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Conditioned medium from human umbilical vein endothelial cells markedly improves the proliferation and differentiation of circulating endothelial progenitors

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ABSTRACT

Circulating endothelial progenitor cells (EPCs) have been suggested as a precious source for generating functionally competent endothelial cells (ECs), candidate for various clinical applications. However, the paucity of these progenitor cells and the technical difficulties for their *in vitro* growth represent a main limitation to their use. In the present study we hypothesized that the paracrine effects of human umbilical vein endothelial cells (HUVECs) may improve endothelial cell generation from cord blood (CB) EPCs. In line with this hypothesis we showed that HUVEC conditioned medium (CM) or co-culture with HUVECs markedly improved the proliferation and differentiation and delayed the senescence of CB EPCs.

The endothelial-promoting effect of CM seems to be related to smaller vesicles including exosomes (sEV/exo) contained in this medium and transferred to CB $CD34^+$ EPCs: in fact, purified preparations of sEV/exo isolated from CM mimicked the effect of CM to sustain endothelial formation. These observations provided the interesting indication that mature ECs exert a stimulatory effect on endothelial cell differentiation from $CD34^+$ cells.

1. Introduction

Studies carried out during the last years have made a great effort in trying to identify, define and characterize cell populations with phenotypic, biochemical, molecular and functional properties of endothelial progenitors cells (EPCs) (reviewed in [1] and [2]). These studies have led to the identification in hematopoietic tissues (blood, bone marrow and cord blood) of very rare EPCs, called endothelial colony-forming cells (ECFCs) or late outgrowth endothelial cells, not issued from bone marrow, capable of generating *in vitro* a large progeny of phenotypically and functionally competent mature endothelial cells (ECs), capable of *in vitro* extensive growth and of sustaining *in vivo* angiogenetic processes (reviewed in [1] and [2]). These EPCs are also present at the level of the vessel wall, particularly in the vascular endothelial intima (reviewed in [1] and [2]). These true EPCs must be distinguished from a population of hematopoietic progenitors capable of generating monocytic cells, unable to generate an endothelial cell progeny, but exerting a pro-angiogenetic effect, mediated by a paracrine mechanism which involves the secretion of various factors and cytokines stimulating angiogenesis; these cells are now called circulating angiogenic cells (CACs) (reviewed in [1] and [2]).

The original method for growing ECFCs was initially described by Ingram and coworkers and consisted in growing mononuclear cells isolated from bone marrow or cord blood (CB) in complete EGM2 medium on collagen-coated dishes: after 1 to 2 weeks a population of adherent cells starts to be detectable, forming endothelial colonies [3,4]. These cells have a high proliferative potential and can achieve at least 100 cell doublings *in vitro* when grown in the same initial medium [3,4].

Martin-Ramirez and coworkers developed a protocol derived from the methodology described by Ingram and coworkers [3,5] and based on the plating of peripheral blood mononuclear cells resuspended in EGM2 medium in tissue cultures coated with type I collagen at 135,000 cells/cm^2 [5]. When plated in this culture condition, mononuclear cells form foci of ECs after 14–28 days [5]. These cells were called blood outgrowth endothelial cells (BOECs) and are comparable to those obtained by other investigators using the same culture conditions [5]. Using this culture system, Joo et al. reported the generation from adult peripheral blood mononuclear cells of human ECs characterized by a peculiar phenotype compared to HUVECs (higher DLL4, VCAM1, CXCR4 and CD34 expression) and by enhanced sprouting angiogenic potential *in vitro* and *in vivo* through up-regulation of the VEGFR-2 signaling pathway [6]. However, other studies have suggested that endothelial progenitor cells derived from CB have a higher *in vivo* potential to form functional long-lasting vessels that EPCs derived from adult peripheral blood [7].

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Cell fractionation studies have shown that ECFCs originate from rare $CD34^+CD45^-$ cells, while $CD34^+CD45^+$ progenitors are totally unable to generate ECs *in vitro* [8]. It was suggested that vascular endothelium and not marrow is the tissue of origin of these rare progenitors [9].

In view of possible clinical applications, this culture system was modified by replacing the fetal bovine serum present in the complete medium with 10% pooled human platelet lysate (pHPL), providing evidence that this humanized system was suitable to obtain ECFCs, capable of achieving more than 30 population doublings [10].

Recent studies have shown that NOTCH signaling is critical for promoting the survival and the vasculogenic activity of CB-derived ECFCs [11]. In parallel, other studies by the same authors provided evidence that human platelet lysate exerts an effect similar to that elicited by NOTCH activation, improving ECFC survival and vasculogenesis in three dimensional collagen matrices [12].

Virtually all the studies carried out on ECFCs involved the use of populations of unpurified cord blood or bone marrow or peripheral blood (total mononuclear cells) and there is evidence that the optimal generation of ECs from $CD34^+$ EPCs requires the presence of accessory $CD34^-$ cells secreting angiogenic cytokines [13].

