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Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from patients with myocardial infarction

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Abstract

Background aims. Endothelial progenitor cells (EPCs) are known to play a beneficial role by promoting postnatal vasculogenesis in pathological events, such as ischemic heart disease and peripheral artery disease. However, little is known about the potential of EPCs to restore heart damage tissue. We compared the cardiac differentiation capacity of EPCs isolated from peripheral blood of patients with acute myocardial infarction (AMI) with EPCs obtained from umbilical cord blood (UCB). *Methods.* EPCs from both origins were isolated by density gradient centrifugation and characterized through the use of endothelial markers (UEA-1lectin, CD133 and KDR) and endothelial cell colony-forming unit assay. Cardiac differentiation capacity of EPCs was assessed by immunofluorescence and reverse transcriptase–polymerase chain reaction after 5-azacytidine (5-aza) induction. *Results.* No significant differences were observed between the number of endothelial cell colony-forming units in peripheral blood of patients with AMI and samples from UCB. Moreover, 5-aza induced the appearance of myotube-like structures and the positive expression of sarcomeric α-actinin, cardiac troponin I and T and desmin in a similar pattern for both cell sources, which indicates a comparable acquisition of a cardiac-like phenotype. *Conclusions.* For the first time, we have compared, *in vitro*, the cardiomyogenic potential of EPCs derived from patients with AMI with UCB-derived EPCs. Our data indicate that EPCs obtained from both origins have similar plasticity and functions and suggest a potential therapeutic efficacy in cardiac cell therapy.

Key Words: *acute myocardial infarction, cardiomyocyte differentiation, endothelial progenitor cells, umbilical cord blood*

Introduction

Myocardial infarction is the result of coronary artery obstruction with the consequent reduction of blood supply to the heart muscle and the massive loss of cardiomyocytes that are replaced by a non-functional scar tissue (1).

In 1997, Asahara *et al.* (2) identified for the first time $CD34^+$ circulating bone marrow (BM)-derived cells, implicated in physiological or pathological neovascularization, that were identified as endothelial progenitor cells (EPC). These blood cells were characterized by the expression of several markers such as CD133, CD34, KDR or VE-cadherin (3). However, controversy regarding overlapping expression of markers by EPCs and other hematopoietic progenitor cells has emerged (4). In fact, EPCs have been subdivided into two main categories (i) hematopoietic or (ii) non-hematopoietic EPCs. The hematopoietic EPCs are a heterogeneous cell population, which include colony-forming EPCs, non–colony-forming ''differentiating'' EPCs, myeloid EPCs and angiogenic cells (5). These progenitor cells,

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regardless their origin, have the potential to differentiate into mature endothelial cells (ECs) and to play a significant role contributing to neovascularization in ischemic tissues (2,6,7). Therefore, the significant increase of progenitor cells after myocardial infarction (3,8–10), indicates that circulating EPCs could represent a useful marker of pathogenesis and prognosis of cardiovascular diseases (11–15).

The angiogenic properties of EPCs, isolated from circulating peripheral blood (PB) and umbilical cord blood (UCB), have been proven in both experimental and *in vivo* settings (16,17). Recent studies in animal models of ischemic cardiomyopathy have suggested that transplanted EPCs could improve heart recovery after injury (18–20). Furthermore, ongoing clinical trials are using EPC-enriched cell populations with the aim to elucidate the therapeutic effects of these progenitor cells on ischemic diseases (21). Although initial results from clinical trials assessing the safety and feasibility of autologous progenitor cell transplantation are promising, a long-term follow-up of these patients must be evaluated.

On the other hand, it has been suggested that EPCs isolated from mouse BM and from human PB could differentiate into cardiomyocytes under certain *in vitro* conditions (22–24). On the basis of this potential plasticity, additional studies are still needed to elucidate the possible beneficial effect of circulating EPCs from patients who have had an infarct in the treatment of ischemic diseases. In the present study, we tested the cardiac differentiation potential of circulating EPCs from PB of patients who had been diagnosed with acute myocardial infarction (AMI) and compared the cells with EPCs from UCB.

