possible utility of autologous BM-derived EPCs in the patients with impaired BM function, an appreciable number of EPCs isolated from umbilical cord blood or differentiated from tissue specific stem/progenitor or embryonic stem cells need to be optimized for EPC therapy. However, the unlimited potential of EPCs along with the emerging concepts of autologous cell therapy with gene modification suggests that they may soon reach clinical fruition.

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References

[1] Asahara T, Murohara T, Sullivan A et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–967.
[2] Shi Q, Rafii S, Wu MH et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362–367.
[3] Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992;267:10931–10934.
[4] Asahara T, Masuda H, Takahashi T et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221–228.
[5] Tamaki T, Akatsuka A, Ando K et al. Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle. J Cell Biol 2002;157:571–577.
[6] Gill M, Dias S, Hattori K et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001;88:167–174.
[7] Shintani S, Murohara T, Ikeda H et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 2001;103:2776–2779.
[8] Risau W, Sariola H, Zerwes HG et al. Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 1988;102:471–478.
[9] Pardanaud L, Altmann C, Kitos P et al. Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development 1987;100:339–349.
[10] Flamme I, Risau W. Induction of vasculogenesis and hematopoiesis in vitro. Development 1992;116:435–439.
[11] Weiss MJ, Orkin SH. In vitro differentiation of murine embryonic stem cells. New approaches to old problems. J Clin Invest 1996;97:591–595.
[12] Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995;11:73–91.
[13] Choi K, Kennedy M, Kazarov A et al. A common precursor for hematopoietic and endothelial cells. Development 1998;125:725–732.
[14] Yin AH, Miraglia S, Zanjani ED et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997;90:5002–5012.
[15] Peichev M, Naiyer AJ, Pereira D et al. Expression of VEGFR2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–958.
[16] Nieda M, Nicol A, Denning Kendall P et al. Endothelial cell precursors are normal components of human umbilical cord blood. Br J Haematol 1997;98:775–777.
[17] Murohara T, Ikeda H, Duan J et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–1536.
[18] Kang HJ, Kim SC, Kim YJ et al. Short-term phytohaemagglutinin-activated mononuclear cells induce endothelial progenitor cells from cord blood CD34+ cells. Br J Haematol 2001;113:962–969.
[19] Crisa L, Cirulli V, Smith KA et al. Human cord blood progenitors sustain thymic T-cell development and a novel form of angiogenesis. Blood 1999;94:3928–3940.
[20] Crosby JR, Kaminski WE, Schatteman G et al. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000;87:728–730.
[21] Murayama T, Tepper OM, Silver M et al. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. Exp Hematol 2002;30:967.
[22] Reyes M, Dudek A, Jahagirdar B et al. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109:337–346.
[23] Boyer M, Townsend LE, Vogel LM et al. Isolation of endothelial cells and their progenitor cells from human peripheral blood. J Vasc Surg 2000;31:181–189.
[24] Lin Y, Weisdorf DJ, Solovey A et al. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000;105:71–77.
[25] Kalka C, Masuda H, Takahashi T et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 2000;97:3422–3427.
[26] Gunsilius E, Duba HC, Petzer AL et al. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. Lancet 2000;355:1688–1691.
[27] Gehling UM, Ergun S, Schumacher U et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000;95:3106–3112.
[28] Fernandez Pujol B, Lucibello FC et al. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000;65:287–300.
[29] Schatteman GC, Hanlon HD, Jiao C et al. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000;106:571–578.
[30] Harraz M, Jiao C, Hanlon HD et al. CD34- blood-derived human endothelial cell progenitors. Stem Cells 2001;19:304–312.
[31] Quirici N, Soligo D, Caneva L et al. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 2001;115:186–194.
[32] Grzelak I, Olszewski WL, Zaleska M et al. Surgical trauma evokes a rise in the frequency of hematopoietic progenitor cells and cytokine levels in blood circulation. Eur Surg Res 1998;30:198–204.
[33] Takahashi T, Kalka C, Masuda H et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–438.
[34] Shalaby F, Rossant J, Yamaguchi TP et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995;376:62–66.
[35] Carmeliet P, Ferreira V, Breier G et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435–439.
[36] Ferrara N, Carver Moore K et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439–442.
[37] Asahara T, Takahashi T, Masuda H et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18:3964–3972.

[38] Kalka C, Masuda H, Takahashi T et al. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000;86:1198–1202.
[39] Moore MA, Hattori K, Heissig B et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann NY Acad Sci 2001;938:36–45; discussion 45–47.
[40] Kureishi Y, Luo Z, Shiojima I et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000;6:1004–1010.
[41] Llevadot J, Murasawa S, Kureishi Y et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. J Clin Invest 2001;108:399–405.
[42] Dimmeler S, Aicher A, Vasa M et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 2001;108:391–397.
[43] Vasa M, Fichtlscherer S, Adler K et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001;103:2885–2890.
[44] Urbich C, Dernbach E, Zeiher AM et al. Double-edged role of statins in angiogenesis signaling. Circ Res 2002;90:737–744.
[45] Davidoff AM, Ng CY, Brown P et al. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. Clin Cancer Res 2001;7:2870–2879.
[46] Lyden D, Hattori K, Dias S et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–1201.
[47] Rivard A, Silver M, Chen D et al. Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. Am J Pathol 1999;154:355–363.
[48] Van Belle E, Rivard A, Chen D et al. Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines. Circulation 1997;96:2667–2674.
[49] Couffinhal T, Silver M, Kearney M et al. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE−/− mice. Circulation 1999;99:3188–3198.
[50] Rivard A, Berthou Soulie L, Principe N et al. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. J Biol Chem 2000;275:29643–29647.
[51] Rivard A, Fabre JE, Silver M et al. Age-dependent impairment of angiogenesis. Circulation 1999;99:111–120.
[52] Murayama T, Kalka C, Silver M et al. Aging impairs therapeutic contribution of human endothelial progenitor cells to postnatal neovascularization [Abstract]. Circulation 2001;104:II-68.
[53] Vasa M, Fichtlscherer S, Aicher A et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001;89:E1–E7.
[54] Kawamoto A, Gwon HC, Iwaguro H et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634–637.
[55] Kocher AA, Schuster MD, Szabolcs MJ et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430–436.
[56] Levenberg S, Golub JS, Amit M et al. Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 2002;99:4391–4396.
[57] Iwaguro H, Yamaguchi J, Kalka C et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 2002;105:732–738.
[58] Shintani S, Murohara T, Ikeda H et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001;103:897–903.
[59] Hamano K, Li TS, Kobayashi T et al. The induction of angiogenesis by the implantation of autologous bone marrow cells: a novel and simple therapeutic method. Surgery 2001;130:44–54.
[60] Kamihata H, Matsubara H, Nishiue T et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001;104:1046–1052.
[61] Fuchs S, Baffour R, Zhou YF et al. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol 2001;37:1726–1732.
[62] Bhattacharya V, McSweeney PA, Shi Q et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. Blood 2000;95:581–585.
[63] Kaushal S, Amiel GE, Guleserian KJ et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001;7:1035–1040.
[64] Shimizu T, Yamato M, Isoi Y et al. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 2002;90:e40.
[65] Shimizu T, Yamato M, Akutsu T et al. Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. J Biomed Mater Res 2002;60:110–117.
[66] Zhang ZG, Zhang L, Jiang Q et al. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. Circ Res 2002;90:284–288.
[67] Hess DC, Hill WD, Martin Studdard A et al. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. Stroke 2002;33:1362–1368.
[68] Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–705.
[69] Orlic D, Kajstura J, Chimenti S et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001;98:10344–10349.
[70] Jackson KA, Majka SM, Wang H et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395–1402.
[71] Condorelli G, Borello U, De Angelis L et al. Cardiomyocytes induce endothelial cells to trans-differentiate into cardiac muscle: implications for myocardium regeneration. Proc Natl Acad Sci USA 2001;98:10733–10738.

Estrogen-Mediated, Endothelial Nitric Oxide Synthase–Dependent Mobilization of Bone Marrow–Derived Endothelial Progenitor Cells Contributes to Reendothelialization After Arterial Injury

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Background—We hypothesized that estrogen-induced acceleration of reendothelialization might be mediated in part by effects involving mobilization and incorporation of bone marrow–derived endothelial progenitor cells (EPCs).

Methods and Results—Carotid injury was induced in ovariectomized wild-type mice receiving either 17β-estradiol or placebo. Estradiol treatment significantly accelerated reendothelialization of injured arterial segments within 7 days and resulted in a significant reduction of medial thickness 14 and 21 days after the injury. Significant increases in circulating EPCs 3 days after the injury were observed in the estradiol group compared with placebo-treated mice. These data were further supported by fluorescence-activated cell sorting analysis, which disclosed a significant increase in Sca-1/Flk-1–positive cells in estradiol versus control mice. To evaluate the effects of estradiol on bone marrow–derived EPC incorporation at sites of reendothelialization, carotid injury was established in ovariectomized wild-type mice transplanted with bone marrow from transgenic donors expressing β-galactosidase transcriptionally regulated by the Tie-2 promoter. Significantly greater numbers of X-gal–positive cells were observed at reendothelialized areas in the estradiol group 3 days after injury as compared with placebo. Fluorescent immunohistochemistry 14 days after the injury documented a marked increase in cells expressing both β-gal, indicating bone marrow origin and Tie-2 expression, and isolectin B4, also indicating endothelial lineage, in the estradiol group compared with control. In contrast, estradiol did not accelerate reendothelialization or augment EPC mobilization into the peripheral circulation after injury in endothelial nitric oxide synthase–deficient mice ($eNOS^{-/-}$). Furthermore, estradiol exhibited direct stimulatory effects on EPC mitogenic and migration activity and inhibited EPC apoptosis.

Conclusions—Estradiol accelerates reendothelialization and attenuates medial thickening after carotid injury in part by augmenting mobilization and proliferation of bone marrow–derived EPCs and their incorporation into the recovering endothelium at the site of injury. (***Circulation.*** **2003;108:3115-3121.)**

Key Words: estrogen ■ endothelium ■ arteries ■ nitric oxide synthase

Disruption of the anatomic and functional integrity of the endothelium has been postulated as a mechanism for the initiation of atherosclerosis.[1] A corollary hypothesis is that restoration of endothelial integrity should inhibit atherogenesis. Accordingly, the finding that estradiol induces endothelial proliferation and migration[2] mediated by the classic estrogen receptor[3,4] suggests a potential mechanism by which estradiol might protect the vasculature. Indeed, mechanical disruption of the endothelium by balloon angioplasty in various animal models has been shown to result in the formation of a neointimal lesion,[5,6] whereas agents that accelerate reendothelialization, including estradiol, have been shown to attenuate intimal hyperplasia.[7,8]

Recently, work in our laboratory[9,10] and others[11,12] has suggested that endothelial cells (ECs) adjacent to the site of balloon injury might not constitute the sole participants in endothelial recovery. These studies suggested that circulating cells, derived from the bone marrow and exhibiting certain features consistent with EC identity, were capable of being recruited to sites of arterial injury and contributing to reestablishing the neoendothelium. These cells, referred to as endothelial progenitors (EPCs) or circulating endothelial progenitors, have also been shown to participate in neovascularization in a variety of settings including ischemic tissue,[10,13] tumors,[14] and the retina.[15] Moreover, preliminary studies in our laboratory indicated that these cells might be regulated by estradiol.[16]

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Accordingly, we performed a series of investigations to test the hypothesis that estrogen accelerates reendothelialization by effects involving mobilization of bone marrow–derived EPCs.

