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Different Expression of NOS Isoforms in Early Endothelial Progenitor Cells Derived From Peripheral and Cord Blood

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Abstract Cord blood and peripheral-adult blood were compared as different sources of early endothelial precursor cells (eEPCs). Total mononuclear cells (MNCs) were obtained from both blood types and committed to eEPCs by exposure to fibronectin, VEGF, IGF-I, and bFGF. Under this condition, MNCs seeded at the density of 3×10^5 cells/cm^2 assumed a spindle shape, which was indicative of developing eEPCs, and expanded in a similar manner irrespective to the blood sources. Ulex europaeus agglutinin (UEA-1) and acetylated low density lipoprotein (acLDL) double staining was present in 90% in both peripheral- and cord-blood eEPCs after 2-week expansion. Also, the ability of eEPCs to form tubule-like structures in Matrigel was independent of their blood source, but dependent on the presence of human umbilical vein endothelial cells (HUVECs). eNOS and nNOS were not detectable by Western blotting in both peripheral and cord-blood eEPCs upon 3 weeks and their mRNA levels were lower than 2% relative to those present in HUVECs. On the contrary, iNOS protein was detectable in peripheral-blood eEPCs, but not in cord-blood eEPCs and HUVECs, as well as iNOS mRNA was more concentrated in peripheral-blood eEPCs than in cord-blood eEPCs and HUVECs. These data suggest that: (a) peripheral and cord blood can be considered comparable sources of eEPCs when they are expanded and differentiated in a short-term period; (b) the extremely low expression of constitutive NOS isoforms in the eEPCs of both blood types should markedly reduce their ability to regulate NO-dependent vasorelaxation; (c) the presence of iNOS in peripheral-blood eEPCs could improve the process of vasculogenesis. J. Cell. Biochem. 102: 992–1001, 2007.

Key words: endothelial precursor cells (EPCs); human umbilical cord blood; human umbilical vein endothelial cells (HUVECs); nitric oxide synthase (NOS); CD133

Endothelial precursor cells (EPCs) are partially committed cells which have been described to differentiate into mature endothelial cells (ECs) under both in vitro and in vivo conditions [Hristov et al., 2003; Urbich and Dimmeler, 2004]. Several studies in animals and humans indicate that EPCs can improve the function of ischemic organs, by means of their ability to induce vasculogenesis and/or to ameliorate the process of angiogenesis [Kalka et al., 2000; Assmus et al., 2002; Lei et al., 2004]. EPCs can be directly isolated from bone marrow or peripheral blood after marrow mobilization. A few markers, such as CD34, CD133, and KDR, are usually utilized to recognize EPCs in an initial immature stage, while other antigens, such as CD31, von Willebrand factor (vWF), VE-cadherin, characterize the lineage progression towards complete differentiation into ECs [Hristov et al., 2003; Urbich and Dimmeler, 2004].

Two main strategies are commonly used to isolate EPCs. One identifies the markers of putative immature EPCs and/or hematopoietic/endothelial stem cells (hemangioblasts). In this case, EPCs are freshly isolated just after sample collection (i.e., fluorescence activated cell sorting, immunomagnetic separation). According to the second method, mononuclear cells (MNCs) are first isolated and then cultured for variable periods (days, weeks, months) in the presence of specific growth factors which commit them

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toward the endothelial phenotype. The antigen-selected immature EPCs are few in bone marrow and in peripheral blood and after isolation they need a long period of in vitro proliferation to become enough numerous to fit the request for therapeutic transplantation. On the contrary, a greater number of EPCs can be more quickly produced from MNCs in vitro differentiation and expansion, although this kind of EPCs seems to be heterogeneous in both structure and function. In particular, two main subpopulations of EPCs derived from MNCs have been today identified: early EPCs (eEPCs) and late or outgrowth EPCs [Gulati and Jevremovic, 2003; Hur et al., 2004; Gulati and Simari, 2005; Yoon et al., 2005]. eEPCs are indeed MNCs which are forced to express endothelial markers; they can be expanded for not longer than 3–4 weeks and poorly incorporate into new vessels or create tubule-like networks. Despite these features, eEPCs can promote in vivo vasculogenesis by means of a putative mechanism related to the production of angiogenic and vasculogenic factors. By contrast, late EPCs are initially very few and begin to grow exponentially only after 2–3 weeks following MNC seeding. Relative to eEPCs, they show a greater proliferation potential and directly incorporate into new vessels lining the internal endothelial wall. Also, the phenotypes of early and late EPCs are quite different. In particular, nitric oxide (NO) is less produced by early than late EPCs after stimulation of eNOS. In the vascular contest, the role of NO relies not only in increasing vasorelaxation but also in stimulating vessel formation. With regard to angiogenesis, that is, the formation of new vessels from preexisting nearby ECs, it is known that NO stimulates migration, proliferation, and survival of the same ECs [Ziche et al., 1994; Carmeliet and Jain, 2000]. Moreover, NO enhances neovascularization by acting as a downstream mediator of VEGF signaling [Fukumura et al., 2001]. By contrast, the role of NO in vasculogenesis has not been yet investigated well. NO appears to promote the mobilization of EPCs from bone marrow and, therefore, to increase their presence in the damaged region [Duda et al., 2004]. Moreover, by unknown mechanisms, iNOS-derived NO increases the expression of VEGF which, in turn, promotes the recruitment of circulating EPCs [Brandes, 2006]. Therefore, eEPCs could improve both angiogenesis and vasculogenesis as a biological source of NO and, eventually via iNOS, of VEGF.