Methods

Patients

We studied 24 patients, admitted with a diagnosis of AMI, defined as: (i) an acute coronary syndrome, with ST-elevation myocardial infarction (STEMI) with suggestive chest pain, (ii) an elevation of at least 3 mm in the ST segment in at least three precordial leads and (iii) within 8 h of symptoms. In addition, the specific biomarker cardiac troponin T was measured in the patient's serum, showing a positive troponin T peak (54.4 ± 13.1 ng/mL). Patient mean age was 59.5 ± 12.4 years; men represent 72% of the patients included in the study. Furthermore, hypertension was present in the 66% and hypercholesterolemia in 44% of the diagnosed patients. Finally, average ejection fraction was 51.9% ± 12.2%. Pre-infarction angina was defined as the presence of at least one chest pain lasting <30 min the week before the onset of the infarction. Patients were excluded if they had had chest pain compatible with angina for more than 1 week before the infarction or if they had underlying structural heart disease (cardiomyopathy or important valve disorders). Moreover, patients with a history of rheumatoid arthritis, hepatic, hematologic, or coagulation disorders, cancer or other acute or chronic inflammatory diseases such diabetes mellitus were not included in the study. Informed consent was obtained from all patients, and heparinized blood samples (20 mL) were drawn from all subjects. Human UCB samples ($n = 25$, 20 mL) were obtained from the Centro Regional de Transfusión Sanguínea y Tejidos de Málaga, Málaga, Spain, according to institutional guidelines. Samples were generally processed within 24–48 h of collection. Myocardial tissue samples were obtained from forensic cadaver autopsies no more than 12 h postmortem as described previously (25).

Quantification of endothelial markers in mononuclear cells

Mononuclear cells (MNCs) isolated by density gradient centrifugation (Histopaque-1077, Sigma, St Louis, MO, USA) from PB of patients with AMI and from UCB were washed and resuspended in phosphate-buffered saline (PBS) with 2% bovine serum albumin (Sigma) and 2 mmol/L ethylene diamine tetra acetic acid (Sigma). Cells were incubated in the dark at 4°C for 45 min with the following fluorochrome-conjugated monoclonal antibodies: CD133-PE (Miltenyi Biotec, Auburn, CA, USA), KDR-APC (R&D Systems, Minneapolis, MN, USA), CD34–fluorescein isothiocyanate (FITC) and CXCR4-APC (BD Biosciences, San Jose, CA, USA). Cells were then washed in PBS and analyzed in a fluorescence-activated cell sorting (FACS) Canto II cytometer equipped with the FACS Diva analysis software (BD Biosciences). Data obtained are expressed as mean ± standard error from four independent experiments performed in triplicate ($P < 0.05$).

Isolation and culture of EPCs

Samples of PB from patients with AMI and from UCB (20 mL) were processed by density-gradient (Histopaque-10771, Sigma) for 25 min at 1500 rpm/min and washed three times in PBS with 2% fetal bovine serum (FBS). Cells were further processed as described before (26). Briefly, 5×10^6 isolated MNCs per well were plated on fibronectin-coated six-well dishes (BD Biosciences) in endothelial cell basal medium, EBM-2 (Lonza, Basel, Switzerland),

supplemented with the EGM-2 Single Quote Kit (Lonza), which contains hydrocortisone, human fibroblast growth factor-B, vascular endothelial growth factor (VEGF), recombinant analog of insulin-like growth factor (R3-IGF-1), ascorbic acid, heparin, FBS, human endothelial growth factor and glycated albumin (GA-1000). After 2 days, non-adherent cells were re-plated on fibronectin-coated 24-well dishes (BD, Franklin Lakes, NJ, USA) at a density of 1×10^6 cells/well with 1 mL of fresh complete endothelial growth media and were incubated at 37°C, 5% CO_2 and 95% humidity.

The numbers of colonies were counted under a phase-contrast microscope after 5 and 10 days of culture. Five randomly selected microscopic fields were evaluated, and colonies were calculated in each PB from patients with AMI or UCB samples.

