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.

GRANTS

This work was supported by the Research Grant for Cardiovascular Disease (16C-6) from the Ministry of Health, Labor and Welfare, Industrial Technology Research Grant Program in 2003 from New Energy and Industrial Technology Development Organization of Japan, Health and Labor Sciences Research Grants-Genome 005, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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.

ELSEVIER

Cardiovascular Research 66 (2005) 543–551

Cardiovascular Research

www.elsevier.com/locate/cardiores

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

Takashi Iwase[a,b], Noritoshi Nagaya[a,*], Takafumi Fujii[c], Takefumi Itoh[a], Shinsuke Murakami[a], Toshio Matsumoto[b], Kenji Kangawa[d], Soichiro Kitamura[e]

[a]*Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[b]*Department of Medicine and Bioregulatory Sciences, University of Tokushima Graduate School of Medicine, Tokushima, Japan*
[c]*Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan*
[d]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan*
[e]*Department of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan*

Received 23 September 2004; received in revised form 3 February 2005; accepted 7 February 2005
Available online 2 March 2005
Time for primary review 19 days

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.

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

* Corresponding author. Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.
E-mail address: nnagaya@ri.ncvc.go.jp (N. Nagaya).

0008-6363/$ - see front matter © 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.cardiores.2005.02.006

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.

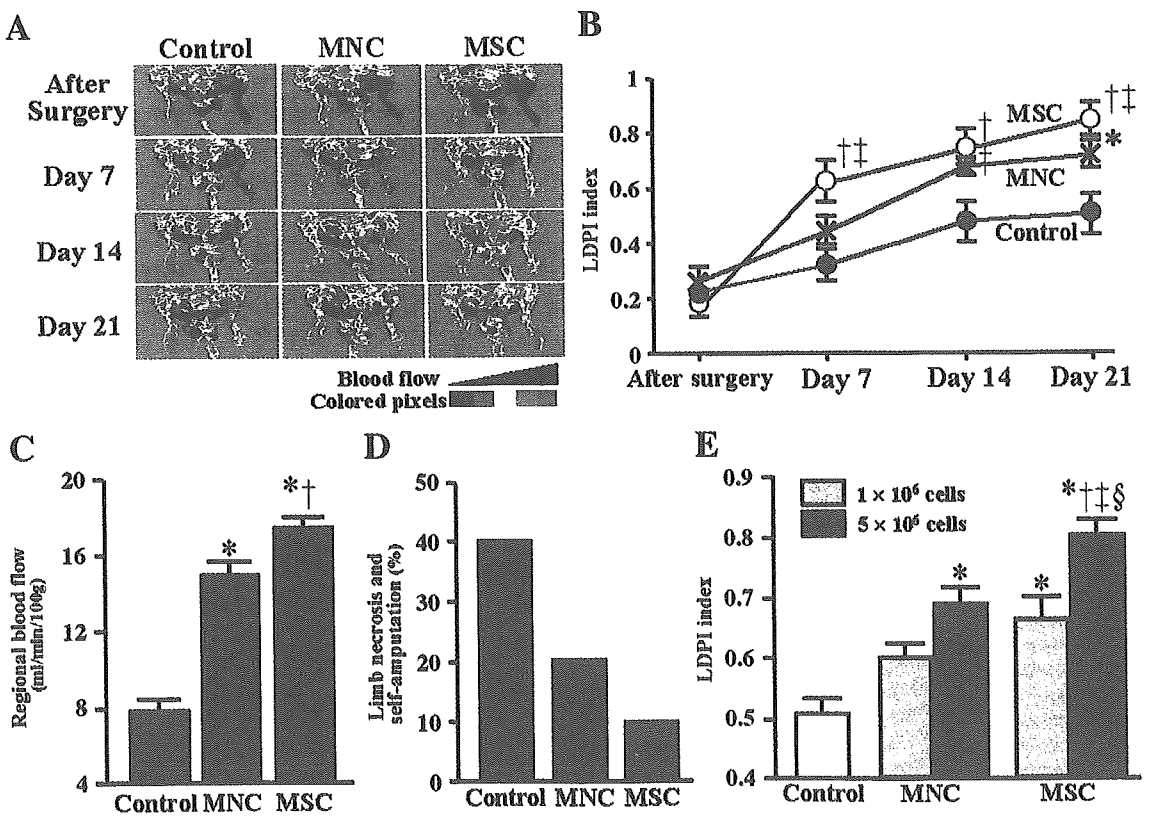

Fig. 1. (A) Representative examples of serial LDPI. Blood perfusion of the ischemic hindlimb markedly increased in the MSC group 3 weeks after transplantation (red to orange). (B) Quantitative analysis of hindlimb blood perfusion. LDPI index was determined as the ratio of ischemic to nonischemic hindlimb 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