Methods

Mouse Carotid Injury

All procedures were performed in accordance with St Elizabeth's Institutional Animal Care and Use Committee. Mice were anesthetized by intraperitoneal injection of 150 mg/kg ketamine for all surgical procedures. Eighty female FVB/N wild-type mice (Jackson Laboratory, Bar Harbor, Maine) were ovariectomized at 4 weeks of age and received either 90-day release 17β-estradiol pellets (1.7 mg 17β-estradiol, Innovative Research of America) or placebo-containing pellets (each n=40) implanted subcutaneously into the backs of the animals 4 weeks after the ovariectomy. This dose was used to achieve levels in the upper range of those occurring in nonpregnant premenopausal humans at midcycle and in the luteal phase of the normal menstrual cycle.[17] These levels are ~3-fold higher than occur in the nonpregnant mouse. We also used 24 bone marrow transplant (BMT) mice created by transplantation of bone marrow from transgenic mice (FVB/N-TgN{TIE2LacZ}, Jackson Laboratory) constitutively overexpressing β-galactosidase regulated by the endothelial specific Tie-2 promoter, as previously described.[18] In brief, female FVB/N wild-type mice were ovariectomized at 4 weeks age. One week after ovariectomy, recipient mice were lethally irradiated with 9.0 Gy, and BMT from the transgenic mice was performed. At 3 weeks after BMT, by which time the bone marrow of recipient mice was reconstituted with the transplanted bone marrow, recipient mice received subcutaneous 17β-estradiol (n=12) or placebo (n=12) pellet implantation. Carotid artery injury was established both in wild-type and BMT animals 1 week after pellet implantation as described previously.[19,20] Briefly, the bifurcation of the left carotid artery was exposed through a midline skin incision of the ventral aspect of the neck, with the use of a dissecting microscope; 6-0 silk sutures were placed around the common carotid and internal and external carotid arteries to temporarily restrict blood flow to the area of surgical manipulation. The artery was injured with a 0.014-inch-diameter flexible angioplasty wire, modified to create a barotraumatic/stretch injury, introduced into the external carotid artery and advanced to the common carotid artery. The wire was advanced and withdrawn 3 times to ensure a reliable effect. The total length of denuded common carotid artery was 4 mm from the bifurcation of carotid arteries in all animals. The wire was removed from the artery, the external carotid artery was permanently ligated, and the temporary ligatures were released to allow blood flow to be restored through the internal carotid artery. The connective tissue and subcutis were closed with continuous 7-0 monofilament suture, and the skin was then closed with the same procedure.

Circulating 17β-estradiol levels were measured 7 and 14 days after carotid injury with a commercially available enzyme-linked immunoassay (estradiol ELISA, Cayman Chemical Co, Inc), according to the manufacturer's instructions.

Histological Assessment of Wild-Type Animals

To measure the reendothelialized area, wild-type animals were perfused in vivo with Evans blue dye (Sigma) 3, 5, 7, and 14 days after the injury, as described previously.[19,20] Briefly, 50 μL of 5% Evans blue diluted with saline was injected into the heart with a 27-gauge needle 10 minutes before the animals were killed, followed by fixation by perfusion of 4% paraformaldehyde (PFA) for 5 minutes. Blood, saline, and fixative were removed through an incision in the right atrium. The common carotid artery was then harvested 4 mm from the carotid bifurcation; the artery was opened longitudinally and placed between slides with Fluoprep. The areas stained and unstained in blue and the total carotid artery area were measured, and the percentage areas were calculated by using the entire injured area, based on anatomic landmarks, as the baseline. For measurement of medial area, carotid arteries 14 and 21 days after the injury were embedded in paraffin after perfusion fixation with 4% PFA, and sections perpendicular to the long axis of the arteries were cut from the proximal and distal sections of the injured artery. Each section was stained with elastic trichrome. Morphometric analysis of digitalized images was performed with the use of NIH Image 1.61 software.

EPC Culture Assay

Total mononuclear cells were isolated from 500 μL of peripheral blood by density gradient centrifugation with Histopaque-1083 (Sigma) and cultured in phenol red–free EC basal medium (EBM, Clonetics) medium supplemented with 5% fetal bovine serum, antibiotics, and growth factors, as previously described.[21] Four days after culture on 4-well glass slides coated with rat plasma vitronectin (Sigma) in 0.5% gelatin solution, EPCs, recognized as attaching spindle-shaped cells, were assayed by costaining with acetylated LDL (acLDL)-DiI (Biomedical Technologies) and FITC-conjugated *Bandeiraea simplicifolia* lectin I (Vector Laboratories), both of which are features characteristic of endothelial lineage.[21] Double-positive cells, identified by fluorescence microscopy by an investigator blinded to treatment, were counted as EPCs in 15 randomly selected fields of each cultured slide. Acknowledging that this technique is not 100% specific for endothelial lineage cells, we performed an additional assay to evaluate EPC kinetics after arterial injury in estradiol-treated and untreated mice.

Fluorescence-Activated Cell Sorting Analysis

The viable mononuclear cell population was analyzed for the expression of Sca-1-FITC (Pharmingen) and Flk-1 (Santa Cruz) conjugated with the corresponding phycoerythrin-labeled secondary antibody (Sigma).[22] Isotype-identical antibodies served as negative controls. Immunofluorescence-labeled cells were fixed with 2% paraformaldehyde and analyzed by quantitative flow cytometry with the use of a FACStar flow cytometer (Becton Dickinson) and Cell Quest Software counting 10 000 events per sample.

Cellular Identification of LacZ-Expressing Cells

The carotid arteries from BMT mice were harvested 3 and 14 days after the injury. X-gal staining was performed on whole mounted vessels to visualize and quantify bone marrow–derived Tie2/LacZ-positive endothelial lineage cells per square millimeter of surface area, as described previously.[23] Target vessels from BMT mice were also embedded in OCT compound (Miles Scientific) and snap-frozen in liquid nitrogen for fluorescence microscopy immediately after the animals were killed. Immunohistochemical staining was performed with the use of antibodies prepared against rabbit anti–β-galactosidase IgG (CORTEX Biochem) and the murine-specific EC marker, biotinylated isolectin B4 (Vector Laboratories).[23]

Endothelial Nitric Oxide Synthase–Deficient Mice

Four-week-old female endothelial nitric oxide synthase–deficient ($eNOS^{-/-}$) mice and C57BL/6J mice as wild-type control mice were purchased from Jackson Laboratories. After ovariectomy, all mice received either 17β-estradiol or a placebo-containing pellet implanted subcutaneously as above, and carotid injury was performed as described. Reendothelialization, assessed by in vivo perfusion with Evans blue dye, was performed 3 and 7 days after injury in $eNOS^{-/-}$ mice treated with or without 17β-estradiol and in wild-type mice treated with 17β-estradiol. EPC mobilization into the peripheral circulation, identified as Sca-1–positive and Flk-1–positive cells by FACS analysis, was assessed before injury and 3 and 7 days after the injury in $eNOS^{-/-}$ and wild-type mice treated with 17β-estradiol.

Effects of Estradiol on Cultured EPC In Vitro (Mitogenic and Migration Activity, Antiapoptosis)

Mitogenic activity was assayed as described previously (CellTiter96 nonradioactive cell proliferation assay (Promega).[21] Briefly, mouse EPCs were harvested after 7 days in culture and reseeded into a 96-well plate in phenol red–free EBM medium supplemented with 0.5% BSA overnight. 17β-Estradiol diluted to serial concentrations (10^{-10}, 10^{-9}, 10^{-9} mol/L) was added to the wells for 48 hours before optical density measurement (562 nm). The EPC proliferation index was calculated by calibrating the density of cells without 17β-estradiol to 1.0.

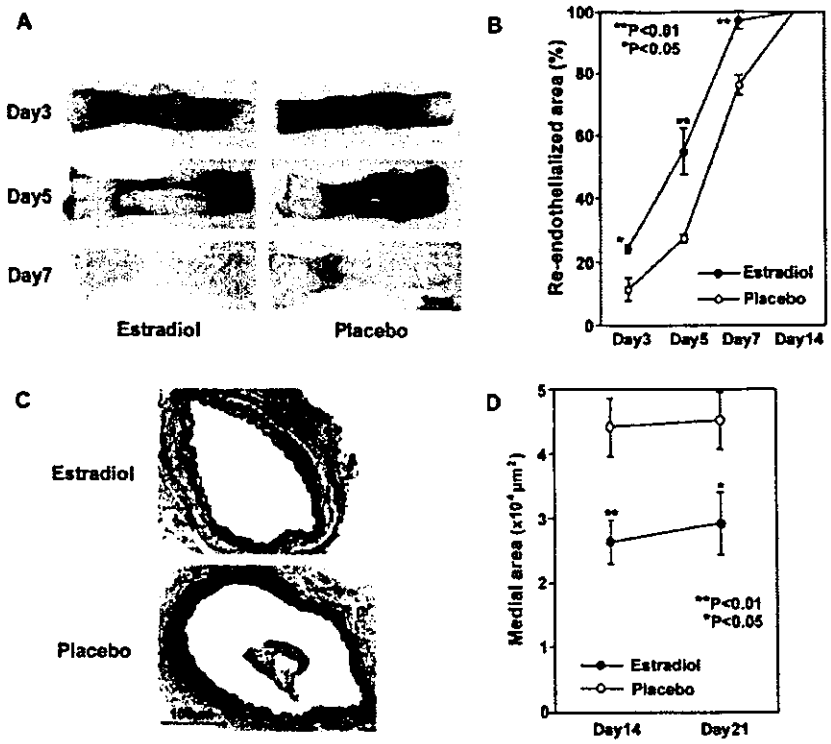

Figure 1. A, Examples of carotid arteries harvested 3 (top), 5 (middle), and 7 (bottom) days after wire injury in estradiol (left) and placebo (right) groups. Evans blue staining identifies segments of each artery that have not recovered functionally intact endothelium. At all time points, estradiol-treated arteries show significantly larger area of recovered endothelium (white areas). B, Quantification of reendothelialized area assessed by Evans blue dye staining of whole-mounted carotid arteries at days 3, 5, 7, and 14 after injury, expressed as mean±SEM (n=5 per each group). C, Elastic tissue–stained histological cross sections of carotid arteries from estradiol-treated (top) and non–estradiol-treated (bottom) animals 14 days after injury. D, Quantification (mean±SEM) of medial area, measured in elastic tissue–stained histological sections 14 and 21 days after injury (n=5 per each group). At both time points, estradiol-treated arteries show significantly smaller medial area.

To investigate EPC migration activity, a modified Boyden chamber assay was performed as previously described.[21] Briefly, 17β-estradiol was diluted to the same serial concentrations in phenol red–free EBM media supplemented with 0.1% BSA in the bottom chamber, and mouse EPCs were reseeded in the upper chamber. The cells on the bottom of the filter, stained with Giemsa solution (Baxter Diagnostics) were counted manually in random fields in each well.

The proportion of apoptotic EPCs after serum starvation was quantified by manually counting pyknotic nuclei after 4′-6′-diaminidmo staining (DAPI, Roche), as described previously.[24] In brief, cultured mouse EPCs (1×10^5 cells per well) underwent serum deprivation for 48 hours and were then incubated in phenol red–free medium alone or supplemented with 17β-estradiol (10^{-8} mol/L) for 3 hours, after which DAPI-stained pyknotic nuclei were counted as a percentage of cells in each well. For DNA cleavage assays, the serum-starved EPCs (4×10^5) were incubated with or without 17β-estradiol (10^{-8} mol/L) for 3 hours, and total DNA was extracted from the cells by using the Puregene DNA isolation kit (Gentra Systems). The pellet was resuspended in TE buffer (10 mmol/L Tris-HCl, pH 8.0; 1 mmol/L EDTA) and treated with DNase-free RNase for 1 hour at 37°C. DNA was ethanol-precipitated and resuspended in distilled water. Total DNA was electrophoretically fractionated on a 1.5% agarose gel and stained with ethidium bromide.[25]

All of the above in vitro studies were performed in triplicate.

Statistical Analysis

All values are expressed as mean±SEM. Statistical significance was evaluated by means of the Wilcoxon rank-sum test for comparisons between two groups. Multiple comparisons were performed through the use of ANOVA with Bonferroni's correction. A value of $P<0.05$ was considered statistically significant.