Assessment of acetylated low-density lipoprotein uptake and Ulex europaeus*—lectin 1 binding*

After 7 days of cell culture, EPCs were identified by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate—labeled acetylated low-density lipoprotein (DiI-ac-LDL, Molecular Probes, Eugene, OR) and adherence of fluorescein isothiocyanate-conjugated lectin from *Ulex europaeus* (FITC—UEA-l, Vector Laboratories, Burlingame, CA, USA). Incubation of adherent cells with 10 ng/mL DiI-Ac-LDL was performed at 37°C for 2 h. Thereafter, cells were

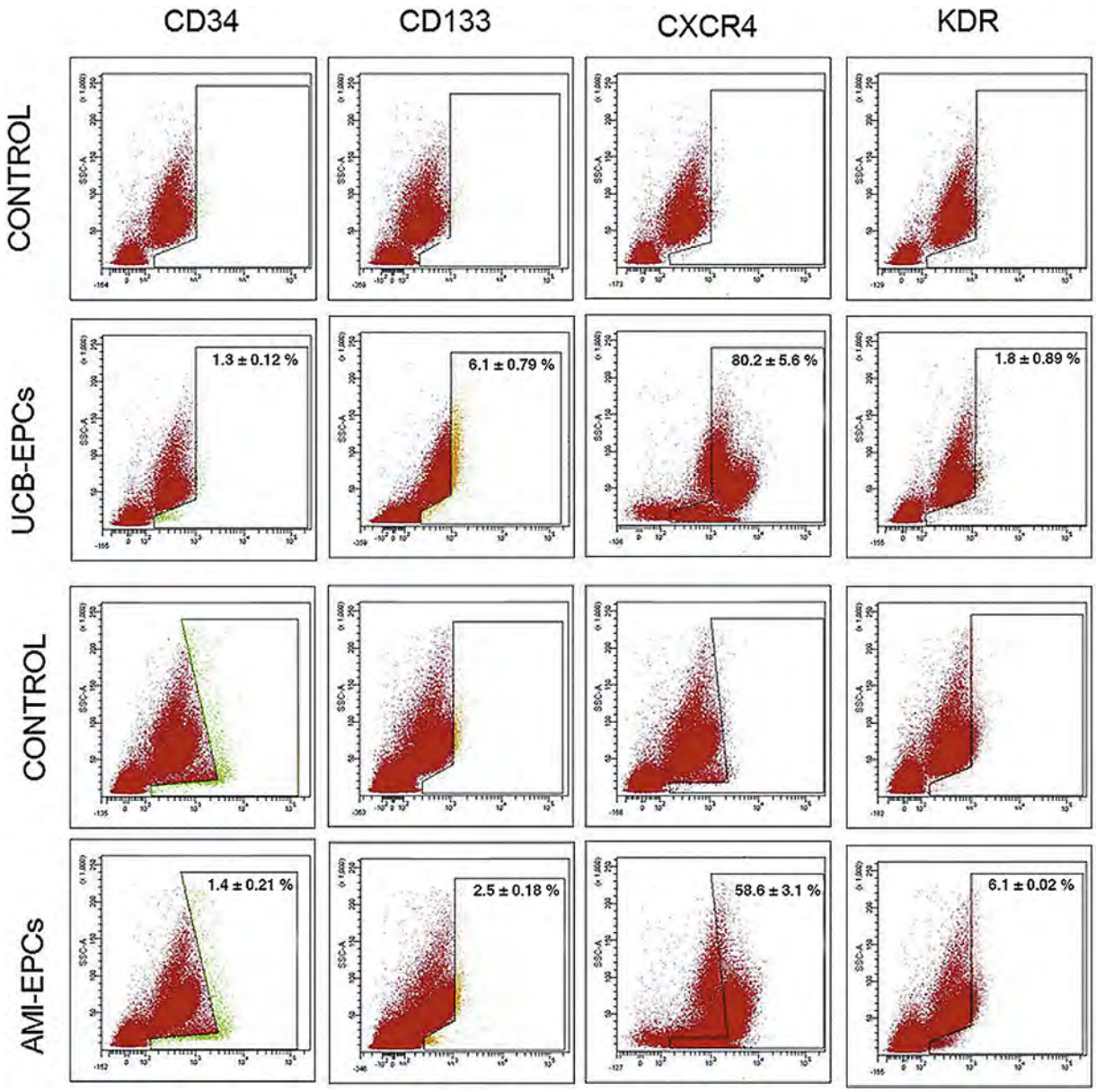
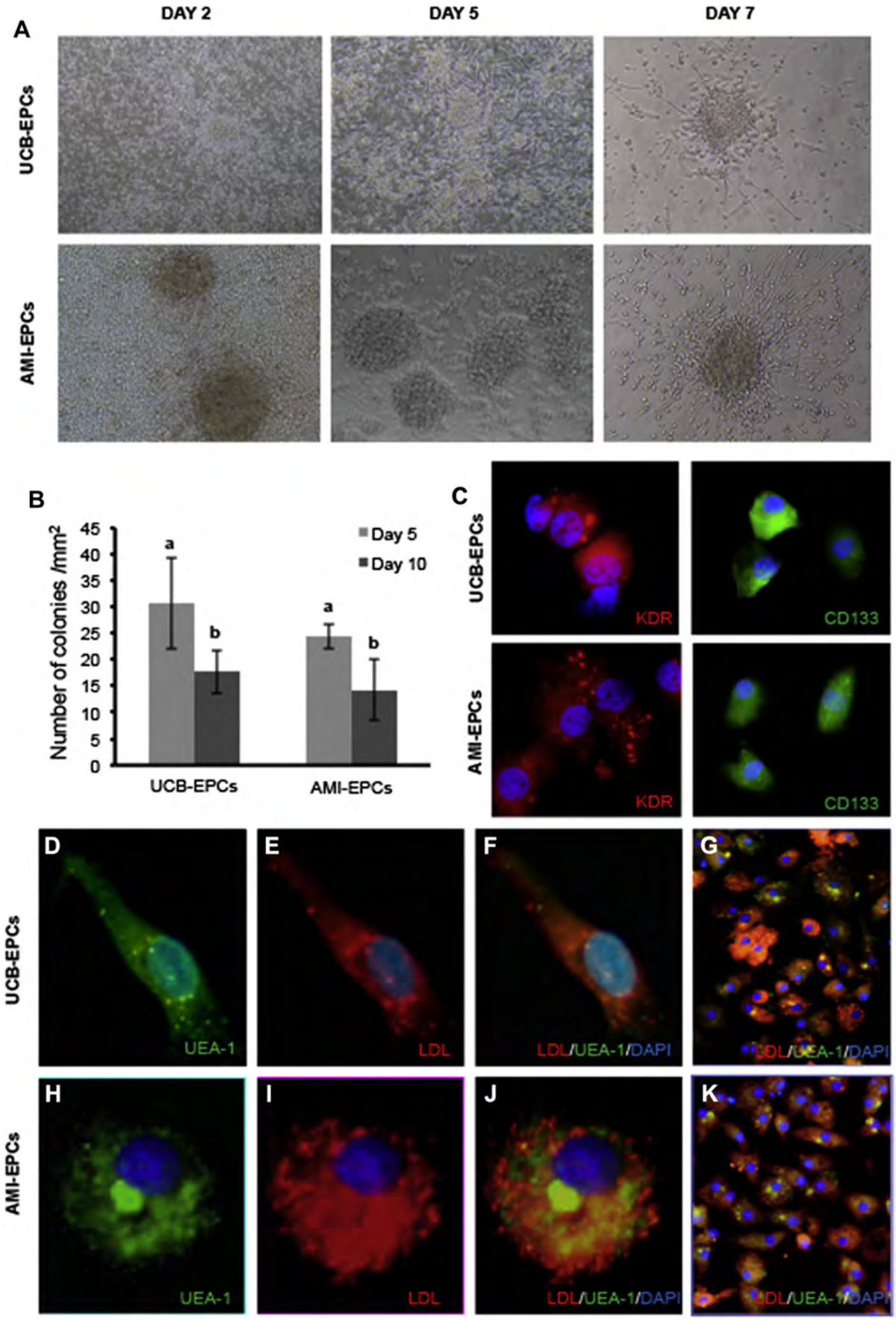


