MAPC Transplantation Confers a More Durable Benefit Than AC133+ Cell Transplantation in Severe Hind Limb Ischemia

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There is a need for comparative studies to determine which cell types are better candidates to remedy ischemia. Here, we compared human AC133+ cells and multipotent adult progenitor cells (hMAPC) in a mouse model reminiscent of critical limb ischemia. hMAPC or hAC133+ cell transplantation induced a significant improvement in tissue perfusion (measured by microPET) 15 days posttransplantation compared to controls. This improvement persisted for 30 days in hMAPC-treated but not in hAC133+-injected animals. While transplantation of hAC133+ cells promoted capillary growth, hMAPC transplantation also induced collateral expansion, decreased muscle necrosis/fibrosis, and improved muscle regeneration. Incorporation of differentiated hAC133+ or hMAPC progeny into new vessels was limited; however, a paracrine angio/arteriogenic effect was demonstrated in animals treated with hMAPC. Accordingly, hMAPC-conditioned, but not hAC133+-conditioned, media stimulated vascular cell proliferation and prevented myoblast, endothelial, and smooth muscle cell apoptosis in vitro. Our study suggests that although hAC133+ cell and hMAPC transplantation both contribute to vascular regeneration in ischemic limbs, hMAPC exert a more robust effect through trophic mechanisms, which translated into collateral and muscle fiber regeneration. This, in turn, conferred tissue protection and regeneration with longer term functional improvement.

Key words: Angiogenesis; Stem cells; Critical limb ischemia; Multipotent adult progenitor cells (MAPC);
AC133+ cells

INTRODUCTION

Peripheral vascular disease (PVD) caused by inadequate blood supply to the limbs is a severe health problem in the Western world, affecting 3–10% of the population above the age of 60 (5). Therapeutic revascularization techniques, like laser revascularization, endovascular treatment, or bypass surgery, can in some cases restore flow and prevent gangrene formation/amputation. Nevertheless, for many patients, these techniques are not applicable due to the diffuse nature or the distal location of the obstructions or coexistence of comorbidities. For these no-option patients, alternatives have been proposed, mainly focused on the induction of tissue revascularization by angiogenic gene/protein delivery or by cell therapy. Unfortunately, clinical trials based on growth factor delivery have not yielded the expected results, thereby exposing the need for alternatives (5,13). Evidence indicating that adult vasculogenesis—vessel formation from endothelial progenitor cells (EPC)—is a process that contributes to therapeutic neovascularization (6) has fueled the interest in the use of this alternative paradigm for therapeutic revascularization.

Different (stem) cell populations have been tested in animals for treatment of hind limb ischemia, like embryonic stem cells (ESC) (10), unselected bone marrow mononuclear cells (MNC) (17), peritoneal macrophages (16), adipose tissue-derived stem cells (ADSC) (26), mesenchymal stem cells (MSC) (18), mature endothelial cells (EC) (19,35), or EPC (34,35). Overall, results have

shown that whereas mature EC transplantation does not induce functional improvement, a variable degree of recovery has been demonstrated when MNC or EPC were transplanted. In contrast to earlier reports, more recent mechanistic studies have revealed that the degree of direct effects of stem/progenitor cells on revascularization may be not as robust, and in some cases even negligible (19). Nevertheless, as a result of the promising preclinical results, at least 40 clinical trials with (stem) cells have already been started in PVD patients (5). Most of them have evaluated the potential of cord blood cells, peripheral blood-derived MNC, or total bone marrow (2). A few clinical trials have been performed using enriched stem cell populations like CD34^{+} or AC133^{+} cells demonstrating a clinical benefit [reviewed in (5,13)]. The mean follow-up time in the majority of these studies was rather short, precluding conclusions on longer term benefits or possible side effects, the latter as reported in one recent study (22).

Taking together the existing preclinical and clinical data, further studies should primarily focus on defining the type of stem cell that can exert a greater benefit in patients with critical limb ischemia as well as identifying the mechanism responsible for this effect. In order to do so, direct comparative studies involving different cell types are required. Along this line, we recently demonstrated the superior effect of murine multipotent adult progenitor cells (mMAPC) compared to unfractionated murine bone marrow cells in a mild hind limb ischemia model, reminiscent of intermittent claudication (4). In the current study, we tested the potential of human MAPC (hMAPC) in a severe model of limb ischemia (resembling critical limb ischemia) and compared it with human AC133^{+} cells, which have already been used in clinical trials. We also compared the main mechanisms of action of both cell types.

MATERIALS AND METHODS

Cell and Tissue Processing

Human bone marrow, muscle biopsies, and umbilical cord blood (UCB) cells were obtained after informed consent from donor or mother according to the guidelines from the Committee on the Use of Human Subjects in Research from the Clínica Universitaria, Pamplona. UCB mononuclear cells were separated by Ficoll Hypaque centrifugation (specific gravity, 1077; Sigma, St. Louis, MO, USA) and hAC133^{+} cells were selected using micromagnetic beads (Miltenyi Biotec, Germany) with autoMACS columns (Miltenyi Biotec) as described (3). More than 90% cell purity was obtained after column selection. hMAPC were obtained according to published protocols (3,4). Primary human umbilical vein endothelial cells (HUVEC), umbilical arterial smooth muscle cells (SMC), and skeletal myoblasts (SkM) were prepared from human umbilical cords and muscle biopsies as previously described (3,15). Briefly, for SkM isolation, human muscle biopsies were minced and digested with trypsin-EDTA (0.5 mg/ml trypsin/0.53 mM EDTA) and collagenase (1.5 mg/ml) and isolated cells were grown in Ham-F12 media (GIBCO-BRL) supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (GIBCO-BRL). HUVEC cells were grown in F12K medium (ATCC) supplemented with serum (10%), heparin (0.1 mg/ml), and ECGS (0.03 mg/ml) and SMC were grown in DMEM high glucose (GIBCO-BRL) supplemented with 10% serum. Cells were grown under normoxic conditions.

