ENDOTHELIAL PROGENITOR CELL-DERIVED MICROVESICLES IMPROVE NEOVASCULARIZATION IN A MURINE MODEL OF HINDLIMB ISCHEMIA

A. RANGHINO[1], V. CANTALUPPI[1], C. GRANGE[1], L. VITILLO[1], F. FOP[1], L. BIANCONE[1], M.C. DEREGIBUS[1], C. TETTA[2], G.P. SEGOLONI[1] and G. CAMUSSI[1]

[1]Department of Internal Medicine, Research Center for Experimental Medicine (CeRMS) and Center for Molecular Biotechnology, and University of Turin, Turin, Italy; [2]Fresenius Medical Care, Bad Homburg, Germany

Received March 24, 2011 – Accepted November 15, 2011

Paracrine mediators released from endothelial progenitor cells (EPCs) have been implicated in neoangiogenesis following ischemia. Recently, we demonstrated that microvesicles (MVs) derived from EPCs are able to activate an angiogenic program in quiescent endothelial cells by a horizontal transfer of RNA. In this study we aim to investigate whether EPC-derived MVs are able to induce neoangiogenesis and to enhance recovery in a murine model of hindlimb ischemia. Hindlimb ischemia was induced in severe combined immunodeficient (SCID) mice by ligation and resection of the left femoral artery and mice were treated with EPC-derived MVs (MVs), RNase-inactivated MVs (RnaseMVs), fibroblast-derived MVs or vehicle alone as control (CTL). Since MVs contained the angiogenic miR-126 and miR-296, we evaluated whether microRNAs may account for the angiogenic activities by treating mice with MVs obtained from DICER-knock-down EPC (DICER-MVs). The limb perfusion evaluated by laserdoppler analysis demonstrated that MVs significantly enhanced perfusion in respect to CTL (0.50±0.08 *vs* 0.39±0.03, $p < 0.05$). After 7 days, immunohistochemical analyses on the gastrocnemius muscle of the ischemic hindlimb showed that MVs but not fibroblast-MVs significantly increased the capillary density in respect to CTL (MVs *vs* CTL: 24.7±10.3 *vs* 13.5±6, $p < 0.0001$) and (fibroblast-MVs *vs* CTL: 10.2±3.4 *vs* 13.5±6, ns); RNaseMVs and DICER-MVs significantly reduced the effect of MVs (RNaseMVs *vs* CTL: 15.7±4.1 *vs* 13.5±6, ns) (MVs *vs* DICER-MVs 24.7±10.3 *vs* 18.1±5.8, $p < 0.05$), suggesting a role of RNAs shuttled by MVs. Morphometric analysis confirmed that MVs enhanced limb perfusion and reduced injury. The results of the present study indicate that treatment with EPC-derived MVs improves neovascularization and favors regeneration in severe hindlimb ischemia induced in SCID mice. This suggests a possible use of EPCs-derived MVs for treatment of peripheral arterial disease.

Peripheral arterial disease, caused by atherosclerotic occlusion of the leg arteries, is an important manifestation of systemic atherosclerosis along with coronary heart disease and cerebrovascular disease. The age-adjusted prevalence of peripheral arterial disease is approximately 12%, and affects men and women equally. The typical clinical manifestation is claudication, nevertheless 5% of patients undergo an amputation within 5 years (1, 2).

Key words: microvesicles, angiogenesis, hindlimb ischemia

Mailing address: Dr. G. Camussi,
Dipartimento di Medicina Interna,
Ospedale Maggiore S. Giovanni Battista,
Corso Dogliotti 14,
10126, Torino, Italy
Tel: +39 011 6336708 Fax: +39 011 6631184
e-mail: giovanni.camussi@unito.it

0394-6320 (2012)

Neovascularization is an important event in rescuing tissues after ischemia. Several studies have shown that stem cells and progenitor cells such as endothelial progenitor cells (EPCs) contribute to neoangiogenesis during hindlimb ischemia (3-7).

The mechanisms by which stem cells or EPCs are able to induce recovery in damaged organs or tissues are not completely understood. Nevertheless, some studies have demonstrated that a transient localization of stem cells in injured tissues might be sufficient to induce functional and regenerative events suggesting the release of paracrine mediators (8-12). The mechanisms underlying cell-to-cell communication involve the secretion of cytokines, chemokines, growth factors, adhesion molecules, tunneling nanotubules and circular membrane fragments called microvesicles (MVs) (13-16).

MVs are released from the cell surface of normal or damaged cells and are able to transfer mRNAs and microRNAs from the cell origin to other cells in a defined microenviroment (17-20). The shedding of MVs from cell surface is a physiological phenomenon that may increase during cell activation, hypoxia or irradiation, oxidative injury and exposure to protein from an activated complement cascade (15, 21-26).