Figure 1. Representative graphics of FACS analysis of MNCs isolated from UCB and from PB of patients diagnosed with AMI.


A
DAY 2
DAY 5
DAY 7
UCB-EPCs
AMI-EPCs
B
Number of colonies /mm²
45
40
35
30
25
20
15
10
5
0
Day 5
Day 10
a
b
a
b
UCB-EPCs
AMI-EPCs
C
UCB-EPCs
KDR
CD133
AMI-EPCs
KDR
CD133
D
E
F
G
UCB-EPCs
UEA-1
LDL
LDL/UEA-1/DAPI
LDL/UEA-1/DAPI
H
I
J
K
AMI-EPCs
UEA-1
LDL
LDL/UEA-1/DAPI
LDL/UEA-1/DAPI

washed with PBS and fixed with 2% formaldehyde for 10 min and washed again with PBS. FITC–UEA-l was added and incubated for 1 h at 25°C. Culture was then washed twice and visualized by means of a Leica DM 5500B (Solms, Germany) fluorescent microscope, with the use of the Meta Systems Isis software.

Cardiomyogenic differentiation of EPCs

EPCs at a concentration of 5×10^4 cells/mL were seeded in per-manox-chamber slides (Nunc, Wiesbaden, Germany). The next day, cells were washed with PBS twice and incubated for 24 h in serum-free endothelial growth media containing 10 µmol/L 5-azacytidine (5-aza, Sigma). Cells were then washed and medium was replaced with fresh endothelial growth media containing FBS to prevent cell death caused by prolonged exposure to 5-aza and incubated in a CO_2 incubator. Cells were cultured for 2 weeks, and medium was changed every 3 days (27).

Immunocytochemistry

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For intracellular staining, cells were permeabilized with 0.1% Triton X-100 (Sigma) for 15 min. Cells were then blocked in 2% blocking buffer solution (Roche, Indianapolis, IN, USA) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C, and, when needed, secondary antibodies were incubated at room temperature for 2 h. Slides were mounted with 4,6-diamino-2-phenylindole (DAPI)-containing mounting solution (Ultra Cruz mounting medium, Santa Cruz Biotechnology). Photographs were taken with a Leica DM 5500B (Solms, Germany) fluorescent microscope equipped with Meta Systems Isis software.

Antibodies used for immunocytochemistry included fluorochrome-conjugated monoclonal antibodies for EPC characterization: KDR-APC (R&D) and CD133-PE (Miltenyi) (dilution 1:200); primary monoclonal antibodies used for cardiac differentiation: desmin (rabbit monoclonal; Sigma), human cardiac-specific troponin I (mouse monoclonal; Research Diagnostics, Flanders, NJ, USA) and sarcomeric α-actinin (mouse monoclonal; Sigma) (dilution 1:100). Secondary antibodies used were FITC or tetramethyl rhodamine isothiocyanate (Santa Cruz, CA, USA) (dilution 1:200).

Reverse transcriptase–polymerase chain reaction

For reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, total RNA from 5-aza–treated UCB-EPCs and from 5-aza–treated AMI-EPCs was extracted with the use of the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA extracted from human heart tissue was used as a positive control. RNA was checked on 1% agarose gel. The complementary DNA reaction was performed from 0.5–2 µg of total RNA with the use of the SuperScriptII kit (Invitrogen, Paisley, United Kingdom), according to the manufacturer's instructions. PCR reactions were performed with ReddyMix PCR Master Mix (Thermo, Waltham, MA, USA) as follows: after the initial denaturation (2 min at 94°C), 35 cycles were performed (20 s at 94°C, 20 s at 53°C, for cardiac troponin T and 20 s at 45°C and 1 min at 72°C for β-actin). Forward and reverse sequence of primers used for cardiac troponin were T 5′-AGA GCG GAA AAG TGG GAA GA-3′ and 5′-CTG GTT ATC GTT GAT CCT GT-3′ (235 bp) and for β-actin, 5′-ATC ATG TTT GAG ACC TTC AA-3′ and 5′-CAT CTC TTG CTC GAA GTC CA-3′ (316 bp). The PCR products were run on 1% agarose gel.

Statistics

Data were subjected to one-way analysis of variance followed by Fisher's test for comparison between any two means. Differences of $P < 0.05$ were considered significant. Values are expressed as mean ± standard error.

Results

The aim of the study was to isolate circulating EPCs from PB of patients who had been diagnosed with AMI and to compare their differentiation potential toward a cardiomyocyte lineage with EPCs isolated from UCB.

