Acknowledgments

This research is supported in part by National Institutes of Health Grants HL57516, HL60911, and HL53354; a Grant-in-Aid from the American Heart Association (to Takayuki Asahara); and a Grant-in-Aid from the Uehara Memorial Foundation (to Jun-ichi Yamaguchi).

This article is dedicated to Dr Jeffrey M. Isner, who died on October 31, 2001. We gratefully acknowledge him for his inspirational leadership, friendship, and encouraging support.

References

1. Nagasawa T, Kikutani H, Kishimoto T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc Natl Acad Sci U S A.* 1994;91:2305–2309.
2. Bleul CC, Farzan M, Choe H, et al. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature.* 1996;382:829–833.
3. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature.* 1996;382:635–638.
4. Tachibana K, Hirota S, Iizasa H, et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature.* 1998; 393:591–594.
5. Zou YR, Kottmann AH, Kuroda M, et al. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature.* 1998;393:595–599.
6. Mohle R, Bautz F, Rafii S, et al. The chemokine receptor CXCR-4 is expressed on CD34$^+$ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. *Blood.* 1998;91:4523–4530.
7. Lataillade JJ, Clay D, Dupuy C, et al. Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. *Blood.* 2000;95:756–768.
8. Hattori K, Heissig B, Tashiro K, et al. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. *Blood.* 2001;97:3354–3360.
9. Salcedo R, Wasserman K, Young HA, et al. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1α. *Am J Pathol.* 1999;154:1125–1135.
10. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964–967.
11. Shi Q, Rafii S, Wu MH, et al. Evidence for circulating bone marrow-derived endothelial cells. *Blood.* 1998;92:362–367.
12. Orlic D, Kajstura J, Chimenti S, et al. Bone marrow cells regenerate infarcted myocardium. *Nature.* 2001;410:701–705.
13. 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.
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. 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.
16. Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A.* 2000;97:3422–3427.
17. 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.
18. McDonald DM, Munn L, Jain RK. Vasculogenic mimicry: how convincing, how novel, and how significant? *Am J Pathol.* 2000;156: 383–388.
19. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, et al. Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. *Circ Res.* 2000;87:378–384.
20. 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.

PHYSIOLOGY 20: 36–42, 2005; 10.1152/physiol.00033.2004

Endothelial Progenitor Cells for Vasculogenesis

Satoshi Murasawa[1] and Takayuki Asahara[1,2]

[1]Department of Regenerative Medicine and Research, Kobe Institute of Biomedical Research and Innovation/RIKEN Center for Developmental Biology, Kobe; and [2]Department of Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Japan
asa777@aol.com
murasawa@ibri-kobe.org

Postnatal vasculogenesis is considered to be involved in neovascularization of adult tissues, because bone marrow-derived endothelial progenitor cells (EPCs) were isolated from circulating mononuclear cells in peripheral blood and were shown to incorporate into sites of physiological and pathological neovascularization and to differentiate into mature endothelial cells. EPCs might have an attractive potential therapeutic application for cardiovascular ischemic diseases as a novel cell-based strategy mainly via a vasculogenesis mechanism.

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago (14). Their work documented the extent to which tumor development was dependent on neovascularization and suggested that this relationship might involve angiogenic growth factors that were specific for neoplasms. Subsequent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. This novel strategy for the treatment of vascular insufficiency was termed "therapeutic angiogenesis" (43).

More recent data suggest that the basis for native as well as therapeutic neovascularization is not restricted to angiogenesis but includes postnatal vasculogenesis as well. Our laboratory (2–4, 20, 22, 42) and others (8, 15–17, 30, 32, 39) have established that bone marrow (BM)-derived endothelial progenitor cells (EPCs) are present in the systemic circulation, are augmented in response to certain cytokines and/or tissue ischemia, and home to as well as incorporate into sites of neovascularization (FIGURE 1). Because of these features, EPCs have been investigated as therapeutic agents in studies of "supply-side" angiogenesis under pathological as well as physiological conditions. This review focuses on EPC isolation from adult peripheral blood, EPC kinetics in vivo, and the therapeutic potential of EPCs for ischemic diseases.

Isolation of EPCs

Available evidence suggests that hematopoietic stem cells (HSCs) and EPCs (33, 36) are derived from a common precursor (hemangioblast) (12, 18, 46). Growth and fusion of multiple blood islands in the yolk sac of the embryo ultimately give rise to the yolk sac capillary network (35); after the onset of blood circulation, this network differentiates into an arteriovenous vascular system (36). The integral relationship between the elements that circulate in the vascular system—the blood cells—and the cells that are principally responsible for the vessels themselves—endothelial cells (ECs)—is implied by the composition of the embryonic blood islands. The cells destined to generate hematopoietic cells are situated in the center of the blood island and are termed HSCs. EPCs, or angioblasts, are located at the periphery of the blood islands. In addition to this spatial association, HSCs and EPCs share certain antigenic determinants, including Flk-1, Tie-2, c-Kit, Sca-1, CD133, and CD34. These progenitor cells have consequently been considered to derive from a common precursor, putatively termed a hemangioblast (12, 18, 46).

The identification of putative HSCs in peripheral blood and BM and the demonstration of sustained hematopoietic reconstitution with these HSC transplants have constituted inferential evidence for HSCs in adult tissues (5, 24, 38, 41). Recently, the related descendents—EPCs—have been isolated along with HSCs in hematopoietic organs. Flk-1 and CD34, shared by embryonic EPCs and HSCs, were used to detect putative EPCs from the mononuclear cell fraction of peripheral blood (3). In vitro, these cells differentiated into endothelial lineage cells, and in animal models of ischemia, heterologous, homologous, and autologous EPCs were shown to incorporate into sites of active neovascularization. This finding was followed by diverse identifications of EPCs by several groups (15, 16, 27, 34, 39) using equivalent or different methodologies. It should be noted that no specific surface markers have been found between HSCs and EPCs in the immature stage. Although no specific markers are yet available, endothelial-specific surface markers, such as vascular endothelial cadherin, Tie-2, and Flk-1, disappear in HSCs; on the

1548-9213/05 8.00

other hand, those markers remain in EPCs following the differentiation step. It should be possible to divide EPCs and HSCs in the downstream by using these markers. The evidence that EPCs are descendents from HSCs is still unclear. Regarding a hierarchy of EPCs, EPCs could be descendents from HSCs or could be transdifferentiated from HSCs. Further precise investigation would be necessary to confirm the hierarchy of EPCs.

Therapeutic Mobilization of EPCs

Having demonstrated the potential for endogenous mobilization of BM-derived EPCs, we considered that iatrogenic expansion and mobilization of this putative EC precursor population might represent an effective means to augment the resident population of ECs that is competent to respond to administered angiogenic cytokines. Such a program might thereby address the issue of endothelial dysfunction or depletion that may compromise strategies of therapeutic neovascularization in older, diabetic, and/or hypercholesterolemic animals and patients. We should take into account that the plasma level of growth factors (e.g., VEGF) is attenuated in older patients with atherosclerosis and that the frequency of EPC mobilization is reduced, suggesting that frequency of EPC mobilization from BM depends on aging or diseases. Our preliminary data suggested that cornea neovascularization in nude mice was impaired by EPC transplants derived from older patients compared with those from healthy young volunteers. Tepper et al. (44) have shown the impairment of EPC incorporation into vascular structures in type 2 diabetic patients. Granulocyte macrophage colony stimulating factor, which stimulates hematopoietic progenitor cells and myeloid lineage cells as well as nonhematopoietic cells, including BM stromal cells and ECs, has been shown to exert a potent stimulatory effect on EPC kinetics (42) (FIGURE 1). Such cytokine-induced EPC mobilization could enhance neovascularization of severely ischemic tissues as well as de novo corneal vascularization (42). The mechanisms whereby these EPCs are mobilized to the peripheral circulation occur in the early stage of definition. Among all growth factors, VEGF is the most critical factor for vasculogenesis and angiogenesis (6, 11, 37). Recent data indicate that VEGF is an important factor for EPC kinetics