Recently, we demonstrated that MVs derived from EPCs are able to promote proliferation and reduce apoptosis *in vitro* of human microvascular endothelial cells and human umbilical vein endothelial cells. These effects require the incorporation of the MVs into endothelial cells with transfer of genetic information. *In vivo,* human umbilical vein endothelial cells pre-incubated with MVs derived from EPCs formed patent vessels in Matrigel when implanted subcutaneously in SCID mice (18).

In the present study we investigated whether EPC-derived MVs could induce neoangiogenesis in a murine model of hindlimb ischemia that mimics a peripheral arterial disease to evaluate whether the MVs may represent a potential therapeutic strategy.

MATERIALS AND METHODS

Human endothelial progenitor cell cultures

EPCs were isolated from PBMCs of healthy donors by density centrifugation, seeded on type I collagen-coated plates (27) and characterized as previously described (18). When homogeneous monolayers with typical cobblestone morphology were obtained, phenotypic characterization and functional evaluation of angiogenic properties were carried out as previously described (18). EPCs from 3-5 passages were used to avoid monocyte and platelet contamination. By FACS, EPCs resulted negative for CD2, CD3, CD4, CD5, CD8, CD16, CD20, CD62E, VEGFR-1, CD14, CD45 but positive for CD34, CD133, Tie-2, VEGFR-3, VEGFR-2, P-selectin and CD42b. EPCs were characterized by dual-staining for 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein and Ulex europaeus agglutinin-1 and lectin and by the expression of endothelial marker proteins VEGFR-2, VE-cadherin, eNOS, and vWF. Endothelial phenotype was further confirmed by Western blot analysis and RT-PCR by expression of markers characteristic for endothelial cells such as Tie-2, VEGFR-2, VEGFR-3, but not of VEGFR-1 (18).

Isolation and characterization of MVs released from EPCs

MVs were obtained from supernatants of EPCs by ultracentrifugation (Optima L-90K, Beckman Coulter, Fullerton, CA) as previously described (18). Transmission and scanning electron microscopy showed the spheroid morphology of MVs with a size ranging from 60-130 nm. By FACS analysis, MVs were detectable under the 1 μm beads and expressed the hematopoietic stem cell marker CD34 and molecules essential for leukocyte adhesion such as α4 and β1 integrins and L-selectin. MVs did not express HLA class I and class II and markers of platelets (P-selectin, CD42b) and monocytes (CD14). RNA extraction from MVs was performed using the mirVana isolation kit (Ambion, Austin, TX). RNA was analyzed by Agilent 2100 bioanalyzer (Agilent Tech. Inc., Santa Clara, CA). miR-126 and miR-296 expression levels were analyzed by qRT-PCR in a 48- well StepOne™ Real Time System (Ambion): 200 ng of RNA was reverse-transcribed and the cDNA was used to detect and quantify miR-126 and miR-296 by qRT-PCR using the miScript SYBR Green PCR Kit (Qiagen, Valencia, CA, USA). In selected experiments, MVs were treated with 1U/ml RNase (Ambion) for 3 h at 37°C. After RNase treatment the reaction was stopped by addition of 10 U/ml RNase inhibitor (Ambion) and MVs were washed by ultracentrifugation (18). The efficacy of RNase treatment was evaluated by MV-RNA analyses by Agilent 2100 bioanalyzer (Agilent) and by evaluation of miR-126 and miR-296 by qRT-PCR. As control, MVs were obtained from human fibroblast as previously described (21).

Knock-down of DICER in EPCs

Knock-down of DICER in EPCs was performed by a specific siRNA according to the manufacturer's

instructions (Santa Cruz Biotech, Santa Cruz, CA, USA). An irrelevant siRNA (vector-MVs) was used as control. Western blot for DICER expression was performed by using an anti-DICER polyclonal antibody (Abcam, Cambridge, UK). The maximum knock-down of DICER was observed four days after transfection. At that time MVs derived from DICER knock-down-EPCs (DICER-MVs) or vector-MVs were collected by supernatants of engineered cells and used for proliferation and angiogenesis assays.

Murine model of hindlimb ischemia

All procedures were approved by the Ethics Committee of the University of Torino and conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. SCID mice (Charles River Laboratories), aged 7 to 8 wks and weighing 18 to 22 g, were anesthetized with i.m. injection of zolazepam 80 mg/Kg. Postoperatively, the animals were closely monitored, and analgesia with Ketorolac (5mg/Kg) was administered if required.