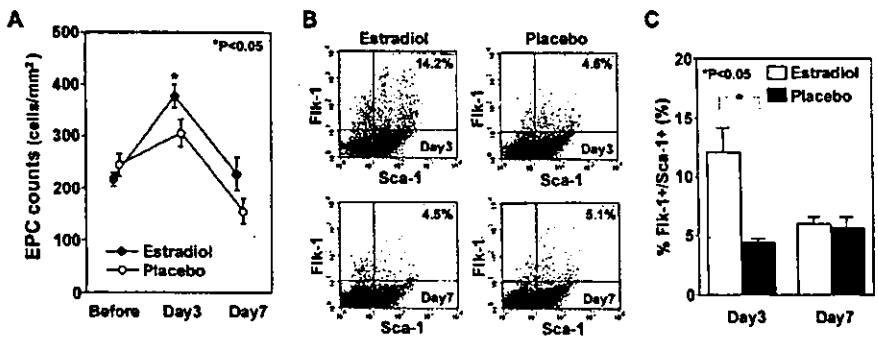

Figure 2. A, Quantification (mean±SEM) of circulating EPCs identified by double-positive staining for DiI-acLDL+ and BS1-lectin+ cells in EPC culture assay (n=5 per each group). Number of circulating EPCs in estradiol-treated animals significantly increased 3 days after carotid injury compared with placebo-treated animals. B, Representative 4-quadrant FACS analysis of circulating mononuclear cells from estradiol-treated and non–estradiol-treated mice identifying cells that are double-positive for Sca-1 (*x* axis) and Flk-1 (*y* axis). C, Quantitative analysis of the percentage of Sca-1/Flk-1–positive cells in peripheral blood (mean±SEM, n=5 per each group) revealing significantly higher percentage of double-positive cells in the circulation of estradiol-treated mice 3 days after arterial injury.

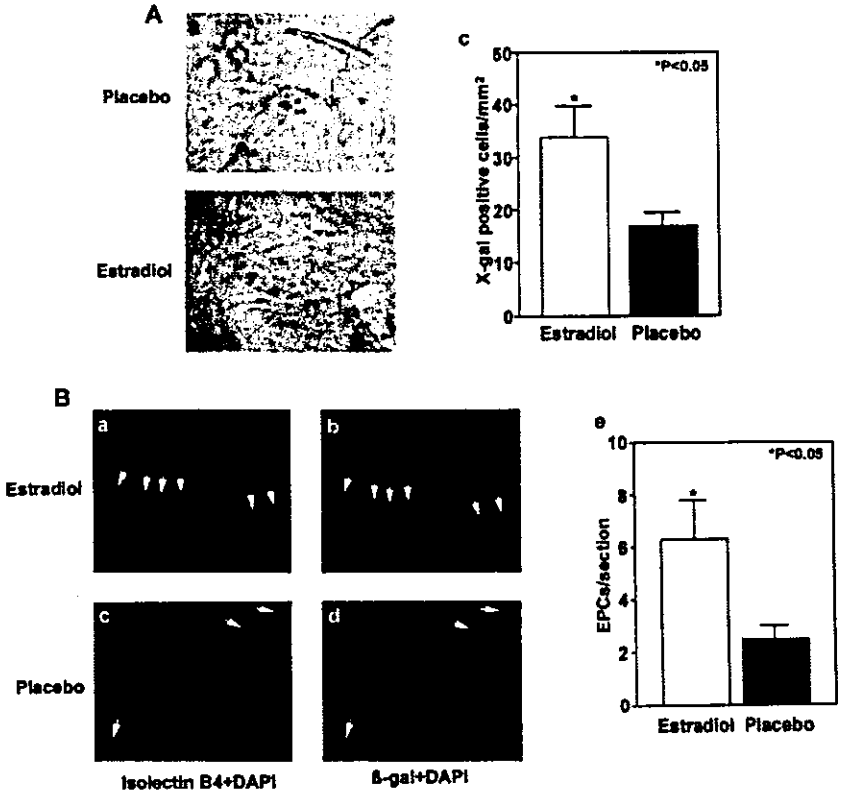

Figure 3. A, Representative photomicrographs of the luminal surface of X-gal–stained injured carotid segments from non–estradiol-treated (a) and estradiol-treated (b) mice 3 days after injury; c, statistical analysis discloses significant increase of X-gal–positive cells at injured site 3 days after injury (mean±SEM, n=5 per each group). B, Representative photomicrographs of carotid arteries from bone marrow–transplanted mice receiving estradiol pellets (a and b) or placebo pellets (c and d) 14 days after injury. Green, Isolectin B4; red, β-galactosidase; blue, 4′-6′-diaminidmo (DAPI) staining. White arrows indicate double-positive cells. e, Quantitative analysis of double-positive cells per section (mean±SEM, n=5 per each group).

Results

Serum levels of 17β-estradiol in the placebo group 7 and 14 days after the carotid injury were <2 pg/mL. In contrast, the levels in the estradiol group after 7 and 14 days after the injury were 580±55 and 559±62 pg/mL, respectively ($P<0.01$ versus placebo).

Effect of Estradiol on the Response to Injury in Wild-Type Animals

Figure 1A shows examples of arteries harvested 3, 5, and 7 days after injury from mice receiving estradiol versus placebo pellets. At all time points after injury, the estradiol-treated arteries showed a larger reendothelialized area as compared with the non–estradiol-treated arteries. Quantification demonstrated that estradiol treatment significantly accelerated reendothelialization compared with the placebo treatment, as has been shown previously in the rat and mouse (Figure 1B).[8,26,27] The inner surface of the carotid artery in each group was completely reendothelialized 14 days after the injury. Also consistent with prior studies, estradiol significantly inhibited the injury-induced increase in medial area (Figure 1, C and D).[28]

Effect of Estrogen on Circulating EPC Kinetics

Before carotid injury, the number of circulating EPCs was similar in the groups. However, the number of EPCs in the circulation of the estradiol group was significantly greater than in the placebo group 3 days after arterial injury (377±22 versus 306±26 cells/mm², $P<0.05$, Figure 2A). Furthermore, the number of EPCs returned to baseline 7 days after injury in both groups.

These data were further supported by FACS analysis for quantification of Sca-1/Flk-1–positive cells. Representative FACS analysis 3 and 7 days after injury in both groups is shown in Figure 2B, revealing a significant increase of EPCs in the estradiol group 3 days after injury as compared with the placebo group (12.1±2.0 versus 4.4±0.3%, $P<0.05$) (Figure 2C).

Enhanced Contribution of Bone Marrow–Derived EPCs to Reendothelialization

As shown in Figure 3A, a and b, whole-mounted X-gal–stained carotid arteries from the estradiol-treated mice displayed greater numbers of X-gal positive cells on the luminal surface than in the placebo-treated mice 3 days after the injury. Quantification of whole-mounted X-gal–stained specimens in the estradiol group revealed a significant increase in X-gal–positive cells 3 days after the injury as compared with the placebo group (34±6 versus 17±3 cells/mm², $P<0.05$, Figure 3A, c). Furthermore, double-fluorescent immunohistochemistry for β-galactosidase and biotinylated isolectin B4 was performed on frozen sections 14 days after injury to identify bone marrow–derived Tie2/LacZ–positive ECs. At the injury site, double-positive cells were detected with greater frequency in the endothelial monolayer in estradiol-treated mice (Figure 3B, a through d). Quantification of bone marrow–derived Tie2/LacZ–positive ECs at the injury site disclosed a significant 3-fold increase in the number of double-positive cells on the reendothelialized luminal surface in cross sections of estradiol-treated versus non–estradiol-treated mice (6.2±1.8 versus 2.2±0.4 cells/section, $P<0.05$)(Figure 3B, e). These data thus demonstrate that accelerated reendothelialization achieved with estradiol involves augmented EPC incorporation into the carotid artery neoendothelium.

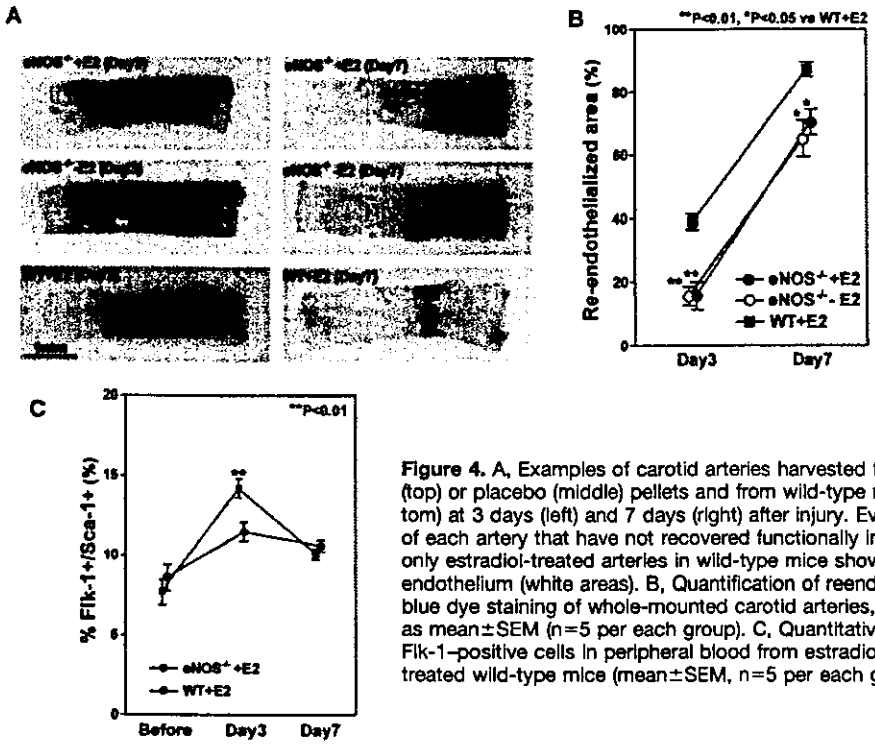

Figure 4. A, Examples of carotid arteries harvested from eNOS$^{-/-}$ mice receiving estradiol (top) or placebo (middle) pellets and from wild-type mice receiving estradiol pellets (bottom) at 3 days (left) and 7 days (right) after injury. Evans blue staining identifies segments of each artery that have not recovered functionally intact endothelium. At both time points, only estradiol-treated arteries in wild-type mice show significantly larger area of recovered endothelium (white areas). B, Quantification of reendothelialized area, assessed by Evans blue dye staining of whole-mounted carotid arteries, at days 3 and 5 after injury expressed as mean±SEM (n=5 per each group). C, Quantitative analysis of the percentage of Sca-1/Flk-1–positive cells in peripheral blood from estradiol-treated eNOS$^{-/-}$ and estradiol-treated wild-type mice (mean±SEM, n=5 per each group).

eNOS Is Required for Estradiol-Induced Acceleration of Reendothelialization

Figure 4A shows examples of carotid arteries harvested from eNOS$^{-/-}$ mice receiving estradiol or placebo pellets and from wild-type mice receiving estradiol pellets 3 days and 7 days after the injury. The reendothelialized areas were similar in eNOS$^{-/-}$ mice receiving estradiol or placebo pellet, whereas wild-type mice receiving estradiol pellets had a greater area reendothelialized than did the eNOS$^{-/-}$ mice receiving estradiol or placebo pellets (Figure 4A). Quantification demonstrated that estradiol treatment significantly accelerated reendothelialization in wild-type mice (Figure 1B) but had no effect in eNOS$^{-/-}$ mice (Figure 4B).

The percentage of Flk-1/Sca-1–positive cells in the circulation in estradiol-treated wild-type and eNOS$^{-/-}$ mice, assessed by FACS analysis, was equivalent before the carotid injury (Figure 4C). However, the percentage of double-positive cells significantly increased 3 days after injury only in the wild-type mice receiving estradiol. Thus, estradiol failed to increase EPC numbers after arterial injury and failed to accelerate reendothelialization in the absence of eNOS expression.