In the present article, we have investigated the expression of either constitutive or inducible NOS isoforms in eEPCs, which were derived from human peripheral adult and cord blood, and compared with those present in human umbilical vein endothelial cells (HUVECs). We have studied cord blood as an alternative source of EPCs because it contains a greater number of multipotent/precursor cells [Zhang et al., 2006], and HUVECs as a model of differentiated ECs representing a hypothetical end-point of NOS expression for eEPCs.

MATERIALS AND METHODS

Peripheral Adult and Umbilical Cord-Blood Samples

Blood samples (50 ml of buffy coat preparation) of healthy human adult volunteers (age range: 29–60 years) of both genders were obtained from the Service of Transfusion Medicine of S. Orsola-Malpighi Hospital of Bologna (Italy). Human umbilical cord blood (40–80 ml) was from healthy newborns of both genders (gestational range: 38–40 weeks) and provided by the Transfusion Cardiovascular Tissue Bank of S. Orsola-Malpighi Hospital of Bologna. Cord-blood samples were collected in citrate-phosphate-dextrose solution within 10 h from delivery, after an informed consent obtained from the mothers. All investigations reported in this article were conducted in conformity with the principles of the Declaration of Helsinki regarding ethical procedures.

Cell Isolation and Culture

Total MNCs were isolated from blood by Ficoll density gradient centrifugation according to manufacturer's instructions (Amhersham Biosciences, Upsala, Sweden). The cells were counted by Trypan blue exclusion procedure for viable cells. MNCs ($1–10 \times 10^6$ cells/ml) were then plated on 24–96 well plates, or on 35–100 mm diameter-culture dishes, all coated with human fibronectin (BD Biosciences, Bedford, MA), and maintained in Medium 199 supplemented with 10% foetal-calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin (both from BioWhittaker-Cambrex Bio Science, Verviers, Belgium), 50 ng/ml vascular endothelial growth

factor (VEGF, Sigma, St. Louis, Missouri), 1 ng/ml basic fibroblast growth factor (b-FGF), and 2 ng/ml insulin-like growth factor I (IGF-I) (both growth factors from PeproTech, London, UK). In this article, we will refer to this cytokine-enriched medium, together with fibronectin, as "differentiating medium" or DM. Both non-adherent and adherent cells were cultured for 14–21 days and every 3–4 days the DM was replaced with a fresh one.

$CD133^+$ MNCs were isolated from total MNCs, just after their separation by Ficoll, using the immunomagnetic method of the MACS CD133 Isolation Kit (Miltenyi Biotec, Bergish-Gladbach, Germany) according to manufacturer's instructions. Immediately after their separation, $CD133^+$ MNCs were counted by Trypan blue exclusion procedure. The recovery was evaluated as a percentage of the total MNC loaded on the separation apparatus.

All data were referred to HUVECs because end-differentiated ECs. The cells were suspended in a culture medium containing M199-RPMI 1640 containing 20% pooled human serum, 2 mM glutamine, 10 mM Hepes 10 mmol/l, and 1% penicillin-streptomycin, and seeded on gelatine-coated culture wells. HUVECs showed the typical cobblestone morphology and reacted positive to endothelial markers.

Fluorescent-Activated Cell Sorting (FACS) Analysis

Immediately after separation, up to 3×10^5 of total or $CD133^+$ enriched MNCs were suspended in 50 μl PEB containing phosphate buffer saline (PBS) supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA). Then cells were incubated with a phycoerythrin-conjugated mouse monoclonal anti-CD133 antibody (1:10) for 10 min at 4°C. The cells were washed by centrifugation in PEB, and the pellet was resuspended in 200 μl of 1.5% paraformaldehyde in PEB. Flow cytometry analysis was performed using an Epics Elite (Beckman Coulter, Fullerton, CA) flow cytometer, equipped with a 15 mW Argon ion laser tuned to 488 nm. Red fluorescence was collected at 605 nm.

Cellular Staining and acLDL Uptake

Fluorescent chemical detection of eEPCs was performed on attached MNC-derived cells after 2, 5, 7,and 14 days of culture. Direct fluorescent staining was used to detect the binding of fluorescein isothiocyanate (FITC)—labeled Ulex europaeus agglutinin (UEA-1, Sigma) and cell uptake of 1,1-dioctadecyl-3,3,3,3-tetramethilindocarbocyanine (Dil)—labeled acetylated low density lipoprotein (acLDL, Molecular Probes, Eugene, Oregon). Cells were incubated with 10 μg/ml acLDL for 4 h, and 10 μg/ml UEA-1, for 2 h, at 37°C and fixed with 3% paraformaldehyde for 15 min. Cells with double-fluorescent staining were identified as differentiating eEPCs and their average number per field was evaluated counting 10 randomly selected high-power fields (20×) by an inverted fluorescent microscope (IX50 Olympus).

