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The atheroma plaque secretome stimulates the mobilization of endothelial progenitor cells *ex vivo*

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ABSTRACT

Endothelial progenitor cells (EPCs) constitute a promising alternative in cardiovascular regenerative medicine due to their assigned role in angiogenesis and vascular repair. In response to injury, EPCs promote vascular remodeling by replacement of damaged endothelial cells and/or by secreting angiogenic factors over the damaged tissue. Nevertheless, such mechanisms need to be further characterized. In the current approach we have evaluated the initial response of early EPCs (eEPCs) from healthy individuals after direct contact with the factors released by carotid arteries complicated with atherosclerotic plaques (AP), in order to understand the mechanisms underlying the neovascularization and remodeling properties assigned to these cells. Herein, we found that the AP secretome stimulated eEPCs proliferation and mobilization *ex vivo*, and such increase was accompanied by augmented permeability, cell contraction and also an increase of cell-cell adhesion in association with raised vinculin levels. Furthermore, a comparative mass spectrometry analysis of control *versus* stimulated eEPCs revealed a differential expression of proteins in the AP treated cells, mostly involved in cell migration, proliferation and vascular remodeling. Some of these protein changes were also detected in the eEPCs isolated from atherosclerotic patients compared to eEPCs from healthy donors.

We have shown, for the first time, that the AP released factors activate eEPCs *ex vivo* by inducing their mobilization together with the expression of vasculogenic related markers. The present approach could be taken as a *ex vivo* model to study the initial activation of vascular cells in atherosclerosis and also to evaluate strategies looking to potentiate the mobilization of EPCs prior to clinical applications.

Abbreviations: AP, atherosclerotic plaque; eEPCs, early endothelial progenitor cells; KDR/VGFR2, vascular growth factor receptor 2; ox-LDL, oxidized low-density lipoprotein; GM-SCF, granulocyte macrophage colony-stimulating factor; SDF-1, stromal cell-derived factor 1; MA, mammary arteries; PBMCs, peripheral blood mononuclear cells; FBS, Fetal bovine serum; UEA-1, *Ulex europaeus* agglutinin-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HMOX1, heme oxygenase 1; VKORC1, vitamin K epoxide reductase complex subunit 1; KCD12, BTB/POZ domain-containing protein KCTD12; UBE2I, ubiquitin conjugating enzyme E2I; PARP1, poly(ADP-ribose) polymerase 1; MCTS1, malignant T-cell amplified sequence 1; AIMP1, aminoacyl TRNA synthetase complex-interacting multifunctional protein 1; SMVC, smooth muscle vascular cells; MMP2, matrix metalloproteinase-2; LPA, lipoprotein A; MIF, macrophage inhibitory factor; CORO1B, coronin 1B; ITGAX, integrin alpha X/CD11c.

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1. Introduction

Since the discovery of endothelial progenitor cells (EPCs) in 1997 [1], these cells have become potential candidates for therapeutic applications pursuing tissue revascularization due to their assigned vascular regenerative properties. They seem to play a pivotal role in the pathogenesis of atherosclerosis and arterial healing after injury [2], by actively participating in vascular repairing, promoting angiogenesis and maintaining a thrombo- and inflammatory resistant surface [3].

Several cardiovascular risk factors, including diabetes, hypertension and hypercholesterolemia, have devastating impacts on EPCs [4]. In atherosclerosis itself, a decrease in the number of circulating EPCs associates with the development of disease [5]. In addition, known protective factors against atherosclerosis have been correlated with increased numbers of EPCs, such as high-density lipoprotein, estrogen, statins and angiotensin II inhibitors [6]. Moreover, CD34, KDR and c-Kit positive cells (characteristics of EPCs) have been detected in human atherosclerotic lesions, suggesting their involvement in the remodeling process [7].

Recent research has highlighted the need to discriminate between several subsets of EPCs, depending on the differentiation status or the capability to form colonies [8–10]. Thus, circulating pro-angiogenic cells, also named "early" endothelial progenitor cells (eEPCs), have been identified in the adult human peripheral blood as cells expressing the CD34 progenitor cell marker [11]. These cells derive from hematopoietic progenitors and share phenotypic traits with monocytes such as CD14 expression. eEPCs are able to promote neovascularization into ischemic tissues [1–3], and participate in vascular homeostasis, contributing to cell turnover by homing in sites with damaged endothelium, where they replace damaged endothelial cells and protect vessels from injury. Moreover, these cells seem to exert their proangiogenic and vasoprotective function mainly in a paracrine fashion by secreting angiogenic hemocytokines [11].

