Brief UltraRapid Communication

Exosomes From Human CD34+ Stem Cells Mediate Their Proangiogenic Paracrine Activity

Susmita Sahoo, Ekaterina Klychko, Tina Thorne, Sol Misener, Kathryn M. Schultz, Meredith Millay, Aiko Ito, Ting Liu, Christine Kamide, Hemant Agrawal, Harris Perlman, Gangjian Qin, Raj Kishore, Douglas W. Losordo

Rationale: Transplantation of human CD34+ stem cells to ischemic tissues has been associated with reduced angina, improved exercise time, and reduced amputation rates in phase 2 clinical trials and has been shown to induce neovascularization in preclinical models. Previous studies have suggested that paracrine factors secreted by these proangiogenic cells are responsible, at least in part, for the angiogenic effects induced by CD34+ cell transplantation.

Objective: Our objective was to investigate the mechanism of CD34+ stem cell–induced proangiogenic paracrine activity and to examine if exosomes, a component of paracrine secretion, are involved.

Methods and Results: Exosomes collected from the conditioned media of mobilized human CD34+ cells had the characteristic size (40 to 90 nm; determined by dynamic light scattering), cup-shaped morphology (electron microscopy), expressed exosome-marker proteins CD63, phosphatidylserine (flow cytometry) and TSG101 (immunoblotting), besides expressing CD34+ cell lineage marker protein, CD34. In vitro, CD34+ exosomes replicated the angiogenic activity of CD34+ cells by increasing endothelial cell viability, proliferation, and tube formation on Matrigel. In vivo, the CD34+ exosomes stimulated angiogenesis in Matrigel plug and corneal assays. Interestingly, exosomes from CD34+ cells but not from CD34− cell–depleted mononuclear cells had angiogenic activity.

Conclusions: Our data demonstrate that human CD34+ cells secrete exosomes that have independent angiogenic activity both in vitro and in vivo. CD34+ exosomes may represent a significant component of the paracrine effect of progenitor cell transplantation for therapeutic angiogenesis. (Circ Res. 2011;109:724-728.)

Key Words: CD34+ cells • paracrine factor • exosomes • angiogenesis

Clinical studies have provided evidence that locally transplanted autologous CD34+ stem cells reduce angina and improve exercise capacity in patients with refractory angina1 and lower amputation rates in patients with critical limb ischemia.2 Preclinical studies indicate that the benefit of human CD34+ cell transplantation after ischemic injury occurs through increases in neovascularization3; however, the mechanisms of new blood vessel formation have not been completely characterized. Incorporation of CD34+ cells into the growing vasculature has been documented in multiple studies4; however, the magnitude of structural contribution of transplanted cells has typically seemed modest compared with the significant overall physiological impact. This discrepancy has led to the assumption that paracrine factors secreted by CD34+ cells contribute significantly to the therapeutic angiogenesis induced by the cells.5 Exosomes, a component of paracrine secretion, are extracellular, membrane-bound nano-vessicles that originate intracellularly in multivesicular bodies (MVBs) and are secreted out when the MVBs fuse with the plasma membrane.6 They often carry proteins, RNAs, and/or microRNAs and mediate some aspects of cell-to-cell signaling.5 We investigated the potential role of exosomes in CD34+ cell–induced neovascularization by determining whether CD34+ stem cells secrete exosomes, and, if so, whether these exosomes can induce angiogenic activity in the absence of CD34+ cells.
Methods

All experimental protocols were approved by the Northwestern University Animal Care and Use Committee. Both CD34+ cells3 and CD34+ cell–depleted mononuclear cells (MNCs)5 were cultured, and exosomes from the conditioned media were obtained as described previously.7 Electron microscopy, dynamic light scattering (DLS), flow cytometry, and immunoblotting analyses were performed according to established protocols.7 The angiogenic activity of cultured human umbilical vein endothelial cells (HUVECs) was evaluated by means of the Matrigel tube-formation assay, proliferation was evaluated through 5-bromo-2-deoxyuridine incorporation, and viability was assessed by means of the MTS assay. In vivo angiogenesis was evaluated in nude (nu/nu) mice by means of the Matrigel plug and corneal angiogenesis assays. Detailed methods are provided in the Online Supplemental Methods available at http://circres.ahajournals.org. Quantified results are presented as mean±SD; comparisons between groups were evaluated with the Student t test; and P<0.05 was considered significant.