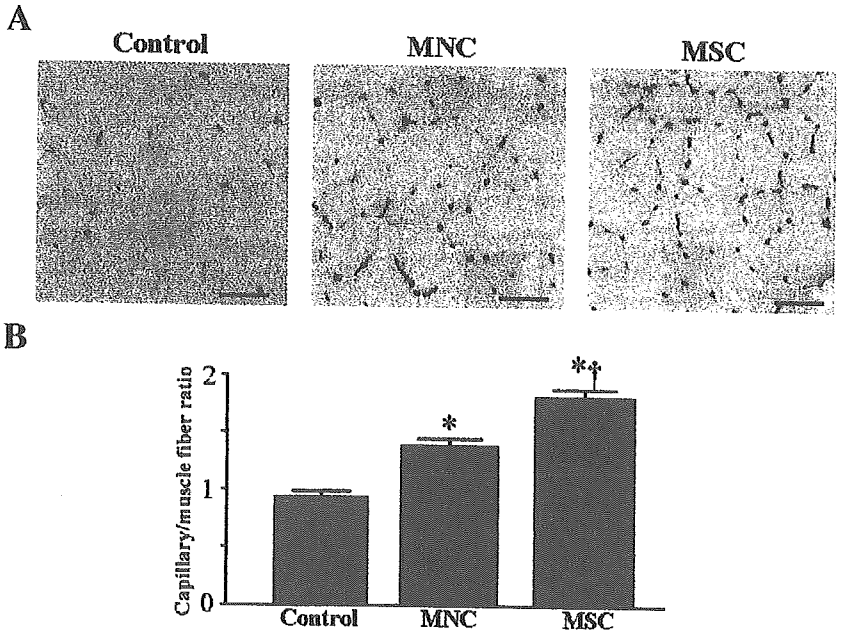

Fig. 2. (A) Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. In the MNC and MSC groups, the number of capillaries markedly increased compared with the Control group. (B) Quantitative analysis of capillary density in ischemic hindlimb muscles. Capillary density is 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