Effect of Estradiol on EPC Mitogenesis, Migration, and Apoptosis

Mitogenic activity of EPCs was increased by estradiol at all but the lowest concentration of estradiol (10^{-10} mol/L) and displayed a dose response (control: 1.00±0.32, 10^{-10} mol/L: 1.05±0.02, 10^{-9} mol/L: 1.32±0.03, 10^{-8} mol/L: 1.17±0.02, $P<0.001$, Figure 5A). Estradiol also induced EPC migration in a dose-dependent manner (control: 901±50, 10^{-10} mol/L: 1446±146, 10^{-9} mol/L: 1468±138, 10^{-8} mol/L estradiol: 1799±38 cells/mm^2, $P<0.01$, Figure 5B). Moreover, estradiol had an antiapoptotic effect on EPCs when assayed on the basis of counting of apoptotic cells (8±3 versus 19±6%, $P<0.01$, Figure 5C, a to c), as has been shown previously in mature ECs.[29] The antiapoptotic effects of estradiol were also verified by documenting inhibition of DNA fragmentation in cultured EPCs subjected to serum starvation (Figure 5C).

Discussion

The present study provides evidence that estrogen mobilizes circulating EPCs from the bone marrow and that these cells incorporate into the neoendothelium, thereby contributing to reendothelialization after arterial injury. This effect is shown to be dependent on eNOS expression, as both EPC mobilization and acceleration of reendothelialization are absent in estradiol-treated eNOS-null mice. Moreover, these data disclose that estradiol has similar direct effects on EPCs, as have been documented in mature ECs,[2,29] inducing proliferation and migration and inhibiting apoptosis. These findings are consistent with those recently reported by Strehlow et al[27] and also add the important finding of an eNOS-dependent mechanism for the effects of estradiol on EPCs and endothelial recovery. Most notably, the present findings suggest that enhanced EPC mobilization and recruitment to sites of arterial injury may be the central feature of the beneficial effect of estradiol.

It has long been thought that endothelial regeneration in response to arterial injury was a local process involving endothelial proliferation and migration from intact ECs adjacent to the site of injury. The acceleration of reendothelialization by estrogen after injury has likewise been interpreted to develop as a result of these same mechanisms.

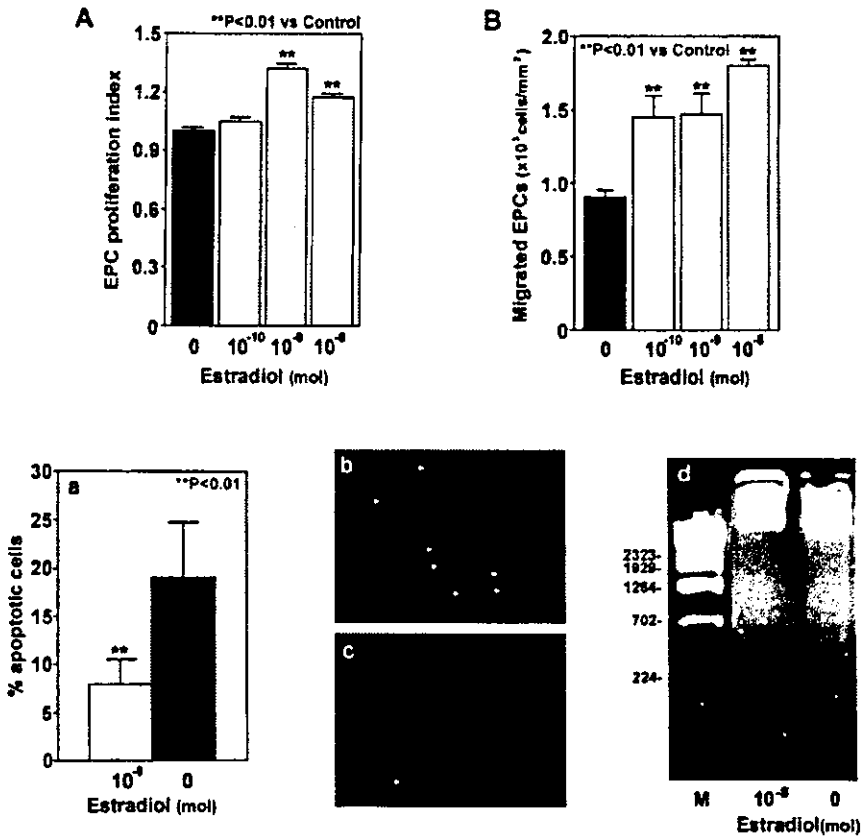

Figure 5. A, Proliferation assays show moderate-dose dependent mitogenic response of EPCs to estradiol (mean±SEM, n=8 per each group). B, Migratory response of EPCs toward different dosages of estradiol measured by modified Boyden chamber migration assay. Cultured EPCs demonstrated potent dose-dependent migratory activity to estradiol (mean±SEM, n=5 per each group). C, Estradiol inhibits EPCs apoptosis induced by serum starvation (a). Quantification of percentage of pyknotic nuclei determined by DAPI staining, after serum starvation with or without estradiol supplementation (mean±SEM, n=5 per each group). b and c, Representative photomicrographs from non–estradiol-treated (b) and estradiol-treated (c) EPCs. c, Fragmentation of total DNA from EPCs subjected to serum starvation. Total DNA isolated from estradiol-treated (10^{-8} mol/L) and non–estradiol-treated EPCs was subjected to gel electrophoresis alongside molecular weight markers (M). Gel demonstrates ladder of DNA bands from non–estradiol-treated EPCs indicate apoptosis of EPCs that was prevented in EPCs in which estradiol was added to culture medium.

These data suggest that an alternative mechanism may be critical to this process.

EPCs have been isolated from circulating mononuclear cells in peripheral blood and shown to incorporate into foci of neovascularization of adults, consistent with the notion of postnatal vasculogenesis.[9,11,30] These bone marrow–derived EPCs are mobilized endogenously in response to tissue ischemia and/or exogenously by cytokine stimulation. Vascular endothelial growth factor and granulocyte macrophage-colony stimulating factor can mobilize EPCs from bone marrow into the peripheral circulation.[10,21] The present data show estradiol to be capable of modulating EPC kinetics under pathological circumstances. The full implications of this finding for our understanding of the effects of estradiol on vascular biology remain to be determined. It is, however, interesting to note the parallel between the effect of estradiol and that of statins. Statins have been shown to reduce coronary event rates and to accelerate endothelial recovery after injury, in part through mobilization and incorporation of EPCs at the site of injury.[23] These actions of statins are mediated through phosphorylation of the protein kinase Akt. Moreover, statins have been shown to modulate EPC proliferation, migration, and survival[31,32] and to accelerate reendothelialization by a mechanism involving EPCs.[23] In this context, it is interesting to note that estradiol has also been shown to activate Akt signaling, suggesting one possible mechanism for modulation of EPCs.[33]

Given the above findings, the central role of Akt signaling in eNOS activity and the regulation of eNOS and NO by estradiol,[34,35] we considered the possibility that EPC mobilization by estradiol might require NO-mediating signaling. We evaluated reendothelialization and EPC kinetics after the injury in $eNOS^{-/-}$ mice and found that the absence of eNOS essentially nullified estradiol-induced mobilization of EPCs and blocked the acceleration of endothelial recovery seen in wild-type mice treated with estradiol. These results suggested that the presence of eNOS might be a key factor of estradiol-induced, EPC-mediated reendothelialization.

The present study reveals that estrogen can augment EPC mobilization into sites of neovascularization in adult organs under pathological conditions. This process appears to require eNOS; however, the precise mechanism by which estrogen enhances EPC mobilization and incorporation remains to be defined. In addition, our data suggest that EPCs may provide a major contribution in the salutary effect of estradiol on endothelial recovery after arterial injury.

In conclusion, estrogen augments mobilization and proliferation of bone marrow–derived EPCs, which contribute to accelerate reendothelialization and attenuate vascular remodeling at sites of arterial injury. These results provide novel insights into potential mechanisms of estrogen effects on vascular biology.

Acknowledgments

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References

1. Ross R. Atherosclerosis: a defense mechanism gone awry. *Am J Pathol.* 1993;143:985–1002.
2. Morales DE, McGowan KA, Grant DS, et al. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation.* 1995;91:755–763.
3. Venkov CD, Rankin AB, Vaughan DE. Identification of authentic estrogen receptor in cultured endothelial cells. *Circulation.* 1996;94: 727–733.
4. Kim-Schulze S, McGowan KA, Hubchak SC, et al. Expression of an estrogen receptor by human coronary artery and umbilical vein endothelial cells. *Circulation.* 1996;94:1402–1407.
5. Clowes AW, Collazzo RE, Karnovsky MJ. A morphologic and permeability study of luminal smooth muscle cells after arterial injury in the rat. *Lab Invest.* 1978;39:141–150.
6. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury, I: smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983;49:327–333.
7. Asahara T, Bauters C, Pastore CJ, et al. Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery. *Circulation.* 1995;91:2793–2801.
8. Krasinski K, Asahara T, Spyridopoulos I, et al. Estradiol accelerates endothelial recovery after arterial injury. *Circulation.* 1997;95: 1768–1772.
9. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964–967.
10. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow–derived endothelial progenitor cells for neovascularization. *Nat Med.* 1999;5:434–438.
11. Shi Q, Rafii S, Wu MH-D, et al. Evidence for circulating bone marrow–derived endothelial cells. *Blood.* 1998;92:362–367.
12. Reyes M, Dudek A, Jahagirdar B, et al. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest.* 2002;109:337–346.
13. Edelberg JM, Tang L, Hattori K, et al. Young adult bone marrow–derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. *Circ Res.* 2002;90:E89–E93.
14. Lyden D, Hattori K, Dias S, et al. Impaired recruitment of bone-marrow–derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med.* 2001;7:1194–1201.
15. Otani A, Kinder K, Ewalt K, et al. Bone marrow–derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat Med.* 2002;8:1004–1010.
16. Masuda H, Kalka C, Takahashi T, et al. Regulatory role of estrogen on endothelial progenitor cell kinetics for cyclic endometrial neovascularization. *Circulation.* 1999;100(suppl I):I-2503.
17. Burger HG, Loriaux DL, Marshall JC, et al. *Endocrinology.* 4th ed. Philadelphia, Pa: WB Saunders Co; 2001.
18. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999;85: 221–228.
19. Lindner V, Fingerle J, Reidy MA. Mouse model of arterial injury. *Circ Res.* 1993;73:792–796.
20. Goukassian D, Kishore R, Dolan C, et al. Engineering the response to vascular injury: divergent effects of deregulated E2F1 expression on vascular smooth muscle cells and endothelial cells result in endothelial recovery and inhibition of neointimal growth. *Circ Res.* 2003;93: 162–169.
21. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow–derived endothelial progenitor cells. *EMBO J.* 1999;18:3964–3972.
22. Werner N, Priller J, Laufs U, et al. Bone marrow–derived progenitor cells modulate vascular reendothelialization and neointimal formation: effect of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibition. *Arterioscler Thromb Vasc Biol.* 2002;22:1567–1572.
23. Walter DH, Rittig K, Bahlmann FH, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow–derived endothelial progenitor cells. *Circulation.* 2002;105:3017–3024.
24. Yamaguchi J, Kusano KF, Masuo O, et al. Stromal cell–derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation.* 2003;107:1322–1328.
25. Aikawa R, Nawano M, Gu Y, et al. Insulin prevents cardiomyocytes from oxidative stress–induced apoptosis through activation of PI3 kinase/Akt. *Circulation.* 2001;102:2873–2879.
26. Brouchet L, Krust A, Dupont S, et al. Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor-alpha but not estrogen receptor-beta. *Circulation.* 2001;103:423–428.
27. Strehlow K, Werner N, Berweiler J, et al. Estrogen increases bone marrow–derived endothelial progenitor cell production and diminishes neointima formation. *Circulation.* 2003;107:3059–3065.
28. Sullivan TR, Karas RH, Aronovitz M, et al. Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J Clin Invest.* 1995; 95:2482–2488.
29. Spyridopoulos I, Sullivan AB, Kearney M, et al. Estrogen-receptor–mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. *Circulation.* 1997;95:1505–1514.
30. Asahara T, Masuda H, Takahashi T, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999;85: 221–228.
31. Kureishi Y, Luo Z, Shiojima I. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med.* 2000;6:1004–1010.
32. Dimmeler S, Aicher A, Vasa M, et al. HMG-CoA–reductase inhibitors (statins) increase endothelial progenitor cells via the PI3 kinase/Akt pathway. *J Clin Invest.* 2001;108:391–397.
33. Simoncini T, Hafezi-Moghadam A, Brazil DP, et al. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature.* 2000;407:538–541.
34. Hishikawa K, Nakaki T, Marumo T, et al. Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells. *FEBS Lett.* 1995; 360:291–293.
35. MacRitchie AN, Jun SS, Chen Z, et al. Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ Res.* 1997;81:355–362.