Results

CD34+ Cells Secrete Exosomes

To investigate the role of exosomes in CD34+ cell-induced neovascularization, we examined if the CD34+ cells produce and secrete exosomes. Electron micrographs identified several MVBs in the cytoplasm of CD34+ cells, carrying bilipidic membrane-bound exosome-like vesicles. The MVB membrane invaginated inward initiating the biogenesis of exosomes as previously shown6; MVB fused to the plasma membrane and released the exosome-like vesicles to the media (Figure 1A). Because CD34+ cells appear to be significantly more potent for inducing angiogenesis in ischemic tissue than unselected MNCs,3 experiments were performed with exosomes isolated both from CD34+ cells (CD34+ exosomes) and from CD34+-depleted MNCs (MNC exosomes). Exosomes isolated from the conditioned media (CM) of both CD34+ cells and MNCs were similar to previous descriptions of exosomes in size (40 to 90 nm in diameter),7 cup-shaped morphology (Figure 1B), and in their unique flotation density (1.127 g/cm3, floated on 30% sucrose-D2O solution). DLS analysis confirmed the purity (100%) and mean hydrodynamic radius (CD34+ : 50±7.8 nm; MNC: 75±0.4 nm) of the exosomes in each preparation (Figure 1C). Exosomes from both CD34+ cells and MNCs displayed the exosomal surface marker proteins CD63 and phosphatidylserine (Figure 2A and 2B) and contained the exosomal luminal protein TSG101 (Figure 2C). Further, CD34 protein was present on the surface of exosomes from CD34+ cells but not on exosomes from MNCs (Figure 2D), which is consistent with previous reports that exosomes carry the same marker proteins that are specific for the secreting cell.6 Collectively, these observations confirm that both CD34+ cells and MNCs secrete exosomes and that the exosomes secreted by each cell population are biochemically distinct.

CD34+ Exosomes Induce Angiogenic Activity in Endothelial Cells In Vitro

To determine whether CD34+ exosomes induce angiogenic activity in vitro, tube formation was evaluated in HUVECs that had been cultured for 8 hours with PBS, CD34+ cells, CD34+ cell-CM, CD34+ exosomes, or the exosome-depleted CM (Figure 3A). Tube length was significantly greater in HUVECs incubated with the CD34+ cell-CM or with CD34+ exosomes than in HUVECs incubated with PBS but was unchanged in HUVECs incubated with the exosome-depleted CM (Figure 3B). This suggests that CD34+ exosomes mediate the in vitro angiogenic activity from the CD34+ cell-CM. Interestingly, CD34+ exosomes, similar to CD34+ cells, induced longer-lasting tubes in HUVECs (Online Figure I).
Tube formation was less pronounced at lower exosome concentrations (Figure 3C). Both CD34+/H11001 exosomes and CD34+/H11001 cells significantly enhanced HUVEC viability (Figure 3D) and proliferation (Figure 3E). Thus, most of the in vitro angiogenic activity associated with CD34+/H11001 cells appears to be mediated by exosomes. HUVECs incubated with MNCs or MNC exosomes did not differ significantly from saline-treated cells in any functional parameter (Figure 3D and 3E and Online Figures I and II). The superior efficacy of CD34+/H11001 exosomes compared with MNC exosomes is consistent with prior in vivo studies documenting the enhanced angiogenic activity of CD34+/H11001 cells versus MNC for therapeutic angiogenesis.3 Although the mechanisms that mediate the enhanced potency of CD34+/H11001 cells versus MNC have not been completely clarified, preliminary data show that the proangiogenic microRNAs 126 and 130a8 are highly expressed in CD34+/H11001 exosomes compared with MNC (Online Figure III).