In Vivo Studies

Surgery. Severe hind limb ischemia was induced under anesthesia (75 mg/kg ketamine/10 mg/kg xylazine) in 6–10-week-old male nude Balbc mice by excision of the left iliac artery as previously described (25). The mice were given postoperative analgesia (5 mg/kg ketoprofen, every day for 3 days). One million cells resuspended in 30 μl of PBS, or PBS alone were injected in four equal fractions in the adductor and quadriceps region of the left limb, 24 h after surgery. As undifferentiated hMAPC express very low levels of MHC-I and are thus sensitive to natural killer (NK) cell-mediated clearance (32), all mice were intraperitoneally injected with 20 μl of a specific anti-NK antibody (28) (anti-asialo GM1 antibody; Wako Chemicals) 1–2 h before transplantation and every 10 days thereafter. Mice were housed in specific pathogen-free conditions and all procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Navarra.

Live Imaging and Assessment of Limb Perfusion. Limb perfusion assessment was performed by microPET as described by our group (25). Briefly, microPET imaging was performed 10 min after ^{13}N-ammonia injection 1, 7, 15, and 30 days after surgery. For quantitative analysis and comparisons among subjects, evaluation of perfusion in both hind limbs was carried out as follows: regions of interest (ROIs) were drawn on coronal 1-mm-thick microPET images over the hind limbs, and activity concentration per area unit calculated as a measurement of perfusion. The ratio between left (ischemic) and right (nonischemic) hind limbs was used in all cases for comparisons and obtained data were exported to the PMOD software package for quantification.

Histological Analysis

At sacrifice (day 15 and day 30), mice were anesthetized with 60 mg/kg Nembutal and perfused with Tris-buffered saline (TBS)-adenosine and followed by zinc-

paraformaldehyde. Following dissection, muscles were postfixed 24 h and divided in two equal pieces to be processed for paraffin embedding. H&E and Sirius red stainings were performed as described (3). Those areas with presence of fat cells replacing muscle fibers, inflammatory infiltrates, or "ghost" cells (muscle cells without nucleus) were considered necrotic areas. For immunofluorescence and immunohistochemistry, antibodies against α-smooth muscle actin (α-SMC, unconjugated, DAKO; or Cy3 or FITC conjugated, Sigma), human CD31 (DAKO or Pharmingen), UEA lectin (biotin, TRITC, or FITC, conjugated; Sigma), mouse BS-I lectin (Sigma), human vimentin (DAKO), and desmin (DAKO) were used as primary antibodies. Secondary antibodies coupled to FITC or peroxidase enzyme were purchased from Molecular Probes. For quantification, analyses were done on cross sections with regular intervals spanning 2 mm. When technically feasible, entire cross sections were evaluated on lower power field images, or when higher magnification was required several (usually 3–4 per section) randomly chosen higher power fields covering a large part of each cross section were analyzed. Pictures for morphometric analysis were taken using a Zeiss Axio Imager connected to an Axiocam MRc5 camera (Zeiss, Zaventem, Belgium) and analysis was performed using Image J, KS300 (Leica, Brussels, Belgium).

In Vitro Proliferation and Apoptosis Assays

Proliferation. hMAPC were cultured in MAPC medium (3) with 2% FCS but without cytokines at a density of 17,500 cells/cm^2, and hAC133^{+} cells were cultured in DMEM high glucose supplemented with 2% FCS at a density of 10^5/cm^2. Conditioned media (CM) were collected after 48 h. Corresponding media incubated without cells for 48 h served as controls [nonconditioned media (NCM)]. Early passages of HUVEC, SMC, or SkM were seeded in 24-well plates at a cell density of 1,250 cells/cm^2 (HUVEC and SkM) or 625 cells/cm^2 (SMC), respectively, and cultured in the presence of hMAPC or hAC133[+] CM or NCM. In order to obtain the same cell concentration as hMAPC, hAC133[+] CM was previously diluted and all media were supplemented with extra serum to reach a 6% FCS concentration. After 72 h, the number of viable cells was quantified by a luminiscent cell viability assay (Cell Titer-Glo™, Promega, USA) following the manufacturer's protocol. Four independent experiments were performed and every cell type grown in CM or NCM was seeded in quadruplicate.

Apoptosis. CM from hMAPC and hAC133[+] were collected as described before, after 12-h culture in non-serum-containing media. Endothelial and skeletal cells

were seeded in 96-well plates at a density of 10^3 cells/cm^2 (HUVEC) or 5,000 cells/cm^2 (SMC and SkM) and cultured under hypoxic conditions (1% O_2) in the presence of CM, NCM, or in normal cell culture conditions (CCM) (20% O_2 and cell culture media) during 24 h for HUVEC and 72 h for SMC and SkM cells. For this assay, no serum was added to the CM. The degree of cell apoptosis was measured by an ELISA kit that quantifies the presence of oligonucleosomal fragments (Roche Applied Science, Barcelona, Spain). The apoptosis control corresponds to cells cultured under hypoxia and NCM. Under this condition apoptosis was clearly detected and considered 100%. The rest of the samples were referred to that value, in order to determine the degree of "protection" conferred by the conditioned media. Four independent experiments were performed on cells seeded in quadruplicate.