Regarding the immunofluorescence analysis, cells were grown on glass coverslips under DM condition, fixed for 15 min at room temperature with 3% paraformaldehyde in PBS and rinsed twice with PBS. Cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature only for the detection of vWF. After blocking with 4% BSA and 0.2% Tween-20 in PBS for 1 h at room temperature, the cells were incubated with primary antibodies against the following antigens: vWF (Sigma) 1:200; KDR (Sigma) 1:20; CD45 (BD Bioscience, Pharmigen, San Diego, CA) undiluted. All primary antibodies, with the exception of anti-vWF, were monoclonal anti-human and diluted in the blocking reagent. The FITC-conjugated anti-mouse antibody (Sigma) or the rhodamine-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Europe) was used as secondary antibodies (diluted in blocking buffer 1:2,000). After immunofluorescent staining, cells were mounted with the bleaching reagent (ProLong Gold antifade reagent, Molecular probes) on standard glass slide and observed at the microscope. Controls were performed by replacing the primary or the secondary antibodies with the blocking reagent in order to confirm that the fluorescence observed was specifically related to the investigated antigen. HUVECs were chosen as positive controls either for direct fluorescence staining (Dil-acLDL, FITC-UEA-1) or immunofluorescence analysis. More than 85% of HUVECs was positively stained for the above-mentioned endothelial markers. H9c2 rat cardiomyoblasts (European Collection of Animal Cell Culture—ECACC) and rat bone marrow stromal cells [Muscari et al., 2005] were used as negative controls. Both types of cells were not substantially positive to endothelial marker

staining, since not more than 2.5% of them showed target-specific fluorescence.

Western Blot Analysis

Whole protein extract (80 μg/sample) was prepared from MNCs and eEPCs (1×10^7 cells/ml) at 7, 14, and 21 days of culture. The samples were electrophoresed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1 h, 150 V) and blotted (1 h, 100 V) onto nitrocellulose membranes (Bio-rad, CA). After washing in TBS-Tween (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20), the membranes were treated for 1 h with 5% non-fat dry milk (Bio-rad) to block non-specific binding sites. The membranes were incubated overnight at 4°C with rabbit polyclonal antibodies against eNOS (NOS3), iNOS (NOS2), and nNOS (NOS1) (Santa Cruz) diluted 1:1,000 in TBS-Tween. The immune reaction was revealed by a peroxidase-labeled goat anti-rabbit antibody (Santa Cruz) diluted 1:2,500 in TBS-Tween. The secondary antibody was added to membranes for 1 h at room temperature, followed by 1 min exposure with the Luminol reagent (Santa Cruz) which was revealed by high-sensitivity photographic film. β-actin was used as internal control.

Quantification of NOS mRNAs by Real-Time PCR

The assay for all isoforms of NOS mRNA expression was optimized for the Light Cycler version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany), with a RT-PCR in two steps. RNA extraction was performed using a High Pure RNA Isolation Kit and reverse transcription using Transcriptor First Strand cDNA Synthesis Kit (all reagents from Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. NOS mRNA expression was quantified relative to the β-actin housekeeping gene. The primer sequences were as follows: β-actin, sense 5′-TTG GCA ATG AGC GGT TCC-3′ and antisense 5′-AGC ACT GTG TTG GCG TAC-3′; PCR fragment of 148 bp [Gago et al., 2003]. eNOS, sense 5′-GGA CAT TTT CGG ACT CAC ATT G-3′ and antisense 5′-GCT GTC GCT CCT GCA AAG A-3′; PCR fragment of 77 bp. iNOS, sense 5′-ACA TTG ATC AGA AGC TGT CCC AC-3′ and antisense 5′-CAA AGG CTG TGA GTC CTG CAC-3′; PCR fragment of 235 bp [Blanco et al., 1995]. nNOS, sense 5′-TCT CCT CCT ACT CTG ACT CC-3′ and antisense 5′-TTG TGG ACA TTG GAT AGA CC-3′; PCR fragment of 424 bp [Jarry et al., 2003]. Real-time PCR was performed in Light Cycler capillaries using a master mix containing FastStart Taq DNA polymerase and SYBR-Green I (LightCycler FastStart DNA Master^{plus} SYBR-Green I, Roche Molecular Biochemicals). DNA fragment amplification was performed after the addition to the master mix of primers (final concentration: 0.5 μM) and template cDNA (10–100 ng). The protocol was the following: 1 cycle at 95°C for 15 min for the initial enzyme activation, then 45 cycles of denaturation (94°C for 10 s), annealing (60–63°C for 10 s) and extension (72°C for 6–15 s). In order to verify the purity of the products, a melting curve was produced after the completion of each PCR amplification by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/s, starting at 60°C for 60 s.