CD34+ Exosomes Induce Angiogenesis In Vivo
The angiogenic potency of CD34+ exosomes was evaluated in vivo by performing the Matrigel-plug and corneal angiogenesis assays in mice. Both CD34+ cells and CD34+ exosomes induced the formation of vessel-like endothelial structures (Figure 4A) and significantly increased the proportion of endothelial cells (Figure 4B) in the Matrigel plug. In the corneal angiogenesis assay, pellets containing CD34+ exosomes but not MNC exosomes were associated with significantly greater vessel growth (Figure 4C and 4D); the effect of CD34+ cells on corneal angiogenesis could not be evaluated because the pellets could not be prepared with viable cells.

Discussion
CD34+ cells have been shown to form a structural component of the neovasculature in ischemic tissue4 and secrete paracrine factors that also stimulate neovascularization.5 We demonstrate that a significant component of the proangiogenic paracrine activity associated with CD34+ cells is mediated by exosomes. The exosomes secreted by CD34+ cells were morphologically similar in size and shape to exosomes described in previous reports, carried known exosomal protein markers, and potently induced angiogenic activity both in vitro and in vivo.

The cell culture medium was supplemented with growth factors and may have contained soluble proteins secreted directly from the cells, which could, in principle, have contributed to the angiogenic effects associated with CD34+ exosomes. However, the MNC exosomes were derived from MNCs cultured with the same growth factors, and the exosome-depleted conditioned media would have contained both the supplemental growth factors and any secreted
soluble proteins. Because none of these treatments stimulated angiogenic activity, our findings indicate that the CD34+ exosomes are the key paracrine component of CD34+ cell–induced vessel growth.

Exosomes can stimulate both receptor-mediated and genetic mechanisms by transferring proteins, RNA, or microRNA directly into the cytoplasm of target cells.9 We have presented data demonstrating that CD34+ exosomes are enriched with proangiogenic microRNAs; the extent to which these microRNAs are transferred and induce any molecular changes in the recipient cells will be clarified in ongoing studies. Indeed, the repertoire of specific molecules transported by CD34+ exosomes remains to be fully characterized, but they are likely to be more stable than molecules secreted directly into the extracellular matrix because the exosomal membrane protects the contents of the exosome from degradation.6,9 Furthermore, the exosomes used in our investigation were sufficiently durable to remain intact and biologically active throughout the isolation procedure, which suggests that the functional radius of CD34+ exosomes could extend beyond the immediate vicinity of the secreting cell. The observation that in some of the in vitro and in vivo assays the exosomes from CD34+ cells appeared more potent than the cells

Figure 3. In vitro assays. A, HUVECs (2.5×10⁴) were treated with PBS, 2.0×10⁴ CD34+ cells, or with conditioned media (CM), exosomes (Exo), or exosome-depleted CM from 2.0×10⁴ CD34+ cells and plated on Matrigel. B, Tube length was measured 8 hours later and expressed as percentage of saline-treated HUVECs (n=6 to 9). C, Representative dose-response of CD34+ exosome tube formation, evaluated in HUVECs incubated with exosomes from 1.5×10⁵ CD34+ cells and serially diluted with saline to the indicated ratios; D, viability, and E, proliferation of HUVECs (1×10⁴) in response to PBS, 2.5×10³ cells, or exosomes from 2.5×10³ cells, measured 20 hours later and expressed as percentage of PBS-treated HUVECs (n=3 to 6). *P<0.001 versus PBS, †P<0.05 versus Exo-depleted CM, ‡P<0.05 versus MNCs or MNC exosomes.

