unprovoked VTD. The last thrombotic episode must have been recorded at least 6 months prior to study initiation. Upon enrollment, all patients were under anticoagulant therapy either with vitamin K antagonists or rivaroxaban at the accepted doses. At entry, none patient received aspirin or statins and D-dimer was always negative. Exclusion criteria were the presence of known neoplastic disease, diseases affecting the immune system, pregnancy, and an ongoing infectious disease. In all women, blood collection was performed during menstruation.

2.2. Preparation of MNCs

One hundred ml of peripheral blood was collected in glass tubes containing 1000 IU of heparin from VTD patients and blood donors (Controls). MNCs preparation was performed as described with minor modifications [8]. Blood was diluted 1:1 (vol:vol) using phosphate-buffered saline (PBS) (Invitrogen, Grand Island, NY). This diluted blood was collocated on Ficoll–Paque Plus (density = 1.077) (Pharmacia Biotech, Uppsala, Sweden) (1:1, vol:vol) and centrifuged for 30 min at room temperature at 740 g in order to obtain low-density MNCs. Once obtained, low-density MNCs were re-suspended in PBS supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 2% penicillin/streptomycin (Gibco, Grand Island, NY), and gentamycin 0.25 μg/ml (Gibco). The number of nucleated and viable MNCs were determined using a Neubauer chamber using Turk's solution and trypan blue stain, respectively.

2.3. Culture of ECs from patients with VTD and controls

MNCs were re-suspended in EGM-2 medium (Lonza, Walkersville, MD, USA), fully supplemented with EGM-2 SingleQuots (Lonza), and seeded in three separate wells using a 6-well tissue culture plate pre-coated with type 1 rat tail collagen (BD Biosciences, Bedford, MA, USA) at 37 °C and 5% CO_2, in a humidified incubator. After 24 h of culture, non-adherent MNCs and debris were aspirated. Adherent cells were washed once with PBS and then 2 ml supplemented EGM-2 medium was added to each well. Medium was changed daily until colonies of ECs appeared. Colonies were identified as well-circumscribed monolayers of cobblestone-appearing cells. These ECFC-ECs were counted by visual inspection using an inverted light microscope (CKX41SF, Olympus Corporation, Tokyo, Japan) under 4× and 10× magnification. ECFC-ECs were released from the original plates with clonation rings (BEL-ART Products, Pequannock, NJ, USA) using trypLE™ Express (Invitrogen, Denmark). The collected cells were re-suspended in complete EGM-2 media and reseeded in 6-well tissue culture plates and 75 cm^2 tissue culture flasks plates pre-coated with type 1 rat tail collagen (BD Biosciences) in further passages.

2.4. Matrigel assays

They were performed as described with minor modifications [9]. Early-passage ECFC-ECs (<3 passages) were seeded in 48-well tissue culture plates coated with 100 μl Matrigel (BD Biosciences) at a cell density of 100,000 cells/well. Cells were analyzed after 24 h using light microscopy at 40× magnification in order to search for capillary-like structures. We used the Image J Program in order to quantify the tubes formed.

2.5. Immunophenotyping of ECFC-ECs

Early-passage ECFC-ECs (5×10^4) were incubated at room temperature for 15 min at different concentrations of the primary antibody (as outlined below) in 200 μl PBS (Invitrogen) and 2% FBS (HyClone). Cells were washed twice and analyzed by means of fluorescence-activated cell sorting (FACS) (FACS Calibur, Becton Dickinson, USA). We used monoclonal antibodies (Bioscience, San Diego, CA, USA) unless otherwise indicated including a FITC-conjugated anti-human anti-CD14 murine monoclonal antibody, a FITC-conjugated mouse anti-human anti-CD45 monoclonal antibody, a FITC-conjugated mouse anti-human anti-CD146 monoclonal antibody, an allophycocyanin (APC)-conjugated mouse anti-human anti-CD31 monoclonal antibody, an APC-

Table 1
General characteristics of controls and patients with VTD.

	Controls(n = 10)	Patients with VTD (n = 21)			P
		Male (n = 9)	Female (n = 12)	All (n = 21)	
Age (years) (mean, range)	41 (24–48)	41 (28–52)	43 (29–54)	43 (28–54)	NS
Male:female ratio	5:5			9:12	
Family history of VTD (n, %)	0	6 (66)	9 (75)	15 (71.4)	
Age at first VTD episode (mean, range)		21 (17–37)	23 (16–33)	22 (16–37)	NS
Number of VTD events (mean, range)		39 (4, 2–7)	50 (4, 2–6)	89 (4, 2–7)	NS
Type of VTD events (n, %)		39	50	89	
DVT LL		19 (48.7)	22 (44.0)	41 (46.1)	NS
DVT RL		11 (28.2)	14 (28.0)	25 (28.1)	NS
Bilateral DVT		4 (10.2)	6 (12.0)	10 (11.2)	NS
DVT + PE		3 (7.7)	5 (10.0)	8 (9.0)	NS
PE		1 (2.6)	2 (4.0)	3 (3.4)	NS
Other		1 (2.6)	1 (2.0)	2 (2.2)	NS

VTD: venous thromboembolic disease; DVT LL: deep venous thrombosis of the left leg; DVT RL: deep venous thrombosis of the right leg; PE: pulmonary embolism; NS: not significant.

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