In humans, BM-EPCs are mobilized after an acute ischemic event in response to growth factors, cytokines and hormones that are released from the target tissue (28,29). First, we analyzed by flow cytometry the expression of different progenitors and endothelial markers in freshly isolated total MNCs from both origins. The cell surface adhesion

Figure 2. Characterization of EPCs derived from UCB or PB of patients with AMI. (A) Phase-contrast light microscopy of UCB-EPCs and AMI-EPCs at days 2, 5 and 7 of culture. (B) Assessment of CFU-EC at day 5 and day 10 of culture. Different lowercase letters stand for significant differences (one-way analysis of variance; $P < 0.05$). (C) Immunofluorescence of the UCB-EPCs and AMI-EPCs show positive staining for endothelial markers CD133 and KDR. (D–K) Cell surface binding of UEA-1 and Dil-Ac-LDL incorporation. Original magnification: ×10 for A; ×20 for G and K; ×40 for C and ×100 for D–F and H–J.

molecule CD34 was expressed by 1.3% ± 0.12% and 1.4% ± 0.21% of total MNC from UCB and AMI-PB, respectively (Figure 1), approximately 10-fold excess of $CD34^+$ cells when compared with MNCs of healthy subjects (30). Recently, Yang *et al.* (31) showed that $CD34^+$ cells could represent a functional EPC population in BM with beneficial therapeutic effects in myocardial infarction. In fact, others studies demonstrated mobilization of $CD34^+$ MNCs in patients with AMI (9). In addition, we found a higher expression of CD133 in UCB (6.1% ± 0.79%) than in AMI-PB (2.5% ± 0.18%) ($P < 0.05$) (Figure 1) that correlates with the immaturity of UCB-MNC, because CD133 is a marker for stemness that represents a hematopoietic/endothelial progenitor fraction (32–34). Furthermore, the chemokine receptor CXCR4 was notably expressed on freshly isolated MNCs from both origins, with an expression of 80.2% ± 5.6% for UCB and 58.6% ± 3.1% for AMI-PB (Figure 1). CXCR4 is highly expressed on hematopoietic stem cells and has previously been shown to play a key role in their homing and mobilization toward ischemic tissue (35). Moreover, over-expression of CXCR4 in mesenchymal stromal cells (MSCs) enhances *in vivo* engraftment into the ischemic heart and subsequently improves functional recovery through increasing myoangiogenesis (36). Finally, we found significant differences when the VEGF receptor KDR was studied (Figure 1), with 1.8% ± 0.89% expression in MNCs from UCB in comparison with a 6.1% ± 0.02% from the PB of patients with AMI. In fact, KDR has been proven to be responsible for vascular permeability factor (VPF)/VEGF-stimulated EC proliferation and migration (37). These results indicate that blood from patients with AMI appears to be a valuable source for obtaining EPCs comparable with UCB.

Isolated MNCs were further processed, and, because mature circulating ECs attach within the first 48 h of culture, we discarded this population by collecting only non-adherent cells and replanting then on fibronectin-coated well plates. Initially, isolated cells had a rounded morphology, and, after 2 days, the formation of colonies was detected (Figure 2A), which consisted of rounded ball-like clusters with elongated sprouting cells at the periphery. These clusters were classified as endothelial cell colony-forming units (CFU-ECs). At day 5, CFU-ECs increased in size and appeared with a more defined morphology, whereas on day 7, spindle-like EPCs sprouted out from the edge of the clusters and spread over the entire culture well surface (Figure 2A). CFU-ECs were counted manually in a minimum of four wells at days 5 and 10, and no significant differences ($P = 0.5$) were found between UCB-EPCs and AMI-EPCs. In addition, CFU-EC number peaked at day 5 and significantly decreased ($P < 0.05$) by day 10 in both cell sources (Figure 2B). Others studies have found that EPC colonies derived from UCB emerged earlier and in a major number than from colonies obtained from healthy adult PB (38). Because in our study we did not detect these variances, our data suggest that the cardiac insult enhances EPC population in PB of patients with AMI.