Figure 4. In vivo assays. A and B, Matrigel plug assay: Matrigel containing PBS, 5×10⁵ CD34+ cells, or exosomes from 5×10⁵ CD34+ cells was subcutaneously injected into mice, and the plugs were harvested 7 days later. A, Sections from the plug were stained with isolectin to identify endothelial cells (brown) and vessel-like endothelial structures (arrows). B, The plug was digested, and CD31+ endothelial cells were quantified by flow cytometry (n=3 to 6). C and D, Corneal angiogenesis assay: Pellets containing PBS or exosomes from 1×10⁶ MNCs or CD34+ cells were implanted in the corneas of mice; corneas were harvested 7 days later, stained with fluorescently labeled lectin identifying vascular structures (C), and the extent of vessel growth was quantified (D) (n=4). *P<0.05 versus PBS, †P<0.01 versus MNC exosomes.
themselves is interesting and might also be a byproduct of the durability of the exosome in culture providing the ability to deliver a high dose of exosomes through collection from culture medium in which exosomes are secreted over a period of time.

In summary, our observations demonstrate for the first time that adult human CD34+ stem cells secrete exosomes and that these exosomes induce angiogenic activity in isolated endothelial cells and in murine models of vessel growth. Thus, the benefit of CD34+ cell therapy on functional recovery after ischemic injury could be induced primarily through the exosome-mediated transfer of angiogenic factors to surrounding cells. Novel therapies designed to exploit this previously unidentified mechanism of paracrine signaling may enhance recovery from ischemic disease or injury.

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Disclosures
None.

References

Novelty and Significance

What Is Known?
- CD34+ cells have been shown to stimulate therapeutic neovascularization in preclinical studies and in phase I and II human clinical trials.
- The potency of CD34+ cells is greater than unselected mononuclear cells.
- The mechanisms by which CD34+ cells induce neovascularization appear to include both direct participation in vessel formation and undefined “paracrine” effects.
- Exosomes are small, membrane-bound vesicles secreted from various cells that contain protein and nucleic acids and are increasingly being shown to mediate cell-to-cell signaling.

What New Information Does This Article Contribute?
- CD34+ cells secrete exosomes that independently induce angiogenesis in vitro and in vivo.
- The proangiogenic activity of CD34+ exosomes is significantly greater than CD34-depleted mononuclear cell exosomes.
- The exosomes from CD34+ cells contain higher levels of proangiogenic microRNAs.

The clinical potential of CD34+ cells for therapeutic neovascularization of ischemic tissue is being evaluated in a series of completed and ongoing clinical trials. Hence, the mechanisms by which CD34+ cells mediate these effects are of high scientific and clinical importance. Although paracrine effects have been assumed to be responsible for a significant proportion of the effects of endothelial progenitor cell–based therapies in general, the precise nature of the paracrine phenomena has not been defined. Our data show that CD34+ cells secrete exosomes that appear to be responsible for much if not most of their paracrine activity. Specifically, the conditioned medium from CD34+ cells exerts proangiogenic effects that are abolished when the exosomes are removed, whereas the exosomes alone, without any of the soluble material from the conditioned medium, exhibit the full potency of the conditioned medium. Complete characterization of the exosome content of endothelial progenitor cells could provide new insights permitting enhancement of their therapeutic potency.
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SUPPLEMENTAL MATERIAL

Exosomes from Human CD34+ Stem Cells Mediate their Pro-angiogenic Paracrine Activity

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Supplemental Figure 1. *In vitro* matrigel tube formation assay at 24h. HUVECs (2.5x10^4) were treated with PBS, with 2.0x10^4 cells, or with the conditioned media (CM), exosomes (Exo), or exosome-depleted conditioned media from 2.0x10^4 cells, plated on Matrigel, tube length was measured 24 hours later and expressed as a percentage of PBS-treated HUVECs; n=3-6. *P<0.005 versus PBS, †P<0.05 versus MNCs or MNC exosomes.
Supplemental Figure 2. *In vitro* matrigel tube formation assay of MNCs at 6h. HUVECs (2.5x10^4) were treated with PBS, 2.0x10^4 MNCs, or with the conditioned media (CM), exosomes (Exo), or exosome-depleted conditioned media from 2.0x10^4 MNCs, plated on Matrigel, tube length was measured 8 hours later and expressed as a percentage of PBS-treated HUVECs; n=3-4.
Supplemental Figure 3. Pro-Angiogenic miRNA's are highly expressed in CD34+ cell exosomes. Total RNA was isolated from the CD34+ cells, CD34+ depleted MNC and their respective exosomes using Qiagen miRNEASY isolation kit; miRNA expression from equal amounts of total RNA was measured by qRT-PCR Taqman assays, data was normalized to the expression of small nuclear RNA U6; miRNA 130a, n=2; miRNA 126, n=6.
Supplemental Methods