Role of Blood Mononuclear Cells in Recanalization and Vascularization of Thrombi: Past, Present, and Future

Nicanor I. Moldovan* and Takayuki Asahara

Recent experimental data suggest a role for blood-borne mononuclear cells, including bone marrow–derived endothelial progenitor cells (EPCs) in organization, vascularization, and recanalization of thrombi. Older studies have described in detail the in situ structural modifications accompanying these processes. The common themes are (a) involvement of pluripotent mononuclear cells, and (b) proteolytic and/or phagocytic activity of monocytes/macrophages (MCs/Mphs) in modifying the thrombus to a proangiogenic state. In addition to a nurturing function, MCs/Mphs may assist the engraftment of thrombus-trapped or incoming EPCs. The differences between the recanalization potency of venous and arterial thrombi suggest that in addition to the organization of the fibrin meshwork, the cellular composition of the venous thrombi may be responsible for the better recanalization seen in venous clots. These observations set the stage for therapeutic manipulation of organization and recanalization of thrombi, by increasing the MC/Mph and/or EPC content. (Trends Cardiovasc Med 2003;13:265–269)

• Background

Clotting, a key mechanism of circulatory homeostasis, is involved in the recovery of vascular integrity following injury. When inappropriate intravascular clotting is triggered by pathologic mechanisms, then thrombi (intravascular clots) are produced. An elegant account of the various instances of formation and the fates of thrombi can be found elsewhere (Majno and Joris 1996).

The restoration of blood flow in a thrombus-occluded vessel may occur spontaneously through recanalization. Pathologists and surgeons have long been aware of this process of potentially great importance. However, its mechanism remained largely unexplored with the tools of modern molecular cell biology.

For example, venous thrombi in humans often recanalize, so that a patent lumen is restored (Flanc 1968, Sevitt 1973). Arterial thrombi also recanalize (Dible 1958, Haudenschild 1998), but less frequently and to a lesser extent (Majno and Joris 1996). When venous vascular grafts are placed in the arterial circulation, their thrombi recanalize poorly, suggesting that it is the type of blood—arterial or venous—rather than the vascular conduit that drives the recanalization (Flanc 1968). The reason for this difference between the arterial and venous thrombi is unknown and even counterintuitive, because the pressure of the blood flow in the arterial circulation would be expected to help recanalization. Recanalization also was described in Buerger's disease (Dible 1958, Hoppe et al. 2002) and may be involved as well in the formation of coaxial arteries that sometimes are present in patients with Kawasaki syndrome (Terashima et al. 2002).

Recanalization is part of thrombus "organization," the process that converts a thrombus into a tissue. From this point of view, organization is similar to wound healing. Pathologists distinguish two sets of vessels in a successfully organized thrombus: (a) vasa vasorum of the outer and middle coats of the organized thrombus, and (b) the vessels that canalize the thrombus. These two sets of vessels are separated by the elastic laminae of the canalizing vessels. They also display morphologic distinctions: the former run in radial directions, whereas the latter—which lie in the center of the parent vessel—run almost exclusively parallel to it (Dible 1958).

Organization of intravascular clots relies on several cellular processes: (a) covering of the surface exposed to blood with monocytes (MCs) and, eventually, with an endothelial layer (Davies et al. 1975); (b) penetration by monocytic cells; (c) occurrence of myofibroblasts and new capillaries (McGuinness et al. 2001); and (d) in some instances, recanalization—that is, the formation of one or more veins (or arteries, depending on the thrombus localization) inside and parallel to the original vessel.

Recent developments in angiogenesis research involved bone marrow–derived, blood-borne (i.e., circulating) endothelial progenitor cells (EPCs) (Asahara et al. 1997, Moldovan 2003, Murayama and Asahara 2002) and possibly smooth muscle progenitor cells (Religa et al. 2002, Simper et al. 2002) in adult vasculogenesis in a variety of normal and pathologic situations. Indications for this possibility occurred previously, particularly in conjunction with the "fallout endothelialization" of synthetic grafts (Mackenzie et al. 1968, Stump et al. 1963).

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- **Infiltration and Recanalization of Thrombi: Old Insights**

Long ago it was observed that the stage for clot revascularization is set by the architecture of the early primary thrombus (Dible 1958). This was described as a spongy structure in which bands of more solid material surround spaces filled with looser fibrin. The latter contains in its meshes large numbers of red blood cells and leukocytes. "Channels" with walls made of (or within) fibrin and containing blood that appears to circulate were found inside of the thrombi. The looser component of the thrombus disappears later, either through autolysis or through the action of leukocytes, leaving the spongework of fibrin bands almost "empty." Next, endothelial (progenitor?) cells "apply themselves to this framework, so that in a short interval the spongy structure becomes endothelialized." Depending on the thickness of the fibrin bands, the resulting structure may have the appearance of either a fibrin lattice covered with endothelium or endothelium-lined spaces within a denser fibrin mass. All these initial processes take place entirely in the thrombus, involving only blood components, and there is no detectable reaction in the arterial wall. The red blood cells included in the fibrin meshes "begin to lose hemoglobin and become faintly stained, making them clearly distinguishable from the circulating, fresh red cells in the spaces between bands" (Dible 1958). The next stage is a "collagenous transformation" (supposedly with the contribution of fibroblasts) of the condensed fibrin with formation of silver-positive reticulin fibers. The further modification of the collagenous, endothelium-lined "lacunae" results in the formation of more mature vascular channels, "which may later acquire the rounded form of ordinary blood vessels" (Dible 1958).

In an ultrastructural study of experimentally produced mural thrombi in the pig aorta (Davies et al. 1975), it was shown that at 3 days, inside of the clots there were, among other constituents, two distinct cell populations: Mphs and undifferentiated mesenchymal cells. Over the subsequent 2 weeks, the mesenchymal cells, which initially appeared to be homogeneous throughout the thrombus, gradually differentiated. On the surface of the thrombus, they formed an endothelial lining. In the superficial layers, myointimal cells and, ultimately, smooth muscle cells occurred and in the deeper layers new capillaries were formed that joined with the endothelium-lined clefts from the surface. Thus, the early stage of organization of the thrombus appears to be effected by pluripotent precursor cells derived from blood. Again, no early evidence was found to suggest a contribution from the cells native to the vessel wall. These cells, however, may be involved in the more evolved stages of organization (Davies et al. 1975).

Later studies confirmed these earlier descriptions and brought further experimental evidence to support the idea that the recanalization of the thrombus, as part of a nonsprouting, vasculogenesis-like formation of vessels, may be entirely a thrombus-supported mechanism (Feigl et al. 1985, Leu et al. 1987, 1988). To prove this concept, Feigl et al. (1985) developed artificial prostheses made of polyurethane with an athrombogenic surface and with a central part consisting of a thrombogenic Dacron (Dupont, Kinston, North Carolina) velour ring. These devices were implanted into the aorta of sheep. In this setting, organization of the central thrombus by local aortic cells could be excluded. Again, the endothelium developed on the surface of the organizing thrombus. In the final stage, a pseudovessel wall also was formed, which followed the pattern of the original vascular conduit. These data can be considered as an experimental demonstration that a thrombus may be organized by cells derived from blood.

The work done by Leu et al. (1987 and 1988) also argues that in organizing thrombi and emboli, this vasculogenesis type of angiogenesis is not dependent on invasion of vasa vasorum from the vascular wall. They stressed that mononuclear cells of the "monohistiocytic system" (i.e., MCs) that always are present within the clotted blood are capable of differentiation into various types of mesenchymal cells, including endothelial cells (ECs). Such a transdifferentiation mechanism is supported by recent in vitro work (Fernandez-Pujol et al. 2001, Rehman et al. 2003, Schmeisser et al. 2001, Zhao et al. 2003). Alternatively, less-differentiated EPCs may evolve toward ECs through cellular stages that express markers, and possibly other properties, of MCs/Mphs (Nakul-Aquaronne et al. 2003).

Leu et al. (1987 and 1988) have found that "autolytic slits and clefts" appear initially in the fibrinous superficial areas of the thrombus. These gradually are lined by spindle-shaped "pre-endothelial" cells that already possess immunohistologic properties of ECs, but ultrastructurally still resemble primitive mesenchymal cells. These cells connect later with each other by pseudopodia, overlapping, and interdigitation until the channels in the fibrinous matrix are covered by an uninterrupted layer of cells. These cells are characterized ultrastructurally by the appearance of specific endothelial organelles (Weibel-Palade bodies). Circulation within the channels then begins from the bloodstream. In addition, some angiogenesis by sprouting from the vascular wall occurs in those areas of the thrombus in contact with the parent vessel. In blood vessels with an unimpaired intimal endothelial layer, angiogenesis by invasion of capillaries occurs at an earlier stage than does capillary formation by mononuclear cells (Leu et al. 1987 and 1988).

- **Proteolytic Activity of Trapped MC During Recanalization of Thrombi**

The previously described studies revealed capillary-like spaces within clots (called "clefts," "slits," or "channels") that occurred before or simultaneously with the microvascular structures themselves. These precapillary lumens developing in clots come to be lined by ECs similar to those covering the clot, but this seems to occur before the surface clefts make contact with the deep "capillaries."

As a step toward the clarification of this mechanism, Singh et al. (2003) recently showed, using knockout mice, that urokinase-type plasminogen activator (uPA) and not its tissue variant is necessary for resolution and vascularization of experimental venous thrombi. Moreover, these authors demonstrated that experimental venous thrombi in $uPA^{-/-}$ mice fail to organize and recanalize. Wild-type bone marrow–derived cells, potentially of a progenitor nature, were instrumental in rescuing the thrombus organizing and recanalizing deficit of $uPA^{-/-}$ mice (Singh et al. 2003). Remarkably, high concentrations of Mphs also were detected previously in the regions of recanalization, both in spontaneous human and in experimental animal thrombi (McGuinness et al. 2001, Singh

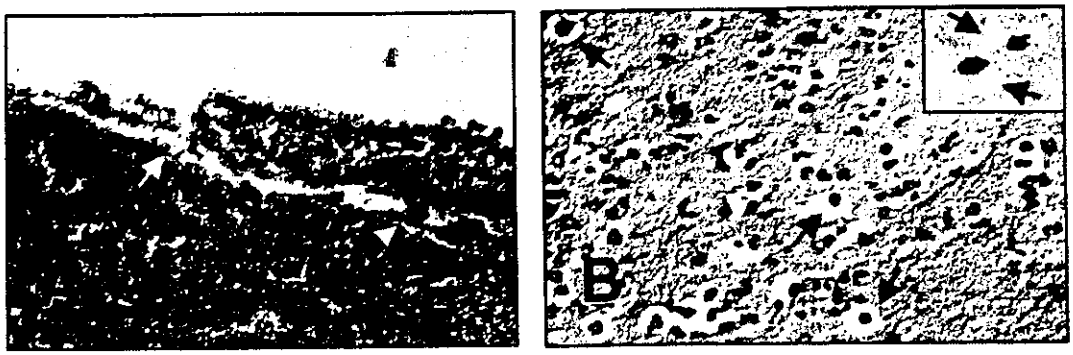

Figure 1. Proteolytic activity of inflammatory cells in organizing thrombi (old atrial clot in a mouse transgenically expressing monocyte chemotactic protein-1 [MCP-1] in the heart). **(A)** Marginal tunnel-like defect (*arrow*) associated with an apparently advancing MC (*arrowhead*). **(B)** Cross-section through a field of leukocytes trapped within the thrombus. Note the sharp proteolysis rims surrounding most of the mononuclear cells. (*Inset*) Clearance of the fibrin around mouse peritoneal macrophages penetrating an artificial fibrin clot in vitro (*arrows*). **(A, B)** Hematoxylin-eosin staining and differential interference contrast microscopy. Original magnification × 120. (*Inset*) Transmission optical microscopy of a semifine section through a glutaraldehyde-fixed specimen embedded in resin and stained with toluidine blue. Original magnification × 200.

et al. 2003). This suggests that actually two cell populations, MCs/Mphs and the progenitors (presumably endothelial), cooperate in restoration of blood flow in occluded thrombi. Such a dual mechanism was proposed to explain the neovascularization of ischemic limbs by a mixture of $CD14^+$ MCs and $CD34^+$ hematopoietic cells (Harraz et al. 2001).