Under sterile conditions, a small skin incision was made overlying the middle portion of the left hindlimb of each mouse. The proximal end of the left femoral artery and the distal portion of the saphenous artery were ligated and dissected free and excised. The overlying skin was closed using a sterilized 6-0 silk suture (28). Immediately before the surgery the mice were treated with 50 μg of proteins of MVs, RNase-inactivated MVs (RNase-MVs), DICER-MVs or vector-MVs, fibroblast-MVs or vehicle alone (CTL) administered intravenously, and after surgery with 100 μg of proteins of the same preparations. Mice were sacrificed on day 7 (T7) after hindlimb ischemia.

Monitoring of hindlimb blood flow

After anesthesia, hair was removed from both legs using a depilatory cream, following which the mice were placed on a heating plate at 37°C for 3 min to minimize temperature variations. Hindlimb blood flow was measured using a Laser Doppler Blood Flow (LDBF) analyzer (PeriScan PIM 3 System, Perimed, Stockholm, Sweden). Immediately before surgery, and on postoperative days 0, 3, 7, LDBF analysis was performed on hindlimbs and feet. Blood flow was displayed as changes in the laser frequency using different color pixels. After scanning, stored images were analyzed to quantify blood flow. To avoid data variations caused by ambient light and temperature, hindlimb blood flow was expressed as the ratio of left (ischemic) to right (non-ischemic) LDBF (28).

Evaluation of capillary density

Capillary density within gastrocnemius muscles was quantified by immunofluorescence analysis. Muscle samples were embedded in OCT compound (Miles) and snap-frozen in liquid nitrogen. Tissue slices (5 μm thick) were prepared and capillary endothelial cells were identified by immunofluorescence using a monoclonal antibody against mouse CD31 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Fifteen randomly chosen microscopic fields from three different sections in each tissue block were examined for the count of capillary endothelial cells for each mouse specimen. Capillary density was expressed as the number of CD-31-positive features per high power field (x400).

Macroscopic evaluation of ischemic severity

One week after the operation, the ischemic limb was macroscopically evaluated by using an ischemia scale assessed to detect less severe levels of ischemia. Tissue grade 0 corresponds to autoamputation of the leg; grade 1 to leg necrosis; grade 2 to foot necrosis; grade 3 relates to two or more toe discoloration; grade 4 to one toe discoloration; grade 5 to two or more nail discoloration; grade 6 to one nail discoloration and grade 7 to the absence of necrosis (29).

Histology

The gastrocnemius muscle from ischemic and non-ischemic limb was dissected out at day 7 after surgery, immediately fixed with 4% paraformaldeide for 8 hours and then embedded in paraffin. Tissue slices were stained with hematoxylin and eosin. Slides were examined under light microscopy at ×200 magnification. Images were acquired from all the injured area of the ischemic limb sections for the count of the total muscle fibers and of the fibers with small-diameter and with multiple central nuclei that are indicative of the regeneration process. Random images from the total non-ischemic limb section were considered as a control (30).

Statistical analysis

Statistical analysis was performed with SPSS (SPSS Inc. Chicago, IL, USA). Differences between groups were analyzed with Student's *t*-test, ANOVA with the Newmann-Keuls multicomparison tests and Mann-Whitney or Kruskal-Wallis non-parametric tests when appropriate. A p value of < 0.05 for all tests was considered statistically significant.

RESULTS

Ischemic hindlimb perfusion

Immediately after left femoral artery resection, the ratio between the ischemic and non-ischemic