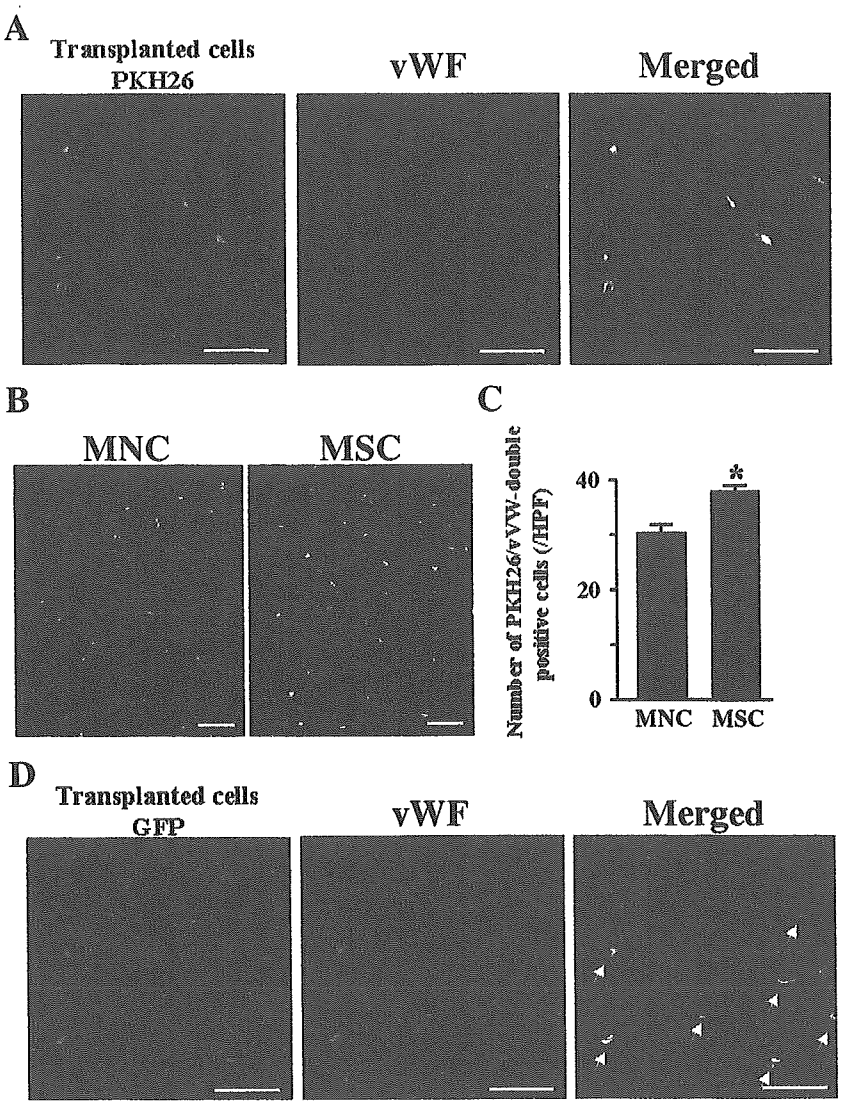

Fig. 3. Endothelial differentiation of transplanted cells. (A) Incorporation and differentiation of transplanted MSC in ischemic thigh muscle. Red fluorescence (PKH26)-labeled MSC were incorporated into interstitial tissues between muscle fibers and were positive for vWF (blue). (B) PKH26/vWF-double-positive 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

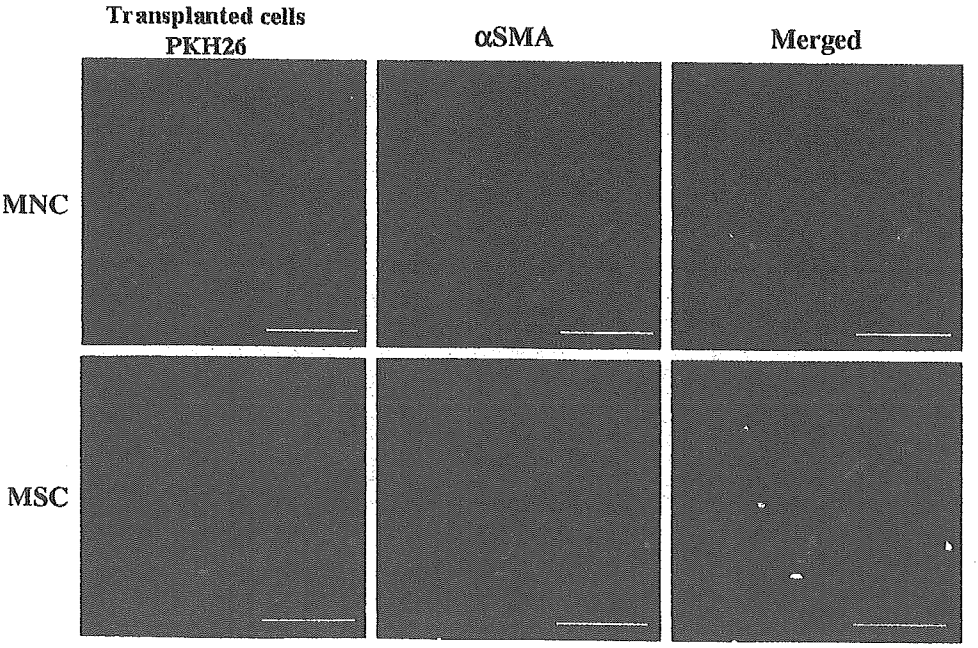

Fig. 4. Differentiation of transplanted cells into vascular smooth muscle cells. Red fluorescence (PKH26)-labeled MSC were stained by αSMA, whereas transplanted MNC did not express αSMA. Bars: 50 μm.