The attached cells were characterized, as previously shown (2), by the expression of the typical endothelial markers KDR and CD133. Immunofluorescence phenotyping revealed that both UCB-EPCs and AMI-EPCs highly expressed these endothelial markers (Figure 2C). In addition, we tested Dil-Ac-LDL uptake and UEA-1 binding as specific functional markers of ECs. Representative images of UEA-1 cell surface binding and Dil-Ac-LDL incorporation are shown in Figure 2D–K, demonstrating that AMI-EPCs and UCB-EPCs displayed, in a similar pattern, the functional phenotypes of EPCs. Although controversy has emerged regarding the endothelial specificity of these markers and that myelomonocytic cells share several properties with ECs such as LDL uptake, lectin binding, and CD31/CD105/CD144 expression (39), nevertheless, the capacity to form CFU-ECs is agreed to be inherent of EPCs (26), proving that in the present study, we isolated an EPC-like cell population.

To investigate the differentiation potential toward cardiomyocytes of circulating EPCs from patients who had an AMI, we treated cells with DNA demethylation agent 5-aza. This agent has been shown before to induce MSC cardiomyocyte differentiation (40–44). After 5-aza exposition, EPC morphology changed dramatically, in both PB-AMI and UCB sources, from spindle-like cells toward elliptical and elongated-shaped cells with a marked size enhancement. Furthermore, cells were arranged in parallel and formed myotube-like structures, whereas control cells maintained their spindle-like morphology (Figure 3A–F).

The acquisition of a cardiomyogenic differentiation phenotype was confirmed by immunocytochemical and RT-PCR analysis. Cardiac-specific markers, troponin I, the myocyte-specific sarcomeric α-actinin, desmin and cardiac troponin T proteins, were detected after 5-aza epigenetic induction in UCB and AMI-EPCs (Figure 3G and 3H–O). On the contrary, expression of cardiac markers was not detected in control non-treated cell (data not shown). Figure 3P shows a representative image of an AMI-EPC–treated cell in which a cardiac striation–like pattern can be appreciated. Although we did not find a full mature cardiomyocyte pattern staining, our results showed that 5-aza–treated