In the present study we have explored the capacity of the ECFC cell culture system [3,4] to generate ECs starting from purified human CB $CD34^+$ cells. Our results showed that the standard EGM2-based cell culture system had a limited capacity to generate ECs from purified $CD34^+$ cells and its efficiency is consistently improved by adding HUVEC-derived cell culture conditioned medium (CM) or by co-culturing $CD34^+$ cells with HUVECs. Since previous studies have shown that HUVECs naturally produce exosomes that contain proteins able to stimulate angiogenesis [14], it seemed logical to evaluate whether the endothelial-promoting effect of CM could be related to exosomes contained in this medium and transferred to $CD34^+$ EPCs. In line with this hypothesis, purified preparations of smaller vesicles containing exosomes (sEV/exo) isolated from CM mimicked the effect of CM to sustain endothelial formation. These observations provided the interesting indication that mature ECs exert a stimulatory effect on endothelial cell differentiation from $CD34^+$ cells.

2. Materials and methods

2.1. Cell purification

Cord blood (CB) was obtained from healthy, full-term placentas according to institutional guidelines A.Fa.R. Centro Trasfusionale, Università La Sapienza, Rome, Italy. The use of human CB samples for research purposes was approved by the Institutional Review Board of the Istituto Superiore di Sanità, Rome, Italy. Low-density mononuclear cells were isolated and $CD34^+$ cells purified as in [15]. The purity of $CD34^+$ cells assessed by flow cytometry was routinely >95%. Each single experiment may include pooled cells derived from different (2/3) cords blood.

2.2. Cell culture

2.2.1. Standard EC cultures

$2{,}5 \times 10^5$ $CD34^+$ cells have been plated at 1×10^5 cells/ml in EGM2 complete medium (Lonza) in a 12-well tissue culture plate coated with human collagen. Each three days the cultures have been inspected under an inverted microscope to detect the formation of foci of elongated adherent cells. Usually adherent cells were detected after 2–4 weeks of culture. When adherent cells reached confluence were trypsinized and grown in the EGM2 medium in the above reported conditions.

2.2.2. EC cultures in CM

In some experiments $CD34^+$ cells have been grown in CM. As a source of CM, HUVECs have been grown in EGM2 medium up to confluence; then the medium was discarded and replaced with fresh medium; after 24 h the CM was recovered, centrifuged twice at 700 ×*g* to remove cells (Fig. 2) and immediately used for $CD34^+$ cell cultivation. In some experiments the centrifuged CM was 0.45 μm filtered before addition to $CD34^+$ cells. During the first days of culture, fresh CM was added to the cells every 4–5 days up to the formation of a layer of adherent ECs (usually occurring after 7–14 days of *in vitro* culture).

2.2.3. Co-culture of $CD34^+$ cells with HUVECs

In some experiments $CD34^+$ cells were co-cultured with HUVECs. For these experiments Corning Transwell permeable microporous insert with a 0.4 μm membrane were used: in the upper chamber HUVECs (8×10^4 cells/well) were grown on collagen-coated membrane of the transwell; in the lower chamber $2{,}5 \times 10^5$ $CD34^+$ cells were layered on the collagen-coated well surface. When grown under this culture condition the first endothelial-like adherent cells in the lower well appeared after 1–2 weeks of culture. Once reached the confluence HUVECs were detached and replaced; in the bottom well fresh EGM2 medium was added every 4–5 days.

2.2.4. Isolation of sEV/exo from HUVEC and culture with $CD34^+$

sEV/exo were prepared according to a procedure previously described with some modifications [16]. Briefly, 25 ml of sub-confluent HUVEC (2×10^6) culture medium were subjected to serial centrifugation steps all at 4 °C (Fig. 2). The first was at 700*g* for 10 min, and the supernatant subjected to 2000*g* for 20 min to remove protein aggregates. Resulting supernatant was centrifuged at 15,000*g* for 20 min in an SW41 rotor to remove macrovesicles (MV). Smaller vesicles containing exosomes were isolated by high speed ultracentrifugation, *i.e.* 130,000*g* for 3 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA). Pellet was washed with PBS without resuspension at 130,000*g* for 1 h.

sEv/exos, resuspended in EGM2 medium in absence of serum, were added to $2{,}5 \times 10^5$ $CD34^+$ cells for 1 h at 37 °C, followed by serum addition. The cells were then grown as above reported.

2.3. Nanoparticle tracking analysis (NTA)

Vesicles pellet isolated as in Fig. 2, was analyzed in size by using the Nanosight NS300 system (Nanosight™ technology, Malvern, UK), configured with a 488 nm laser and a high sensitivity sCMOS camera. Videos were collected and analyzed using the NTA software (version 3.0). Multiple videos of 60 second duration were recorded and averaged as represented.

2.4. Western blot analysis

Total Cell lysate (control) was obtained after lysis of 5×10^6 HUVEC with lysis buffer (10 mM TrisHCl, 150 mM NaCl, 1% NP40, 10% glycerol, pH 7.4 supplemented with proteinase inhibitor cocktail) for 20 min at 4 °C, followed by 10,000*g* centrifugation. Supernatant and sEV/exo were quantified by Bradford assay.

20 μg protein for both samples were resolved by 10% SDS-PAGE under reducing and denaturing conditions and transferred to nitrocellulose filter. The blots were blocked using 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, followed by over/night incubation in TBST, 2% BSA with primary antibodies (anti-alix (3A9) mouse monoclonal, Thermoscientific, Waltham, MA USA; anti-TSG101 mouse monoclonal, GeneTex, Irvine, CA). After washing with TBST, the filters were incubated with mouse horseradish-peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h at room temperature. Immunoreactivity was revealed by using an ECL detection kit (Pierce).