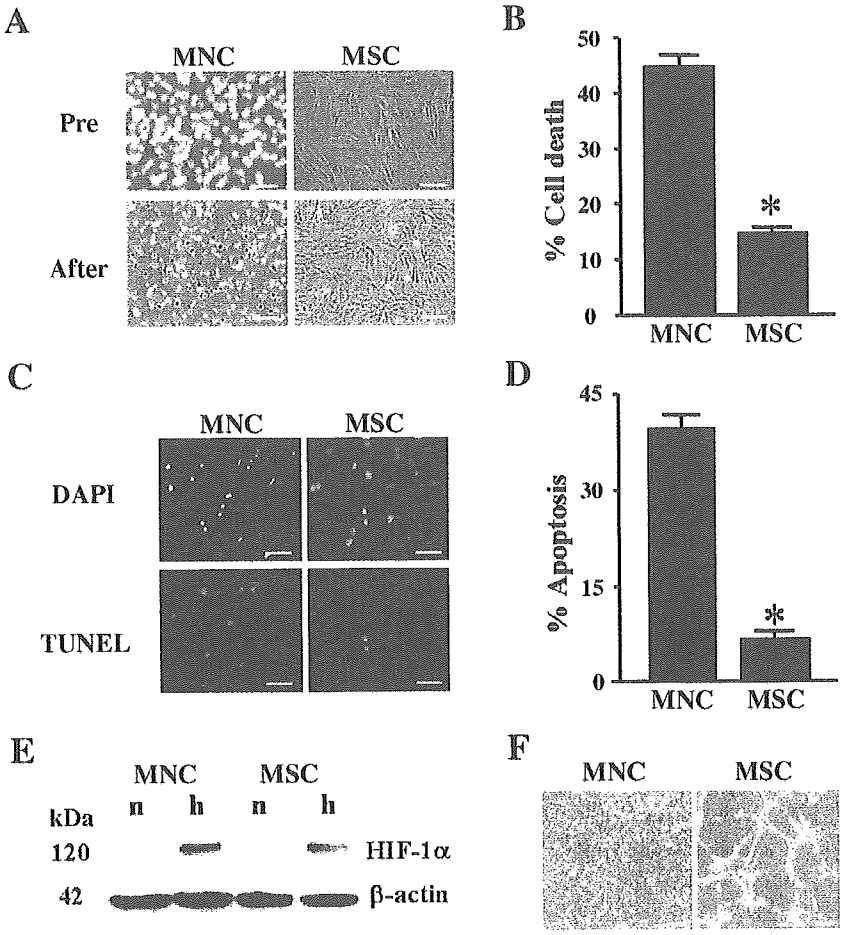

Fig. 5. (A–D) In vitro cell viability assay and apoptosis assay. (A) Representative photographs of MSC and MNC. (B) Quantitative analysis of cell viability by trypan blue staining. The percentage of dead cells in MSC was significantly lower than that in MNC. (C) Representative photographs of apoptotic MSC and MNC. Apoptosis of MSC or MNC was detected by TUNEL staining (green). Nuclei were stained with DAPI (blue). Serum starvation and hypoxia substantially induced MNC apoptosis. (D) Quantitative analysis of TUNEL-positive cells. The percentage of TUNEL-positive cells in MNC was significantly higher than that in MSC. Data are mean±S.E.M. *$P<0.01$ vs. Control. (E) Western blot analysis for hypoxia-inducible factor (HIF)-1α and β-actin. The expression of HIF-1α protein was not detected in MSC and MNC under the condition of normoxia (n). However, HIF-1α protein was expressed in both cell types after exposure to serum-free hypoxia (h). (F) Representative photographs of in vitro Matrigel assay. After 6-h incubation in serum-free hypoxia, MSC formed typical tube-like structures. In contrast, MNC did not show any morphological change. Bars: 100 μm.

percentage of TUNEL-positive cells was significantly higher in MNC than in MSC (Fig. 5D). The expression of HIF-1α protein was observed in both MSC and MNC under serum-free and hypoxic conditions (Fig. 5E). The ratios of HIF-1α/β-actin did not significantly differ between MSC and MNC (data not shown).

3.6. Tube formation under serum starvation and hypoxia

After 6-h incubation on Matrigel, tube formation was observed in MSC, whereas MNC did not show any morphological change (Fig. 5F).

3.7. Secretion of angiogenic factors from MSC and MNC

VEGF and bFGF were detected in conditioned medium of cultured MSC and MNC. Compared with MNC, MSC secreted significantly greater amounts of VEGF and bFGF (VEGF: 817±36 vs. 188±32 pg/10^6 cells, $P<0.01$; bFGF: 47±5 vs. 4±1 pg/10^6 cells, $P<0.01$). Although SDF-1α was not detected in conditioned medium of MNC, MSC secreted a large amount of SDF-1α (17±1 ng/10^6 cells).