RNA Isolation, qRT-PCR, and ELISA

Total RNA from hAC133[+] and hMAPC was extracted using the RNAeasy minikit (Qiagen). mRNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and cDNA underwent 40 cycles of amplification (ABI PRISM 7700, Perking Elmer/ Applied Biosystems). SYBR Green Universal Mix PCR reaction buffer (Applied Biosystems) was used for quantification, normalizing data using *GAPDH* as housekeeping gene. Cytokine levels present in hMAPC or hAC133[+] conditioned and nonconditioned medias were quantified by ELISA. For human VEGF, bFGF, and Ang-2, ELISA kits were purchased from R&D Systems and ELISA procedures were followed according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as mean ± SEM or median (Q1; Q3). Comparisons for repeated measurements were performed by ANOVA or Kruskal-Wallis tests. Shapiro-Wilk test was used to verify that the data had a Gaussian distribution, which justifies the use of a parametric test. Tukey, Tukey's b, and Tamhane's T2 post hoc tests were performed after ANOVA and *U*-Mann-Whitney 1: 1 with signification correction by Bonferroni's method for the nonparametric tests. Statistical analysis was performed with Instat 3.0 software and differences were considered statistically significant when $p < 0.05$.

RESULTS

hMAPC Have a More Durable Effect on Limb Reperfusion Than hAC133[+] Cells

The revascularization potential of hAC133^{+} cells and hMAPC was compared in a severe ischemia model induced by iliac artery excision. After 24 h, such injury caused a dramatic decrease in blood perfusion (~70%)

in the ischemic limb (Fig. 1). By day 15, PBS-injected animals presented a spontaneous recovery in blood flow ($54 \pm 3\%$ at 15 days vs. $23 \pm 3\%$ at 24 h). In comparison with this group, a significantly greater recovery was detected in the animals transplanted either with hAC133^{+} cells or hMAPC (PBS: $54 \pm 3\%$; hAC133[+]: $74 \pm 7\%$, $p < 0.05$; hMAPC: $64 \pm 4\%$, $p < 0.05$). Importantly, 30 days posttransplantation, while animals treated with hMAPC presented a significantly higher blood flow, perfusion recovery was no longer significantly different from controls in the animals treated with hAC133[+] (PBS: $66 \pm 3\%$; hAC133^{+} cells: $73 \pm 5\%$, $p = NS$; hMAPC: $77 \pm 3\%$, $p < 0.05$) (Fig. 1), suggesting that hMAPC exerted a more robust and prolonged effect on reperfusion than hAC133^{+} cells.

hMAPC and hAC133[+] Cells Have a Differential Effect on Blood Vessel Growth

We first examined whether the different effects on reperfusion could be explained by histological assessment of blood vessel growth. In the ischemic adductor region, vascular expansion occurs by a combination of three mechanisms: vasculogenesis (vascular precursor incorporation into growing vessels), angiogenesis (capillary growth), and adaptive arteriogenesis (collateral expansion). First, direct contribution of hMAPC or hAC133[+] vascular progeny was studied at 30 days after surgery. As we have previously reported, direct contribution of hMAPC to both EC (human specific CD31 and UEA positive) and SMC layers (human vimentin and α-actin double positive) of the newly formed vessels was confirmed in the adductor region. However, this contribution was limited (Fig. 2A–C) (4). On the other hand, hAC133^{+} cells also contributed to the EC lineage, although they did not differentiate into SMC (Fig. 2D–F). The remaining hAC133[+] cell fraction at 30 days was too low to allow for reliable quantification. Thus, for both cell types, vascular incorporation was low, which makes it unlikely that vasculogenesis was the main revascularization mechanism.

Next, we quantified the effect of cell transplantation on capillary growth and collateral expansion. Both cell types had a significant effect on both parameters; however, they had a differential effect on these processes. While hAC133^{+} cells mainly affected capillary growth (small CD31^{+} vessels/mm^2: 290 ± 36 in PBS; 759 ± 96 in hAC133^{+}, $p < 0.01$ and 493 ± 45 in hMAPC, $p < 0.01$; hMAPC vs. hAC133^{+}, $p < 0.05$) (Fig. 2G–J), the effect of hMAPC on collateral expansion was much more apparent (α-actin-coated vessel area %: 0.32 ± 0.10 in PBS; 0.59 ± 0.10 in hAC133[+], $p < 0.05$ and 0.72 ± 0.14 in hMAPC, $p < 0.05$; hMAPC vs. hAC133[+], $p < 0.05$) (Fig. 2K–N).

Unlike hAC133[+] Cells, hMAPC Significantly Improve Muscle Viability and Regeneration

In a next series of analyses, we determined whether there was a correlation between revascularization and the degree of tissue damage or regeneration, by analyzing muscle necrosis, fibrosis, and regeneration 30 days posttransplantation. Necrosis and fibrosis were significantly lower in the hMAPC-treated but not in hAC133[+]-treated animals, compared to PBS [% necrosis: 31 ± 5 in PBS; 27 ± 5 in hAC133[+], $P = NS$ and 12 ± 5 in hMAPC, $p < 0.05$; hMAPC vs. hAC133[+], $p < 0.05$ (Fig. 3A–D); % fibrosis: 15 ± 2 in PBS; 12 ± 3 in hAC133[+], $P = NS$ and 8 ± 2 in hMAPC, $p < 0.05$; hMAPC vs. hAC133[+], NS (Fig. 3E–H)]. Moreover, also by day 15, necrosis and fibrosis were significantly lower in the hMAPC-treated animals than in the hAC133[+]-treated ones (data not shown). Finally, we evaluated the degree of muscle regeneration (myogenesis), defined by the presence of fibers with strong positivity for desmin (14). Unlike hAC133[+] cell-injected muscles, at day 30, hMAPC-treated muscles showed a statistically significant higher expression of this marker compared with nontreated limbs [% desmin area: 1.15 ± 0.15 in PBS; 1.51 ± 0.13 in hAC133[+], $p = NS$; 1.71 ± 0.17 in hMAPC, $p < 0.05$; hMAPC vs. hAC133[+], NS) (Fig. 3I–L), a difference that was already detected at day 15 (data not shown). These results suggest a unique capacity of hMAPC to contribute to muscle regeneration compared to hAC133^{+} cells.