The Roche software uses the second derivative maximum method to calculate the fractional cycle numbers where the fluorescence rises above background at the fastest rate (crossing point, C_p). The amount of mRNA was calculated according to Pfaffl [2001] relative to the housekeeping gene β-actin. All the values were normalized to the corresponding mRNA of NOS isoforms expressed by HUVECs at passages between 3 and 6.

In Vitro Vasculogenesis Assay

In vitro vasculogenesis assay was performed on Matrigel (BD Biosciences) according to manufacturer's instructions. Briefly, ECmatrix™ solution was thawed on ice overnight and placed in a 96-well tissue culture plate at 37°C for 1 h to allow the matrix solution to solidify. Dil-labeled eEPCs at day 7, 14, and 21 of culture were detached using 1mM PBS-EDTA at 37°C for 20 min, harvested by centrifugation and replated (20.000 cells/well) alone or with HUVECs (20.000 cells/well) on top of the solidified matrix solution. Cells were incubated in Medium 199 at 37°C for 24 h. Tubule formation was inspected under an inverted light microscope.

Statistical Analysis

All data were represented as mean ± SEM. Differences between groups were assessed by unpaired Student's t-test for single comparison. Values of $P < 0.05$ were considered significant.

RESULTS

Morphological and Antigenic Characterization of Peripheral and Cord Blood-Derived MNCs

Total MNCs were isolated by Ficoll layering, and the $CD133^+$ subpopulation was obtained from the MNCs by further immunomagnetic separation. The viability of the $CD133^+$ MNCs after separation was assessed by counting the cells in the presence of trypan blue: the percentage of dead cells did not significantly increase and was never higher than 5%. The enrichment in $CD133^+$ MNCs was evaluated by fluorescent-activated cell sorting (FACS) analysis and the percentage of positive cells was greater than 90% of the separated population for both peripheral and cord blood. $CD133^+$ MNCs were counted and their amount expressed as percent of total MNCs. The recovery of $CD133^+$ MNCs was about three-four fold higher in cord blood than peripheral blood (Fig. 1a). In either case, these cells were less than 0.2% of total MNCs irrespective to the blood source. No variation was observed between male and female subjects and with respect to age of the adult population. The morphological and antigenic features of total MNCs were evaluated during 2 weeks of expansion in DM. The appearance of spindle-shaped attaching (AT) cells, evaluated at contrast-phase microscopy, was considered as indicative of developing eEPCs (Fig. 1b). When the originally round-