4. Discussion

In the present study, we demonstrated that (1) transplantation of MSC as well as MNC induced angiogenesis in a rat model of hindlimb ischemia, (2) the extent of neovascularization was significantly greater in MSC transplantation than in MNC transplantation, (3) transplanted MSC highly differentiated into endothelial cells compared with transplanted MNC, and (4) only MSC differentiated into vascular smooth muscle cells in ischemic tissue. We also demonstrated in vitro that (5) MSC were more tolerant to an ischemic stimulus than

MNC and that (6) MSC secreted large amounts of angiogenic factors compared with the amounts secreted by MNC.

Earlier studies have shown that MNC transplantation enhances neovascularization by supplying endothelial progenitor cells and multiple angiogenic factors such as VEGF, bFGF, and angiopoietin-1 [3,4,16]. In fact, MNC transplantation significantly augmented blood perfusion and capillary density in the ischemic hindlimb in the present study. Other studies have shown that transplanted MSC differentiate into endothelial cells, secrete angiogenic factors, and thereby induce neovascularization in ischemic tissue [9,17,18]. However, it remains unclear whether the angiogenic potency of MSC transplantation is comparable or superior to that of MNC transplantation. In the present study, we injected equal numbers of MSC or MNC into ischemic muscle to compare the therapeutic effects of the two types of cells. Interestingly, MSC transplantation markedly increased blood perfusion and capillary density in the ischemic hindlimb compared with MNC transplantation. Moreover, perfusion recovery of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. These results suggest that MSC transplantation is more potent in therapeutic angiogenesis than MNC transplantation.

The underlying mechanisms responsible for the superiority of MSC in therapeutic angiogenesis remain unknown. Earlier studies have shown that many transplanted cells undergo apoptosis immediately after transplantation because of a lack of oxygen and nutrition, although they should survive for a sufficiently long period to induce angiogenesis [3,19]. In fact, the present study showed in vitro that MNC readily underwent cell death and apoptosis under conditions of serum starvation and hypoxia. These findings raise the possibility that the therapeutic potency of transplanted MNC is considerably attenuated by an ischemic environment. In contrast, MSC survived well under these conditions. Thus, MSC may be more appropriate for cell transplantation with respect to cell survival than MNC.

The present study showed that transplanted MSC and MNC participated in vascular structures and expressed vWF, an endothelial cell marker. The number of MSC-derived vWF-positive cells in ischemic muscle was significantly higher than that of MNC-derived vWF-positive cells. Previous studies have shown that both transplanted MSC and MNC are capable of differentiating into endothelial cells in ischemic tissue [3,9]. However, the present study showed that a combination of serum starvation and hypoxia greatly reduced MNC viability. Furthermore, only MSC induced tube formation in serum-free and hypoxic conditions. Taking these results together, it is interesting to speculate that transplanted MSC survive well and differentiate into endothelial cells in an ischemic environment and thereby induce angiogenesis more efficiently than transplanted MNC.

During the process of neovascularization, vascular smooth muscle cells play an important role in vessel maturation [20,21]. In the present study, none of the transplanted MNC expressed αSMA, which is consistent with recent findings that MNC-derived CD34-positive cells rarely expressed a vascular smooth muscle cell marker and highly differentiated into endothelial cells in ischemic muscle [22]. On the other hand, earlier studies have shown that MSC readily acquire vascular smooth muscle properties in vitro and that transplanted MSC differentiate into vascular smooth muscle cells in ischemic tissue [9,23]. The present study also demonstrated that some transplanted MSC were positive for αSMA, a vascular smooth muscle cell marker, and formed vascular structures as mural cells. Thus, unlike MNC, transplanted MSC may contribute to vessel maturation.

Recent studies have demonstrated that the angiogenic potential of MSC and MNC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [4,16–18]. The present study demonstrated that MSC secreted large amounts of VEGF and bFGF compared with the amounts secreted by MNC. Interestingly, only MSC significantly secreted SDF-1α, which also has been shown to induce angiogenesis in vivo and in vitro [24,25]. These findings suggest that MSC transplantation induces angiogenesis more efficiently than MNC transplantation partly through the release of angiogenic factors.