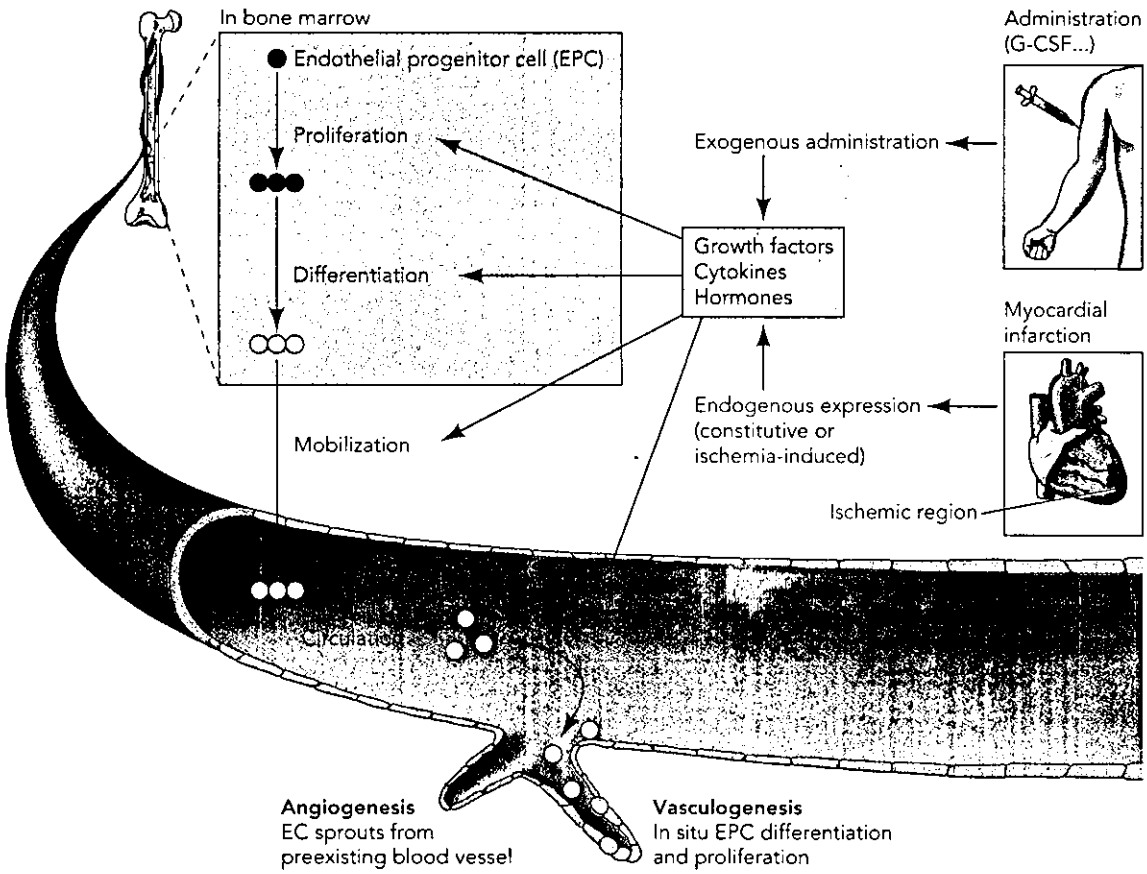


FIGURE 1. **Kinetics of endothelial progenitor cells for neovascularization**
Endothelial progenitor cells (EPCs) circulate in adult human peripheral blood and are mobilized from bone marrow by cytokines, growth factors, and ischemic conditions. Vascular injury is repaired by both angiogenesis and vasculogenesis mechanisms. Circulating EPCs contribute to repair of injured blood vessels mainly via a vasculogenesis mechanism.

too. Our studies performed first in mice (4) and subsequently in patients undergoing VEGF gene transfer for critical limb ischemia (20) and myocardial ischemia (22) established that a previously unappreciated mechanism by which VEGF contributes to neovascularization is via mobilization of BM-derived EPCs. A similar mobilization of EPCs has been observed in response to other hematopoietic stimulators, such as granulocyte colony stimulating factor (15) and stromal-derived factor-1 (34). In a pathological situation (e.g., ischemia, wound), the plasma level of cytokines and growth factors should be systemically augmented depending on the ischemic size or severity of the wound. The evidence that EPCs are mobilized from BM to peripheral blood confirms the elevation of the plasma level of cytokines or growth factors. Shintani et al. (40) have reported that plasma VEGF levels positively correlated with the number of CD34-positive cells derived from circulating mononuclear cells.

This therapeutic strategy of EPC mobilization has recently been implemented not only by using natural hematopoietic or angiogenic stimulants but also by using antihypercholesterolemia drugs. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibit the activity of HMG-CoA reductase, which catalyzes the synthesis of mevalonate, a rate-limiting step in cholesterol biosynthesis. The statins rapidly activate Akt signaling in ECs, and this stimulates EC bioactivity in vitro and enhances angiogenesis in vivo (26). Recently, we and Dimmeler et al. demonstrated a novel function for HMG-CoA reductase inhibitors that contributes to postnatal neovascularization by augmented mobilization of BM-derived EPCs through stimulation of the Akt signaling pathway (9, 29, 45). Because of their well-established pharmacological safety and their effectiveness against hypercholesterolemia, one of the risk factors for atherogenesis, the statins might thus become a potent medication against atherosclerotic vascular diseases both for patients with normal and with high cholesterolemia.

Therapeutic Vasculogenesis of EPC Transplantation

Recently the regenerative potential of stem cells has been under intense investigation. In vitro, stem and progenitor cells possess the capability for self-renewal and differentiation into organ-specific cell types. In vivo, transplantation of these cells may reconstitute organ systems, as shown in animal models of diseases (1, 3, 10, 13, 21, 28).

Direct repopulation capability of EPCs was investigated in murine and rodent models of hindlimb and myocardial ischemia, respectively. One day after the operative excision of one femoral artery, athymic nude mice (n = 17), in which angiogenesis is characteristically impaired (7), received an intracardiac injection of 5×10^5 culture-expanded human EPCs (hEPCs). Two control groups were similarly injected with either human microvascular ECs (HMVECs) (n = 12) or medium from the culture plates employed for hEPC ex vivo expansion (n = 14). Time-course studies demonstrated that peak hEPC incorporation into sites of neovascularization was achieved within 3–7 days after administration of hEPCs. Histological evaluation of skeletal muscle sections retrieved from the ischemic hindlimbs of mice killed 7, 14, and 28 days later showed that capillary density, an index of neovascularization, was markedly increased in hEPC-transplanted mice.

Enhanced neovascularization in mice transplanted with hEPCs led to important biological consequences. Among mice in which induction of hindlimb ischemia was followed by administration of HMVECs, limb salvage was limited to 1 (8.3%) of 12 animals, whereas the remainder developed extensive forefoot necrosis (n = 5, 41.7%), leading in 6 (50%) to spontaneous amputation. Likewise, a preserved limb was observed in only 1 (7.1%) of 14 mice treated with culture medium, whereas foot necrosis and/or autoamputation developed in 7 (50%) and 6 (42.9%) mice, respectively.

In contrast, hEPC transplantation was associated with successful limb salvage in 10 (58.8%) of 17 animals. Foot necrosis was limited to five (29.4%) mice, and only two (11.8%) experienced spontaneous limb amputation. The difference in outcomes between the hEPC-treated mice and both control groups was statistically significant (for hEPC vs. HMVEC, P = 0.006; for hEPC vs. control medium, P = 0.003). The outcomes in mice receiving HMVECs vs. culture medium were similar (P = 0.9).

Similar outcomes have now been demonstrated in rats with myocardial ischemia (23). In this case, peripheral blood mononuclear cells obtained from healthy human adults were cultured in EPC medium and were harvested 7 days later. Myocardial ischemia was induced by ligation of the left anterior descending coronary artery in male Hsd:RH-rnu (athymic nude) rats. In two rats, 10^6 EPCs labeled with DiI were injected intravenously 3 h after induction of myocardial ischemia. Seven days later, fluorescence-conjugated BS-1 lectin, a murine-specific EC marker, was administered intravenously and the rats were immediately killed. Fluorescence microscopy revealed that transplanted EPCs accumulated in the ischemic area and incorporated into foci of myocardial neovascularization.

To determine the impact on left ventricular (LV) function, five rats (EPC group) were injected intra-

venously with 10^6 EPCs 3 h after induction of ischemia. Five other rats (control group) received culture medium. Echocardiography, performed just before and 28 days after induction of ischemia, disclosed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater in the EPC vs. the control group by day 28 (diastole = 0.87 ± 0.03 vs. 0.93 ± 0.01 cm, $P < 0.05$; systole = 0.68 ± 0.03 vs. 0.79 ± 0.02 cm, $P < 0.01$; fractional shortening = 21.3 ± 0.6 vs. 15.3 ± 2.2%, $P < 0.001$). Regional wall motion was better preserved in EPC vs. control group (absolute value 25.3 ± 0.8 vs. 30.6 ± 1.0, $P < 0.01$). Following death on day 28, necropsy examination disclosed that capillary density was significantly greater in the EPC group than in controls (290.1 ± 21.5 vs. 191.1 ± 17.8/mm^2, $P < 0.001$). Moreover, the extent of LV scarring was significantly lower in rats receiving EPCs than in controls (8.9 ± 0.9 vs. 17.8 ± 1.4% of LV, $P < 0.01$). Immunohistochemistry revealed capillaries that were positive for human CD31 and UEA-1 lectin. Thus ex vivo-expanded EPCs administered intravenously to rats with myocardial ischemia incorporate into foci of myocardial neovascularization and have a favorable impact on the preservation of LV function.

Recently, Kocher et al. attempted intravenous infusion of freshly isolated (not cultured) human CD34$^+$ mononuclear cells (EPC-enriched fraction) into nude rats with myocardial ischemia (25). This strategy resulted in preservation of LV function associated with inhibition of cardiomyocyte apoptosis. These experimental findings using immunodeficient animals suggest that both cultured and freshly isolated human EPCs have therapeutic potential in peripheral and coronary artery diseases.

It should be noted that ischemic or wound stimulus causes proliferation, migration, and mobilization of EPCs, and then mobilized EPCs are incorporated into the foci of neovascularization. The mechanism of angiogenesis is thought to be involved at any time, and it is not completely excluded when vasculogenesis is induced. Vasculogenesis is dominant in the case of severe ischemic or wound condition. In the foci of neovascularization, mobilized EPCs derived from a vasculogenesis mechanism act as both the provider for repairing the injured vessels and the producer of cytokines for stimulating other cells.

Gene Therapy using EPCs

Given these findings, together with the limited quantity of EPCs available even under healthy, physiological conditions, one must consider a

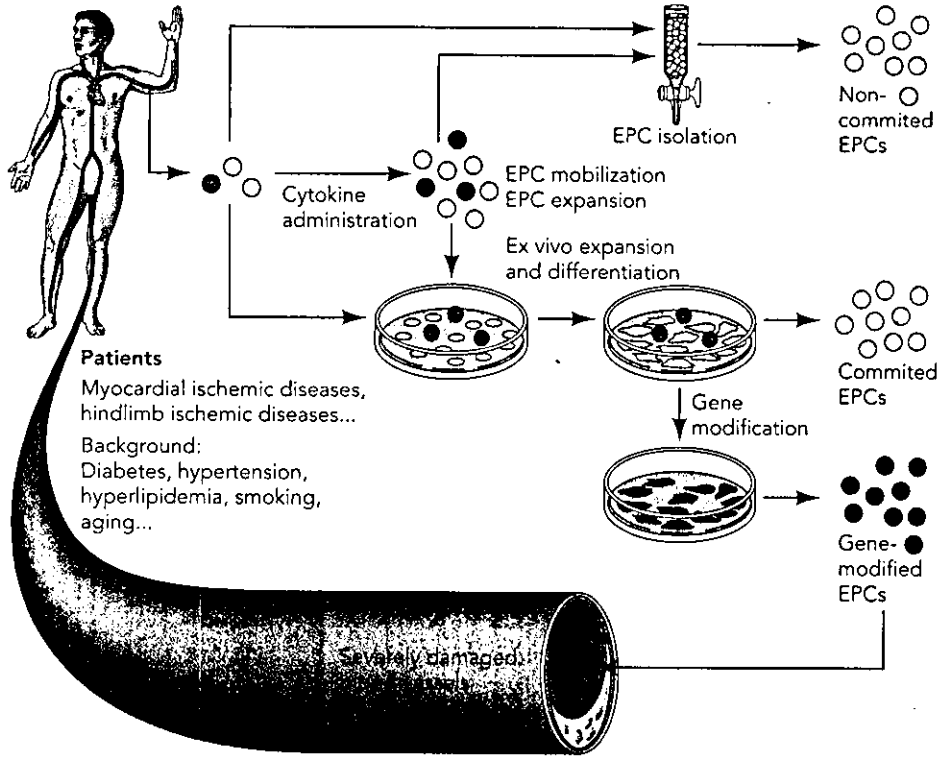


FIGURE 2. **Strategies for EPC transplantation**
Patients with ischemic disorders often experience complications, e.g., hypertension, hyperlipidemia, and/or diabetes in addition to aging. To overcome the cell-functional impairment, it is necessary to increase the cell number or improve the cell quality. Cytokine administration or gene modification of ex vivo-expanded cells might be effective for clinical application.

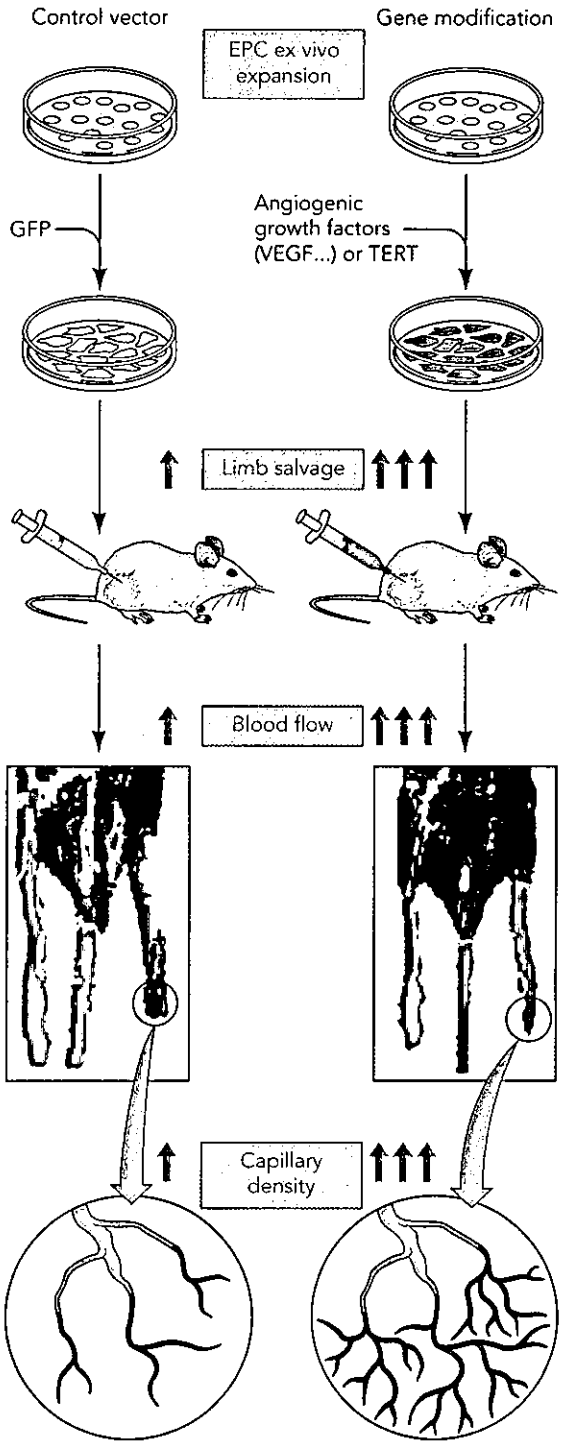


FIGURE 3. **Enhancement of neovascularization by gene-modified EPCs**
Constitutive expression of telomerase reverse transcriptase (TERT) induces delay in senescence and recovers/enhances regenerative properties of EPCs. EPCs were isolated from healthy human volunteers and were cultivated for 7 days. An adenoviral TERT or green fluorescent protein (GFP) construct was introduced into EPCs the next day. Immunodeficient mice with hindlimb ischemia received TERT-EPCs or GFP-EPCs systemically. TERT-EPC transplantation resulted in superior rescue of ischemic legs and improvement of blood flow compared with GFP-EPC transplantation.

strategy that addresses this shortfall and mitigates the possibility of dysfunctional EPCs for therapeutic vasculogenesis in ischemic disorders complicated by aging, diabetes, hypercholesterolemia, and/or hyperhomocysteinemia. Genetic modification of EPCs to overexpress angiogenic growth factors, enhance signaling activity of the angiogenic response, and rejuvenate the bioactivity and/or extend the life span of EPCs constitutes one potential strategy that might address these limitations of EPC transplantation and thereby optimize therapeutic neovascularization (FIGURE 2).

Our recent findings provide the first evidence that exogenously administered, gene-modified EPCs augment naturally impaired neovascularization in an animal model of experimentally induced limb ischemia (19, 31) (FIGURE 3). Most somatic cells of humans and other mammals undergo a finite number of cell divisions, ultimately entering a nondividing state, termed senescence. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence. In contrast to somatic cells, true stem cells and germline cells highly express the catalytic subunit of telomerase (human telomerase reverse transcriptase; hTERT), thus maintaining telomerase activity and full replication of telomeric DNA; these cells (by definition) are thereby able to divide indefinitely. Although they have demonstrated regenerative potentials for vascular development, EPCs are not pluripotent, self-renewing stem cells but rather are lineage-committed progenitors and thus are subject to a Hayflick life span via replicative senescence. Accordingly, we have deduced that constitutive expression of hTERT might induce a delay in senescence and recover/enhance regenerative properties of EPCs. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF or hTERT (Ad/VEGF, Ad/TERT) not only improved neovascularization and blood flow recovery but also had meaningful biological consequences: limb necrosis and autoamputation were reduced compared with controls. The dose of EPCs used in the current in vivo experiments was subtherapeutic, i.e., this dose of EPCs was 30 times less than that required in previous experiments done with nontransduced cells. Thus transplantation of EPCs transduced with Ad/VEGF or Ad/TERT successfully combines VEGF or hTERT gene therapy and stem cell therapy; it constitutes an attractive option to address the limited number of EPCs that can be isolated from peripheral blood before ex vivo expansion and subsequent autologous readministration Although the potential risk is the evidence of malignant transformation or loss of functional and morphogenetic characteristics of the parental cells in the case of hTERT gene modification, no such evidence was observed in the experi-

mental procedure. To minimize these risks, we tested only temporary overexpression of hTERT.

Summary

EPCs were isolated from circulating mononuclear cells and shown to enhance neovascularization. EPC transplantation in ischemic diseases could be a future therapeutic strategy. ■

References

1. Anklesaria P, Kase K, Glowacki J, Holland CA, Sakakeeny MA, Wright JA, FitzGerald TJ, Lee CY, and Greenberger JS. Engraftment of a clonal bone marrow stromal cell line in vivo stimulates hematopoietic recovery from total body irradiation. *Proc Natl Acad Sci USA* 84: 7681–7685, 1987.
2. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearney M, Magner M, and Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 85: 221–228, 1999.
3. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, and Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964-967, 1997.
4. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, and Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 18: 3964–3972, 1999.
5. Brugger W, Heimfeld S, Berenson RJ, Mertelsmann R, and Kanz L. Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. *N Engl J Med* 333: 283–287, 1995.
6. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Kendraprasad H, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, and Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435–439, 1996.
7. Couffinhal T, Silver M, Kearney M, Sullivan A, Witzenbichler B, Magner M, Annex B, Peters K, and Isner JM. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE$^{-/-}$ mice. *Circulation* 99: 3188–3198, 1999.
8. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, and Bowen-Pope DF. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circ Res* 87: 728–730, 2000.
9. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, and Zeiher AM. HMG-CoA-reductase inhibitors (statins) increase endothelial progenitor cells via the P13 kinase/Akt pathway. *J Clin Invest* 108: 391–397, 2001.
10. Evans JT, Kelly PF, O'Neill E, and Garcia JV. Human cord blood CD34+CD38– cell transduction via lentivirus-based gene transfer vectors. *Hum Gene Ther* 10: 1479–1489, 1999.
11. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hilan KJ, and Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380: 439–442, 1996.
12. Flamme I and Risau W. Induction of vasculogenesis and hematopoiesis in vitro. *Development* 116: 435–439, 1992.
13. Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, and Snyder EY. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* 16: 1033–1039, 1998.
14. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285: 1182–1186, 1971.
15. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schunch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, and Fiedler W. In vivo differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 95: 3106–3112, 2000.
16. Gunsilius E, Duba HC, Petzer AL, Kahler CM, Grunewald K, Stockha G, Gabl C, Dirnhofer S, Clausen J, and Gastl G. Evidence from a leukaemia model for maintenance of vascular endothelium by bone-marrow-derived endothelial cells. *Lancet* 355: 1688–1691, 2000.
17. Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, and Rosenberg RD. Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* 125: 1457–1468, 1998.
18. His W. Leoithoblast und angioblast der wirbelthiere. *Abhandl K S Ges Wiss Math Phys* 22: 171–328, 1900.
19. Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, and Asahara T. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation* 105: 732–738, 2002.
20. Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, and Asahara T. Vascular endothelial growth factor 165 gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res* 86: 1198–1202, 2000.
21. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, and Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* 97: 3422–3427, 2000.
22. Kalka C, Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T, and Symes JF. Mobilization of endothelial progenitor cells following gene therapy with VEGF165 in patients with inoperable coronary disease. *Ann Thorac Surg* 70: 829–834, 2000.
23. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, and Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 103: 634–637, 2001.
24. Kessinger A and Armitage JO. The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies. *Blood* 77: 211–213, 1991.
25. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkoff D, Wang J, Homma S, Edwards NM, and Itescu S. Neovascularization of ischemic myocardium by human bone marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7: 430–436, 2001.
26. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, and Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase akt and promotes angiogenesis in normocholesterolemic animals. *Nat Med* 6: 1004–1010, 2000.
27. Lin Y, Weisdorf DJ, Solovey A, and Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 105: 71–77, 2000.
28. Lindvall O, Brundin P, Widner H, Rehncrona S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, and Marsden CD. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247: 574–577, 1990.
29. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, and Asahara T. HMG-CoA reductase inhibitor mobilizes bone-marrow derived endothelial progenitor cells. *J Clin Invest* 108: 399–405, 2001.
30. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, and Kolattukudy PE. Contribution of monocytes/macrophages to compensatory neovascularization. The drilling of metalloelastase-positive tunnels in ischemic myocardium. *Circ Res* 87: 378–384, 2000.
31. Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW, and Asahara T. Constitutive hTERT expression enhances regenerative properties of endothelial progenitor cells. *Circulation* 106: 1133–1139, 2002.
32. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K, and Imaizumi T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest* 105: 1527–1536, 2000.
33. Pardanaud L, Altman C, Kitos P, and Dieterien-Lievre F. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. *Development* 105: 473–485, 1989.

34. Peichev M, Naiyer AJ, Pereira D, Ahu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, and Moore MAS. Expression of VEGFR-2 and AC133 by human CD34+ cells identifies a population of functional endothelial precursors. *Blood* 95: 952–958, 2000.

35. Risau W and Flamme I. Vasculogenesis. *Annu Rev Cell Dev Biol* 11: 73–91, 1995.

36. Risau W, Sariola H, Zerwes HG, Sasse J, Ekblom P, Kemler R, and Doetschman T. Vasculogenesis and angiogenesis in embryonic stem cell-derived embryoid bodies. *Development* 102: 471–478, 1988.

37. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, and Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. *Nature* 376: 62–66, 1995.

38. Sheridan WP, Begley CG, and Juttener C. Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *Lancet* 339: 640–644, 1992.

39. Shi Q, Rafii S, Wu MHD, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, and Hammond WP. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 92: 362–367, 1998.

40. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki S, Shimada T, Oike Y, and Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 103: 2776–2779, 2001.

41. Shpall EJ, Jones RB, and Bearman SI. Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy. *J Clin Oncol* 12: 28–36, 1994.

42. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, and Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 5: 434–438, 1999.

43. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, and Isner JM. Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest* 93: 662–670, 1994.

44. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, and Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 106: 2781–2786, 2002.

45. Vasa M, Fichtischerer S, Adler K, Aicher A, Martin H, Zeiher AM, and Dimmeler S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 103: 2885–2890, 2001.

46. Weiss M and Orkin SH. In vitro differentiation of murine embryonic stem cells: new approaches to old problems. *J Clin Invest* 97: 591–595, 1996.

ANNUAL REPORT

2003年における遺伝子・再生医学研究

村澤 聡・浅原 孝之*
先端医療センター再生医療研究部主任研究員／部長*
東海大学医学部基盤診療学系再生医療科学教授*

はじめに

再生医学とは，細胞移植や，サイトカインや成長因子などの投与により，失われた器官や組織(あるいはその一部)を再生するための，基礎・臨床研究，および臨床応用に関わる学問である。近年，心血管領域での再生医学に関する研究が進んでいるが，これには，血管再生や心筋再生の概念が含まれる。世界に先駆けて，ボストン・タフツ大学のIsner教授らがVEGF(Vascular Endothelial Growth Factor)プラスミドの遺伝子治療を末梢動脈閉塞症患者に試みて血管再生による効果を報告して以来，再生医療と遺伝子治療が密接な関連をもつことが示唆されるようになった。本稿では心血管領域における最近の遺伝子・再生医学研究の動向について紹介をする。

遺伝子治療の動向

1994年にIsner教授らによってVEGF$_{165}$プラスミド遺伝子導入による閉塞性動脈硬化症(ASO)への臨床研究が開始されて以来，細胞増殖因子を用いた血管新生療法の概念が臨床応用に適用されるようになった。1998年にはBaumgartner[1]らが重症ASO患者に対して，またLosordo[2]らが，重症狭心症患者に対してそれぞれ，VEGF$_{165}$プラスミド遺伝子治療を行い，自覚症状の改善とともに，客観的評価による側副血行路の増加，および末梢血流の改善をもたらすことを報告した。

虚血性心疾患に対する遺伝子導入経路はその後，胸部小切開法による心筋内注入から，血管内を経由したカテーテルによる選択的注入へと変わり，より低侵襲の治療が可能になった。その後，Losordo[3]らは，血管内を経由するカテーテルを用いて，VEGF-2プラスミドを注入する第Ⅰ/Ⅱ相試験を開始した。カテーテルを用いた方法による安全性が確認され，第Ⅲ相試験への試みがなされている。一方，開胸によるVEGF-2プラスミドの心筋への直接注入による第Ⅰ相試験が重症狭心症患者に対して行われ，1年のフォローアップの報告がFortuin[4]らによって最近なされたが，この中では，遺伝子治療による臨床所見の改善は認めたものの，客観的評価による血管新生の所見は得られず，プラセボ効果の関与が示唆されている。これらの効果を明らかにするため，現在第Ⅲ相試験が行われているものと思われる。一方，国内ではVEGFと同様に血管内皮増殖作用が報告されているHGF(Hepatocyte Growth Factor)を用いた重症下肢虚血患者に対する臨床応用が大阪大学の森下らによって進められている。

細胞増殖因子を用いた血管新生療法には，VEGFの遺伝子治療のほかに，FGF(Fibroblast Growth factor)蛋白による治療が行われている。1998年のSchumacherら[5]，1999年にLaham[6]ら，また2000年にHendel[7]らがいずれも重症狭心症患者に対して臨床研究を行い，有効性を報告している。しかし一方で，動物実験レベルで確認される血管新生治療効果が，重症冠動脈疾患患者で十分に発揮されないことが，これまでのデータから示唆されるようになった。Ruelらは[8]，局所のNO(nitric oxide)が血管新生に関与するという基礎研究の結果をもとにして，高コレステロール食による内皮障害ミニブタと通常食によるミニブタに対して，それぞれ慢性心筋虚血を作製し，FGF-2蛋白を投与して心筋の血流改善を比較したところ，内皮障害モデルにおいて明らかにFGF-2に対する血管再生の反応が障害されているという結

果が得られた。したがって，臨床のプロトコールにおいて，より有効な方法を工夫することが今後の課題となっている。実際，最近の生活習慣病の増加に伴い血管細胞がすでに障害を受けている場合が多くなってきている。このため従来の治療法が奏効しない症例が増加し，血管新生療法が次世代の治療として注目されているが，同時に問題点も残されている(図1)。

細胞治療の動向

遺伝子治療とともに，再生医学において細胞移植療法が注目を浴びている。1997年にわれわれのグループは，成人の末梢血中に血管内皮前駆細胞(EPC)が存在することを明らかにし[9]，この細胞を用いた細胞移植による，虚血性疾患への応用を実現するために，基礎研究および，小動物，大動物を用いた細胞移植実験を積み重ねてきた。

ヒトの血管内皮前駆細胞は骨髄から末梢循環血中に，さまざまな刺激によって動員されることがわかっている。サイトカインや成長因子などによる刺激のほか，虚血[10]によっても骨髄から末梢血に動員される。具体的にはG-CSFによって末梢血中の血管内皮前駆細胞の数を十分に確保し細胞移植することにより虚血性疾患(ASO，狭心症，心筋梗塞)への臨床効果が期待される。われわれの最近のデータでは，HMG-CoA還元酵素阻害薬(スタチン製剤)が血管内皮前駆細胞を骨髄から末梢血中に動員し分化させることがβ-ガラクトシダーゼ発現骨髄移植マウスにおける角膜血管形成モデルと*in vitro*実験により示され，心筋虚血や脳虚血後の末梢血管再構築に利点があると考えられている。このように*ex vivo*で増殖・分化誘導を受けた血管内皮前駆細胞が治療に有効と考えられている[11][12]。

細胞移植の有効性についてはこれまで動物モデルを用いた実験で明らかになってきた。これまでの研究では，免疫不全マウスの下肢虚血モデルにヒト血管内皮前駆細胞を投与すると，新生血管の増加がもたらされ，虚血筋肉組織内の血流改善の促進が確認された[13]。この治療法は，免疫不全ラットの心筋梗塞モデルにも応用され，組織学的に新生血管の増生・心筋壊死の減少を誘導し，心筋の機能改善にも役割を果たすことが判明した[14]。この方法は，血管内皮前駆細胞の前駆細胞としての増殖能・分化能を利用した強力かつ生理的な再生治療として注目を受けている。血管内皮前駆細胞は臍帯血中にも豊富に存在し，下肢虚血動物モデルへの投与によって血管新生が増強されることが明らかになった[15]。

これらの基礎検討によって，動物レベルでの血管新生療法に血管内皮前駆細胞が有効であることは証明されたが，実際に血管内皮前駆細胞のマーカーの1つであるCD34陽性細胞は通常，末梢血中に1%以下しか存在していない。そこで実際に臨床応用するためには，細胞の数を確保するとともに，細胞の質を高める工夫が必要になってくる。この点を明らかにするため血管内皮前駆細胞の

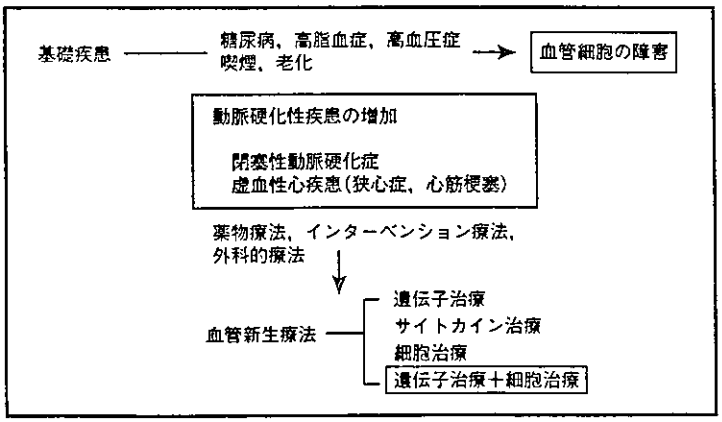


図1
生活習慣病の増加
生活習慣病の増加に伴い，わが国の閉塞性動脈硬化症の患者数は年々増加の傾向にある。重症度の診断に基づいて，薬物療法や，インターベンション療法，および外科的治療が選択されてきたが，重症虚血肢に対しては有効な治療がなく下肢切断を余儀なくされてきた。その後，海外で遺伝子による血管新生療法が臨床応用され，重症虚血肢に対して症状の改善が期待できるようになった。さらに最近では細胞移植による治療が開始されその有効性が確認されている。血管内皮前駆細胞移植や，さらに治療効果を高めるため，遺伝子導入を行った細胞移植の検討が進められており今後臨床応用が期待される。

細胞移植と遺伝子治療を組み合わせた方法の臨床応用への検討が注目を浴びている。われわれが留学していた米国の施設では重症心虚血，下肢動脈硬化症の患者に遺伝子治療による血管新生療法が続けられてきたが，その最大の問題点は，いかに安全に効率よく患部で遺伝子発現できるかにあった。血管内皮前駆細胞による遺伝子治療は，細胞移植単独よりもさらに有効な治療法であることが動物モデルを用いた実験で明らかになってきた。具体的には虚血部位，あるいは障害動脈部位に血管内皮細胞増殖因子や老化の遅延に関連した因子の遺伝子治療を施した血管内皮前駆細胞を投与することが可能と考えられる。これまでの研究で，ヒトの血管内皮前駆細胞VEGF遺伝子を強制発現させ，細胞治療よりはるかに少ない量の細胞を投与したところ，血管新生・血流改善の面で単独細胞治療をしのぐ治療効果が確認されている[16]。

最近われわれは，一般にヒト体細胞の老化に伴いテロメラーゼ活性が低下することに注目し，テロメラーゼを活性化させるテロメラーゼ逆転写酵素(TERT)の遺伝子導入を血管内皮前駆細胞に施すことにより分裂寿命の延長を誘導することを明らかにし報告してきた。すなわちコントロールの細胞群に比べてTERTを導入した細胞群では継代に伴うテロメラーゼ活性低下は抑制されており，実際に老化(senescence)に移行する細胞の割合も減少していた。興味深いことにTERTを導入した細胞群においてのみ内皮への分化を示唆するコロニー形成が認められた。細胞機能の評価ではTERTの導入群で増殖，遊走がともに増強し，アポトーシスは抑制されていた。動物実験において，TERTを強制発現させた血管内皮前駆細胞を下肢虚血動物モデルに投与したところVEGFの場合と同様，単独細胞治療をしのぐ治療効果が確認された。つまり“若返り”させた血管内皮前駆細胞が少量で虚血に対する治療効果を示したと考えられる[17]。

心筋虚血の改善に関しては，血管再生の概念とともに，最近，心筋そのものの再生の概念が生まれてきた。血管内皮前駆細胞や，CD34陽性細胞についても，血管内皮細胞のみならず，心筋細胞に分化することが報告されており[18][19]，われわれのグループでも同様の現象を確認している。ただし，臨床応用に結びつけるための効率のよい心筋再生への誘導条件については今後，さらに検討する必要があると思われる。

最近の細胞治療

細胞移植による血管新生療法は，末梢血に比べ骨髄中には豊富な血管内皮前駆細胞が含まれているため，近年，自己骨髄単核球細胞を用いた血管再生療法が動物モデルで検討され[20][21]有効性が確認された。その後末梢性血管疾患患者に対して臨床応用が開始され，有意な血管新生を認めている[22]。血管内皮前駆細胞によ

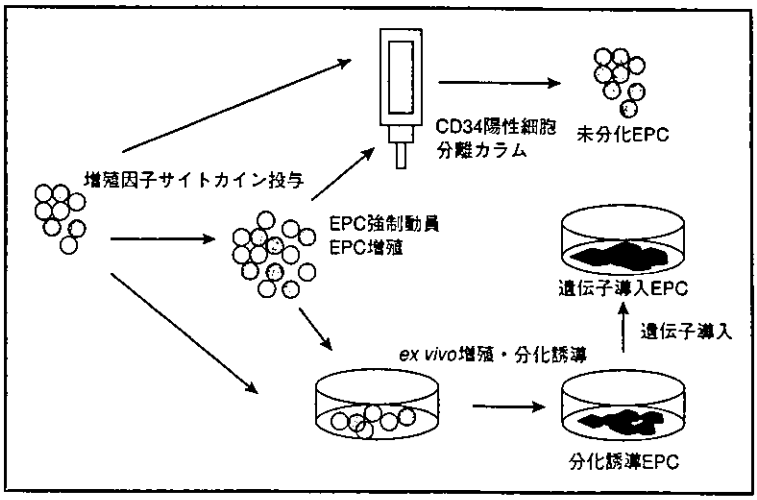


図2
血管内皮前駆細胞(EPC)による細胞移植治療
血管内皮前駆細胞(EPC)による細胞移植治療には大きく分けて以下の3つの戦略がある。①サイトカインにより単核球を骨髄から動員後，CD34陽性細胞分離カラムを用いて純化したものをそのまま用いる方法。②末梢血単核球を体外培養し，増殖させた後用いる方法。③末梢血単核球を体外培養し増殖させた後，血管内皮増殖因子(VEGF)などの遺伝子導入を行い，遺伝子導入されたEPCを用いる方法。これらの方法はすべて，研究レベルでは虚血に対する効果がすでに明らかになっているが，実際に臨床研究として始まっているのは①の方法のみである。その他の方法については，細胞精製や，遺伝子導入などに対して今後さらに慎重な検討が必要である。

る細胞移植治療にはいくつかの戦略があるが(図2)，われわれのグループでは，サイトカインを用いて患者末梢血中の血管内皮前駆細胞を十分量動員し，CD34のmagnetic beadsを用いて，CD34陽性細胞を純化することにより得られた細胞を，末梢性血管疾患患者に移植する治療を開始したばかりである。具体的には，G-CSFの投与によって，末梢血中のCD34陽性細胞を動員し，アフェレーシスによって，大量の末梢血単核球を確保し，この細胞をCD34抗体で分離することにより，治療に必要なCD34陽性細胞を得ることができた。これらの細胞の分画は，FACSを用いて98%以上の割合で純化されていることが確認されている。具体的な効果については今後の経過を待ちたい。

おわりに

心血管領域における最近の遺伝子・再生医学研究の動向について概要を述べた。遺伝子治療，細胞治療ともに共通していることは，血管新生を期待する患者の背景が，特に動脈硬化性疾患の場合，一般に高齢であるため細胞そのものの機能が低下しているため，効果を高めるための検討が必要になるという点であろう。細胞移植に関しては，今後，遺伝子導入により細胞機能を高めた細胞を体外培養によって増殖させた産物を，臨床に応用することが考えられ，そのための検討が現在進められている。

文　献

1) Baumgartner I, Pieczek A, Manor O, et al : Constitutive expression of phVEGF$_{165}$ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation **97**(12) : 1114-1123, 1998
2) Losordo DW, Vale PR, Symes JF, et al : Gene therapy for myocardial angiogenesis ; initial clinical results with direct myocardial injection of phVEGF$_{165}$ as sole therapy for myocardial ischemia. Circulation **98**(25) : 2800-2804, 1998
3) Losordo DW, Vale PR, Hendel RC, et al : 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 **105**(17) : 2012-2018, 2002
4) Fortuin FD, Vale P, Losordo DW, et al : One-year follow-up of direct myocardial gene transfer of vascular endothelial growth factor-2 using naked plasmid deoxyribonucleic acid by way of thoracotomy in no-option patients. Am J Cardiol **92**(4) : 436-439, 2003
5) Schumacher B, Pecher P, von Specht BU, et al : Induction of neoangiogenesis in ischemic myocardium by human growth factors ; first clinical results of a new treatment of coronary heart disease. Circulation **97** : 645-650, 1998
6) Laham RJ, Sellke FW, Edelman ER, et al : Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery ; results of a phase I randomized, double-blind, placebo-controlled trial. Circulation **100**(18) : 1865-1871, 1999
7) Hendel RC, Henry TD, Rocha-Singh K, et al : Effect of intracoronary recombinant human vascular endothelial growth factor on myocardial perfusion ; evidence for a dose-dependent effect. Circulation **101**(2) : 118-121, 2000
8) Ruel M, Wu GF, Khan TA, et al : Inhibition of the cardiac angiogenic response to surgical FGF-2 therapy in a Swine endothelial dysfunction model. Circulation **108**(Suppl. 1) : II 335-340, 2003
9) Asahara T, Murohara T, Sullivan A, et al : Isolation of putative progenitor endothelial cells for angiogenesis. Science **275**(5302) : 964-967, 1997
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 **5**(4) : 434-438, 1999
11) Llevadot J, Murasawa S, Kureishi Y, et al : HMG-CoA reductase inhibitor mobilizes bone marrow—derived endothelial progenitor cells. J Clin Invest **108**(3) : 399-405, 2001
12) 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 **108**(3) : 391-397, 2001
13) Kalka C, Masuda H, Takahashi T, et al : Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A **97**(7) : 3422-3427, 2000
14) Kawamoto A, Gwon HC, Iwaguro H, et al : Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation **103**(5) : 634-637, 2001
15) Murohara T, Ikeda H, Duan J, et al : Transplanted cord blood-derived endothelial precursor cells augment

postnatal neovascularization. J Clin Invest **105**(11): 1527-1536, 2000
16) Iwaguro H, Yamaguchi J, Kalka C, et al : Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation **105**(6): 732-738, 2002
17) Murasawa S, Llevadot J, Silver M, et al : Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. Circulation **106**(9): 1133-1139, 2002
18) Badorff C, Brandes RP, Popp R, et al : Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active. Circulation **107**(7): 1024-1032, 2003
19) Yeh ET, Zhang S, Wu HD, et al : Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation **108**(17): 2070-2073, 2003
20) Shintani S, Murohara T, Ikeda H, et al : Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation **103**(23): 2776-2779, 2001
21) 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 **104**(9): 1046-1052, 2001
22) Tateishi-Yuyama E, Matsubara H, Murohara T, et al : Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells ; a pilot study and a randomised controlled trial. Lancet **360**(9331):427-435, 2002

Synergistic Effect of Bone Marrow Mobilization and Vascular Endothelial Growth Factor-2 Gene Therapy in Myocardial Ischemia

Atsuhiko Kawamoto, MD, PhD*; Toshinori Murayama, MD, PhD*; Kengo Kusano, MD, PhD; Masaaki Ii, MD, PhD; Tengiz Tkebuchava, MD; Satoshi Shintani, MD, PhD; Atsushi Iwakura, MD, PhD; Ingrid Johnson, BS; Patrick von Samson, MD; Allison Hanley, BS; Mary Gavin, BS; Cindy Curry, BS; Marcy Silver, BS; Hong Ma, BS; Marianne Kearney, BS; Douglas W. Losordo, MD

Background—We performed a series of investigations to test the hypothesis that combining angiogenic gene therapy and cytokine (CK)-induced endothelial progenitor cell mobilization would be superior to either strategy alone for treatment of chronic myocardial ischemia.

Methods and Results—A swine model of chronic myocardial ischemia and a murine model of acute myocardial infarction were used in this study. In both models, animals were randomly assigned to 1 of 4 treatment groups: Combo group, intramyocardial vascular endothelial growth factor (VEGF)-2 gene transfer plus subcutaneous injection of CKs; VEGF-2, VEGF-2 gene transfer plus saline subcutaneously injected; CK, empty vector transfer plus CKs; and control, empty vector plus subcutaneous saline. Acute myocardial infarction was also induced in wild-type mice 4 weeks after bone marrow transplantation from enhanced green fluorescent protein transgenic mice to permit observation of bone marrow–derived cells in the myocardium after acute myocardial infarction. In chronic myocardial ischemia, combination therapy resulted in superior improvement in all indexes of perfusion and function compared with all other treatment groups. In the bone marrow transplant mice, double immunofluorescent staining revealed that the combination of CK-induced mobilization and local VEGF-2 gene transfer resulted in a significant increase in the number of bone marrow–derived cells incorporating into the neovasculature, indicating that recruitment and/or retention of bone marrow–derived progenitors was enhanced by mobilization and that local VEGF-2 gene transfer can provide signals for recruitment or incorporation of circulating progenitor cells.

Conclusions—Mobilization of endothelial progenitor cells with cytokines potentiates VEGF-2 gene therapy for myocardial ischemia and enhances bone marrow cell incorporation into ischemic myocardium. (***Circulation.* 2004;110:1398-1405.**)

Key Words: cytokines ■ endothelial cells ■ ischemia ■ stem cells ■ vascular endothelial growth factor

Catheter-based, intramyocardial vascular endothelial growth factor (VEGF) gene transfer has been shown to induce therapeutic angiogenesis in preclinical models of myocardial ischemia[1,2] and to increase exercise tolerance time and decrease the incidence of anginal episodes in pilot studies in patients with chronic myocardial ischemia.[3] These favorable effects of VEGF gene transfer were accompanied by objective evidence of improvement of myocardial perfusion in a recent placebo-controlled, double-blind pilot clinical trial.[4] The principal mechanism of these effects was initially thought to be the formation of new blood vessels by sprouting and migration of preexisting endothelial cells in ischemic tissue.[5–8] However, extensive preclinical data have suggested that a portion of the effect of angiogenic cytokines (CKs) involves the mobilization and recruitment of precursor cells, capable of differentiation into endothelial cells, from the bone marrow (BM).[9,10] Recent clinical data have revealed that VEGF gene transfer is also accompanied by the mobilization of BM-derived endothelial progenitor cells (EPCs).[11] Moreover, extensive preclinical and early clinical data have revealed that EPCs may exert a therapeutic effect on ischemic tissue when administered systemically or locally.[12–16] Together, these data suggest that administration of exogenous angiogenic growth factors may stimulate both angiogenesis and vasculogenesis for therapeutic neovascularization.

Granulocyte colony stimulating factor (G-CSF) and stem cell factor (SCF) are CKs that have been clinically applied for mobilization of BM-derived hematopoietic stem cells into the systemic circulation.[17–19] Administration of G-CSF and SCF

Received November 11, 2003; de novo received March 16, 2004; revision received June 8, 2004; accepted June 14, 2004.
From the Division of Cardiovascular Research, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Mass.
*Drs Kawamoto and Murayama contributed equally to this study.
Correspondence to Douglas W. Losordo, MD, Division of Cardiovascular Research, St. Elizabeth's Medical Center, 736 Cambridge St, Boston, MA 02135. E-mail douglas.losordo@tufts.edu

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000141563.71410.64

has been reported to improve left ventricular (LV) function in mice with acute myocardial infarction (MI) through increased homing of mobilized, BM-derived EPCs and cardiomyogenic progenitor cells into ischemic myocardium,[20] providing direct evidence that mobilization of BM progenitors might represent a viable strategy for preserving the integrity and restoring function in ischemic tissue. In the present study, we performed experiments to test the hypothesis that BM mobilization can augment VEGF gene transfer–induced therapeutic neovascularization by enhancing the contribution of BM-derived precursor cells.

Methods

Experimental Animals

All animals were handled in accordance with the guidelines of the Animal Care and Use Committee at St Elizabeth's Medical Center (Boston, Mass).

Thirty-two male Yorkshire swine (Pine Acre Rabbitry Farm, Norton, Mass) weighing 20 to 25 kg were used to induce chronic myocardial ischemia. After left thoracotomy, an ameroid constrictor (Research Instruments SW) was placed around the proximal portion of the left circumflex coronary artery (LCx) as previously detailed.[21,22]

Thirty BM transplant (BMT) animal models were also prepared as previously described as a means of documenting the kinetics of BM-derived cells in the ischemic myocardium.[23] In brief, female C57BL/6 mice received BM mononuclear cells from transgenic mice constitutively overexpressing enhanced green fluorescent protein (eGFP mice, C57BL/6-TgN[ACTbEGFP]1Osb, Jackson Laboratory)[24] after sublethal irradiation. Four weeks after BMT, by which time the BM of the recipient mice was reconstituted, BMT mice were used for experiments.

Mice were anesthetized with 2,2,2-Tribromoethanol (200 μL/g body weight IP), orally intubated with a 22G IV catheter, and ventilated with a respirator (Harvard Apparatus). A left intercostal thoracotomy was performed, and the ribs were retracted with 5-0 polypropylene sutures to open the chest. After the pericardium was opened, the left anterior descending coronary artery (LAD) was ligated distal to the bifurcation between the LAD and diagonal branch with 8-0 polypropylene sutures through a dissecting microscope. After positive end-expiratory pressure was applied to fully inflate the lung, the chest was closed with 7-0 polypropylene sutures. The overall survival ratio after MI was 86% at 4 weeks.

Administration of Plasmid Human VEGF-2 Gene and CKs

In the swine study, NOGA nonfluoroscopic LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia 5 weeks after constrictor placement. The NOGA system (Cordis) of catheter-based mapping and navigation and has been previously described in detail.[2,25,26] Ischemic myocardium was defined as a zone with unipolar voltage higher than an automatically determined cutoff and linear local shortening of <3%.[21] This definition was consistent in all examinations throughout this study. Immediately after the ischemic territory was identified by NOGA mapping, 800 μg plasmid human VEGF-2 gene (phVEGF-2) in 3 mL PBS or 800 μg empty vector in 3 mL PBS was injected into 6 sites within the ischemic myocardium (500 μL to each site) using the NOGA injection catheter (MyoSTAR, Cordis). The rhG-CSF (5 $\mu g \cdot kg^{-1} \cdot d^{-1}$) and rhSCF (20 $\mu g \cdot kg^{-1} \cdot d^{-1}$) or control saline was injected subcutaneously in swine with myocardial ischemia for 7 days starting immediately after the intramyocardial gene transfer. Swine were randomly assigned to 1 of 4 treatment groups: Combo group (n=8), catheter-based intramyocardial gene transfer of 800 μg of phVEGF-2 and subcutaneous rhG-CSF (5 $\mu g \cdot kg^{-1} \cdot d^{-1}$) and rhSCF (20 $\mu g \cdot kg^{-1} \cdot d^{-1}$) for 7 days immediately after the gene transfer; VEGF-2 group (n=8), phVEGF-2 gene transfer and saline injection; CK group (n=8), empty vector transfer and subcutaneous rhG-CSF and rhSCF injection; and control group (n=8), empty vector transfer and saline injection.

In the mouse study, 100 μg phVEGF-2 or empty vector as control (both dissolved in 100 μL saline) was administered intramyocardially with a 30G needle distal to the LAD occlusion site immediately after LAD ligation. The combination of CKs with recombinant human (rh) G-CSF (50 ng/g body weight) and rhSCF (200 ng/g) or control saline was administered (subcutaneously) daily for a week (days 0 to 6) after MI (rhG-CSF and rhSCF were supplied by Amgen, Inc). The mice were randomly assigned to 4 subgroups (n=6 or 7 in each group): empty vector plus saline (control group), empty vector plus CKs (CK group), phVEGF-2 plus saline (VEGF-2 group), or phVEGF-2 plus CKs (Combo group).

Physiological Assessment of LV Function and Ischemia

In the swine study, transthoracic echocardiography (SONOS 5500), selective left coronary angiography, and NOGA LV electromechanical mapping were performed 5 weeks after constrictor placement (just before injection of genes) and 4 weeks after gene injection. Echocardiographic fractional shortening (FS) and regional wall motion scores[27] were quantified by use of the LV short-axis view at the midpapillary muscle level. Collateral flow to the LCx territory was graded angiographically in a blinded fashion with the Rentrop scoring system.[28] The area of ischemia was quantified by NOGA mapping as previously described.[2]

All data were evaluated by blinded observers (echocardiography by K.K., coronary angiography by S.S., and postprocessing analysis of the NOGA mapping by I.J.).

Histological Assessment of Neovascularization and LV Remodeling

All swine were killed 4 weeks after gene transfer. At necropsy, swine hearts were sliced in a bread-loaf fashion into 4 transverse sections from apex to base, and each section was separated to anterior, lateral, posterior LV free wall, interventricular septum, and right ventricular free wall. All tissues obtained from each segment were fixed in 100% methanol. Immunohistochemistry for isolectin B4 was also performed to evaluate capillary density in the ischemic myocardium identified by NOGA mapping.

Double Immunofluorescence Histochemistry

The hearts of GFP-BM transplanted mice were harvested at predetermined times after surgery and prepared for frozen tissue sections. Frozen cross sections (6-μm thickness) were air dried and fixed with 4% paraformaldehyde for 5 minutes. After washing with PBS, double immunohistochemistry was performed with antibodies against GFP and isolectin B4. Nonspecific protein binding was blocked with 10% normal goat serum. Rabbit polyclonal anti-GFP antibody (1:200 dilution; Molecular Probe) was used at 4°C overnight, followed by goat anti-rabbit IgG conjugated with Cy2 (1:500 dilution; Jackson ImmunoResearch) as a secondary antibody for 30 minutes at room temperature. The endothelial cell–specific marker, biotinylated isolectin-B4 (1:100 dilution; Vector Laboratories), was used as a second primary antibody for capillary staining and visualized by binding with rhodamine-conjugated streptoavidin (1:500 dilution; Jackson ImmunoResearch) for 30 minutes at room temperature. Normal rabbit IgG served as a negative control for GFP detection. Nuclei were counterstained with DAPI (1:5000) and mounted in aqueous mounting medium. Images were examined with a fluorescent microscope (Nikon ECLIPSE TE200).

Double-positive cells were quantified in 5 randomly selected fields from 5 sections from each heart. All morphometric studies were performed by 3 examiners (H.M., A.H., and M.I.) who were blinded to treatment assignment.

Statistical Analysis

All values are expressed as mean±SE. Student's paired t test was used to compare data before and after treatment. ANOVA was

performed to compare data among the 4 treatment groups. A value of $P<0.05$ was considered statistically significant.

Results

Two pigs died in the control and CK groups before the final assessment. There were no deaths in the VEGF-2 or Combo groups.

BM Mobilization Augments the Effects of VEGF-2 Gene Transfer for Attenuation of Chronic Myocardial Ischemia

In the swine study, the ischemic area determined by NOGA mapping before gene transfer was not significantly different between the Combo, VEGF-2, CK, and control groups (35.4±4.4%, 34.7±5.6%, 43.0±8.7%, and 26.5±6.2%, respectively). Four weeks after treatment, the reduction in ischemic area was significantly better in the Combo therapy group (−85.4±2.2%) compared with all other treatment groups (VEGF-2, −24.3±21.5%; CK, 29.5±48.3%; and control, 40.7±25.1%; $P<0.05$ versus VEGF-2, $P<0.01$ versus CK and control). Ischemic area was also significantly improved in the VEGF-2 group compared with the control group ($P<0.05$). The ischemic area in the CK group was not significantly different from that in the control group (Figure 1).

Cytokine-Induced BM Mobilization Enhances Therapeutic Neovascularization by VEGF-2 Gene Transfer

In the swine study, selective left coronary angiography was performed to evaluate collateral development before and after treatment. The mean Rentrop score of collateral development to the LCx territory at baseline was 1.3±0.3 in the Combo group, 1.4±0.3 in the VEGF-2 group, 2.0±0.4 in the CK group, and 1.0±0.3 in the control group (P=NS). The change in the Rentrop score after treatment was significantly greater in the Combo group than in the control group ($P=0.01$). The change in the Rentrop score in the Combo group was similar to those in VEGF-2 and CK groups; however, neither the VEGF-2 nor the CK group showed an improvement in Rentrop scores that was significantly greater than the controls (Figure 2). These data indicated that there was anatomic evidence of improved collateral formation in the Combo therapy group compared with all other treatment groups.

In the swine study, histochemical staining for isolectin B4 was performed to identify capillaries in the ischemic myocardium 4 weeks after treatment. Capillary density was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (879.9±44.8, 717.0±75.7, 326.4±14.1, and 345.0±20.4/mm^2, respectively; $P=0.03$ versus VEGF-2, $P<0.0001$ versus CK and control). Capillary density was also significantly greater in the VEGF-2 group than in the CK and control groups ($P<0.0001$). Capillary density in the CK group was similar to that in the control group (Figure 3). These data reveal that in addition to augmenting the angiographically visible collateral supply, combined VEGF-2 myocardial gene therapy plus CK-induced mobilization of BM progenitors resulted in a significant increase in microvascular capillary density compared with monotherapy.

BM Mobilization Augments the Effects of VEGF-2 Gene Transfer on LV Function in Chronic Myocardial Ischemia

In the swine study, echocardiographic FS and regional wall motion score before treatment were similar in all groups (FS: Combo, 27.6±1.3%; VEGF-2, 29.6±0.9%; CK, 30.5±1.4%; control, 29.4±1.2%; regional wall motion score: Combo, 22.4±1.0; VEGF-2, 20.8±0.7; CK, 20.4±0.5; control, 20.5±0.8). The improvement in FS after treatment was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (5.3±0.9%, 1.0±1.2%, −1.1±0.8%, and −1.1±1.3%, respectively; $P=0.03$ versus VEGF-2, $P=0.01$ versus CK, $P=0.001$ versus control). Changes in FS were similar in the VEGF-2, CK, and control groups. Regional wall motion score after treatment was significantly improved in the Combo group compared with the VEGF-2, CK, and control groups (−3.9±1.0, −1.3±0.9, 0.4±0.5, and 1.2±0.9, respectively; $P=0.04$ versus VEGF-2, $P=0.009$ versus CK, and $P=0.0004$ versus control; Figure 4a and 4b).

Cytokine Mobilization Increases Recruitment and Incorporation of BM Cells Into Myocardial Neovasculature

Immunohistochemistry was performed on the hearts from BMT mice 1 week after MI to assess BM-derived cell incorporation into the neovasculature. Double immunofluorescent staining for eGFP and isolectin B4 permitted identification of BM-derived cells that also expressed a marker of endothelial cell identity (Figure 5A). The double-positive cells were quantified and were found to be most abundant in the border zones between ischemic and nonischemic tissue in the Combo group (50.7±5.8), followed by the VEGF-2 group (19.8±3.7). Both groups had significantly greater numbers of double-positive cells than the control group ($P<0.001$), and the number of double-positive cells in the Combo group was significantly greater than in the VEGF group ($P<0.01$; Figure 5B). As shown in Figure 5A, some of the double-positive cells were incorporated into tubular structures, consistent with vasculogenesis. These data provide evidence that VEGF gene therapy stimulates vasculogenesis in the myocardium and that this effect can be augmented by BM mobilization.

Discussion

The concept of therapeutic angiogenesis by administration of angiogenic genes or proteins has been established in numerous preclinical models.[1,5,8] Recently, pilot clinical trials of therapeutic angiogenesis using some of these growth factors have been reported in patients with coronary artery disease.[4,29–31] Although subjective symptoms have been significantly improved in these phase I and II trials, some studies have failed to demonstrate significant improvement in objective findings such as myocardial perfusion and exercise tolerance. Analysis of the data generated in all these pilot studies reveals at least 2 common features: (1) In each study, the effect of a single agent was evaluated, and (2) certain patients are "nonresponders." The absence of a response in certain individuals is a consistent feature of all therapies and is the basis for the concept of pharmacogenomics, the science of designing drugs based on genetic features of individual

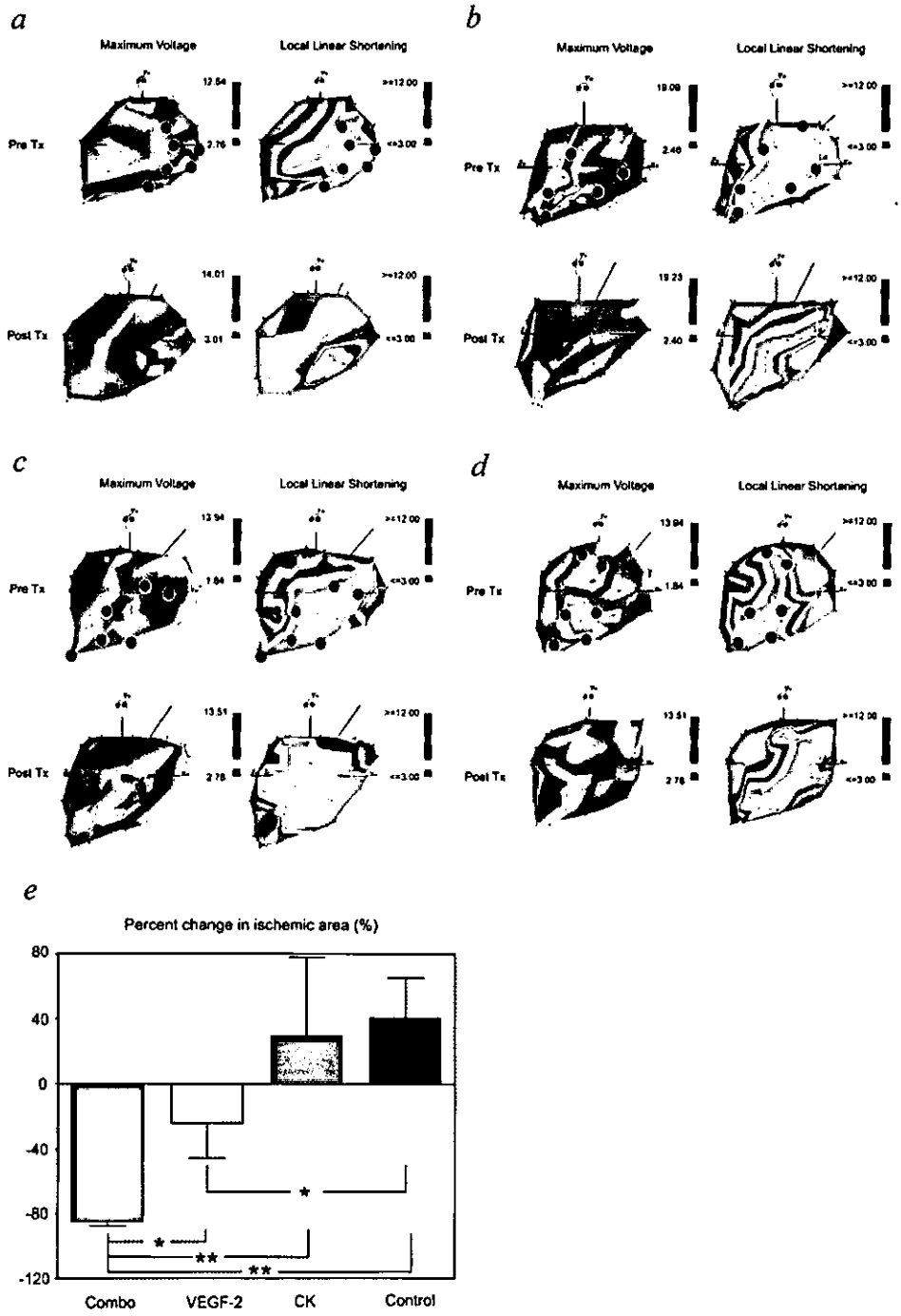


Figure 1. Representative recordings of NOGA electromechanical mapping immediately before (pre TX) and 4 weeks after (post Tx) gene transfer in porcine model of chronic myocardial ischemia. Black dots in pretreatment map show sites of gene transfer. Red area on pretreatment linear local shortening map (top right) indicates area of decreased wall motion in lateral wall of left ventricle, consistent with ischemia in territory of LCx. Four weeks after gene transfer, this area of ischemia improved in representative case in Combo therapy group (a) and moderately in case from VEGF-2 group (b), whereas no improvement was observed in cases from CK group (c) and control group (d). e, Percent change in ischemic area during 4 weeks after gene transfer. $^{*}P<0.05$; $^{**}P<0.01$.

patients. Lacking this tailored approach to drug development, physicians have traditionally tried combining drugs to achieve therapeutic effects in patients with conditions refractory to single agents.

In parallel with studies attempting neovascularization by administration of angiogenic CKs, the use of progenitor or stem cells as therapeutic agents in ischemic diseases has emerged.[15,16,32] These studies are based on observations indicating that circulating cells, some of which appear to originate in the BM, are capable of homing to and augmenting neovascularization of ischemic tissue.[33–35] More recent data have indicated that at least part of the effect of locally administered angiogenic CKs results from recruitment of progenitor cells and that the failure of native or therapeutic

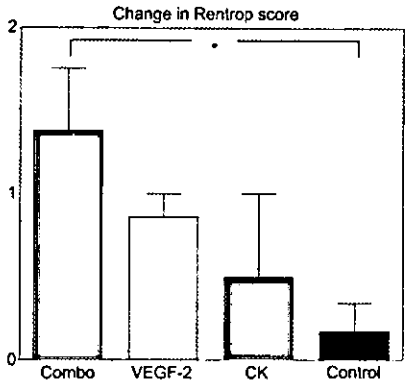


Figure 2. Change in Rentrop grade of collateral development 4 weeks after gene transfer in porcine model of chronic myocardial ischemia. *$P<0.05$.

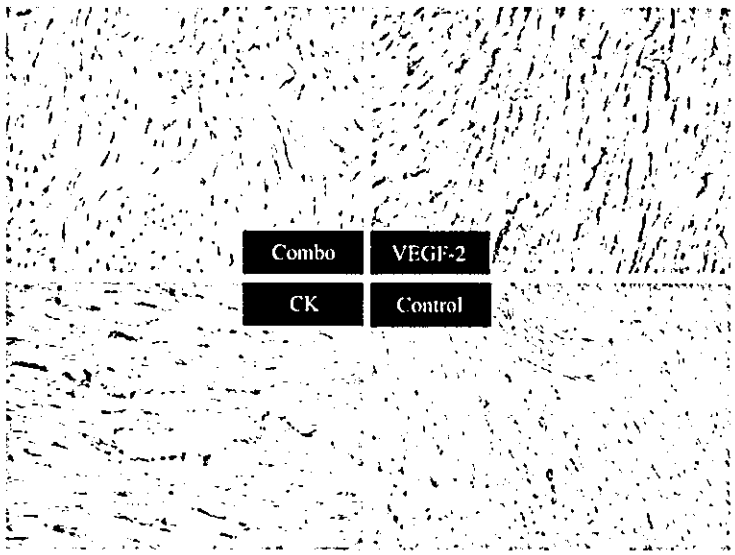
neovascularization might result in part from a deficiency in the quantity or quality of these cells.[11,36–38]

This constellation of findings raised an important fundamental question regarding VEGF gene therapy for therapeutic neovascularization: Is the mechanism of local VEGF predominantly via local effects, enhancing the proliferation and migration of EC in pre-existing blood vessels, or is it possible that VEGF, expressed after gene transfer in the local tissue environment, is acting as a chemokine, recruiting progenitor cells from remote sites to deliver a more varied repertoire of CKs[39] in addition to providing parent cells for the neovasculature?[33] The latter possibility is well illustrated in studies by Orlic et al[40] in the setting of acute ischemia in which the local homing signals for circulating cells are apparently robust, obviating the need for induction of local CK expression.

Accordingly, we hypothesized that the effect of transient local expression of VEGF, mediated by gene transfer of naked plasmid DNA, might be amplified by increasing the circulating supply of progenitor cells by systemically administered hematopoietic stem cell mobilization using GCSF and SCF. This is consistent with a report demonstrating superiority of a combination of growth factor therapy and cell transplantation. In this previous study,[41] the combination of hepatocyte growth factor gene transfer and neonatal rat cardiomyocyte transplantation had more potent therapeutic efficacy in a model of rat MI compared with either single treatment.

Although the therapeutic potential of systemically administered, mobilizing CKs has been reported in the setting of acute MI,[20] the efficacy of the same approach in chronic myocardial ischemia has not been defined in animal models. Interestingly, this approach has been attempted in a single human pilot study of granulocyte-macrophage CSF administration.[42] This study revealed potential benefit by a novel

a



b

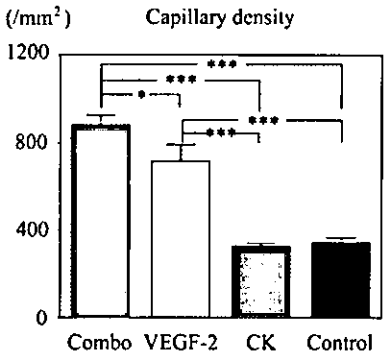


Figure 3. a, Representative immunohistochemistry for isolectin B4 in specimens of ischemic porcine myocardium from 4 treatment groups. These specimens were obtained 4 weeks after gene transfer. b, Capillary density 4 weeks after gene transfer. *$P<0.05$; ***$P<0.001$.