hMAPC Secrete Factors That Protect Against Apoptosis and Stimulate Proliferation

Because direct contribution to vessels was limited, we hypothesized that the beneficial effects on revascularization and muscle regeneration were due to trophic effects on endogenous cells. Therefore, we measured growth factor secretion and performed experiments with CM on vascular and muscular cells. Quantification by qRT-PCR showed a higher expression of proangiogenic and arteriogenic factors (VEGF$_{165}$, bFGF, and PlGF) in hMAPC versus hAC133^{+} cells (hMAPC vs. hAC133^{+} cells: % expression vs. universal RNA: VEGF$_{165}$: 236 ± 144 vs. 8.4 ± 0.4; PlGF: 1828 ± 2407 vs. 10 ± 5; bFGF: 452 ± 106 vs. 12 ± 17). Protein secretion, determined by ELISA, confirmed the higher secretion of VEGF$_{165}$, bFGF, and PlGF by hMAPC (hMAPC vs. hAC133^{+} cells: pg/10^5 cells: VEGF$_{165}$: 8066 ± 636 vs. undetectable; bFGF: 20 ± 3 vs. undetectable; PlGF: 11 ± 5 vs. undetectable).

To determine whether the release of growth factors by the transplanted cells could be responsible for the observed beneficial effect on vascular/muscular regeneration in vivo, we analyzed the potential of hAC133[+] cell-
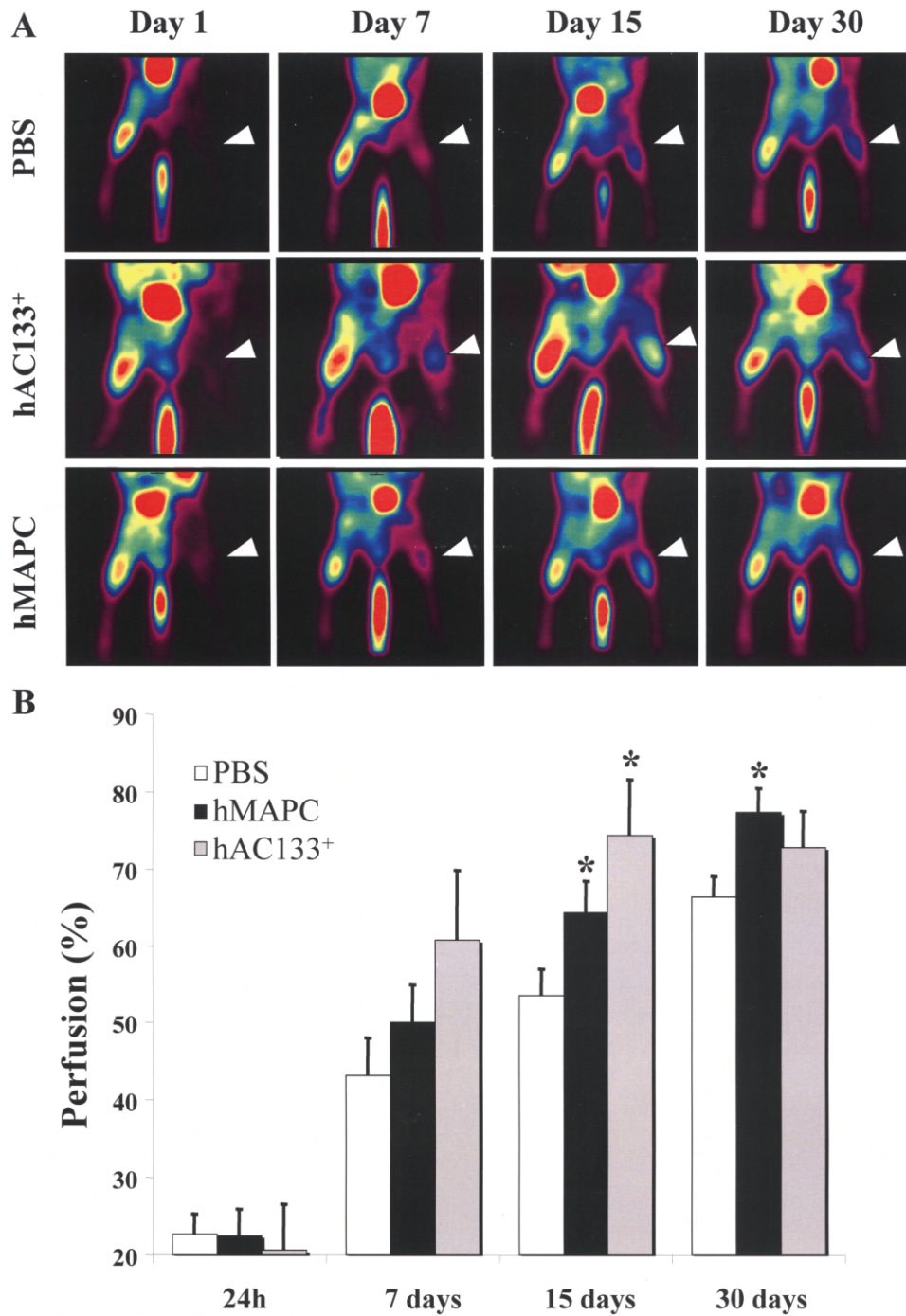
Figure 1. hMAPC have a more durable effect on limb reperfusion than hAC133^{+} cells. (A) Representative ^{13}N-ammonia PET images taken 1, 7, 15, and 30 days after ischemia and transplantation of PBS, hAC133^{+} cells, or hMAPC are shown. Arrowheads indicate the ischemic limb. (B) ^{13}N-Ammonia uptake in animals treated with PBS (white bars), hAC133^{+} cells (gray bars), and hMAPC (black bars) are shown at the different time points as the mean% $\pm$ SEM blood perfusion relative to the nonischemic limbs ($N = 7–10$ mice/group). *$p < 0.05$.