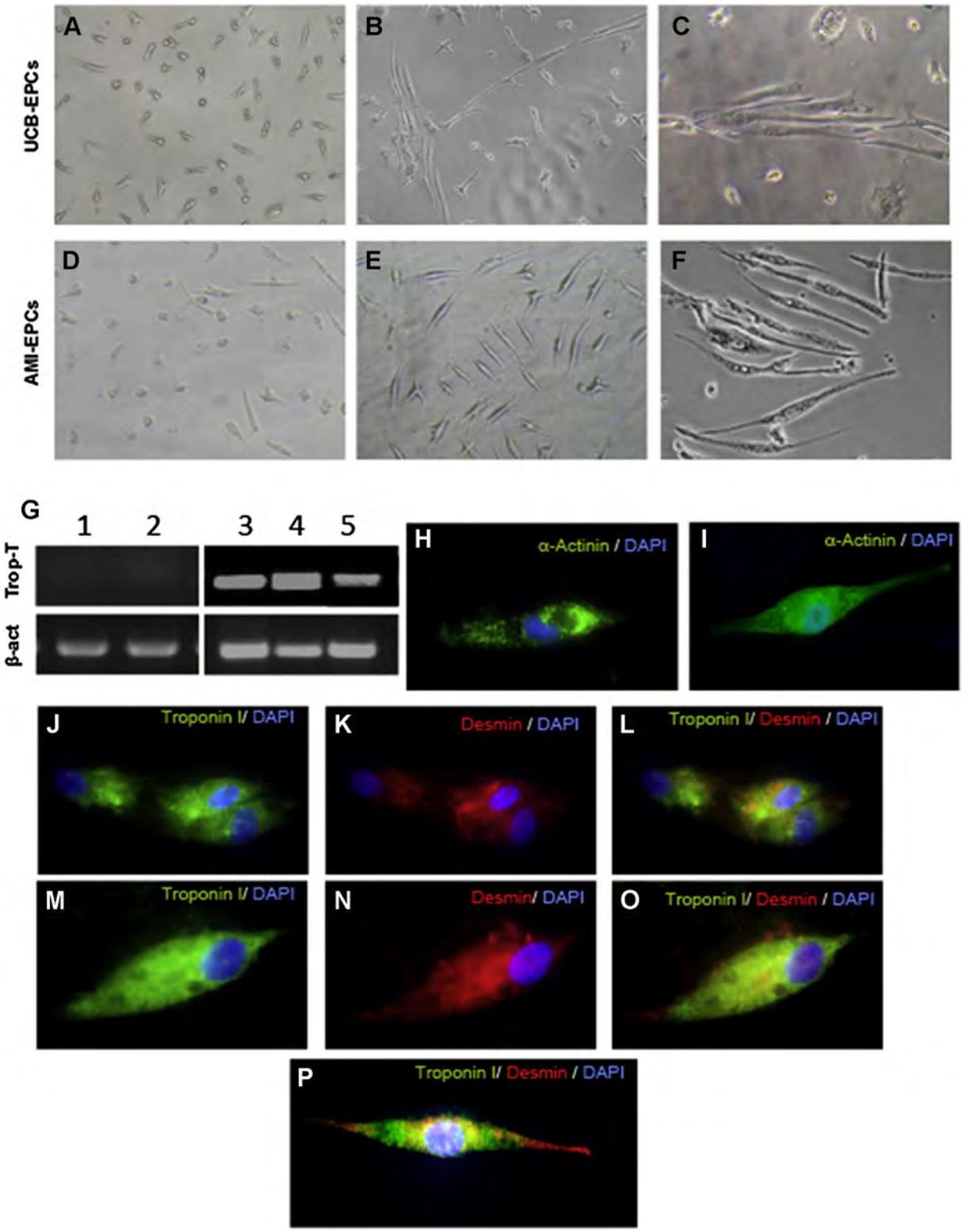


Figure 3. Treatment with 5-aza induces UCB-EPC and AMI-EPC differentiation toward cardiomyocyte-like cells. (A–F) Phase-contrast light microscopy of non-treated EPCs (A, D) and 5-aza–exposed UCB-EPCs (B, C) and AMI-EPCs (E, F). (G) RT-PCR analysis of treated UCB-EPCs (line 3) and AMI-EPCs (line 5) revealed expression of cardiac troponin T in contrast to non-treated cells (lines 1 and 2). Human heart tissue was used as positive control (line 4). (H–O) Immunofluorescence of UCB-EPCs (H, J–L) and AMI-EPCs (I, M–P) after 5-aza induction. Green labels indicate sarcomeric α-actinin and troponin I expression and red labels indicate desmin expression. Nuclei are stained with 4′-6-diamidino-2-phenylindole. Original magnification: ×10 for A, B, D and E; ×20 for C and F; ×40 for H–L and ×63 for M–P. Results are representative of three independent experiments.

EPCs have entered the cardiomyocyte differentiation program. In agreement with our data, the potential of human UCB-MSCs to differentiate into cells with cardiomyocyte characteristics has been shown before (27,45). Moreover, EPCs obtained from PB of healthy adult volunteers and patients with coronary artery disease have also been proven to transdifferentiate into cardiac myocytes after coculture with neonatal rat cardiomyocytes (22). Because ECs, vascular smooth muscle cells, and cardiomyocytes all differentiate from a common progenitor in the mesoderm, evidence suggests that reprogramming ECs back to an earlier state in mesodermal development could recapitulate their cardiomyogenic potential (24). This fact also could explain why EPCs, when cultured under appropriate culture conditions such as paracrine factors (growth factors and cytokines) and epigenomic agents (5-aza) or exposed to the proper microenvironment *in vivo*, differentiate into cardiomyocyte-like cells. The process of tissue auto-regeneration is highly complex, including cell dedifferentiation, transdifferentiation or differentiation from stem cells sitting within the adult tissue (46).

We show for the first time that EPCs isolated from patients with AMI have cardiogenic potential similar to that of EPCs isolated from UCB. Figure 3 clearly shows that cells isolated from both sources showed a similar pattern of cardiac marker expression. In agreement, intracoronary infusion of autologous EPCs in patients with AMI appears to enhance myocardial regeneration (47).

In conclusion, our finding suggests that EPCs isolated from patients who have had a myocardial insult are a valuable EPC source with the potential to differentiate toward cardiac-like cells. Because the ultimate aim of cardiac regenerative medicine is to replace both microvasculature and lost cardiomyocytes, further studies to identify long-term differentiation potential of autologous AMI-EPCs could be relevant for a future potential cell-based therapy application.

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