Cells and culture
CD34+ cells and the CD34- cell–depleted mononuclear cells (MNCs) were obtained from Baxter Healthcare (Deerfield, IL, USA). The cells were purified from mobilized peripheral-blood mononuclear cells (AllCells LLC, Emeryville, CA, USA) with an Isolex 300i device (Baxter Healthcare); cell purity (85-95%) was determined via flow cytometry. Both CD34+ cells and MNCs (250,000 cells/mL) were cultured in X-VIVO 10 serum-free cell-culture medium (Lonza Group Ltd, Basel, Switzerland) containing 0.25% human serum albumin and supplemented with 100 ng/mL Flt-3L, 100 ng/mL stem-cell factor, and 20 ng/mL vascular endothelial-growth factor. Human umbilical-vein endothelial cells (HUVECs) (Cambrex Corporation, East Rutherford, NJ, USA) were maintained in endothelial growth medium-2 (EGM™-2; Cambrex Corporation) and starved in EBM-2 medium containing 0.25% fetal bovine serum for 24 hours before cell assays were performed.

Exosome purification
Cells were cultured for 40 hours, and then exosomes were collected and ultrapurified as described previously. Briefly, the cells and conditioned media were separated by centrifugation (800g for 5 minutes); the conditioned media was clarified by centrifugation (14,000g for 20 minutes) and the exosomes were collected by ultracentrifugation (100,000g for 1 hour) on a 30% sucrose-D2O solution (density ~1.127g/cm3), washed in PBS and pelleted. The purified exosome fraction was re-suspended in PBS for use.

Electron microscopy
Cells were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Electron Microscopy Sciences, Hatfield, PA, USA) for 3 hours at room temperature, washed with cacodylate buffer, postfixed in 1% osmium tetroxide, progressively dehydrated in a graded ethanol series (50–100%), and embedded in Epon. Thin (1-mm) and ultrathin (70- to 80-nm) sections were cut from the polymer with a Reichert (Depew, NY, USA) Ultracut S microtome, placed on copper grids, and briefly stained with uranyl acetate and lead citrate. Exosomes were fixed with 2% paraformaldehyde, loaded on 300-mesh formvar/carbon-coated electron microscopy grids (Electron Microscopy Sciences, PA, USA), post-fixed in 1% glutaraldehyde, and then contrasted and embedded as described previously. Transmission electron microscopy images were obtained with an FEI (Hillsboro, OR, USA) Tecnai Spirit G2 transmission electron microscope operating at 120 kV.

Dynamic light scattering
Exosomes were suspended in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA); then, dynamic light-scattering measurements were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Intensity, volume, and distribution data for each sample were collected on a continuous basis for 4 minutes in sets of three. At least three different measurements from three different samples were performed for each exosome population.