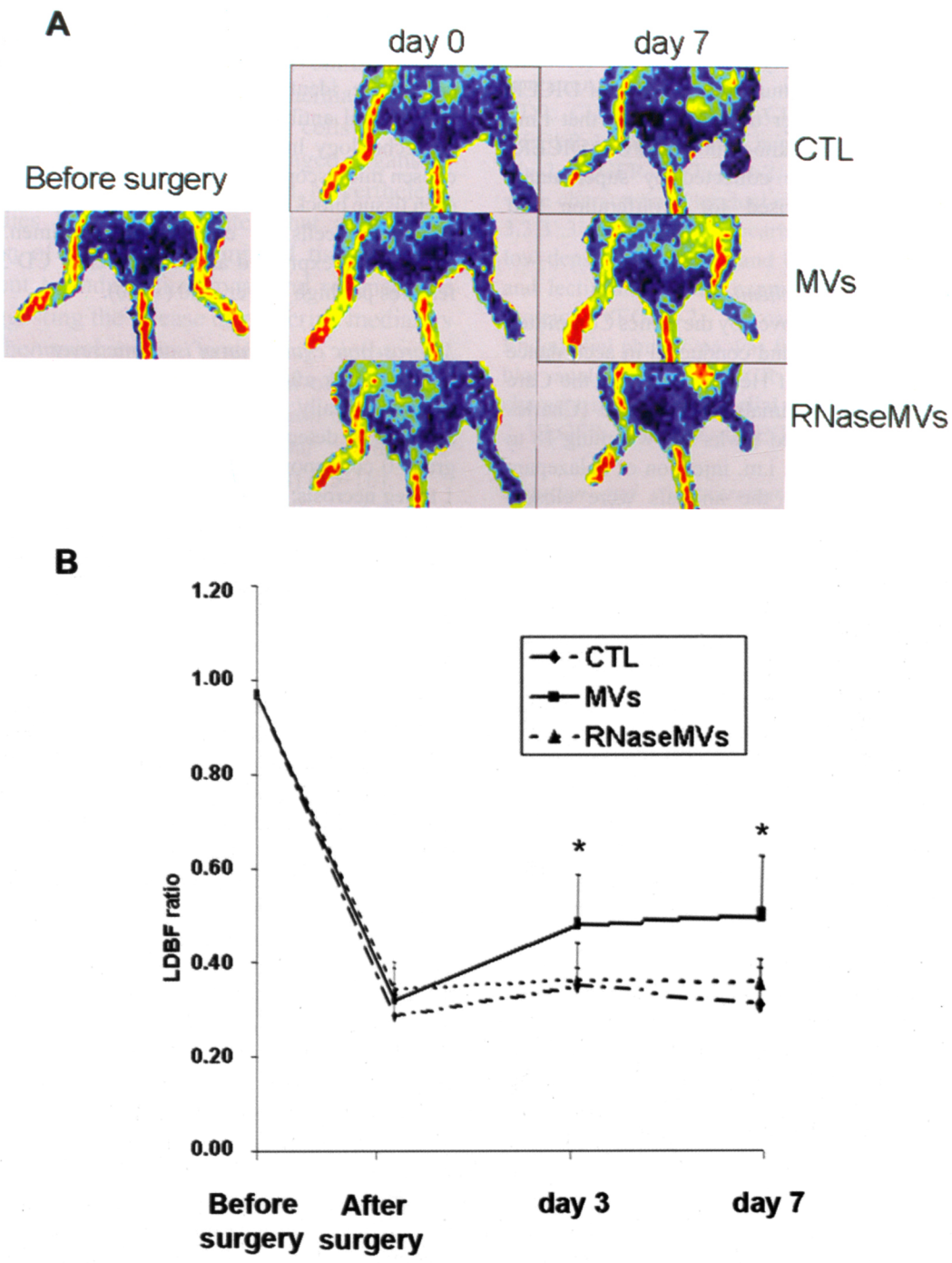

Fig. 1. *Effect of MVs on blood perfusion. **A)** Representative images of laser doppler blood flow obtained before surgery, immediately after surgery (day 0) and 7 days after surgery (day 7) in CTL, and in mice treated with MVs and RNase inactivated MVs. Hues indicating regions from lower to higher perfusion are as follows: dark blue, blue, green, yellow, red, dark red. **B)** Quantitative analysis of perfusion measured by laser doppler blood flow (LDBF) ratio indicates that MVs significantly enhanced perfusion in respect to CTL and RNaseMVs-treated groups. Data are expressed as mean±SD; *p value < 0.05, (Mann-Whitney non-parametric test).*