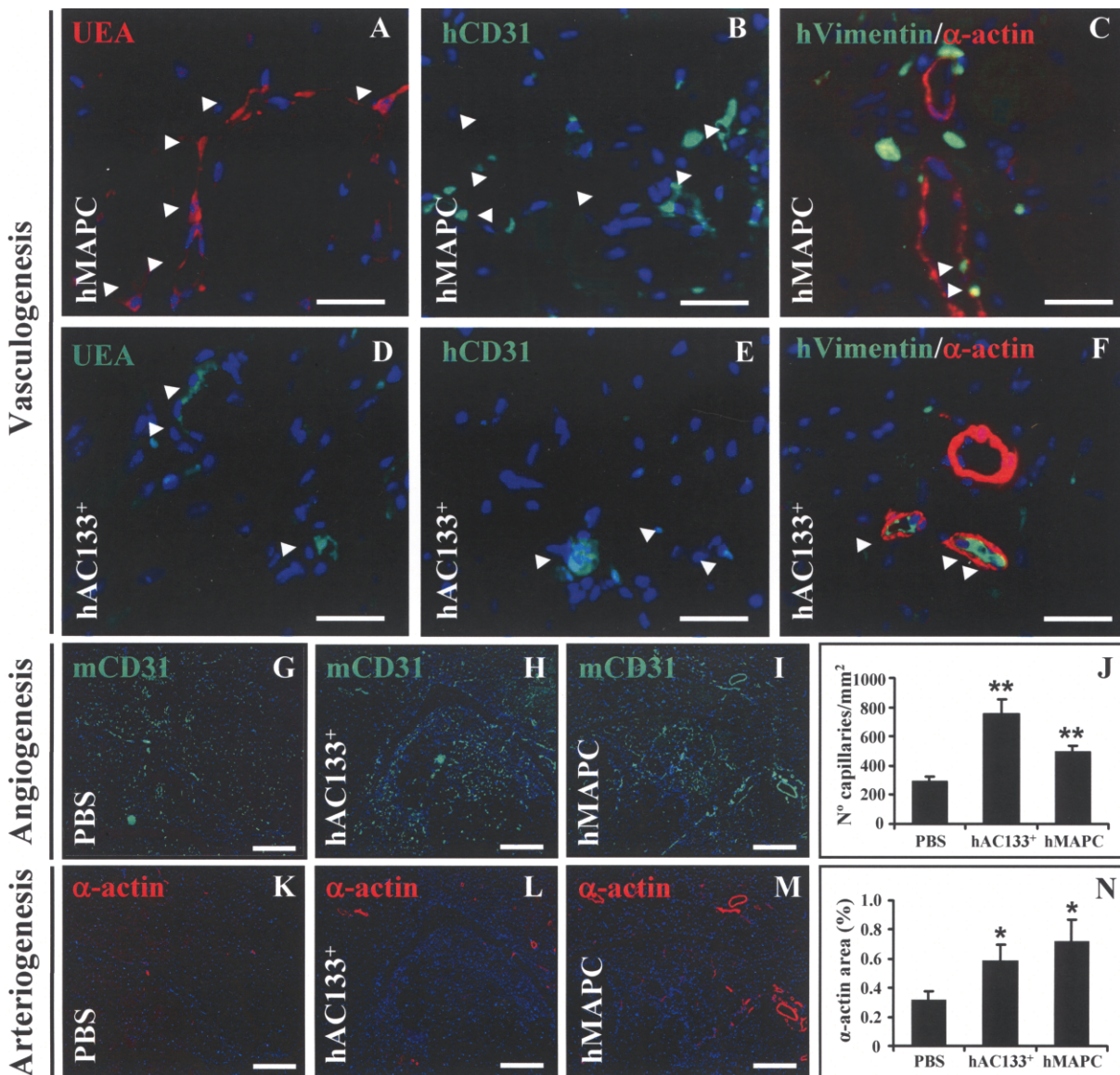
Figure 2. hMAPC and hAC133^{+} cells have a differential effect on blood vessel growth. (A–F) Analysis of direct contribution (vasculogenesis) by immunofluorescence-stained cross sections of hMAPC-transplanted (A–C) or hAC133[+]-transplanted (D–F) adductors for UEA lectin, only recognizing human EC (red in A, green in D), human-specific CD31 (green in B, E) indicating the endothelial identity of the transplanted cells (arrowheads), and double staining for human vimentin (recognizing human mesodermal cells like EC and smooth muscle cells) (green in C, F) and smooth muscle α-actin (recognize both human and mouse smooth muscle cells) (red in C, F) showing colocalization in hMAPC but not in hAC133^{+} cells. (G–J) Analysis of endogenous angiogenesis status. Representative pictures of capillary density (revealed by mouse-specific CD31 in green) in PBS (G), hAC133[+] (H), or hMAPC-treated (I) quadriceps muscles and corresponding quantification (J; data expressed as number of capillaries/muscle area in mm^2; mean ± SEM). (K–N) Representative pictures of collaterals (arteriogenesis) [revealed by (mouse and human) smooth muscle α-actin in red] in PBS (K), hAC133[+] (L), or hMAPC-treated (M) quadriceps muscles and corresponding quantification (N; data expressed as fractional area in % ± SEM; $N = 4$–6 mice per group). DAPI was used for nuclear staining in (A–I, K–M). *$p < 0.05$ and **$p < 0.01$ versus PBS. Scale bars: 50 μm (A–F) and 400 μm (G–I, K–M).

or hMAPC-derived CM to induce proliferation and/or to inhibit apoptosis of vascular/muscular cells. Culture of EC and SMC in the presence of hMAPC but not hAC133[+] CM induced a significant increase in their proliferation rate in comparison with the NCM (EC proliferation relative to NCM: 1.0 ± 0.2-fold for hAC133[+] CM, $p = $ NS and 6.5 ± 0.8-fold for hMAPC-CM, $p < 0.05$; SMC proliferation relative to NCM: 0.9 ± 0.1-fold for hAC133[+] CM, $p = $ NS and 1.4 ± 0.2-fold for hMAPC-CM, $p < 0.05$) (Fig. 4A, B. In addition, while hMAPC-CM almost completely abrogated the apoptosis induced by hypoxia (1% O_2) and serum deprivation in the EC and SMC, no significant protection was observed when cells were cultured in the presence of hAC133[+] CM [% EC apoptosis vs. NCM (100%): 7 ± 3 for CCM (normoxia and 10% FCS), $p < 0.01$, 84 ± 8 for hAC133[+] CM, $p = $ NS and 35 ± 12 for hMAPC-CM, $p < 0.05$; % SMC apoptosis vs. NCM (100%): 18 ± 1 for CCM, $p < 0.01$, 87 ± 5 for hAC133[+] CM, $p = $ NS and 21 ± 10 for hMAPC CM, $p < 0.05$] (Fig. 4C). Finally, the effect of CM on SkM proliferation was moderate in the case of

hMAPC CM with a trend towards statistical significance (data not shown); however, unlike hAC133[+] CM, hMAPC CM exerted a potent antiapoptotic effect on SkM when these were exposed to hypoxia or serum deprivation [% SkM apoptosis vs. NCM (100%): 3 ± 3 for CCM, $p < 0.01$, 97 ± 16 for hAC133[+] CM, $p = $ NS, and 20 ± 8 for hMAPC CM, $p < 0.01$] (Fig. 4C). Thus, hMAPC, but not hAC133^{+} cells, had a beneficial effect on vascular/muscular cell proliferation and/or survival through paracrine effects.

DISCUSSION

The already high incidence of PVD will further increase due to a growing number of patients that carry risk factors (e.g., diabetes, hypertension) predisposing them for developing PVD. At the same time, for many of these patients these risk factors may limit the therapeutic options for revascularization. The demonstration of postnatal vasculogenesis (6) fueled a boost in preclinical studies as well as a rapid clinical translation of this concept. However, many issues need to be resolved be-
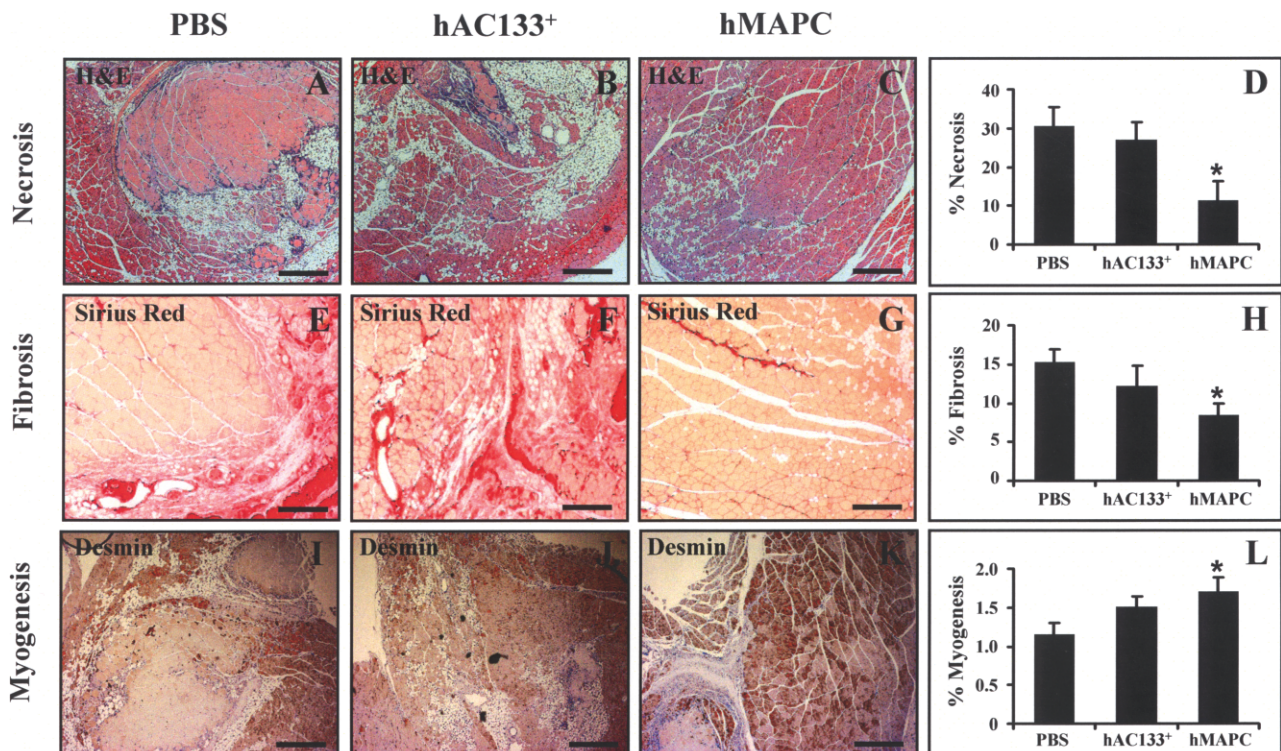

Figure 3. Unlike hAC133^{+} cells, hMAPC significantly improve muscle viability and regeneration. (A–D) Representative pictures of necrosis (defined by dead muscle cells, fat, and inflammatory clusters on H&E) in PBS (A), hAC133[+] (B), or hMAPC-treated (C) quadriceps muscles and corresponding quantification (D); data expressed as fractional area in % ± SEM ($N = 4$–6 mice per group). (E–H) Representative pictures of fibrosis (revealed by Sirius red staining) in PBS (E), hAC133[+] (F), or hMAPC-treated (G) quadriceps muscles and corresponding quantification (H); data expressed as fractional area in % ± SEM ($N = 4$–6 mice per group). (I–L) Representative pictures of myogenesis (revealed by desmin staining) in PBS (I), hAC133[+] (J), or hMAPC-treated (K) quadriceps muscles and corresponding quantification (L; data expressed as fractional area in % ± SEM; $N = 4$–6 mice per group). *$p < 0.05$ versus PBS. Scale bars: 200 µm (E–G) and 400 µm (A–C, I–K).
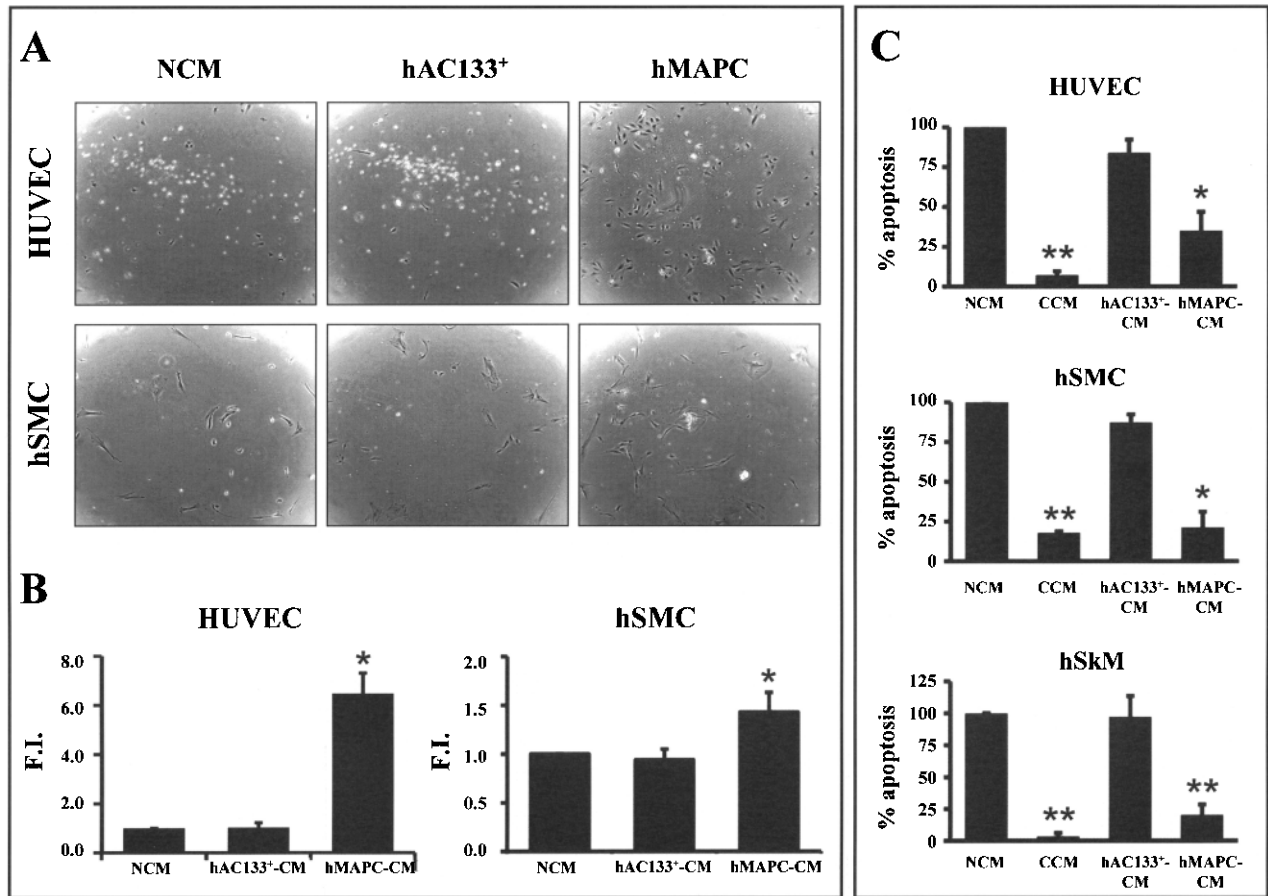
Figure 4. hMAPC secrete factors that protect against apoptosis and stimulate proliferation. (A, B) HUVEC and SMC were cultured in NCM, hAC133+ CM, or hMAPC CM for 72 h. Cell proliferation is shown as fold increase in comparison with proliferation in NCM. The mean ± SEM of four different experiments in quadruplicate is shown as well as representative pictures showing the cell density differences after 72 h. *$p < 0.05$. (C) Apoptosis of HUVEC, SMC, and skeletal myoblasts (SkM) induced by hypoxia and serum deprivation was measured by ELISA after 24 h (HUVEC) or 72 h (SMC and SkM) in culture in the presence of NCM, CCM (cell culture media and normoxia), hAC133+ CM, or hMAPC-CM. Values are expressed as percentage of apoptosis relative to the control group (cells in hypoxic conditions cultured with NCM) where apoptosis was considered 100%. The mean ± SEM of four different experiments in quadruplicate is shown. *$p < 0.05$; **$p < 0.01$.

fore cell transplantation becomes routine practice for PVD treatment. In this context, we performed a recent study in which we compared murine unfractionated bone marrow cells with mMAPC in a model reminiscent of intermittent claudication, a milder PVD form (4). The current study was intended to further extend these studies in order to increase our understanding of the role of stem cells in ischemic disease: by comparing two populations of human-derived cells; by following up the effects of transplantation for longer term; by testing the cells in a severe limb ischemia model, reminiscent of critical limb ischemia (which is the main target for cell therapy); and by performing mechanistic studies to get insight into the way these cells may have beneficial effects. We found that hMAPC had a more durable effect on revascularization than hAC133+ cells. In addition, un-

like hAC133+ cells, hMAPC stimulated muscle regeneration to a statistically significant extent in this severe ischemic setting.