Flow cytometry
Flow cytometry analysis was performed as described previously. Exosomes were conjugated to 4-µm latex beads for analysis, because their diameter (<0.1 nm) is smaller than the detection limit (0.1-0.2 nm) of the flow cytometer. Briefly, exosomes from 5x10⁶ cells were incubated overnight at 4°C with 2.5x10⁵ aldehyde/sulfate latex beads (Invitrogen, Carlsbad, CA, USA) and
then blocked with 100 mM glycine for 30 minutes at room temperature to saturate any free binding sites that remained on the beads. To detect the presence of CD63 and CD34, the exosome-coated beads were resuspended in 500 µL PBS containing 0.5% human serum albumin (HSA) and 2 mM EDTA; then, 100 µL of the beads were incubated with fluorescein-isothiocyanate (FITC)–conjugated anti-CD63 or FITC-conjugated anti-CD34 antibodies (Beckman Coulter, Inc., Brea, CA, USA) for 30 minutes at 4°C. For phosphatidylserine detection, the beads were resuspended in 100 µL of Annexin-V-FLUOS labeling solution (Annexin-V-FLUOS Staining Kit, F. Hoffmann-La Roche Ltd, Basel, Switzerland) and incubated for 10 minutes at 25°C. Non-specific signaling was inhibited by the addition of FcR blocking reagent (Miltenyi Biotec Inc., Auburn, CA, USA); the threshold for negative staining was obtained by incubating exosome-free, glycine-blocked beads with each antibody; and additional experiments were performed with identical concentrations of control IgG antibodies to correct for non-specific binding. Flow cytometry data were acquired on a BD LSRII (BD Franklin Lakes, NJ USA) flow cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

**In-vitro Matrigel tube formation assay**

HUVECs (2.5x10⁴, serum-starved overnight) were seeded with PBS, with 2.0x10⁴ CD34⁺ cells or MNCs, or with the conditioned media, exosomes, or exosome-depleted conditioned media from 2.0x10⁴ CD34⁺ cells or MNCs into 48-well plates that had been coated with 150 µL of growth-factor–reduced Matrigel™ (BD). Tube formation was examined by phase-contrast microscopy 6-8 hours, or, 24 hours later. Each condition in each experiment was assessed in duplicates, and tube length was measured as the mean summed length of capillary-like structures in 2 wells, per high-power fields (HPFs, 2.5x) per well. 6-9 experiments were performed for each condition.

**In vitro proliferation and viability assays**

Cell proliferation was evaluated via 5-bromo-2-deoxyuridine (BrdU) incorporation. Serum-starved HUVECs (1x10⁵) were incubated with 10 µM BrdU and 2.0x10⁴ CD34⁺ cells, 2.0x10⁴ MNCs, or exosomes from 2.0x10⁵ CD34⁺ cells or MNCs for 24 hours, and then washed and fixed with 4% paraformaldehyde at 4 °C. Ten minutes later, the HUVECs were washed in PBS with 1% TritonX100 for 5 minutes, incubated on ice in 1N HCl for 10 minutes, incubated at room temperature in 2N HCl for 10 minutes, and incubated at 37°C for 20 minutes. The HCl was neutralized via three 5-minute washes with borate buffer (0.1M), and then the HUVECs were washed in PBS with 1% TritonX100 at room temperature for 3 minutes, blocked with 5% normal goat serum and 1% Triton X in PBS for 1 hour, and incubated overnight with immunofluorescent sheep anti-BrdU antibodies (Abcam Inc., Cambridge, MA, USA); nuclei were counterstained with DAPI. Cells were viewed at 10x magnification, and BrdU⁺ cells were counted in 10 HPFs per well, 2 wells per condition.

Cell viability was evaluated via the MTS assay. HUVECs (1x10⁴ cells/well) were seeded on 96-well flat-bottomed plates and incubated with 2.0x10⁴ CD34⁺ cells or MNCs, or with exosomes from 2.0x10⁴ CD34⁺ cells or MNCs, for 20 hours at 37°C; then, the MTS assay reagent (Promega Corporation, Madison, WI, USA) was added to the wells, and HUVECs were incubated for 3 hours at 37°C. Viability was evaluated by measuring absorbance at 490-nm wavelength with a 96-well ELISA plate reader (spectrophometer SpectraMaxPlus; Molecular Devices, Sunnyvale, CA, USA) in at least 6 wells per experiment, 3-7 experiments per condition.