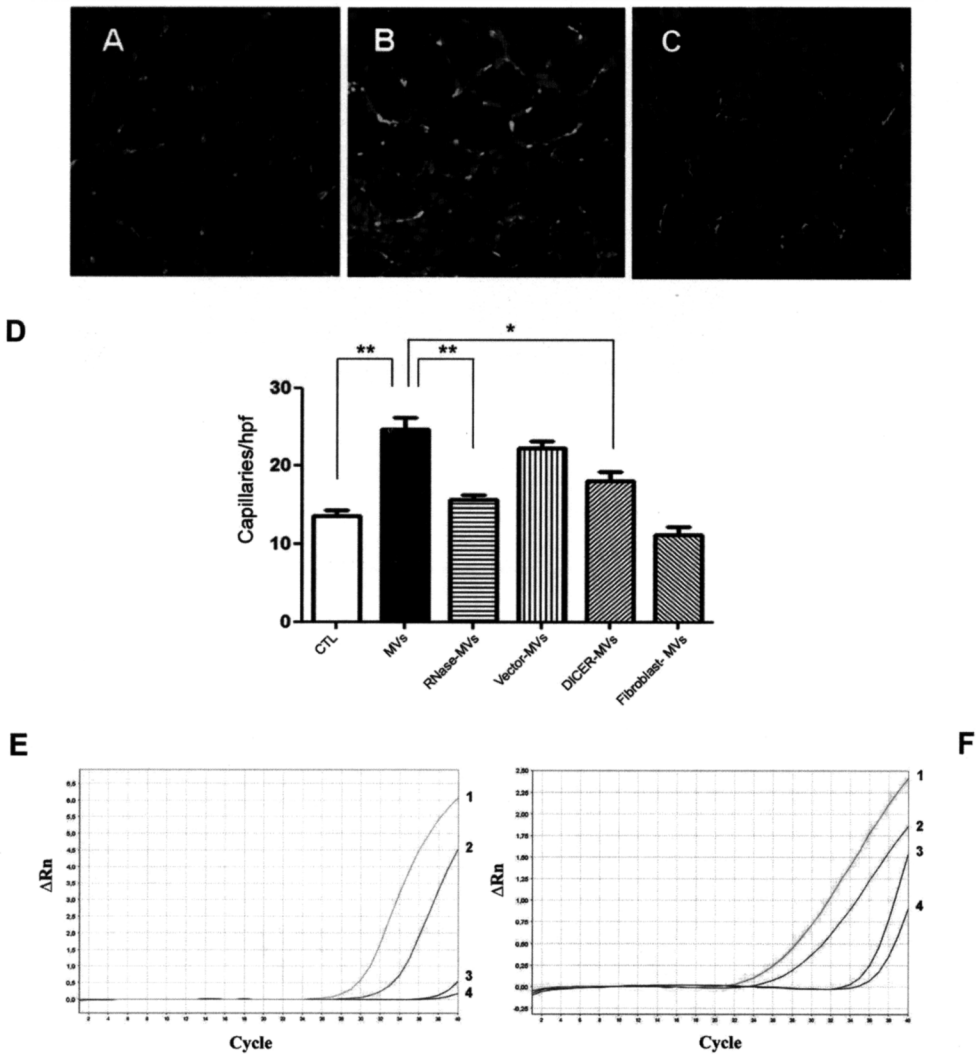

Fig. 2. *Increased capillary density in MVs-treated mice after hindlimb ischemia. Representative confocal images of capillaries stained with mAbs anti-CD31 in CTL (**A**), MVs-treated (**B**) and RnaseMVs-treated (**C**) mice (Original magnification x400). **D)** Quantitative analysis of capillary density in the ischemic hindlimb. Data are expressed as mean±SD; **p value < 0.0001, *p value < 0.05, (analysis of variance, ANOVA). **E)** Representative qRT-PCR analysis for miR-126 (1 and 3) and miR-296 (2 and 4) in MVs incubated with vehicle alone (1 and 2) or with 1 U/ml RNase (3 and 4) to inactivate RNAs. **F)** Representative qRT-PCR analysis for miR-126 (1 and 3) and miR-296 (2 and 4) in MVs derived from EPCs transfected with Vector siRNA as control (1 and 2) or DICER siRNA (3 and 4).*

hindlimb decreased to 0.32±0.06 in the CTL group, 0.35±0.06 in the MVs group and 0.37±0.05 in the RNase-MVs group, (n=10/group) indicating that the severity of the induced ischemia was similar in the three experimental groups. Fig. 1A shows representative LDBF images of hindlimb blood flow before, immediately after (day 0), at 3 and 7 days after surgery in the three groups (CTL, MVs and RNaseMVs). In the MV-treated group hindlimb perfusion ratio significantly increased 3 days after surgery compared with the CTL group (0.50±0.08 *vs* 0.39±0.03, $p < 0.05$) and the RNase-MVs treated group (0.50±0.08 *vs* 0.41±0.06, $p < 0.05$). This significant increase in the perfusion ratio observed in the MV-treated group compared to the CTL and the RNase-MVs treated groups still persisted at day