We chose to compare hMAPC with hAC133+ cells because the latter are already used in clinical PVD trials (8,9,20). AC133 (or prominin-1) is a surface antigen expressed on precursors with hematopoietic, neuronal, endothelial, or myogenic potential (30). Our study is not the first to evaluate these cells in a preclinical PVD model; however, in most of the studies, their potential was not compared to that of other stem cell sources and the study settings were also different (7,27,31). Unlike in the majority of these studies, we used a severe model (by more proximal ligation/excision combined with an immune-deficient and low collateral reserve BalbC genetic background) and followed the effects on blood

flow recovery beyond 3 weeks of transplantation. While hMAPC had a sustained and progressive effect on perfusion recovery, the initial benefit of hAC133[+] cell transplantation was lost at 1 month, with a regression of the capillary vessels from day 15 to day 30. Interestingly, the most recent clinical study with the latter cells also showed that the initially observed functional improvement was no longer significant after a 1-year follow-up (8).

Several reasons may explain the temporary nature of the hAC133[+] cell treatment effect. One may be the limited engraftment of the cells in the inflammatory ischemic environment. This may be overcome by delivering the cells on a matrix scaffold, as recently demonstrated in ischemic rat limbs (31). As for the clinical studies, another problem relates to the functional deficit of EPC derived from patients with risk factors (12,23). Recent studies have revealed that these functionally deficient cells can be "reeducated" by pretreatment with (angiogenic) growth factors (33). In the current study we propose another reason for the temporal benefit of hAC133^{+} cells compared to the sustained effect of hMAPC (i.e., a differential mechanism of revascularization). While hAC133[+] mainly affected capillary growth, hMAPC had a significant effect on collateral growth. The latter is responsible for delivering the bulk flow and hence may represent a more crucial requirement for long-term revascularization of the ischemic tissue.

The importance of muscle regeneration is often not considered when evaluating cell therapy studies for limb ischemia. However, from our earlier comparative studies, it was apparent that, even in case of a milder form of ischemia, cells with a combined vascular and muscular regeneration effect, such as undifferentiated mMAPC, had a more durable effect on limb functional recovery compared to cells with a restricted vascular regenerative effect, such as vascular predifferentiated mMAPC (4). Most likely, muscle regenerative effects are even more desirable in more severe forms of ischemia, such as in the model used in the current study. While this has not been shown in a limb ischemia model, hAC133^{+} cells have been recently reported to contribute to skeletal muscle regeneration in a model of muscle injury (29). Here, there was a trend towards stimulation of muscle regeneration (estimated by the appearance of desmin-positive regenerating fibers) following hAC133[+] cell treatment; however, this did not reach statistical significance. hMAPC, on the other hand, did stimulate myogenesis to a significant extent, confirming our previous observations (4). Therefore, despite higher perfusion capacity and greater capillary density induced by day 15 in the AC133^{+}-treated tissues, no significant effect on muscle necrosis was observed, suggesting that the indirect effect exerted by the hMAPC in muscle regeneration was critical for limb recovery. Muscle regeneration

was observed even macroscopically in hMAPC-treated limbs, suggesting a more durable effect of this cell population in comparison with AC133^{+} cells. Additional studies in large animal models will contribute valuable information for future application of hMAPC in patients.

The direct contribution of hAC133[+] or hMAPC to muscle fibers has not been analyzed in the current study. However, we have previously demonstrated a limited contribution of mMAPC to skeletal muscle in a similar hind limb model, even though mostly due to fusion processes (4). Although we cannot rule out a direct muscle (trans)differentiation of hMAPC or even hAC133^{+} cells, the limited engraftment suggests that muscle regeneration was mainly derived from stimulation of endogenous cells induced by factors secreted from hMAPC.

The existence of stem/progenitor cells with broad differentiation potential, such as ESC, and more recently induced pluripotency cells has raised many hopes for regenerative approaches, because one cell type would be sufficient to supply cells for many different damaged tissue types. However, in the last 5 years this paradigm has been shifting, because many studies have now shown that the main benefit of stem cell transplantation may rather result from their trophic activity. Also in the context of ischemia, the potential of stem cells to confer functional and perfusional improvement by the production of cytokines is by no means a new concept (18,19). In our previous study (4), we pointed out that also mMAPC have a beneficial effect on vessel and muscle regeneration mainly by secreting growth factors such as VEGF and IGF-1. Here, we show that also hMAPC secrete significant amounts of VEGF$_{165}$, PlGF, and bFGF, while hAC133^{+} cells did not produce these factors at levels that were detectable by our ELISA assays. Accordingly, CM from hMAPC but not hAC133^{+} cells stimulated EC, SMC, and SkM proliferation and/or survival. Our failure to detect VEGF or other cytokines in hAC133[+] CM may appear in conflict with a recent study; however, they used cells from a different source (fetal aorta versus cord blood in our study) (7).

Patients with myocardial infarction (MI) can also benefit from treatment with endothelial progenitor cells. In fact, functional improvement has been shown after MAPC or AC133 treatment (1,11,21,24), although unfortunately, a direct comparison between the different cell types has not been performed. In accordance with our results in the limb ischemia model, low/absent engraftment and differentiation potential of both cell types have been demonstrated, suggesting that paracrine mechanisms are responsible of the benefit observed. In fact, we have shown that secretion of angio/arteriogenic factors by MAPC are associated with revascularization of the ischemic tissue (24).

In summary, our study demonstrates that locally in-

jected hMAPC but not hAC133⁺ cells have a long-term effect on limb revascularization and, in addition, contribute to muscle regeneration. We believe that these results set the stage for future clinical trials with hMAPC in patients with critical limb ischemia.

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