The intercellular cooperation between MCs/Mphs and EPCs is at the core of a novel angiogenic model based on the ability of MCs/Mphs to penetrate the extracellular matrices and create tubular spaces ("tunnels") of lower density (Moldovan 2002). This model was derived from the observations on the ischemic cardiomyopathy in transgenic mice, in which a chronic inflammatory infiltrate (mostly composed of MCs) was induced by expression of MC chemotactic protein-1 (MCP-1) under the cardiac-specific myosin heavy-chain promoter (Kolattukudy 1998). In these hearts, cells and tunnels staining positive for the Mph-specific metalloelastase (MMP-12) were observed (Moldovan et al. 2000). Later, Anghelina et al. (2002) also detected, intermixed with "normal" capillaries, sparse blood conduits positive for CD18 (a pan-leukocyte marker) and CD34 (a hematopoietic marker). These observations suggest that the tunnels may be covered with the mononuclear precursors of ECs (Rehman et al. 2003, Zhao et al. 2003). The presence of MMP-12 secreting cells associated with the growing capillaries in excisional wounds (Madlener et al. 1998) and of leading $CD45^+$ Mphs at the tips of sprouting capillaries in the forming embryo microvasculature (Takakura et al. 2000) suggest a cooperative, rather than unicellular, nature of penetration of the extracellular matrix by ECs.

Formation of tunnels was described previously in a study addressing the migration of activated Mphs in fibrin gels in vitro (Castellucci and Montesano 1988). It should be stressed, however, that the occurrence of tunnels, and the ability to detect them, depends on the density of the matrix, because the Mphs also are able to simply push apart the fibrin strands, with very little fibrinolytic activity, when they migrate in lax fibrin gels (Ciano et al. 1986). In thrombi composed of well-organized fibrin strands, the MCs/Mphs rather may engulf interfibrillar material, supposedly removing debris of shorter-lived cells such as polymorphonuclear cells and platelets; thus, they contribute to the creation of spaces of lower density between the fibrils (tubular or round when they are not migrating, as shown in Figure 1.

A mechanism of vasculogenesis based on the engraftment of circulating EPCs assisted by the proteolytic activity of MCs/Mphs would explain an important feature of recanalization that is other-

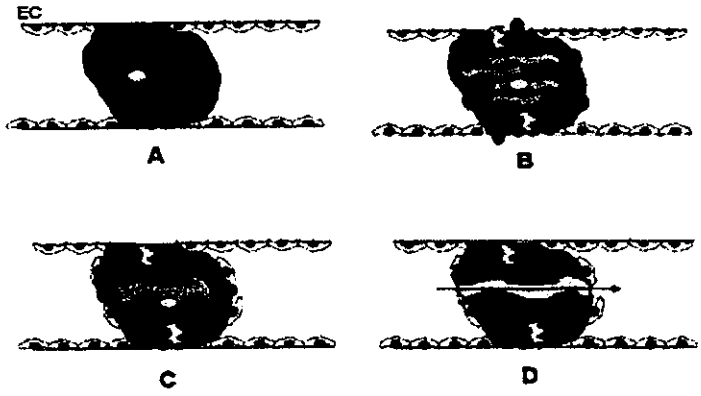

Figure 2. Diagram presenting tentative stages in the recanalization of intravascular thrombi, as suggested by the current review. **(A)** A newly formed thrombus incorporates leukocytes, including monocytes (MCs; *blue*) and endothelial progenitor cells (EPCs; *pink*). **(B)** MCs/macrophages (Mphs) lyse the fibrin meshwork and clean up debris and defunct cell corpses from the interfibrillar spaces, forming "tunnels." At the same time, more penetration of the clot by cells derived from blood or the vessel wall and by sprouts may occur. **(C)** The tunnels may undergo a maturation process, some being closed, others becoming enlarged, and covered with transdifferentiating MCs/Mphs and/or with EPCs. The surface of the clot also may become covered with MC- or with EPC-derived endothelial cells (ECs). **(D)** Circulation across the thrombus would be established with further proteolytic contribution of infiltrating MCs.

wise difficult to account for: the lumen of recanalizing neovessels run within, and parallel to, but not across the thrombosed vessel. This imposes a topological constraint on the angiogenesis process, making it unlikely that the neovessels would develop by sprouting from the wall of the parent vessel as shown in Figure 2.

EPCs derived from bone marrow are released initially into the venous microcirculation; therefore, their concentration may be higher locally in the venous rather than the arterial blood. Additionally, the venous clots may benefit from a stronger infiltrate of MC/Mph cells, which is facilitated by a thinner vessel wall. In both instances, a higher density of either one of these interacting cell populations in venous thrombi may be reflected in an increased propensity to recanalization.

• Perspectives for Therapeutic Manipulation of Recanalization of Thrombi

A detailed understanding of the roles played by MCs/Mphs and precursor cells (for endothelium, vascular smooth muscles cells, and myofibroblasts) in vascularization and recanalization of thrombi opens promising therapeutic perspectives. As previously discussed, venous thrombi are more prone to spontaneous recanalization than are arterial thrombi. Therefore, therapeutic interventions aimed at mimicking the mechanisms operating in veins in the arterial settings should help to increase the speed of recanalization.

In this respect, MCP-1 is known to contribute to the organization and resolution of venous thrombi. To this end, the endogenous levels of MCP-1 were measured in naturally resolving experimental rat venous thrombi and in the adjacent vessel wall after thrombus formation. Endogenous MCP-1 increased in the first week in the thrombus and vein wall. At 2 weeks, the thrombus became incorporated into the vein wall and total MCP-1 levels remained high. Moreover, MCP-1 injected into thrombus increased the thrombus organization scores, reduced the thrombus area, and increased the MC content of the surrounding vessel wall (Humphries et al. 1999).

The angiogenic chemokine interleukin 8 (IL-8) has been found in thrombi months after their initiation. It has been hypothesized that given the fact that thrombus organization involves leukocyte influx and neovascularization, IL-8 administered at the induction of thrombus will promote its organization (Wakefield et al. 1999). Indeed, neovascularization and inflammation (including MCs and cells that were defined as "spindle shaped," fibroblasts, and ECs) were increased significantly in the animals treated with IL-8 compared with the animals treated with vehicle alone. These results were correlated with a significant increase in thrombus perfusion (Wakefield et al. 1999). This study was reconfirmed by showing that IL-8 administration indeed enhances the resolution of venous thrombi (Henke et al. 2001). The same benefit was obtained from intraclot injection of VEGF in a rat model of inferior vena cava thrombosis. In animals treated with 10 ng of VEGF there was an area of recanalization lumen about twice as large as in controls. In this model, the proportion of MCs migrating to the center of the thrombus increased in a dose-related manner (Waltham et al. 2003).

Another compound useful for the stimulation of thrombus resorption via manipulation of its cellular composition could be Mph colony-stimulating factor (M-CSF). The treatment with recombinant human M-CSF produced clot resorption and restored osteoclastogenesis in heterotopic bone induced by partially purified native bone morphogenetic protein in osteopetrotic (op/op) mice (Miyazawa and Urist 1997). Induction or improvement of angiogenesis in tissues by administration of cell suspensions enriched in EPCs has been suggested recently (Murayama and Asahara 2002), and its feasibility has been demonstrated (Iba et al. 2002, Kawamoto et al. 2003, Strauer et al. 2002). Based on the data presented here, the same procedure may be used to increase the speed of vascularization and recanalization of venous clots or induction of these processes in arterial clots, if devoid of such mechanisms. A justification for this approach can be found in the study by McGuinness et al. (2001). Peripheral rat blood MC were labeled fluorescently and injected intravenously into rats prior to thrombus induction in the vena cava. By assessing the fluorescence levels in the clot, the researchers found that there was a steady increase in thrombus MC content and at 3 weeks the percentage of MCs in the thrombus had increased 35-fold. MCs appeared around the edge of the thrombus and became more evenly distributed through the thrombus as resolution progressed (McGuinness et al. 2001).

Recently, Tepper et al. (2002) demonstrated the presence of EPCs in carotid and caval experimental clots formed in lethally irradiated mice reconstituted with bone marrow from mice expressing β galactosidase under the control of the endothelial-specific Tie-2 promoter. The same group was able to prove that the direct systemic delivery of fluorescently labeled human EPCs in athymic rats significantly increased the number of vascular channels and the perfusion of experimental clots (Tepper et al. 2002).

In conclusion, recanalization of thrombi, as part of their organization, is a mechanism that is dependent on circulating mononuclear cells, in which two major processes (proteolytic activity of MCs/Mphs and engrafting of EPCs) seem to concur. Both mechanisms can be exploited therapeutically to increase the speed of clot organization and reperfusion, minimizing the time of exposure of downstream tissues to ischemia. In the future, this approach may complement the surgical and pharmacologic treatments for reperfusion of occluded blood vessels.

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References

Anghelina M, Schmeisser A, Krishnan P, et al.: 2002. Migration of monocytes/macrophages in vitro and in vivo is accompanied by MMP12-dependent tunnels formation and by neo-vascularization. Cold Spring Harb Symp Quant Biol LXVII: 209–215.

Asahara T, Murohara T, Sullivan A, et al.: 1997. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967.

Castellucci M, Montesano R: 1988. Phorbol ester stimulates macrophage invasion of fibrin matrices. Anat Rec 220:1–10.

Ciano PS, Colvin RB, Dvorak AM, et al.: 1986. Macrophage migration in fibrin gel matrices. Lab Invest 54:62–70.

Davies MJ, Ballantine SJ, Robertson WB, et al.: 1975. The ultrastructure of organising experimental mural thrombi in the pig aorta. J Pathol 117:75–81.

Dible HJ: 1958. Organization and canalization in arterial thrombosis. J Pathol Bacteriol LXXV:1–7.

Feigl W, Susani M, Ulrich W, et al.: 1985. Organisation of experimental thrombosis by blood cells. Evidence of the transformation of mononuclear cells into myofibroblasts and endothelial cells. Virchows Arch A Pathol Anat Histopathol 406:133–148.

Fernandez Pujol B, Lucibello FC, Zuzarte M, et al.: 2001 Dendritic cells derived from peripheral monocytes express endothelial markers and in the presence of angiogenic growth factors differentiate into endothelial-like cells. Eur J Cell Biol 80:99–110.

Flanc C: 1968. An experimental study of the recanalization of arterial and venous thrombi. Br J Surg 55:519–524.

Harraz M, Jiao C, Hanlon HD, et al.: 2001. $CD34^-$ blood-derived human endothelial cell progenitors. Stem Cells 19:304–312.

Haudenschild CC: 1998. Angiogenesis in a coronary thrombus partially restores the circulation. 30th Lofland Conference on Arterial Wall Metabolism, Rockville, MD, May 30 (Poster).

Henke PK, Wakefield TW, Kadell AM, et al.: 2001. Interleukin-8 administration enhances venous thrombosis resolution in a rat model. J Surg Res 99:84–91.

Hoppe B, Lu JT, Thistlewaite P, et al.: 2002. Beyond peripheral arteries in Buerger's disease: angiographic considerations in thromboangiitis obliterans. Catheter Cardiovasc Interv 57:363–366.

Humphries J, McGuinness CL, Smith A, et al.: 1999. Monocyte chemotactic protein-1 (MCP-1) accelerates the organization and resolution of venous thrombi. J Vasc Surg 30:894–899.

Iba O, Matsubara H, Nozawa Y, et al.: 2002. Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs. Circulation 106:2019–2025.

Kawamoto A, Tkebuchava T, Yamaguchi J, et al.: 2003. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 107: 461–468.

Kolattukudy PE, Quach T, Bergese S, et al.: 1998. Myocarditis induced by targeted expression of the MCP-1 gene in murine cardiac muscle. Am J Pathol 152:101–111.

Leu HJ, Feigl W, Susani M: 1987. Angiogenesis from mononuclear cells in thrombi. Virchows Arch A Pathol Anat Histopathol 411:5–14.

Leu HJ, Feigl W, Susani M, et al.: 1988. Differentiation of mononuclear blood cells into macrophages, fibroblasts and endothelial cells in thrombus organization. Exp Cell Biol 56:201–210.

Mackenzie JR, Hackett M, Topuzlu C, Tibbs DJ: 1968. Origin of arterial prosthesis lining from circulating blood cells. Arch Surg 97:879–885.

Madlener M, Parks WC, Werner S: 1998. Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. Exp Cell Res 242:201–210.

Majno G, Joris I: 1996. Cells, Tissues, and Disease. Principles of General Pathology. Cambridge, Blackwell Science.

McGuinness CL, Humphries J, Waltham M, et al.: 2001. Recruitment of labelled monocytes by experimental venous thrombi. Thromb Haemost 85:1018–1024.

Miyazawa K, Urist MR: 1997. Treatment with recombinant human macrophage colony-stimulating factor resorbs blood clot and restores osteoclastogenesis in heterotopic bone induced by partially purified native bone morphogenetic protein in osteopetrotic (op/op) mice. J Orthop Res 15:456–462.

Moldovan NI: 2002. Role of monocytes and macrophages in adult angiogenesis: a light at the tunnel's end. J Hematother Stem Cell Res 11:179–194.

Moldovan, NI: 2003. Current priorities in the research of circulating pre-endothelial cells. *In* Moldovan NI, ed. Novel Angiogenic Mechanisms: Role of Circulating Progenitor Endothelial Cells. New York, Kluwer/Plenum Academic Publishers, pp 1–8.

Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, et al.: 2000. Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. Circ Res 87:378–384.

Murayama T, Asahara T: 2002. Bone marrow-derived endothelial progenitor cells for vascular regeneration. Curr Opin Mol Ther 4:395–402.

Nakul-Aquaronne D, Bayle J, Frelin C: 2003. Coexpression of endothelial markers and CD14 by cytokine mobilized $CD34^+$ cells under angiogenic stimulation. Cardiovasc Res 57:816–823.

Rehman J, Li J, Orschell CM, March KL: 2003. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 107:1164–1169.

Religa P, Bojakowski K, Maksymowicz M, et al.: 2002. Smooth-muscle progenitor cells of bone marrow origin contribute to the development of neointimal thickenings in rat aortic allografts and injured rat carotid arteries. Transplantation 74:1310–1315.

Schmeisser A, Garlichs CD, Zhang H, et al.: 2001. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in matrigel under angiogenic conditions. Cardiovasc Res 49:671–680.

Sevitt S: 1973. The mechanisms of canalization in deep vein thrombosis. J Pathol 110:153–165.

Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM: 2002. Smooth muscle progenitor cells in human blood. Circulation 106:1199–1204.

Singh I, Burnand KG, Collins M, et al.: 2003. Failure of thrombus to resolve in urokinase-type plasminogen activator gene-knockout mice: rescue by normal bone marrow-derived cells. Circulation 107: 869–875.

Strauer BE, Brehm M, Zeus T, et al.: 2002. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 106:1913–1918.

Stump MM, Jordan JL, DeBakey ME, Halpert B: 1963. Endothelium grown from circulating blood on isolated intravascular Dacron hub. Am J Pathol 43:361–367.

Takakura N, Watanabe T, Suenobu S, et al.: 2000. A role for hematopoietic stem cells in promoting angiogenesis. Cell 102:199–209.

Terashima M, Awano K, Honda Y, et al.: 2002. Images in cardiovascular medicine. "Arteries within the artery" after Kawasaki disease: a lotus root appearance by intravascular ultrasound. Circulation 106: 887.

Tepper O, Murayama T, Kearney M, et al.: 2002. Therapeutic neovascularization as a novel approach to thrombus recanalization and resolution. Circulation Abstract book (Supplement No. 19): SII–65.

Wakefield TW, Linn MJ, Henke PK, et al.: 1999. Neovascularization during venous thrombosis organization: a preliminary study. J Vasc Surg 30:885–892.

Waltham M, Burnand KG, Collins M, et al.: 2003. Vascular endothelial growth factor enhances venous thrombus recanalisation and organization. Thromb Haemost 89: 169–176.

Zhao Y, Glesne D, Huberman E: 2003. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc Natl Acad Sci USA 100:2426–2431.

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CARDIOVASCULAR RADIATION MEDICINE

Transplantation of endothelial progenitor cells for therapeutic neovascularization

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Abstract Endothelial progenitor cells (EPCs), which were first identified in adult peripheral blood mononuclear cells (MNCs), play an important role in postnatal neovascularization. Tissue ischemia augments mobilization of EPCs from bone marrow into the circulation and enhances incorporation of EPCs at sites of neovascularization. Two methods to obtain EPCs from bone marrow, peripheral blood or cord blood MNCs have been evaluated for therapeutic neovascularization: (1) fresh isolation using anti-CD34, anti-KDR or anti-AC133 antibody, and (2) ex vivo expansion of total MNCs. In an immunodeficient mouse model of hindlimb ischemia, systemic transplantation of human ex vivo expanded EPCs improves limb survival through the enhancement of blood flow in the ischemic tissue. A similar strategy also leads to histological and functional preservation of ischemic myocardium of nude rats. Recently, a preclinical study of catheter-based, intramyocardial transplantation of autologous EPCs in a swine model of chronic myocardial ischemia demonstrated the therapeutic potential of cell-based therapy, with attenuation of myocardial ischemia and improvement in left ventricular function. These favorable outcomes strongly suggest a therapeutic impact of EPC transplantation in clinical settings. Further basic research, with improved understanding of the mechanisms governing homing and incorporation of EPCs, will be still necessary to optimize the methodology of the cell therapy.

Keywords: Coronary artery disease; Endothelial progenitor cell; Ischemia; Therapeutic neovascularization; Transplantation; Peripheral artery disease

1. Therapeutic neovascularization by angiogenesis and vasculogenesis mechanism

It has been well documented that the development of a collateral circulation can attenuate tissue ischemia in peripheral and coronary artery diseases. The classical mechanism of postnatal neovascularization had been considered to be limited to angiogenesis, i.e., proliferation and migration of preexisting mature endothelial cells (ECs). Therefore, approaches by which one may augment collateral circulation in ischemic diseases in preclinical and clinical studies have been referred to as "therapeutic angiogenesis" [1–4].

Vasculogenesis, which indicates incorporation of circulating endothelial progenitor cells (EPCs) into foci of neovascularization and their proliferation, migration and differentiation into mature ECs, was believed to occur only during embryonic neovascularization, until *postnatal* vasculogenesis was first reported in 1997 [5]. EPCs are mobilized from the bone marrow into the circulation in response to tissue ischemia [6]. In addition to the mobilization of intrinsic EPCs in ischemic diseases, we have also reported that intravenous transplantation of exogenous EPCs, isolated as CD34+ cells from adult peripheral blood, incorporate into foci of neovascularization in hindlimb ischemia [5]. These findings encouraged exploration of the therapeutic use of EPCs to augment collateral formation in ischemic diseases. On the other hand, administration of vascular endothelial growth factor (VEGF) was shown to increase the number of differentiated EPCs in vitro and to augment their incorporation into foci of neovascularization in vivo [7]. Recently, a pilot clinical study provided evidence of a beneficial effect of VEGF gene transfer in human patients with

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severe peripheral vascular disease. Of note, the number of circulating EPCs increased following VEGF gene transfer in these patients [8]. These findings suggest that *vasculogenesis* may be a key component in what was previously termed therapeutic *angiogenesis*. Thus, discerning the role of vasculogenesis in the pathophysiology and therapeutic neovascularization of ischemic diseases is now recognized as essential.

2. Isolation of EPCs for therapeutic neovascularization

Recently, EPCs were successfully isolated from the mononuclear cell (MNC) fraction of peripheral blood, bone marrow and cord blood using KDR, CD34 or AC133 antigens, all of which are shared by hematopoietic stem cells (HSCs). Cells selected by these methods differentiate into endothelial lineage cells in vitro and incorporate into sites of neovascularization in vivo. Another method to obtain EPCs is ex vivo expansion. Culture of MNCs in EC basal medium allows the cells to proliferate and differentiate into endothelial lineage cells [9]. EPCs obtained by these methods have been utilized for experimental therapeutic neovascularization.

3. Intravenous transplantation of ex vivo expanded EPCs in hindlimb ischemia

Therapeutic application of EPCs was first attempted in murine model of hindlimb ischemia [9]. One day following operative excision of one femoral artery, athymic nude mice received an intracardiac injection of culture-expanded human EPCs (hEPCs). Two control groups were identically injected with either human microvascular ECs (HMVECs) or media from the culture plates employed for hEPC ex vivo expansion. Serial examination of hindlimb perfusion by laser Doppler perfusion imaging disclosed that the ratio of ischemic/normal blood flow at Day 28 in hEPC-transplanted mice had significantly improved than in mice receiving HMVECs and culture media, respectively. Histological evaluation of skeletal muscle sections retrieved from the ischemic hindlimbs showed that capillary density was markedly increased in hEPC-transplanted mice. Among mice in which induction of hindlimb ischemia was followed by administration of HMVECs, limb salvage was limited to 8% of animals. Likewise, a limb preservation was observed in only 7% of mice treated with culture media. In contrast, hEPC transplantation was associated with successful limb salvage in 59% of animals.

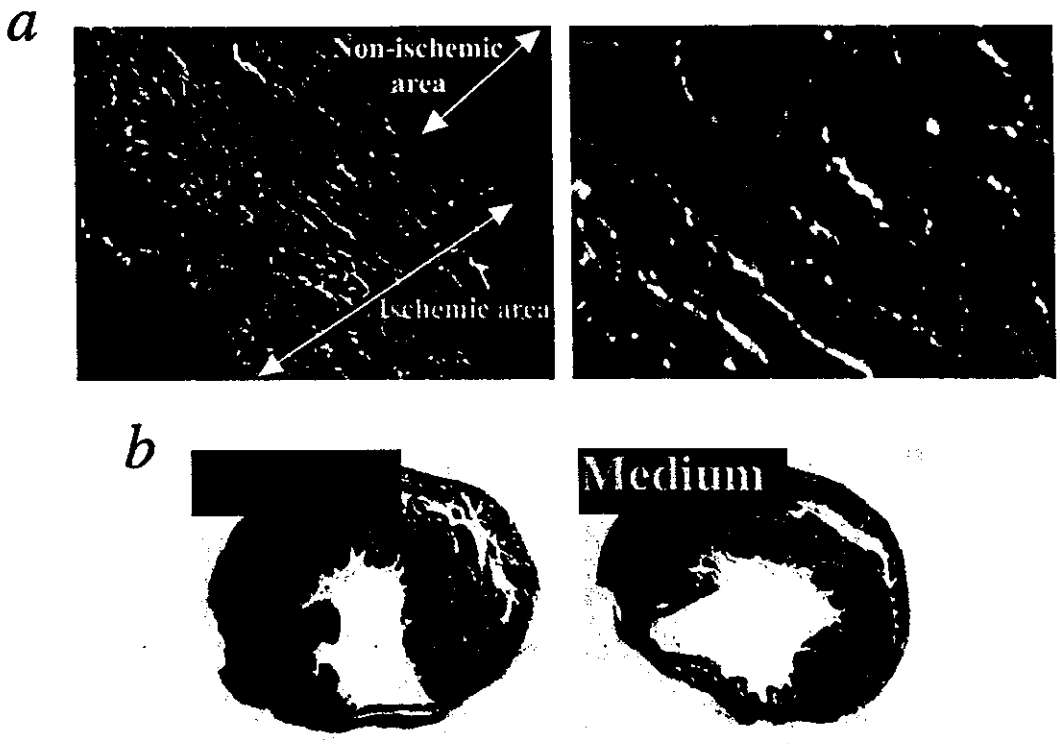

Fig. 1. (a) Representative findings of fluorescent microscopy in the heart sections of nude rats with myocardial ischemia. DU-labeled human cultured EPCs (red), which were intravenously transplanted 3 h after ischemia, specifically homed to site of neovascularization and participated new blood vessel formation with rat intrinsic ECs, which were positive for BS-1 lectin (green). (b) Representative findings of elastic tissue trichrome staining of the heart samples obtained 4 weeks after ischemia. Fibrosis area was significantly smaller in the EPC-treated rats than in control.

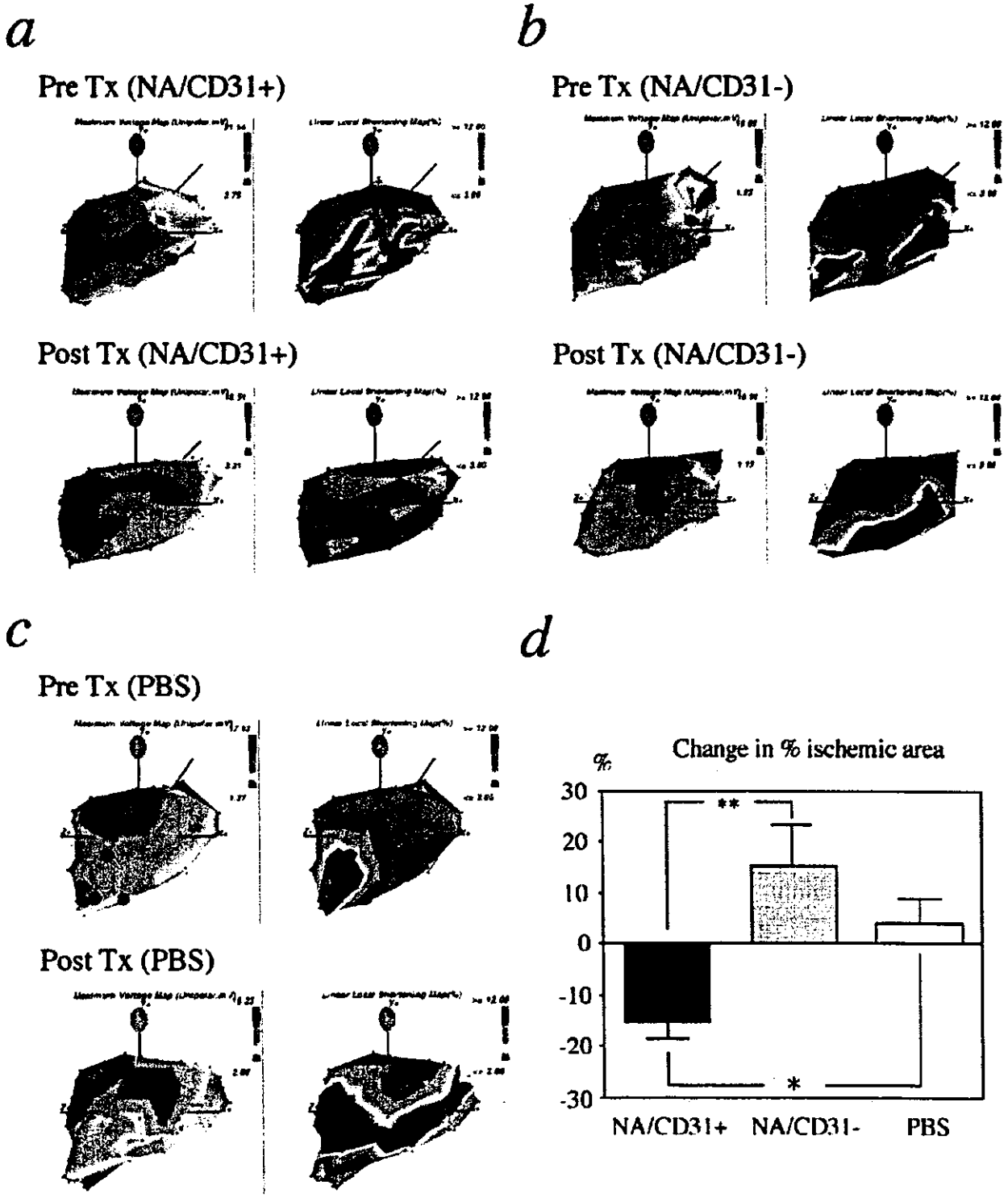

Fig. 2. (a–c) Representative findings of NOGA electromechanical mapping pre (top) and 4 weeks post (bottom) NA/CD31+ MNCs transplantation (a), NA/CD31− MNCs transplantation (b) and PBS injection (c). (d) Change in percent ischemic area during 4 weeks after the treatment. NA/CD31+, swine receiving NA/CD31+ MNCs; NA/CD31−, swine receiving NA/CD31− MNCs. * $P<.05$; ** $P<.01$.

4. Intravenous transplantation of ex vivo expanded EPCs in myocardial ischemia

We have demonstrated similar outcomes in rats with myocardial ischemia [10]. Myocardial ischemia was induced by ligation of the left anterior descending coronary artery in male athymic nude rats. Intravenous transplantation of DiI-labeled hEPCs demonstrated that transplanted EPCs accumulated specifically in the ischemic zone and incorporated into foci of myocardial neovascularization. Ex vivo expanded hEPCs transplanted into rat ischemic myocardium have a favorable impact on preservation of LV function and inhibition of LV remodeling following myocardial ischemia (Fig. 1).

5. Preclinical studies of EPC transplantation for therapeutic neovascularization of myocardial ischemia

Chronic myocardial ischemia was induced by ameroid constrictor placement around the left circumflex artery of male Yorkshire swine. Four weeks after constrictor placement, the EPC-enriched fraction (nonadhesive CD31+ (NA/CD31+) MNCs) was freshly isolated from peripheral blood MNCs of ischemic swine (antiswine CD34 antibody is not available). CD31− MNCs were treated similarly. On the same day as the EPC isolation, NOGA nonfluoroscopic LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia. Immediately after the ischemic territory was identified by NOGA mapping, 10^7 NA/CD31+ MNCs, 10^7 NA/CD31− MNCs or PBS without cells were injected into five sites within the ischemic myocardium using the NOGA injection catheter.

A decrease in the size of the ischemic area by NOGA mapping was observed only after NA/CD31+ injection. Similarly, the change in % ischemic area after transplantation was significantly improved only in the CD31+ group (Fig. 2). The mean value of the Rentrop score of collateral development to the LCX territory was significantly improved only after NA/CD31+ transplantation, and not after NA/CD31− or PBS injection. Capillary density 4 weeks after treatment was significantly greater in the NA/CD31+ group than in the NA/CD31− and PBS groups. LV ejection fraction measured by echocardiography significantly improved only after NA/CD31+ transplantation, not after NA/CD31− or PBS injection [11].

The porcine model of chronic myocardial ischemia was chosen for the above preclinical studies to evaluate the strategy of local delivery using the NOGA injection catheter. Although CD34+ MNCs could be used in future clinical situations, antiswine CD34 antibody is not available. To complement these studies and to verify that human CD34 selected cells could also yield similar clinical benefit, we transplanted freshly isolated human CD34+ cells incorporated into foci of myocardial neovascularization, differentiated into mature ECs, enhanced vascularity in the ischemic myocardium, preserved LV systolic function and inhibited LV fibrosis [11].

Taken together with the favorable outcomes in the swine study, percutaneous delivery of autologous, freshly isolated CD34+ MNCs targeted to sites of ischemia may represent a practical strategy for revascularization in patients with chronic myocardial ischemia.

6. Conclusion

The discovery of EPCs in adults, increasing knowledge of the pathophysiological role of EPCs in ischemic tissue, and favorable outcomes of EPC transplantation in experimental peripheral and coronary artery diseases suggest a promising future for clinical applications of this cell-based regeneration strategy. However, there are still some issues to be addressed:

1. The optimal dose of EPCs to be transplanted has not been clarified. Dose–response studies should yield important data to guide initial clinical applications.
2. While safety issues in both acute and chronic phases following EPC transplantation have not been fully explored, the established track record for autologous cell transplantation offers a basis upon which to initiate clinical studies in ischemic diseases.
3. Some of the risk factors of ischemic diseases, such as diabetes and hypercholesterolemia, were reported to impair EC function. In the case of autologous EPC transplantation, the therapeutic potential of EPCs derived from the patients with these and other risk factors remains to be determined.
4. The most effective methods of EPC isolation, expansion and administration need to be established for optimal cell transplantation.

Considering these issues, clinical trials of EPC transplantation should be performed based on carefully examined preclinical research. Needless to say, discreet observation of adverse events following the cell transplantation is essential.

References

[1] Isner JM. Myocardial gene therapy. Nature 2002;415:234–9.
[2] Losordo DW, Vale PR, Hendel RC, Milliken CE, Fortuin FD, Cummings N, Schatz RA, Asahara T, Isner JM, Kuntz RE. Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia. Circulation 2002;105:2012–18.
[3] Morishita R, Nakamura S, Hayashi S, Taniyama Y, Moriguchi A, Nagano T, Taiji M, Noguchi H, Takeshita S, Matsumoto K, Nakamura T, Higaki J, Ogihara T. Therapeutic angiogenesis induced

by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 1999; 33:1379–84.

[4] Yanagisawa-Miwa A, Uchida Y, Nakamura F, Tomaru T, Kido H, Kamijo T, Sugimoto T, Kaji K, Utsuyama M, Kurashima C. Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. Science 1992;257:1401–3.

[5] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275: 964–7.

[6] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.

[7] Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18:3964–72.

[8] Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000;86: 1198–202.

[9] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97:3422–7.

[10] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634–7.

[11] Kawamoto A, Tkebuchava T, Yamaguchi JI, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107:461–8.

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Stem Cell Review Series

Endothelial progenitor cells: past, state of the art, and future

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Abstract

Recent evidences suggest that endothelial progenitor cells (EPCs) derived from bone marrow (BM) contribute to *de novo* vessel formation in adults occurring as physiological and pathological responses. Emerging preclinical trials have shown that EPCs home to sites of neovascularization after ischemic events in limb and myocardium. On the basis of these aspects, EPCs are expected to develop as a key strategy of therapeutic applications for the ischemic organs. Such clinical requirements of EPCs will tentatively accelerate the translational research aiming at the devices to acquire the optimized quality and quantity of EPCs. In this review, we attempt to discuss about biological features of EPCs and speculate on the clinical potential of EPCs for therapeutic neovascularization.

Keywords: endothelial progenitor cell (EPC) • vasculogenesis • angiogenesis • therapeutic neovascularization • cardiovascular disease • cell therapy

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