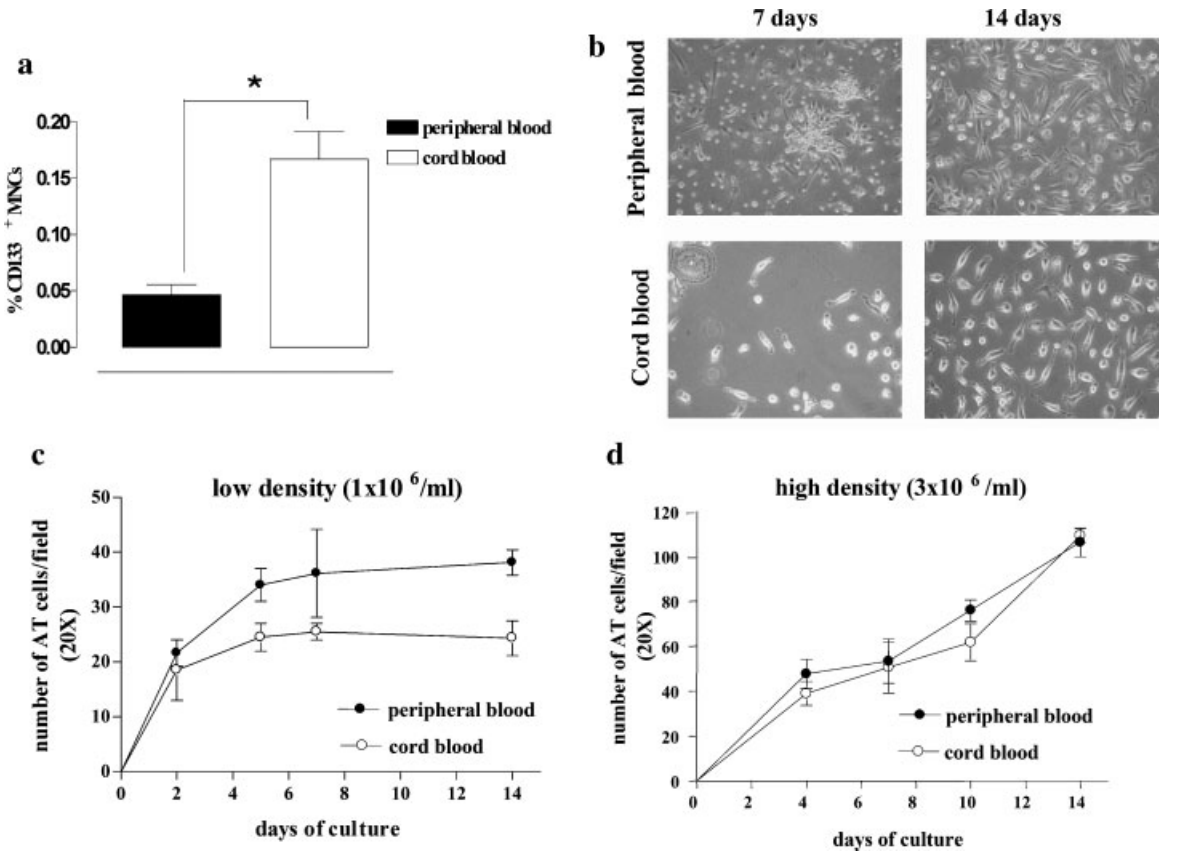

Fig. 1. Percentage of $CD133^+$ MNCs in peripheral and cord blood and characterization of total MNCs under exposure to DM. **a**: The percentage of $CD133^+$ MNCs with respect to total MNCs was extremely low and threefold higher in cord ($n = 3$) than peripheral ($n = 9$) blood ($^*P < 0.05$). $CD133^+$ MNCs were obtained by immunomagnetic separation as described in the Materials and Methods section. **b**: MNCs were seeded at the density of 10^6 cells/ml in the presence of DM and their morphology observed by phase contrast microscopy. The number of spindle-shaped attaching (AT) cells, which were indicative of developing eEPCs, increased during the first 2 weeks of differentiation; (**c**) 1×10^6/ml; and (**d**) 3×10^6/ml MNCs ($n = 2$ at least) were seeded in triplicate and the average number of AT-MNCs per field was evaluated by counting 10 randomly selected high-power fields ($20\times = 0.35\ mm^2$). The amount of AT-MNCs at 1×10^6/ml density reached a plateau after the first week and, at day 14, was significantly lower for cord than peripheral blood-derived AT-MNCs ($^*P < 0.05$). On the contrary, the number of AT-MNCs increased linearly up to 14 days when they were seeded at the density of 3×10^6 cells/ml, without observing differences between the two blood sources.

shaped MNCs were plated at density of 1×10^6 cells/ml per 35 mm culture dish ($\cong 10^5$ cells/cm^2) they gradually assumed the morphological features of AT cells, reaching a plateau after the first week of expansion (Fig. 1c). The maximal amount of AT-MNCs derived from peripheral blood was significantly higher than the corresponding value of cord-blood cells. The trend of proliferation changed by increasing the initial number of MNCs from 1×10^6 to 3×10^6 cells/ml per dish (Fig. 1d). At the higher density, the AT-MNCs proliferated linearly with time, without reaching a plateau upon the 2 weeks of observation. Moreover, no difference in this early rate of proliferation was observed between peripheral and cord-blood AT-MNCs.

MNCs expressed some typical markers of ECs when exposed to the DM. In particular, they were stained for acLDL, UEA-1, vWf, and KDR (Fig. 2a). CD45, a marker of blood white cells, was expressed in part by MNCs just after their isolation from blood (day 0), and also by AT-MNCs under the differentiating treatment, suggesting that at least a component of eEPCs was derived from adherent leucocytes, that is, monocytes. About 80% of the peripheral blood AT-MNCs were double stained with acLDL and UEA-1 just after 2 days of differentiation, while

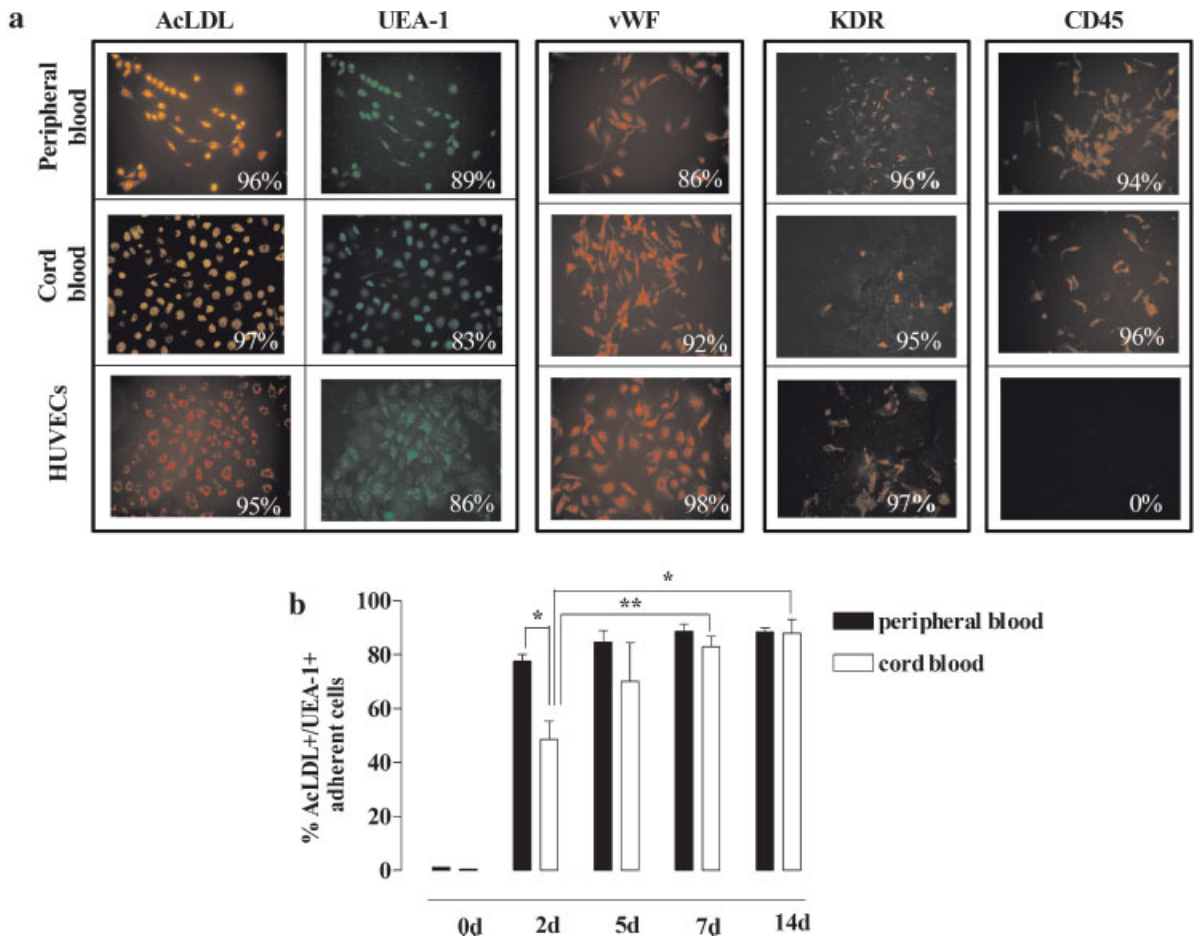

Fig. 2. Functional and immunocytochemical characterization of MNCs differentiating into eEPCs. **a**: Micrographs shows the uptake of acLDL by MNCs and their expression of EC markers (UEA-1, vWF, KDR) and CD45 under exposure to DM. For 14 days, 3×10^6 MNCs/ml were seeded into 35 mm culture dishes ($n = 3$) and grown. Cells were stained with fluorochromes as described in the Materials and Methods section. Fluorescence intensity and distribution relative to ECs markers were similar in 2-week differentiated MNCs and HUVECs. On the contrary, at that time CD45 immunostaining was only detected in MNCs but not in HUVECs. **b**: The contemporary uptake of acLDL and expression of UEA-1, which identifies eEPCs, is shown in the bar graph. Just after 2 days of DM exposure, the percentage of double-stained cells was greater in peripheral blood MNCs than MNCs cord blood ($^*P < 0.05$). About 80% of peripheral blood MNCs were double stained at day 2 and became more than 90% at day 7. The percentage of double-stained cord blood MNCs gradually increased from 50% at day 2, to 90% after 2 weeks of DM exposure.

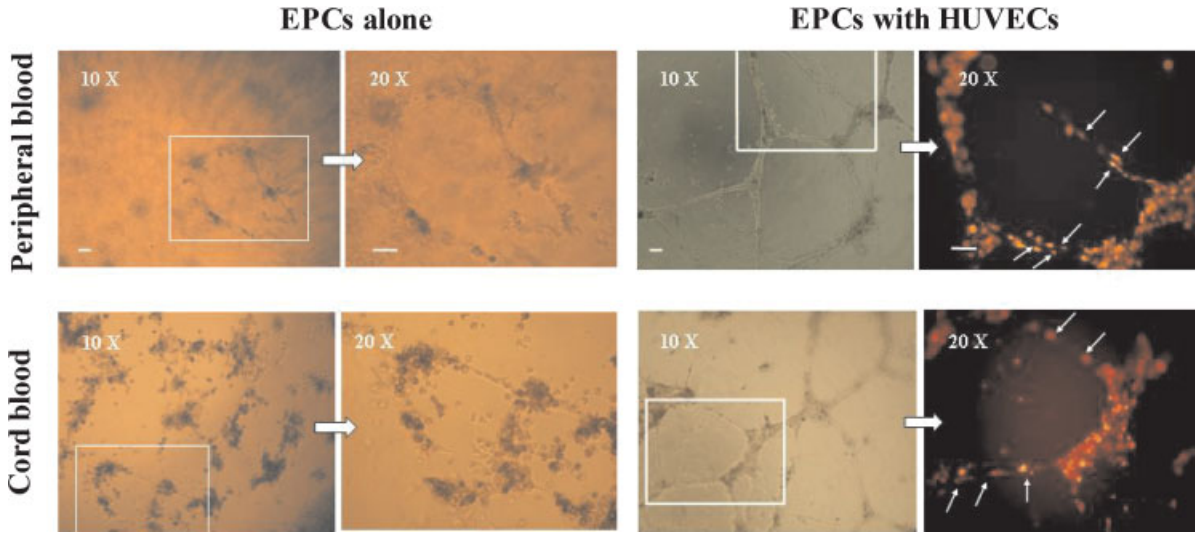

Fig. 3. Representative micrographs of tubule-like structures derived from eEPC organization. eEPCs (n = 6) were stained with Dil-acLDL (red fluorescence) and detached after 7 days of culture in DM. Then, 2×10^4 eEPCs grown alone in Matrigel, or together with the same number of HUVECs, for 24 h. Well-defined tubule-like structures were only formed by eEPCs when cultured with HUVECs. Arrows indicate spindle-shaped eEPCs intercalated with HUVECs along a formed cord. No particular differences were observed between peripheral and cord blood-derived eEPCs.

only 50% of cord-blood AT-MNCs presented these markers at this time (Fig. 2b). AT-MNCs became almost completely double stained for acLDL and UEA-1 (more than 90%) after 2 weeks of differentiation, irrespective to their blood source.

Formation of Tubule-Like Structures in Matrigel

The MNCs derived from both peripheral adult and cord blood were exposed to the DM for 3 weeks and then detached to investigate their ability to form tubular-like structures in vitro (Fig. 3). When eEPCs were cultured on the Matrigel, they formed a very poorly organized capillary-like network. On the contrary, eEPCs together with HUVECs generated complete tubular structures, but no difference in this process was observed between peripheral and cord blood-derived cells. In particular, after 24 h of incubation in Matrigel, both kind of eEPCs assumed a spindle shape and interacted closely with HUVECs.

Expression of NOS Isoforms

The expression of NOS isoforms (eNOS, nNOS, iNOS) was studied in eEPCs during their in vitro differentiation. Figure 4a shows representative results of Western blot analysis. Constitutive NOS isoforms were undetectable in eEPCs within 2 weeks of growth and differentiation. On the contrary, in HUVECs the eNOS protein was abundant and a low level of nNOS was even detectable. iNOS protein was detectable in some samples of peripheral-blood eEPCs, but neither in cord-blood eEPCs nor in HUVECs.

The mRNA levels of NOS isoforms were expressed as percentage of the corresponding mRNA levels in HUVECs (Fig. 4b). The eNOS mRNA was about 3.5–4.0% at time zero, irrespective to the blood source, and was lower than 100% during the following 3 weeks of exposure to DM. The nNOS mRNA was never higher than 2.0% in eEPCs of both blood origins. By contrast, in peripheral-blood eEPCs the iNOS mRNA was 300–400% at time zero, reached the lowest value after 1 week of DM exposure, and increased again up to 400% in the following 2 weeks. The iNOS mRNA percentages showed a similar trend in cord-blood eEPCs, although with lower variation relative to the corresponding level in HUVECs. The higher values were present at time zero (150–200%) and after 3 weeks of exposure to DM (200%).

In order to evaluate whether gender affects the transcription of NOS isoforms, it was performed a comparison between male and female mean values evaluated at each time of DM exposure (0, 7, 14, 21 days). Although we could not appreciate any significant statistical variation, the trend of eNOS mRNA levels

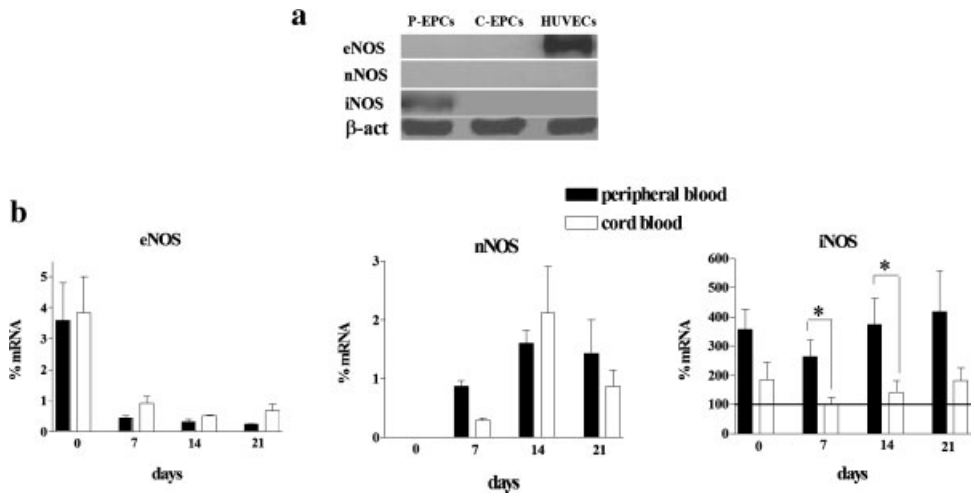

Fig. 4. Expression of NOS isofoms in eEPCs during their in vitro differentiation. **a**: The lanes show the Western blot analysis of eNOS, nNOS, and iNOS produced by eEPCs after 2 weeks of differentiation (n = 2 at least for each blood source), and by HUVECs. eEPCs did not produce detectable amount of constitutive NOS isoforms irrespective to their blood origin. On the contrary, iNOS protein was detectable in eEPCs derived from peripheral blood (P-EPCs), but not from cord blood (C-EPCs). As expected, HUVECs produced eNOS and a low amount of nNOS, but not iNOS. **b**: Bar graphs show the time course of mRNA expression of all three NOS isoforms, determined in eEPCs up to 3 weeks of DM exposure (n = 5 for cord blood eEPCs; n = 3 for peripheral blood eEPCs), with respect to the corresponding mRNAs of HUVECs. Real-time PCR of NOS-isoform mRNAs confirmed that constitutive NOSs were poorly expressed in eEPCs, whilst the levels of iNOS mRNAs were greater in these cells than in HUVECs, especially in peripheral blood eEPCs.

observed during the overall period of in vitro differentiation was constantly lower, while that of iNOS mRNAs was higher in eEPCs from male relative to female subjects (data not shown). This opposite behaviour suggests that beyond gender specific features, when EPCs express low levels of eNOS there is a compensative increase in iNOS synthesis. Moreover, we observed that iNOS transcript levels peaked in the age range of 30–40 years ($P < 0.05$ vs. yonger and oldest decades), indicating that in this period of life eEPCs are more prone to produce iNOS.

DISCUSSION

Although the pattern of specific surface antigens, together with the morphological and functional features, were consistent with the differentiation of blood MNCs to eEPCs; their expression of NOS isoforms was not comparable with that of HUVECs, that is, mature ECs. In particular, both eNOS and nNOS proteins were not detectable by Western blot analysis and the contents of their transcripts were extremely lower than in HUVECs (less than 2.0%). The role of eNOS in ECs has been widely underlined in several functional, pharmacological, and pathological conditions, as a fundamental target for rapid and effective regulation of NO production by specific receptor-mediated signal transduction pathways [Fish and Marsden, 2006]. By contrast, our data indicate that eEPCs cannot be considered functionally competent as mature ECs at least within 3 weeks of culture expansion, due to their extremely low expression of eNOS. Anyway, we cannot exclude that physical stimulations exerted in vivo on the EPC surface, such as sheer stress or haemodynamic forces, can efficaciously induce the synthesis of eNOS [Davis et al., 2004].

Differently from eNOS, the expression of iNOS was more elevated in peripheral-blood eEPCs than HUVECs. A possible explanation is that iNOS was related to the presence of monocytes in the initial pool of MNCs that we identified as $CD45^+$ cells throughout the differentiation period [Rehman et al., 2003]. An increased expression of iNOS in ECs is usually correlated to pathological conditions which are characterized by elevated and dangerous levels of NO [Kleinert et al., 2004]. However, beneficial effects related to iNOS have been also recently described with respect to the EPC functions. For example, iNOS produced from eEPCs appears to play a crucial role in cardioprotection, possibly by increasing angiogenesis [Ti et al., 2005]. More recently, it has been

demonstrated that the iNOS isoform is important for the recruitment of EPCs near the damaged endothelium. In particular, smooth muscle cells and macrophages are particularly prone to produce NO by iNOS, especially in response to cytokine stimulation, and consequently to release VEGF which, in turn, increases the homing and differentiation of EPCs [Mayr et al., 2006]. However, the attracted cells do not incorporate in the newly formed vasculature, indicating that new blood vessel formation induced by VEGF occurs mainly via angiogenesis which could be improved by infiltrating EPCs [Zentilin et al., 2006].

In relation to the emerging role of eEPCs as a source of factors stimulating angiogenesis and vasculogenesis, we speculate that eEPCs derived from peripheral rather than cord blood could better stimulate the recruitment of circulating progenitor cells due to their greater level of intracellular iNOS. Moreover, we suggest that cord-blood eEPCs are not a better source of NO, since they produce a similar amount of constitutive NOS transcripts and even a lower level of iNOS mRNA with respect to the adult counterpart. Finally, there are no substantial differences between peripheral- and cord-blood eEPCs relative to their in vitro ability to grow and expand; also, peripheral-blood eEPCs seem to differentiate quickly than cord-blood eEPCs (Fig. 2b).

Our data are only apparently in contrast with those of Murohara et al. [2000] since, in this work, MNCs were grown in the presence of a bovine pituitary extract whose composition can not be precisely known and that varies depending on different commercial lots. On the contrary, we used well-defined concentrations of specific growth factors (VEGF, bFGF, IGF-I) which are described to selectively and efficiently commit immature cells toward the endothelial phenotype [Quirici et al., 2001].

Thus, these results taken together suggest that there is not a real advantage to use cord blood when the aim is to obtain eEPCs. Moreover, the greater but absolutely low number of immature and clonogenic [Ingram et al., 2004] precursor cells that has been evidenced in cord blood relative to peripheral blood cannot be immediately exploited for therapeutic vasculogenesis, but only after a long period of in vitro expansion. We can also speculate that eEPCs derived from both peripheral and cord blood are not still competent for a proper regulation of vascular tone by NO due to their extremely low levels of eNOS. However, the expression of iNOS, especially in peripheral-blood eEPCs, suggests that NO can be produced as well, but likely exerting a different physiological role with respect to mature ECs.

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