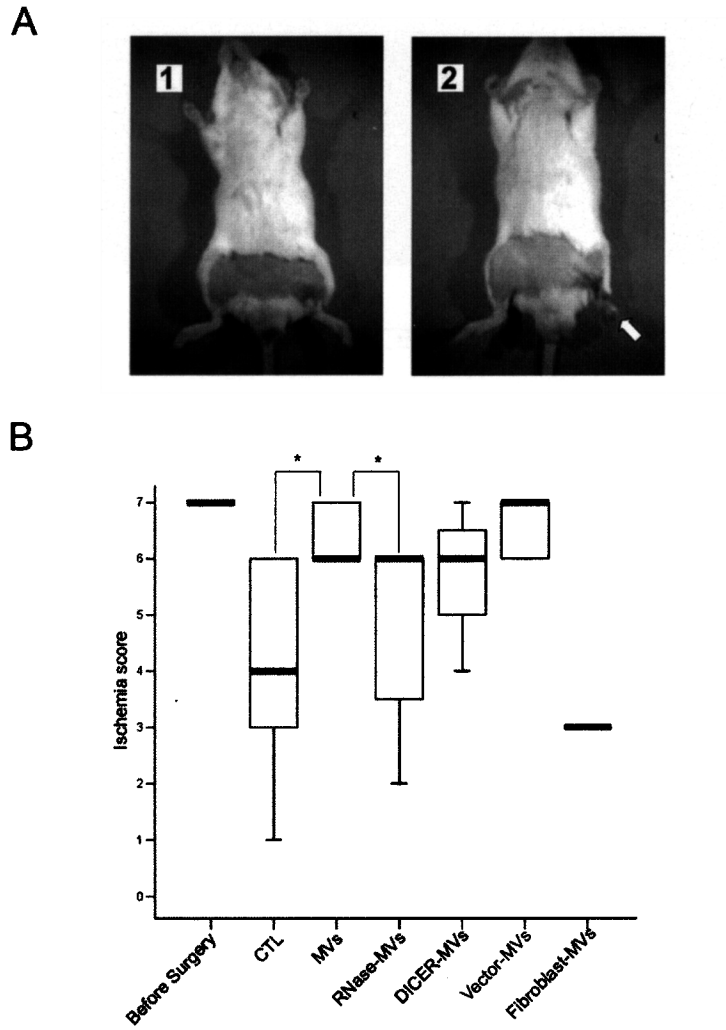

Fig. 3. *Administration of MVs reduces limb loss and increases limb salvage.* ***A)*** *Representative macroscopic photographs of mice showing the positive outcome of MV-treated mice (1, limb salvage) compared to CTL mice (2, foot necrosis, as indicated by white arrow).* ***B)*** *Quantitative analysis of morphological changes in the different experimental conditions evaluated with the ischemia score as described in Methods section. Continuous variables did not follow a normal distribution and are presented as median (min-max); *p value < 0.05. The difference between groups was analyzed with Mann-Whitney and Kruskal-Wallis non-parametric tests.*

7 (MVs 0.52±0.08 *vs* CTL 0.41±0.05, $p < 0.05$; MVs 0.52±0.08 *vs* RNaseMVs 0.43±0.03, $p < 0.05$) (Fig. 1B).

Capillary density

Quantitative analysis of CD31 positive cells in muscle sections obtained from ischemic hindlimb (n= 10/group) revealed that the capillary density was significantly increased in the MV-treated group compared with the CTL group (24.7±10.3 *vs* 13.5±6, $p < 0.0001$) (Fig. 2, A-D). Fibroblast-derived MVs did not increase the capillary density in the ischemic hindlimb compared with CTL group (10.2±3.4 *vs* 13.5±6, ns). Despite MVs protecting RNA from inactivation by physiological concentration of RNase, a previous study demonstrated that treatment with a high concentration of RNase inactivate RNAs shuttled by MVs and inhibited MV biological activities (18, 20). Mice treated with RNase inactivated MVs did not show a significant increase

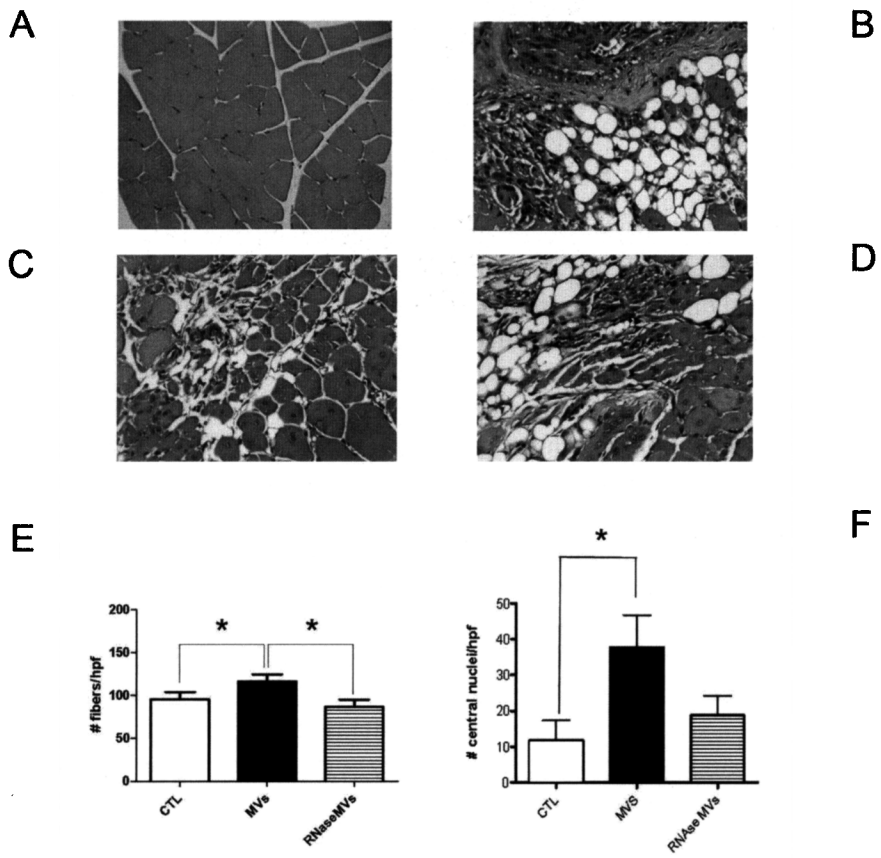

Fig. 4. *MV treatment protects from ischemic muscle damage and promotes muscle regeneration. Representative images of hematoxylin-eosin stained muscle sections before surgery (**A**), and 7 days after surgery of CTL (**B**), MVs-treated (**C**) and RNaseMVs-treated (**D**) mice. **E**) Quantitative analysis of the total number of muscle fibers. **F**) Quantitative analysis of regenerative muscle fibers in the different experimental conditions. Data are expressed as mean±SD; *p value < 0.05, (ANOVA with the Newmann-Keuls multicomparison tests).*

in capillary density compared with the CTL group (15.7±4.1 *vs* 13.5±6, ns), (Fig. 2 C,D), suggesting that the biological activity of EPC-derived MVs was mediated by MV-shuttled RNA.

Role of miRNAs in MV-induced angiogenesis

As shown by RT-PCR, MVs derived from EPC contained miR-126 and miR-296 that are known to be angiogenetic (Fig. 2E). RNase treatment significantly reduced the content of miR-126 and miR-296 (Fig. 2E). To study the role of miRNAs in MV-induced angiogenesis, we engineered EPCs to knock-down DICER, the intracellular enzyme essential for miRNA synthesis. For these experiments, we used MVs derived from EPCs. qRT-PCR analysis showed reduced levels of miR-126 and miR-296 in DICER-MVs but not in Vector-MVs (Fig. 2F). We found that DICER-MVs but not Vector-MVs significantly decreased capillary density (24.7±10.3 *vs* 18.1±5.8, $p < 0.05$) (Fig. 2D).

Severity of ischemic changes

One week after the operation, the ischemic limb was macroscopically evaluated (CTL, MVs and RNase-MVs, n=10/group and DICER-MVs, Vector-MVs, Fibroblast-MVs n=5/group) by using an ischemia scale assessed to detect the levels of ischemia, according to Westvik, et al. (29). Mice treated with EPC-derived MVs exhibited significantly less severe degrees of limb ischemia compared to the CTL group injected with vehicle alone (5.8±2 *vs* 4.1±1.8, $p < 0.05$) and to the RNase-

MVs treated group (5.8±2 *vs* 4.6±1.6, $p < 0.05$). Animals treated with RNase-MVs or DICER-MVs showed a quite similar degree of ischemia compared to the CTL group (4.6±1.7 *vs* 4.1±1.8, n.s. and 5±1.5 *vs* 4.1±1.8, ns, respectively), (Fig. 3 A,B).

Muscle histology

We examined muscle histology of the ischemic limb dissected out at day 7 after surgery (Fig. 4 A-D). Control mice demonstrated a significant reduction of muscle fibers per area compared to MV-treated mice (95.7±37.8 *vs* 116.3±40.7, $p < 0.05$) (Fig. 4E). No significant differences were noted between CTL and RNaseMVs-treated mice. In addition, the number of muscle fibers characterized by a small-diameter and with multiple central nuclei that are hallmarks of the muscle regeneration process was significantly increased in MV-treated mice compared with the CTL group (39±28 *vs* 11±9, $p < 0.05$) (Fig. 4F).

DISCUSSION

The results of the present study demonstrate that MVs derived from EPCs promote neovascularization in an animal model of experimentally induced limb ischemia as a model of peripheral arterial disease.