**Western blotting**

Cells or purified exosomes were lysed with 0.1M Tris, 0.3 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100 in a cocktail of antiproteases (Sigma-Aldrich Corporation,
St. Louis, MO, USA); then, the nuclei and membranes were cleared by centrifugation (15,000 g for 10 minutes). Protein extracts were separated on an 8% SDS-PAGE gel, blotted on Immobilon (Millipore, Billerica, MA, USA) with TSG101 (4A10; Abcam Inc.), and visualized with enhanced chemoluminescence substrate (Thermo Fisher Scientific, Rockford, IL, USA). Images were acquired with a Chemidoc XRS (Kodak, Rochester, NY, USA).

In-vivo Matrigel-plug assay

Ice-cold Matrigel (0.5 mL/plug; BD) was mixed with heparin (1 mg/mL) and with PBS, 0.5x10^6 CD34+ cells, or exosomes from 0.5x10^6 CD34+ cells and then subcutaneously injected into the flanks of 6- to 8-week-old male nude mice (Nu/J; The Jackson Laboratory, Bar Harbor, ME, USA); mice were anesthetized with inhaled isoflurane (2-4%) before injection. Two weeks later, the plug was excised and washed with PBS. To visualize vessel-like endothelial structures, the plug was fixed in methanol and sectioned; then, endothelial cells were stained with biotinylated isolectin B4 (Vector Laboratories Inc, Burlingame, CA, USA), and nuclei were stained with hematoxylin. Images were acquired with an Olympus Vanox bright microscope. For flow-cytometry analysis of endothelial-cell migration, the plug was digested with 0.1% collagenase/dispase (F. Hoffmann-La Roche), 10 mm MgCl₂, and 200 units/mL DNase I (F. Hoffmann-La Roche) in 10% fetal calf serum/PBS for 1 hour at 37°C. After digestion, cells were dispersed 4-5 times with a 21g needle, passed through a 70-mm filter (BD), and stained with phycoerythrin-conjugated rat anti-mouse-CD31 antibodies (BD); control assessments were performed with phycoerythrin-conjugated rat immunoglobulin G2a isotype (Invitrogen). Flow cytometry data were acquired on a FACScan (BD) flow cytometer and analyzed with FlowJo software (Tree Star).

Mouse corneal angiogenesis assay

Pellets were prepared and implanted in the corneas of 6- to 8-week-old male nude mice (Nu/J; The Jackson Laboratory) as described previously.² Briefly, 5 mg sucrose octasulfate-aluminum complex (Sigma-Aldrich Corporation) and 10 µL of 12% hydron in ethanol was mixed and partially dried; then, exosomes from 5x10^5 CD34+ cells or MNCs were added, the mixture was pelleted on a 400-µm nylon mesh (Sefar America Inc., Depew, NY, USA), and the pellets were dried for 5-10 minutes. Pellets were implanted in the corneas of mice that had been anesthetized via intraperitoneal injection of 125 mg/kg Avertin. One week after implantation, the mice were intravenously injected with 50 µL of fluorescein-conjugated BS1-Lectin I (Vector Laboratories) and sacrificed 15 minutes later. Eyes were harvested and fixed with 1% paraformaldehyde; then, the corneas were excised and mounted; angiogenesis was evaluated via BS1-Lectin I fluorescence and quantified with ImageJ software.

MicroRNA quantification

Total RNA from the CD34+ cells, CD34-depleted MNCs and their respective exosomes were extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol including a DNase step. RNA concentrations were verified on the NanoDrop Spectrophotometer (NanoDrop) and the quality of total RNA was assessed using Agilent 2100 Bioanalyzer Pico Chips (Agilent). Equal amount of RNA (5ng) was reverse transcribed using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) using a specific miRNA primer to generate cDNA for use with individual Taqman MicroRNA Assays (Applied Biosystems). Real-time Reactions were performed in triplicate on a 7500FAST Real-Time PCR system (Applied Biosystems). Ct values were averaged and normalized to RNU6B. Relative expression was determined by the ddCt comparative threshold method.
Supplemental References:

