HUVECs, indicating that AM does not function indirectly through upregulation of VEGF. Interestingly, AM and CRLR are both upregulated under hypoxic conditions in microvascular endothelial cells, although expression of RAMPs is not activated by hypoxia in microvascular cells (54). The activity of the CRLR promoter under hypoxic conditions is regulated at least in part through hypoxia-responsive regulatory element binding transcription factor HIF-1. Thus the simultaneous transcriptional upregulation of CRLR and its ligand AM in endothelial cells might play a significant role in the vascular responses to hypoxia and ischemia by creating a potent survival loop.

SMCs are essential for the generation of functional and mature blood vessels (26). We demonstrated in vivo that intramuscular administration of AM increased the number of αSMA-positive cells involved in the formation of vascular structures (25). In vitro, AM enhanced SMC migration, which was inhibited by wortmannin, a PI3K inhibitor. Recent studies using homozygous AM knockout mice have suggested that AM is essential for vascular morphogenesis (6, 21, 70). Taking these findings together, it is possible that AM contributes to vessel maturation through enhancement of SMC migration via a PI3K/Akt-dependent pathway (Fig. 1). This feature of AM-induced angiogenesis is different from VEGF-induced angiogenesis, which is not associated with vessel muturation.

In tumor cells, inflammation and hypoxia increase AM expression, and the elevated expression of AM is associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma (12, 86). AM also acts as a tumor cell survival factor underlying human carcinogenesis. Thus hypoxia-induced AM plays a part in tumor angiogenesis in conjunction with VEGF, and facilitates tumor growth under hypoxic conditions. As angiogenesis is an essential process in tumor-host interactions for tumor growth, maintenance, and metastasis, finding ways to regulate the action of AM may provide a new avenue for developing anticancer therapy (16).

THERAPEUTIC ANGIOGENESIS

A variety of studies have demonstrated that AM gene delivery serves as therapeutic tool to protect the cardiovascular system, including the heart (9, 32, 85), kidney (83), and vasculature (2, 84). In this section, we describe the angiogenic potential of AM gene transfer using novel gene delivery systems.

Nonviral gene transfer. Peripheral vascular disease is a crucial health issue affecting an estimated 27 million people (5). Despite recent advances in medical interventions, the symptoms of some patients with critical limb ischemia fail to be controlled. Although gene therapy has been shown to be an effective approach for angiogenesis (10, 24, 72), it is still unsatisfactory because of the biohazard of viral vectors, low transfection efficiency, and premature tissue-targeting. Therefore, highly efficient and safe gene transfer is desirable. Recently, we developed a novel nonviral vector, gelatin hydrogel, which allows highly efficient and long-lasting gene transfer (13, 30, 81). Gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation (76, 77). Plasmid DNA is known to be negatively charged. Thus we used gelatin as a vector for gene therapy. Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of ~30 μm, water content of 95% and an isoelectric point of 9 after swelling in water (76, 77). After 2-h incubation, positively charged gelatin held negatively charged plasmid DNA in its positively charged lattice structure. DNA particles are released from the gelatin through its degradation. As a result, DNA-gelatin complexes can delay gene degradation, leading to efficient gene transfer (13, 30, 44, 81).

We examined whether nonviral vector gelatin-mediated AM gene transfer induces therapeutic angiogenesis in a rabbit model of hindlimb ischemia (81). Seven days after intramuscular injection of AM DNA-gelatin complexes, there was intense AM immunoreactivity surrounding the gelatin in the skeletal muscles. AM production in the AM-gelatin group was enhanced compared with that in the naked AM DNA group, which received plasmid AM DNA alone. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 wk. Importantly, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hindlimb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, and laser Doppler flow. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. AM DNA-gelatin complexes were distributed mainly in connective tissues. It is interesting to speculate that the delay of gene degradation by gelatin may have been responsible for the highly efficient gene transfer. Thus gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

Cell-based gene transfer. Recently, transplantation of stem cells or progenitor cells has been shown to regenerate a variety of tissues. Endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood (4, 79). EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ (15, 34). Transplantation of EPC induces therapeutic angiogenesis in the ischemic heart or limb (34, 42, 71). However, some patients are refractory to conventional cell therapy because of insufficient cell number, poor survival, or impaired differentiation. Thus a novel therapeutic strategy to enhance the angiogenic properties of EPCs is desirable. Considering the variety of protective effects of AM on vascular endothelial cells, we hypothesized that AM gene transfer into EPCs would strengthen the therapeutic potential of EPCs. Genetically modified EPCs may serve not only as a tissue-engineering tool to reconstruct the vasculature but also as a vehicle for gene delivery to injured endothelium.

Here, we present a new concept for cell-based gene delivery into the vasculature, consisting of three processes (44). First, positively charged gelatin is readily complexed with negatively charged plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs with high efficiency. Third, intravenously administered gene-modified EPCs are incorporated into injured vascular beds. This novel gene delivery system has great advantages over conventional gene therapy; it is nonviral and noninvasive, and it provides highly efficient gene targeting into the vasculature. These benefits

may be achieved mainly by the capability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium. Genetically modified EPCs markedly secreted AM into the culture medium, and AM overproduction lasted for more than 2 wk. The proliferative activity of AM DNA-transduced EPCs exceeded that of nontransduced EPCs. Furthermore, AM gene transfer inhibited apoptosis of EPCs in vivo and in vitro. Thus ex vivo AM gene transfer strengthened the therapeutic potential of EPCs.

Primary pulmonary hypertension (PPH) is a rare, but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (67). Median survival in patients with PPH is considered to be 2.8 years from the time of diagnosis. Thus novel and effective therapy is needed for the treatment of pulmonary hypertension. Because endothelial dysfunction may play a role in the pathogenesis of pulmonary hypertension such as PPH (3), pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. We have demonstrated that administration of AM peptide decreases pulmonary vascular resistance in patients with PPH (45, 46, 48, 51). Thus we investigated the effects of AM gene-modified EPCs on pulmonary hypertension in rats (44). AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature. Immunohistochemical analyses demonstrated that the transplanted EPCs were of endothelial lineage and formed vascular structures. Intravenous administration of AM-expressing EPCs significantly decreased pulmonary vascular resistance compared with EPCs alone (−39%). Kaplan-Meier survival curves demonstrated that rats with pulmonary hypertension transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone. These findings suggest that AM gene-modified EPCs using gelatin may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature, but also as a vehicle for gene delivery to injured pulmonary endothelium. This hybrid cell-gene therapy may be applicable for intractable cardiovascular disease, including ischemic heart disease. Thus genetic manipulation of stem cells opens new avenues for regenerative medicine.

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REFERENCES

1. **Abe M, Sata M, Nishimatsu H, Nagata D, Suzuki E, Terauchi Y, Kadowaki T, Minamino N, Kangawa K, Matsuo H, Hirata Y, and Nagai R.** Adrenomedullin augments collateral development in response to acute ischemia. *Biochem Biophys Res Commun* 306: 10–15, 2003.
2. **Agata J, Zhang JJ, Chao J, and Chao L.** Adrenomedullin gene delivery inhibits neointima formation in rat artery after balloon angioplasty. *Regul Pept* 112: 115–120, 2003.
3. **Archer S and Rich S.** Primary pulmonary hypertension: a vascular biology and translational research "work in progress". *Circulation* 102: 2781–2791, 2000.
4. **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.
5. **Belch JJ, Topol EJ, Agnelli G, Bertrand M, Califf RM, Clement DL, Creager MA, Easton JD, Gavin 3rd JR, Greenland P, Hankey G, Hanrath P, Hirsch AT, Meyer J, Smith SC, Sullivan F, Weber MA, Prevention of Atherothrombotic, and Disease Network.** Critical issues in peripheral arterial disease detection and management: a call to action. *Arch Intern Med* 163: 884–892, 2003.
6. **Caron KM and Smithies O.** Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *Proc Natl Acad Sci USA* 98: 615–619, 2001.
7. **Chun TH, Itoh H, Ogawa Y, Tamura N, Takaya K, Igaki T, Yamashita J, Doi K, Inoue M, Masatsugu K, Korenaga R, Ando J, and Nakao K.** Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. *Hypertension* 29: 1296–1302, 1997.
8. **Cueille C, Pidoux E, de Vernejoul MC, Ventura-Clapier R, and Garel JM.** Increased myocardial expression of RAMP1 and RAMP3 in rats with chronic heart failure. *Biochem Biophys Res Commun* 294: 340–346, 2002.
9. **Dobrzynski E, Wang C, Chao J, and Chao L.** Adrenomedullin gene delivery attenuates hypertension, cardiac remodeling, and renal injury in deoxycorticosterone acetate-salt hypertensive rats. *Hypertension* 36: 995–1001, 2000.
10. **Feldman LJ, Steg PG, Zheng LP, Chen D, Kearney M, McGarr SE, Barry JJ, Dedieu JF, Perricaudet M, and Isner JM.** Low-efficiency of percutaneous adenovirus-mediated arterial gene transfer in the atherosclerotic rabbit. *J Clin Invest* 95: 2662–2671, 1995.
11. **Fernandez-Sauze S, Delfino C, Mabrouk K, Dussert C, Chinot O, Martin PM, Grisoli F, Ouafik L, and Boudouresque F.** Effects of adrenomedullin on endothelial cells in the multistep process of angiogenesis: involvement of CRLR/RAMP2 and CRLR/RAMP3 receptors. *Int J Cancer* 108: 797–804, 2004.
12. **Fujita Y, Mimata H, Nasu N, Nomura T, Nomura Y, and Nakagawa M.** Involvement of adrenomedullin induced by hypoxia in angiogenesis in human renal cell carcinoma. *Int J Urol* 9: 285–295, 2002.
13. **Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, and Tabata Y.** Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J Control Release* 80: 333–343, 2002.
14. **Garayoa M, Martinez A, Lee S, Pio R, An WG, Neckers L, Trepel J, Montuenga LM, Ryan H, Johnson R, Gassmann M, and Cuttitta F.** Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. *Mol Endocrinol* 14: 848–862, 2000.
15. **Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, and Rafii S.** Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res* 88: 167–174, 2001.
16. **Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, Seybold J, Seeger W, Rascher W, Schutte H, and Suttorp N.** Adrenomedullin reduces endothelial hyperpermeability. *Circ Res* 91: 618–625, 2002.
17. **Hirata Y, Hayakawa H, Suzuki Y, Suzuki E, Ikenouchi H, Kohmoto O, Kimura K, Kitamura K, Eto T, Kangawa K, Matsuo H, and Omata M.** Mechanisms of adrenomedullin-induced vasodilation in the rat kidney. *Hypertension* 25: 790–795, 1995.
18. **Hofbauer KH, Jensen BL, Kurtz A, and Sandner P.** Tissue hypoxygenation activates the adrenomedullin system in vivo. *Am J Physiol Regul Integr Comp Physiol* 278: R513–R519, 2000.
19. **Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, and Eto T.** Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett* 338: 6–10, 1994.
20. **Iimuro S, Shindo T, Moriyama N, Amaki T, Niu P, Takeda N, Iwata H, Zhang Y, Ebihara A, and Nagai R.** Angiogenic effects of adrenomedullin in ischemia and tumor growth. *Circ Res* 95: 415–423, 2004.
21. **Imai Y, Shiindo T, Maemura K, Kurihara Y, Nagai R, and Kurihara H.** Evidence for the physiological and pathological roles of adrenomedullin from genetic engineering in mice. *Ann NY Acad Sci* 947: 26–33, 2001.
22. **Ishimitsu T, Nishikimi T, Saito Y, Kitamura K, Eto T, Kangawa K, Matsuo H, Omae T, and Matsuoka H.** Plasma levels of adrenomedullin, a newly identified hypotensive peptide, in patients with hypertension and renal failure. *J Clin Invest* 94: 2158–2161, 1994.
23. **Ishizaka Y, Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, Matsuo H, and Eto T.** Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 200: 642–646, 1994.

24. **Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, and Symes JF.** Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischemic limb. *Lancet* 348: 370–374, 1996.
25. **Iwase T, Nagaya N, Fujii T, Itoh T, Ishibashi-Ueda H, Yamagishi M, Miyatake K, Matsumoto T, Kitamura S, and Kangawa K.** Adrenomedullin enhances angiogenic potency of bone marrow transplantation in a rat model of hindlimb ischemia. *Circulation* 111: 356–362, 2005.
26. **Jain RK.** Molecular regulation of vessel maturation. *Nat Med* 9: 685–693, 2003.
27. **Jiang BH, Zheng JZ, Aoki M, and Vogt PK.** Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci USA* 97: 1749–1753, 2000.
28. **Jougasaki M, Wei CM, McKinley LJ, and Burnett JC Jr.** Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. *Circulation* 92: 286–289, 1995.
29. **Kakishita M, Nishikimi T, Okano Y, Satoh T, Kyotani S, Nagaya N, Fukushima K, Nakanishi N, Takishita S, Miyata A, Kangawa K, Matsuo H, and Kunieda T.** Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clin Sci (Lond)* 96: 33–39, 1999.
30. **Kasahara H, Tanaka E, Fukuyama N, Sato E, Sakamoto H, Tabata Y, Ando K, Iseki H, Shinozaki Y, Kimura K, Kuwabara E, Koide S, Nakazawa H, and Mori H.** Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia. *J Am Coll Cardiol* 41: 1056–1062, 2003.
31. **Kato H, Shichiri M, Marumo F, and Hirata Y.** Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* 138: 2615–2620, 1997.
32. **Kato K, Yin H, Agata J, Yoshida H, Chao L, and Chao J.** Adrenomedullin gene delivery attenuates myocardial infarction and apoptosis after ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 285: H1506–H1514, 2003.
33. **Kawai J, Ando K, Tojo A, Shimosawa T, Takahashi K, Onozato ML, Yamasaki M, Ogita T, Nakaoka T, and Fujita T.** Endogenous adrenomedullin protects against vascular response to injury in mice. *Circulation* 109: 1147–1153 2004.
34. **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.
35. **Kim W, Moon SO, Sung MJ, Kim SH, Lee S, So JN, and Park SK.** Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells. *FASEB J* 13: 1937–1939, 2003.
36. **Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, and Eto T.** Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 192: 553–560, 1993.
37. **Marx M, Perlmutter RA, and Madri JA.** Modulation of platelet-derived growth factor receptor expression in microvascular endothelial cells during in vitro angiogenesis. *J Clin Invest* 93: 131–139, 1994.
38. **McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, and Foord SM.** RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393: 333–339, 1998.
39. **Miller MJ, Martinez A, Unsworth EJ, Thiele CJ, Moody TW, Elsasser T, and Cuttitta F.** Adrenomedullin expression in human tumor cell lines. Its potential role as an autocrine growth factor. *J Biol Chem* 271: 23345–23351, 1996.
40. **Miyao Y, Nishikimi T, Goto Y, Miyazaki S, Daikoku S, Morii I, Matsumoto T, Takishita S, Miyata A, Matsuo H, Kangawa K, and Nonogi H.** Increased plasma adrenomedullin levels in patients with acute myocardial infarction in proportion to the clinical severity. *Heart* 79: 39–44, 1998.
41. **Miyashita K, Itoh H, Sawada N, Fukunaga Y, Sone M, Yamahara K, Yurugi-Kobayashi T, Park K, and Nakao K.** Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both in vitro and in vivo. *FEBS Lett* 544: 86–92, 2003.
42. **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.
43. **Nagae T, Mukoyama M, Sugawara A, Mori K, Yahata K, Kasahara M, Suganami T, Makino H, Fujinaga Y, Yoshioka T, Tanaka I, and Nakao K.** Rat receptor-activity-modifying proteins (RAMPs) for adrenomedullin/CGRP receptor: cloning and upregulation in obstructive nephropathy. *Biochem Biophys Res Commun* 270: 89–93, 2000.
44. **Nagaya N, Kangawa K, Kanda M, Uematsu M, Horio T, Fukuyama N, Hino J, Harada-Shiba M, Okumura H, Tabata Y, Mochizuki N, Chiba Y, Nishioka K, Miyatake K, Asahara T, Hara H, and Mori H.** Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. *Circulation* 108: 889–895, 2003.
45. **Nagaya N, Kyotani S, Uematsu M, Ueno K, Oya H, Nakanishi N, Shirai M, Mori H, Miyatake K, and Kangawa K.** Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. *Circulation* 109: 351–356, 2004.
46. **Nagaya N, Miyatake K, Kyotani S, Nishikimi T, Nakanishi N, and Kangawa K.** Pulmonary vasodilator response to adrenomedullin in patients with pulmonary hypertension. *Hypertens Res* 26 Suppl: S141–S146, 2003.
47. **Nagaya N, Nishikimi T, Horio T, Yoshihara F, Kanazawa A, Matsuo H, and Kangawa K.** Cardiovascular and renal effects of adrenomedullin in rats with heart failure. *Am J Physiol Regul Integr Comp Physiol* 276: R213–R218, 1999.
48. **Nagaya N, Nishikimi T, Uematsu M, Satoh T, Oya H, Kyotani S, Sakamaki F, Ueno K, Nakanishi N, Miyatake K, and Kangawa K.** Hemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart* 84: 653–658, 2000.
49. **Nagaya N, Nishikimi T, Uematsu M, Yoshitomi Y, Miyao Y, Miyazaki S, Goto Y, Kojima S, Kuramochi M, Matsuo H, Kangawa K, and Nonogi H.** Plasma adrenomedullin as an indicator of prognosis after acute myocardial infarction. *Heart* 81: 483–487, 1999.
50. **Nagaya N, Nishikimi T, Yoshihara F, Horio T, Morimoto A, and Kangawa K.** Cardiac adrenomedullin gene expression and peptide accumulation after acute myocardial infarction in rats. *Am J Physiol Regul Integr Comp Physiol* 278: R1019–R1026, 2000.
51. **Nagaya N, Okumura H, Uematsu M, Shimizu W, Ono F, Shirai M, Mori H, Miyatake K, and Kangawa K.** Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am J Physiol Heart Circ Physiol* 285: H2125–H2131, 2003.
52. **Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, and Kangawa K.** Hemodynamic, renal, and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation* 101: 498–503, 2000.
53. **Nakayama M, Takahashi K, Murakami O, Shirato K, and Shibahara S.** Induction of adrenomedullin by hypoxia and cobalt chloride in human colorectal carcinoma cells. *Biochem Biophys Res Commun* 243: 514–517, 1998.
54. **Nikitenko LL, Smith DM, Bicknell R, and Rees MC.** Transcriptional regulation of the CRLR gene in human microvascular endothelial cells by hypoxia. *FASEB J* 17: 1499–501, 2003.
55. **Nishikimi T, Saito Y, Kitamura K, Ishimitsu T, Eto T, Kangawa K, Matsuo H, Omae T, and Matsuoka H.** Increased plasma levels of adrenomedullin in patients with heart failure. *J Am Coll Cardiol* 26: 1424–1431, 1995.
56. **Nishikimi T, Tadokoro K, Mori Y, Wang X, Akimoto K, Yoshihara F, Minamino N, Kangawa K, and Matsuoka H.** Ventricular adrenomedullin system in the transition from LVH to heart failure in rats. *Hypertension* 41: 512–518, 2003.
57. **Nishikimi T, Yoshihara F, Horinaka S, Kobayashi N, Mori Y, Tadokoro K, Akimoto K, Minamino N, Kangawa K, and Matsuoka H.** Chronic administration of adrenomedullin attenuates transition from left ventricular hypertrophy to heart failure in rats. *Hypertension* 42: 1034–1041, 2003.
58. **Nishimatsu H, Suzuki E, Nagata D, Moriyama N, Satonaka H, Walsh K, Sata M, Kangawa K, Matsuo H, Goto A, Kitamura T, and Hirata Y.** Adrenomedullin induces endothelium-dependent vasorelaxation via the phosphatidylinositol 3-kinase/Akt-dependent pathway in rat aorta. *Circ Res* 89: 63–70, 2001.
59. **Nishio K, Akai Y, Murao Y, Doi N, Ueda S, Tabuse H, Miyamoto S, Dohi K, Minamino N, Shoji H, Kitamura K, Kangawa K, and Matsuo H.** Increased plasma concentrations of adrenomedullin correlate with relaxation of vascular tone in patients with septic shock. *Crit Care Med* 25: 953–957, 1997.

60. **Oehler MK, Hague S, Rees MC, and Bicknell R.** Adrenomedullin promotes formation of xenografted endometrial tumors by stimulation of autocrine growth and angiogenesis. *Oncogene* 21: 2815–2821, 2002.
61. **Okumura H, Nagaya N, Itoh T, Okano I, Hino J, Mori K, Tsukamoto Y, Ishibashi-Ueda H, Miwa S, Tambara K, Toyokuni S, Yutani C, and Kangawa K.** Adrenomedullin infusion attenuates myocardial ischemia/reperfusion injury through the phosphatidylinositol 3-kinase/Akt-dependent pathway. *Circulation* 109: 242–248, 2004.
62. **Rademaker MT, Cameron VA, Charles CJ, Lainchbury JG, Nicholls MG, and Richards AM.** Adrenomedullin and heart failure. *Regul Pept* 112: 51–60, 2003.
63. **Rademaker MT, Charles CJ, Cooper GJ, Coy DH, Espiner EA, Lewis LK, Nicholls MG, and Richards AM.** Combined angiotensin-converting enzyme inhibition and adrenomedullin in an ovine model of heart failure. *Clin Sci (Lond)* 102: 653–660, 2002.
64. **Rademaker MT, Charles CJ, Espiner EA, Nicholls MG, and Richards AM.** Long-term adrenomedullin administration in experimental heart failure. *Hypertension* 40: 667–672, 2002.
65. **Rademaker MT, Charles CJ, Lewis LK, Yandle TG, Cooper GJ, Coy DH, Richards AM, and Nicholls MG.** Beneficial hemodynamic and renal effects of adrenomedullin in an ovine model of heart failure. *Circulation* 96: 1983–1990, 1997.
66. **Ribatti D, Guidolin D, Conconi MT, Nico B, Baiguera S, Parnigotto PP, Vacca A, and Nussdorfer GG.** Vinblastine inhibits the angiogenic response induced by adrenomedullin in vitro and in vivo. *Oncogene* 22: 6458–6461, 2003.
67. **Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, Levy PC, Reid LM, Vreim CE, and Williams GW.** Primary pulmonary hypertension : a national prospective study. *Ann Intern Med* 107: 216–223, 1987.
68. **Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, and Gospodarowicz D.** Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* 325: 257–259, 1987.
69. **Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, Ando K, and Fujita T.** Adrenomedullin, an endogenous peptide, counteracts cardiovascular damage. *Circulation* 105:106–111, 2002.
70. **Shindo T, Kurihara Y, Nishimatsu H, Moriyama N, Kakoki M, Wang Y, Imai Y, Ebihara A, Kuwaki T, Ju KH, Minamino N, Kangawa K, Ishikawa T, Fukuda M, Akimoto Y, Kawakami H, Imai T, Morita H, Yazaki Y, Nagai R, Hirata Y, and Kurihara H.** Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. *Circulation* 104: 1964–1971, 2001.
71. **Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, and Imaizumi T.** Augmentation of postnatal neovascularization with autologous bone marrow transplantation. *Circulation* 103: 897–903, 2001.
72. **St George JA.** Gene therapy progress and prospects: adenoviral vectors. *Gene Ther* 10: 1135–1141, 2003.
73. **Sugo S, Minamino N, Shoji H, Kangawa K, Kitamura K, Eto T, and Matsuo H.** Interleukin-1, tumor necrosis factor and lipopolysaccharide additively stimulate production of adrenomedullin in vascular smooth muscle cells. *Biochem Biophys Res Commun* 207: 25–32, 1995.
74. **Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, and Yancopoulos GD.** Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171–1180, 1996.
75. **Suzuki Y, Horio T, Hayashi T, Nonogi H, Kitamura K, Eto T, Kangawa K, and Kawano Y.** Plasma adrenomedullin concentration is increased in patients with peripheral arterial occlusive disease associated with vascular inflammation. *Regul Pept* 118: 99–104, 2004.
76. **Tabata Y and Ikada Y.** Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. *J Pharm Pharmacol* 39: 698–704, 1987.
77. **Tabata Y, Nagano A, and Ikada Y.** Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng* 5: 127–138, 1999.
78. **Tadokoro K, Nishikimi T, Mori Y, Wang X, Akimoto K, and Matsuoka H.** Altered gene expression of adrenomedullin and its receptor system and molecular forms of tissue adrenomedullin in left ventricular hypertrophy induced by malignant hypertension. *Regul Pept* 112: 71–78, 2003.
79. **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.
80. **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 hind limb model. *J Clin Invest* 93: 662–670, 1994.
81. **Tokunaga N, Nagaya N, Shirai M, Tanaka E, Ishibashi-Ueda H, Harada-Shiba M, Kanda M, Ito T, Shimizu W, Tabata Y, Uematsu M, Nishigami K, Sano S, Kangawa K, and Mori H.** Adrenomedullin gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia: benefits of a novel nonviral vector, gelatin. *Circulation* 109: 526–531, 2004.
82. **Totsune K, Takahashi K, Mackenzie HS, Murakami O, Arihara Z, Sone M, Mouri T, Brenner BM, and Ito S.** Increased gene expression of adrenomedullin and adrenomedullin-receptor complexes, receptor-activity modifying protein (RAMP)2 and calcitonin-receptor-like receptor (CRLR) in the hearts of rats with congestive heart failure. *Clin Sci (Lond)* 99: 541–546, 2000.
83. **Wang C, Dobrzynski E, Chao J, and Chao L.** Adrenomedullin gene delivery attenuates renal damage and cardiac hypertrophy in Goldblatt hypertensive rats. *Am J Physiol Renal Physiol* 280: F964–F971, 2001.
84. **Yamasaki M, Kawai J, Nakaoka T, Ogita T, Tojo A, and Fujita T.** Adrenomedullin overexpression to inhibit cuff-induced arterial intimal formation. *Hypertension* 41: 302–307, 2003.
85. **Yin H, Chao L, and Chao J.** Adrenomedullin protects against myocardial apoptosis after ischemia/reperfusion through activation of Akt-GSK signaling. *Hypertension* 43: 109–116, 2004.
86. **Zudaire E, Martinez A, and Cuttitta F.** Adrenomedullin and cancer. *Regul Pept* 112: 175–183, 2003.

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Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia

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Abstract

Objective: Mesenchymal stem cells (MSC) are pluripotent cells that differentiate into a variety of cells including endothelial cells and vascular smooth muscle cells. Although transplantation of bone marrow-derived mononuclear cells (MNC) has already been applied for the treatment of critical limb ischemia, little information is available regarding comparison of the angiogenic potency between MSC and MNC. Accordingly, we injected equal numbers of MSC or MNC in a rat model of hindlimb ischemia and compared their therapeutic potential.
Methods and results: Immediately after creating hindlimb ischemia, rats were randomized to receive MSC transplantation (MSC group), MNC transplantation (MNC group), or vehicle infusion (Control group). Three weeks after transplantation, the laser Doppler perfusion index was significantly higher in the MNC group than in the Control group (0.69 ± 0.1 vs. 0.57 ± 0.06, $P<0.01$). Furthermore, there was a marked improvement in blood perfusion in the MSC group (0.81 ± 0.08). Capillary density was highest in the MSC group. The number of transplanted cell-derived endothelial cells was higher in the MSC group than in the MNC group. Transplanted cell-derived vascular smooth muscle cells were detected only in the MSC group. In vitro, MSC were more tolerant to apoptotic stimulus (serum starvation and hypoxia) than MNC.
Conclusions: MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well under an ischemic environment, and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.
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Keywords: Angiogenesis; Apoptosis; Cell therapy

1. Introduction

Peripheral vascular disease is a major health care problem in an aging society [1]. In the late stages of peripheral vascular disease, progression of tissue hypoperfusion results in ischemic ulceration and gangrene. Unfortunately, amputation is required in more than a third of these patients. Transplantation of bone marrow-derived mononuclear cells (MNC) has been shown to induce therapeutic neovascularization in critical limb ischemia [2–4]. Thus, MNC have been established as a tool for cell therapy. However, MNC transplantation requires harvesting a large

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amount of bone marrow under general anesthesia, which may impose a load on some patients with severe complications such as myocardial ischemia, heart failure, cerebral disease, or renal failure.

Mesenchymal stem cells (MSC), which reside in the bone marrow stroma, possess pluripotency and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [5–7]. MSC have an adherent nature and are expandable in culture. Thus, it would be easy to obtain a sufficient number of MSC for cell therapy. MSC have been shown to form capillary-like structures in an in vitro Matrigel assay [8]. Furthermore, MSC transplantation has been shown to induce neovascularization in a rat model of hindlimb ischemia [9]. These findings suggest that, like MNC, transplantation of MSC may have beneficial effects in patients with critical limb ischemia. However, there has been no study on the therapeutic potency of MSC compared with that of MNC.

Thus, the purposes of this study were (1) to compare the therapeutic potencies of MSC and MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the mechanisms underlying the angiogenic potential of MSC.

2. Methods

2.1. Animal model of hindlimb ischemia

Male Lewis rats weighing 250 to 275 g were used in this study. These isogenic rats served as donors and recipients to simulate autologous implantation of MSC or MNC. To create a hindlimb ischemia model, the left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg i.p.). The distal portion of the saphenous artery and all side branches as well as veins were dissected free and excised. The right hindlimb was kept intact and used as a nonischemic limb. All protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Expansion of MSC and isolation of MNC

MSC expansion was performed as described previously [7]. In brief, we sacrificed male Lewis rats and harvested bone marrow by flushing the cavity of the femurs and tibias with phosphate-buffered saline (PBS). Bone marrow cells were introduced into 100-mm dishes and cultured in α-MEM supplemented with 10% FBS and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. Nonadherent hematopoietic cells were removed and the medium was replaced. The adherent, spindle-shaped MSC population expanded to over 50 million cells at approximately 4 to 5 passages after first culturing the cells. MNC were isolated from whole marrow cells by Ficoll density gradient centrifugation (Lymphoprep, Nycomed) as described previously [3].

2.3. MSC and MNC transplantation

Immediately after resection of the left common iliac artery, 30 rats were randomized to the following three groups: (1) MSC transplantation (MSC group, n=10), (2) MNC transplantation (MNC group, n=10), and (3) PBS injection (Control group, n=10). In each group, 5×10^6 MSC, 5×10^6 MNC or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at five different points.

2.4. Assessment of blood perfusion

A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments) was used to measure serial blood flow over a period of 3 weeks. Low or no blood perfusion was displayed as dark blue, whereas the highest perfusion was displayed as red. After blood flow had been scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification using stored images. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion [10].

Blood flow of the ischemic hindlimb was also assessed by use of colored microspheres 3 weeks after transplantation as reported previously [11,12]. In brief, 2×10^6 microspheres (Dye-Trak, Triton Technology) were injected into the left ventricle under anesthesia and artificial ventilation. Reference blood samples were withdrawn from the right carotid artery at a rate of 0.3 ml/min. Rats were then killed, and muscle samples from the adductor, semimembranous and gastrocnemius muscles were harvested and weighed. These samples were processed according to the manufacturer's instructions, and the absorbance of dye from muscle samples (AU_{muscle}) and reference blood samples (AU_{sample}) was measured with a spectrophotometer. The regional blood flow of ischemic muscle was calculated as $0.3\times(AU_{muscle})/(AU_{sample})$ and presented normalized per 100 g body weight.

2.5. Histological assessment

Rats were killed 3 weeks after transplantation. Four pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase using an indoxyltetrazolium method to detect capillary endothelial cells [3,10]. Five fields from four tissue sections were randomly selected, and the number of capillaries was counted in each field. To avoid overestimation or underestimation of capillary density as a result of myocyte atrophy or interstitial edema, the

capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the three groups [3].

2.6. Monitoring of transplanted MSC or MNC in ischemic hindlimb muscle

An additional 10 rats were used to examine whether transplanted MSC or MNC differentiate into endothelial cells or vascular smooth muscle cells in ischemic muscle. Suspended MSC and MNC were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma Chemical) as reported previously [13]. Red fluorescence-labeled MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in rats (MSC and MNC groups, n=5 each). This subgroup of rats was killed 3 weeks after MSC or MNC transplantation, and frozen tissue sections were obtained from the ischemic muscle. The tissue sections were incubated with anti-von Willebrand factor polyclonal antibody (vWF, DAKO) or anti-α-smooth muscle actin monoclonal antibody (αSMA, DAKO), followed by incubation with Alexa Fluor® 633 IgG antibody (Molecular Probes) or fluorescein isothiocynate (FITC)-conjugated IgG antibody (BD Pharmingen), respectively. Five high power fields (40×) of each section were randomly selected to count the number of transplanted cells, vWF-positive cells, and αSMA-positive cells.

Green fluorescent protein (GFP)-expressing MSC or MNC were also transplanted to examine cell differentiation. MSC and MNC were isolated from male GFP-transgenic rats, which were provided by Dr. Masaru Okabe (Osaka University, Japan) [14,15]. GFP-expressing MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in male Spargue–Dawley rats (MSC group, n=3; MNC group, n=3). Immunohistochemical analysis for vWF was performed 3 weeks after cell transplantation.

2.7. Cell viability assay

MSC or MNC were plated in serum-free medium on 12-well plates (1×10^5 cells per well) and cultured in hypoxic conditions (1% O_2/5% CO_2/94% N_2) for 24 h.

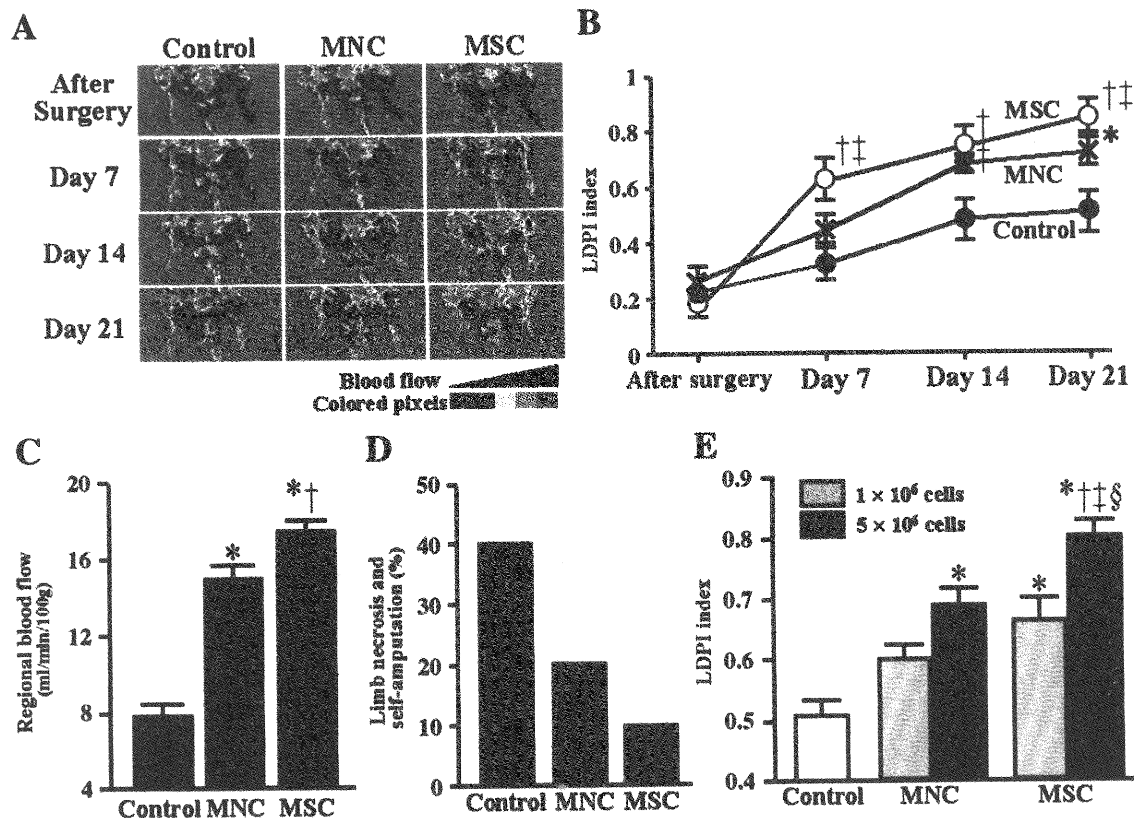

Fig. 1. (A) Representative examples of serial LDPI. Blood perfusion of the ischemic hindlimb markedly increased in the MSC group 3 weeks after transplantation (red to orange). (B) Quantitative analysis of hindlimb blood perfusion. LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion. The increase in the LDPI index was not significant in the Control group. LDPI index was significantly higher in the MNC and MSC groups than in the Control group 3 weeks after surgery. LDPI index in the MSC group was highest among the three groups. Data are mean±S.E.M. *P<0.05 and †P<0.01 vs. Control; ‡P<0.01 vs. MNC. (C) Blood flow assessment of ischemic muscle by use of microspheres. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.05 vs. MNC. (D) Incidence of limb necrosis and self-amputation 3 weeks after transplantation. (E) Comparison between the number of transplanted cells and perfusion recovery of the ischemic hindlimb. LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.01 vs. 1×10^6 MNC; ‡P<0.05 vs. 5×10^6 MNC; §P<0.05 vs. 1×10^6 MSC.

Hypoxic conditions were obtained by the use of a CO_2/multigas incubator (ASTEC). After exposure to serum-free hypoxia, the cells were suspended using trypsin and mixed with 0.4% trypan blue solution (Sigma Chemical). Percentages of viable cells were evaluated by two blinded observers and normalized to the total cell number in the field.

2.8. In vitro apoptosis assay

Terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation) was performed to evaluate apoptosis of MSC or MNC induced by the combination of serum starvation and hypoxia. After incubation for 24 h, MSC or MNC were fixed in 1% paraformaldehyde, and TUNEL was performed for detection of apoptotic nuclei according to the manufacturer's instructions. The cells were then mounted in medium containing 4′,6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields (n=5) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

2.9. Western blot analysis of hypoxia-inducible factor (HIF)

To identify the protein expression of HIF-1α, Western blotting was performed using a mouse monoclonal antibody raised against HIF-1α (clone H1α67, Novus Biologicals). Serum-starved MSC or MNC were cultured in normoxic or hypoxic conditions for 24 h. Cells were homogenized on ice in 150 μl lysis buffer with a protease inhibitor. Then 15 μg of protein was transferred to sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane. After being blocked for 60 min, the membrane was incubated with a primary antibody in blocking buffer (1:1000), followed by incubation with a peroxidase-conjugated secondary antibody. Positive protein bands were visualized by chemiluminesence using an ECL kit (Amersham). Western blot analysis using a monoclonal antibody raised against β-actin (Sigma Chemical) was used as a protein loading control.

2.10. In vitro tube formation assay

In vitro Matrigel assay was performed to investigate whether MSC or MNC induce tube formation under the condition of serum-free hypoxia [6,8]. MSC or MNC were plated in serum-free medium on 12-well plates (1×10^6 cells per well) coated with Matrigel (Becton Dickinson). After 6-h incubation in hypoxia, tube formation was examined with a phase-contrast microscope.

2.11. Measurements of angiogenic factors

To compare the secretion of angiogenic factors from MSC with that from MNC, a total of 1×10^6 MSC or MNC were plated in serum-free medium on 6-well plates. After 24-h incubation, the conditioned medium was

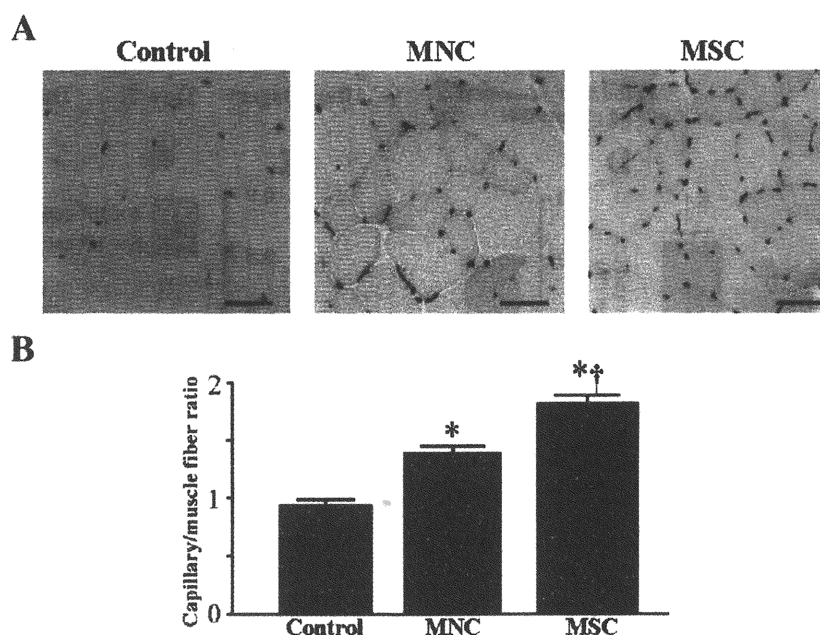

Fig. 2. (A) Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. In the MNC and MSC groups, the number of capillaries markedly increased compared with the Control group. (B) Quantitative analysis of capillary density in ischemic hindlimb muscles. Capillary density is shown as capillary/muscle fiber ratio. The capillary/muscle fiber ratio of ischemic hindlimb muscle was highest in the MSC group, followed by the MNC group and Control group. Data are mean±S.E.M. *P<0.01 vs. Control; †P<0.01 vs. MNC. Scale Bars: 50 μm.

collected and, levels of VEGF, basic FGF (bFGF), and stromal cell-derived factor-1α (SDF-1α) were measured using enzyme immunoassay kits (Quantikine, R&D Systems).

2.12. Statistical analysis

All values are expressed as mean±S.E.M. Comparisons of parameters among the three groups were made by one-way ANOVA followed by Scheffe's multiple comparison test. Student's unpaired *t*-test was used to compare differences between two groups. A probability value <0.05 was considered statistically significant.

3. Results

3.1. Blood perfusion of ischemic hindlimb after transplantation

Blood perfusion of the ischemic hindlimb was considerably impaired 3 weeks after surgery (Control group, Fig. 1A). On the other hand, improvement of hindlimb ischemia was observed in the MNC and MSC groups compared with the Control group. Hindlimb ischemia was markedly improved in the MSC group. Quantitative analysis demonstrated that the LDPI index was highest in the MSC group, followed by the MNC group and

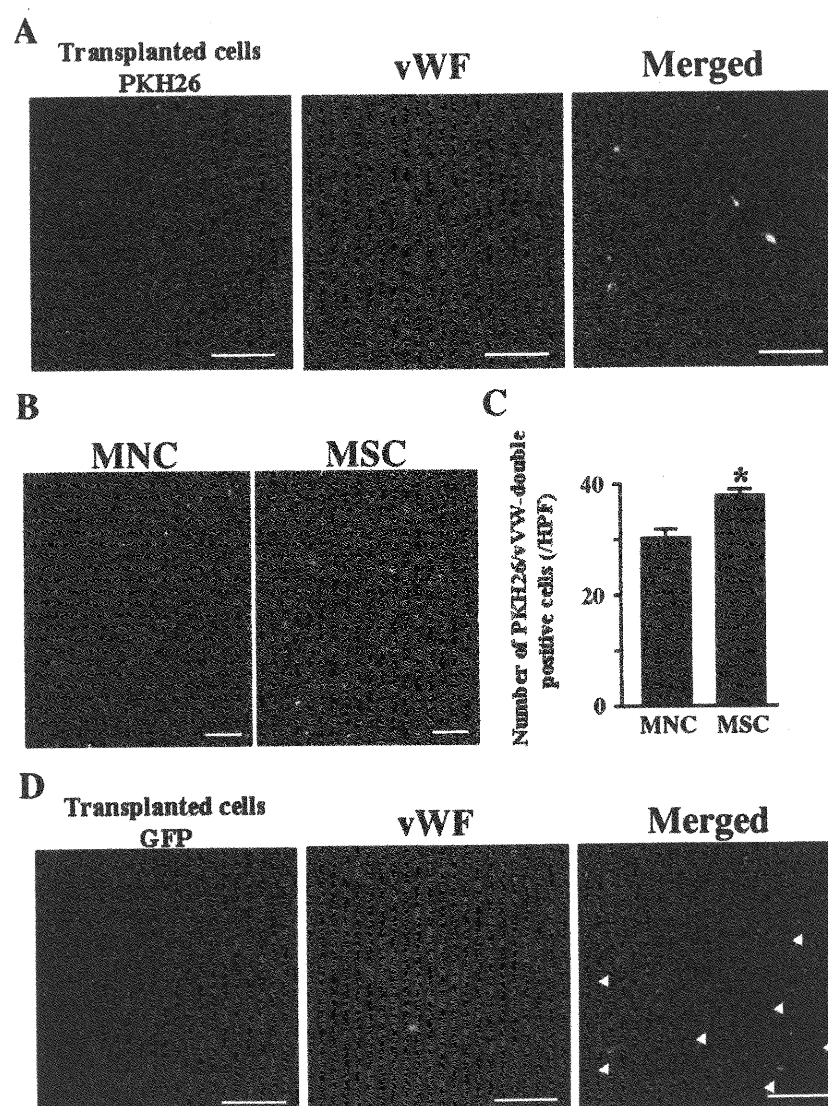

Fig. 3. Endothelial differentiation of transplanted cells. (A) Incorporation and differentiation of transplanted MSC in ischemic thigh muscle. Red fluorescence (PKH26)-labeled MSC were incorporated into interstitial tissues between muscle fibers and were positive for vWF (blue). (B) PKH26/vWF-double-positive cells (pink) were frequently observed in the MSC group. (C) The number of PKH26/vWF-double-positive cells (MNC-derived endothelial cells) was significantly higher in the MSC group than in the MNC group. Data are mean±S.E.M. *$P<0.01$ vs. MNC. Bars: 50 μm. (D) Representative photographs of MSC-derived vascular structures. Green fluorescent protein (GFP)-expressing MSC were transplanted into ischemic thigh muscle. GFP (green)/vWF (red)-double positive cells (orange, arrows) were observed 3 weeks after transplantation. Bars: 50 μm.

Control group (Fig. 1B). The regional blood flow of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 1C). The incidence of limb necrosis and self-amputation of ischemic hindlimb was minimal in the MSC group, followed by the MNC group and Control group (Fig. 1D).

To examine the relationship between the number of transplanted cells and their angiogenic potential, a smaller number of MSC or MNC (1×10^6 cells per animal) was transplanted into the ischemic thigh muscle ($n=10$ each). Transplantation of 1×10^6 MNC tended to improve blood perfusion of the ischemic hindlimb 3 weeks after transplantation, but these changes did not reach statistical significance. On the other hand, transplantation of 1×10^6 MSC significantly improved hindlimb ischemia. The LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation (Fig. 1E).

3.2. Capillary density of ischemic hindlimb

A large number of capillaries were detected in the ischemic muscle of the MSC and MNC groups (Fig. 2A). Importantly, quantitative analysis demonstrated that the capillary/muscle fiber ratio of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 2B).

3.3. Endothelial differentiation of transplanted cells

Red fluorescence-labeled MSC and MNC were detected in the interstitial tissues between muscle fibers 3 weeks after transplantation (Fig. 3A). PKH26-positive cells expressed vWF, an endothelial marker, in both the MSC and MNC groups. Importantly, PKH26/vWF-double-positive cells were frequently observed in the MSC group (Fig. 3B). Quantitative analysis demonstrated that the number of PKH26/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (Fig. 3C). GFP-expressing MSC and MNC were also detected in the ischemic muscle and were positive for vWF 3 weeks after transplantation (Fig. 3D). The number of GFP/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (data not shown).

3.4. Differentiation of transplanted cells into vascular smooth muscle cells

Some of the transplanted MSC were positive for αSMA, a marker of vascular smooth muscle cells. They participated in the formation of vascular structures as mural cells. In contrast, none of the MNC was stained by αSMA (Fig. 4).

3.5. Cell survival under serum starvation and hypoxia

After 24-h incubation under serum-free and hypoxic conditions, the majority of cultured MNC revealed cytoplasmic shrinkage, disintegration into small vesicles, and membrane blebbing (Fig. 5A). In contrast, these morphological changes were rarely observed in MSC. The percentage of dead cells, assessed by the trypan blue exclusion test, was significantly higher in MNC than in MSC (Fig. 5B). TUNEL staining showed that serum-free hypoxia markedly induced MNC apoptosis, whereas only a small number of MSC were TUNEL-positive (Fig. 5C). The

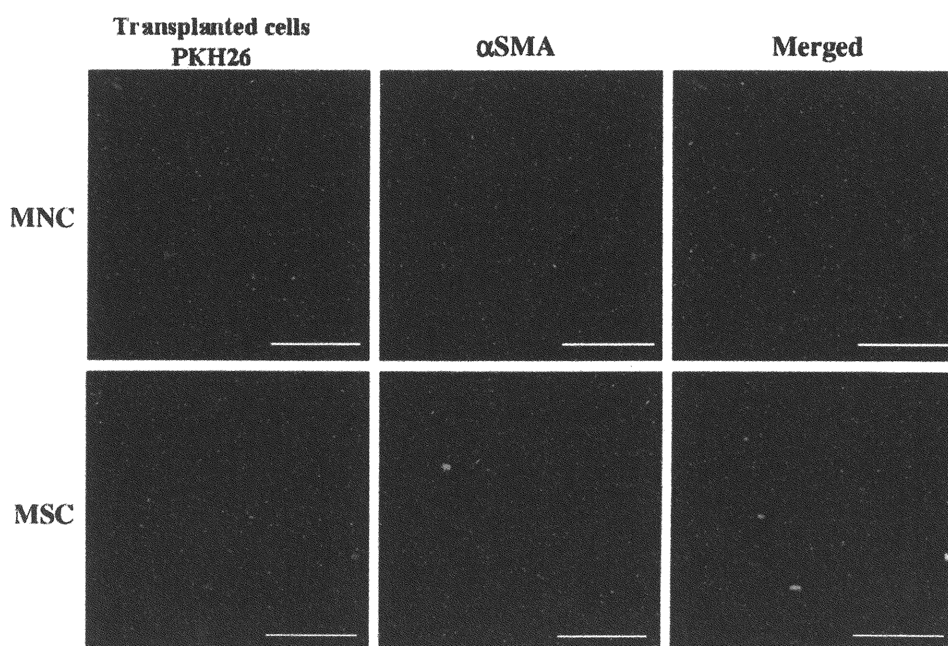

Fig. 4. Differentiation of transplanted cells into vascular smooth muscle cells. Red fluorescence (PKH26)-labeled MSC were stained by αSMA, whereas transplanted MNC did not express αSMA. Bars: 50 μm.