Many studies have focused on the analysis of how eEPCs become activated and how they participate in the neovascularization process. The goal is to be able to manipulate them, increasing their mobilization and other related functions, and therefore to use them for cell therapy purposes. *In vitro* assays have shown that eEPCs are susceptible to inflammatory and pro-atherogenic factors such as ox-LDL, GM-CSF and SDF-1, which thereby augment the neovascularization of ischemic tissues [12,13]. All these assays have evaluated individual or combined inflammatory and atherogenic factors but, to our knowledge, the effect of directly released factors from atherosclerotic plaques (AP) onto eEPCs has not been evaluated. Accordingly, the development of an experimental model to study the molecular and cellular mechanisms by which the AP interacts directly with the eEPCs is of great interest, and it could provide new insights about their physiological or pathological relevance [14].

In the current work we describe a new approach to evaluate the initial response of eEPCs following atherosclerotic damage, by *ex vivo* incubation of eEPCs derived from healthy individuals with the AP secretome obtained from atherosclerotic patients undergoing carotid endarterectomy. Since we first demonstrated that the AP secrete proteins to the surrounding media [15], different approaches have been carried out in which several proteins have been identified in the AP secretome as potential markers of atherosclerosis [16,17] and the effect of different drugs on the secretome composition has been evaluated [18]. Ultimately, the AP secretome constitutes a rich source of information to understand de atherosclerotic phenomenon since it represents the first factors that plaques send to the circulating media as a sign of damage. Herein, we show how eEPCs are activated and mobilized in response to initial contact with the molecules secreted by the APs to the circulating media.

2. Materials and methods

A detailed description is available in the Supplementary material online.

2.1. Sample acquisition

eEPCs were isolated from the blood of healthy donors and atherosclerotic patients undergoing carotid endarterectomy. Carotid artery segments complicated with AP from the same patients were also collected. In addition, non-atherosclerotic mammary arteries (MA) were obtained from patients undergoing cardiac valve replacements. All volunteers provided informed consent prior sample collection. This study was approved by the local Ethic Committee, and it follows the principles outlined in the Declaration of Helsinki.

2.2. eEPCs isolation and culture

eEPCs were isolated from peripheral blood mononuclear cells (PBMCs) following standard procedures, as described [19]. Mononuclear cells were plated in fibronectin coated plates (10 μg/ml) and incubated in 2% FBS/EBM-2V media containing Single Quots growth factors (basal medium). After 4 days, non-adherent cells were discarded by removing cell media and attached cells were allowed to grow until day 7 in fresh media. Different sets of isolated eEPCs were used for the assays. By day 7, cells were confirmed as early EPCs by flow cytometric analysis, UEA-1 lectin labeling and DiI-acLDL up-take, as described [20]. Detailed description of cell isolation and culture are provided in the Supplementary material online.

2.3. Atherosclerotic plaque isolation and culture

Carotid endarterectomies complicated with AP and MA taken as non-atherosclerotic controls were cultured as described [15,21]. Briefly, samples were dissected with a scalpel, separating the non-complicated area, the area complicated with atheroma plaque but not thrombosed region and the ruptured and thrombosed region of the carotid material, as described [15]. For this study, we used the secretome of the complicated but not thrombosed segments, comparing it with the secreted factors of mammary arteries, taken as controls. The surgical pieces (about 0.3–0.4 cm length and 200 mg weight on average) were washed several times with RPMI-1640 medium containing Penicilin/Streptomycin and cultured for 72 h before collecting the supernatants, which were either used immediately or stored at −80 °C.

2.4. Viability and proliferation assays

At day 6, 5×10^4 cells were transferred to fibronectin pre-coated 96-well plates in 2%FBS medium. By day 7, cells were incubated with either fresh medium (eEPCs cont) or a mixture (1:1) of basal medium and AP (eEPCs + AP) or MA (eEPCs + MA) supernatants. Cell viability was measured 48 h and 72 h later by adding 20 μl MTT (5 mg/ml in PBS) and incubating for another 4 h at 37 °C, 5% CO_2. Cells were finally incubated with DMSO for 10 min before measuring optical density (OD).

eEPC proliferation in response to the AP secretome was measured by immunofluorescence staining of the proliferation marker KI67 [22]. Cells, 5×10^5 were plated by day 6 in fibronectin pre-treated coverslips into 24 well plates and incubated at day 7 with either 700 μl of basal media (eEPCs cont), or a mixture (1:1) of AP (eEPCs + AP) or MA secretome (eEPC + MA) with basal medium. After 48 h, supernatants were discarded and cells fixed with 4% PFA, and incubated with anti-human KI67 and the secondary Alexa 488- antibodies followed by nuclei staining with DAPI. Experiments were performed in duplicate for each condition and repeated 3 times with AP or MA secretomes and eEPCs control cells from different individuals (n:6). Quantification of KI67 positive staining was done manually (ten random fields per insert) under a fluorescence microscope.