Rapid revascularization after ischemia is essential to restore tissue function. Several studies suggested that neoangiogenesis after an ischemic injury may involve the recruitment of EPCs by chemokines released from the injured tissues (31). Transplantation of EPCs was shown to facilitate revascularization of various tissues including limb ischemia and postmyocardial infarction (4, 32). It is unclear whether EPCs directly contribute to the formation of neovessels by generation of mature endothelial cells or just by producing angiogenic factors. It has been recently shown that transient localization of EPCs in the injured tissues may be sufficient to induce functional and regenerative events, suggesting a paracrine mechanisms (8-12). Furthermore, pro-angiogenic EPCs may release MVs capable of exerting effects on surrounding cells. Recent studies indicate that MVs play an important role in cell-to-cell communication by direct stimulation of target cells or by transferring proteins or mRNAs and microRNAs (17-20). We recently demonstrated that MVs released from EPCs were able to activate *in vitro* and *in vivo* an angiogenic program in endothelial cells via a horizontal transfer of mRNAs. A proof of translation of MV-shuttled mRNA by recipient cells was obtained using MVs carrying GFP mRNA (18). EPC-derived MVs were shown to be incorporated in endothelial cells by interaction with α4 and β1 integrins expressed on the MV surface. In addition, MVs were shown to activate in the recipient endothelial cells phosphatidyl inositol-3 kinase (PI3K) and eNOS, known to be involved in angiogenic and antiapoptotic programs. mRNA MVs were shown to carry biologically active miRNAs (19, 20). We herein showed that MVs derived from EPC contained miRNA-126 and miRNA-296 that are known to be proangiogenic mainly by regulating response to VEGF in endothelial cells (33, 34). However, MVs might contain other miRNAs that could contribute to the neoangiogenetic effect observed. It has been suggested that the pro-angiogenic potential of EPC obtained from the circulation may depend on the contamination with platelet-derived products (35). To avoid contamination, we used MVs isolated from EPCs after the 3rd passage in culture. Moreover, the cells used and the derived MVs expressed markers of stem cells (CD34, CD133) and of endothelium (VEGFR2, CD31) but not of monocytes (CD14) and platelets (P-selectin, CD41, CD42b).

In the present study we found that i.v. administration of EPC-derived MVs improved the revascularization after ischemic damage in a mouse ischemic hindlimb model (Figs. 1 and 2). Animals treated with MVs had a significantly lower incidence of the biological consequences of ischemia such as foot and limb necrosis compared to the animals injected with vehicle alone (Fig. 3). The enhanced perfusion was associated with the reduced muscle damaged in ischemic limb of mice treated with MVs, suggesting a protective effect. In addition, there were signs of muscle regeneration as inferred by a significant increase in the number of small rounded myofibers with central nuclei in the damaged muscle area of MV-treated mice (Fig. 4). The specificity of the biological effect of MVs derived from EPC was indicated by the absence of protective effect of MVs derived from fibroblasts.

The observation that the RNase inactivation of EPC-derived MVs resulted in reduction of their

protective effect, suggests an involvement of RNA delivery. When MVs derived from DICER knock-down-EPCs were used the proangiogenic effect was also significantly reduced suggesting that the proangiogenic effect is at least in part mediated by MV miRNAs cargo.

Peripheral arterial disease along with coronary heart disease and cerebrovascular disease represent a serious and growing health problem in Western countries. Peripheral arterial disease causes intractable ischemia, impaired mobility, compromised wound healing, ulceration, and amputations (1, 2). EPC transplantation has been suggested as an angiogenic strategy. EPCs are able to promote repair of endothelium (reendothelization) and postnatal formation of new capillaries (neovascularization) (4, 31). Nevertheless, cell therapy for limb ischemia using EPCs imply autologous transplantation of cells because of the expression of HLA antigens, and EPCs obtained from peripheral blood require *ex vivo* expansion. The need of both autologous cells and *in vitro* expansion of the EPCs isolated from peripheral blood limit this procedure because the amount of cells required for transplantation is achieved only after several days of culture (6, 7). Our findings demonstrate the ability of EPC-derived MVs to induce a neoangiogenic program after ischemia. In contrast with EPCs, MVs do not express HLA antigens, therefore MVs could be produced in large amounts using EPCs collected from healthy volunteers and promptly infused in patients with severe ischemia irrespective of their HLA pattern.

In conclusion, the proangiogenic effect of EPCs is mimicked by EPC-derived MVs, suggesting a paracrine mechanism. Further studies are needed to investigate whether MVs might be a valid and safety therapeutic option for peripheral arterial diseases.

ACKNOWLEDGEMENTS

We thank Professor Emilio Hirsh and Professor Mara Brancaccio for technical advice.

This work was supported by Italian Government Miur PRIN project, 'Regione Piemonte, Piattaforme Biotecnologiche PiSTEM project, and Converging Technologies NanoIGT project and Ricerca Finalizzata and Local University Grants ('ex60%).

Disclosures: V.C., M.C.D. and G.C. are inventors named in a related patent (publication number: WO/2009/050742). C.T. (Fresenius Medical Care) is employed by a commercial company and contributed to the study as a researcher.

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