From a clinical standpoint, MNC transplantation is considered to be an established procedure that is easy to implement without any immunosuppressive agents and expensive facilities [2,4,26–31]. In contrast, MSC transplantation requires time and considerable cost to obtain an adequate number of MSC under strictly aseptic conditions. Nevertheless, MSC are an attractive source for cell therapy because they are easily isolated from a small amount of bone marrow and rapidly expand in culture. Thus, MSC transplantation may be one of the most attractive cell therapies in the treatment of critical limb ischemia.

In conclusion, MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well in 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.

Acknowledgements

We thank Dr. Masaru Okabe for providing us with GFP-transgenic rats. This work was supported by the Research Grant for Cardiovascular Disease (16C-6) from the Ministry of Health, Labor and Welfare, the Industrial Technology Research Grant Program in '03 from the New Energy and

Industrial Technology Development Organization (NEDO) of Japan, Health and Labor Sciences Research Grants-genome 005, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

References

[1] Ouriel K. Peripheral arterial disease. Lancet 2001;358:1257–64.
[2] Higashi Y, Kimura M, Hara K, Noma K, Jitsuiki D, Nakagawa K, et al. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. Circulation 2004;109:1215–8.
[3] Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001;103:897–903.
[4] Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, 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 2002;360:427–35.
[5] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
[6] Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002;109:337–46.
[7] Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995;18:1417–26.
[8] Annabi B, Lee YT, Turcotte S, Naud E, Desrosiers RR, Champagne M, et al. Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. Stem Cells 2003;21:337–47.
[9] Al-Khaldi A, Al-Sabti H, Galipeau J, Lachapelle K. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. Ann Thorac Surg 2003;75:204–9.
[10] Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–36.
[11] Mortola JP, Merazzi D, Naso L. Blood flow to the brown adipose tissue of conscious young rabbits during hypoxia in cold and warm conditions. Pflugers Arch 1999;437:255–60.
[12] Smith Jr RS, Lin KF, Agata J, Chao L, Chao J. Human endothelial nitric oxide synthase gene delivery promotes angiogenesis in a rat model of hindlimb ischemia. Arterioscler Thromb Vasc Biol 2002;22:1279–85.
[13] Messina LM, Podrazik RM, Whitehill TA, Ekhterae D, Brothers TE, Wilson JM, et al. Adhesion and incorporation of lacZ-transduced endothelial cells into the intact capillary wall in the rat. Proc Natl Acad Sci U S A 1992;89:12018–22.
[14] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313–9.
[15] Ohta M, Suzuki Y, Noda T, Ejiri Y, Dezawa M, Kataoka K, et al. Bone marrow stromal cells infused into the cerebrospinal fluid promote functional recovery of the injured rat spinal cord with reduced cavity formation. Exp Neurol 2004;187:266–78.
[16] Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, et al. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 2001;104:1046–52.
[17] Al-Khaldi A, Eliopoulos N, Martineau D, Lejeune L, Lachapelle K, Galipeau J. Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo. Gene Ther 2003;10:621–9.
[18] Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 2004;109:1543–9.
[19] Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. J Mol Cell Cardiol 2001;33:907–21.
[20] Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, et al. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J 2002;21:1939–47.
[21] Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, et al. VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. Circ Res 2003;92:1098–106.
[22] Pesce M, Orlandi A, Iachininoto MG, Straino S, Torella AR, Rizzuti V, et al. Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues. Circ Res 2003;93:e51–62.
[23] Kashiwakura Y, Katoh Y, Tamayose K, Konishi H, Takaya N, Yuhara S, et al. Isolation of bone marrow stromal cell-derived smooth muscle cells by a human SM22alpha promoter: in vitro differentiation of putative smooth muscle progenitor cells of bone marrow. Circulation 2003;107:2078–81.
[24] Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, 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-1alpha. Am J Pathol 1999;154:1125–35.
[25] Salvucci O, Yao L, Villalba S, Sajewicz A, Pittaluga S, Tosato G. Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. Blood 2002;99:2703–11.
[26] Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002;106:3009–17.
[27] Fuchs S, Satler LF, Kornowski R, Okubagzi P, Weisz G, Baffour R, et al. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study. J Am Coll Cardiol 2003;41:1721–4.
[28] Hamano K, Nishida M, Hirata K, Mikamo A, Li TS, Harada M, et al. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. Jpn Circ J 2001;65:845–7.
[29] Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 2003;107:2294–302.
[30] Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002;106:1913–8.
[31] Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet 2003;361:47–9.

Adrenomedullin Regenerates Alveoli and Vasculature in Elastase-induced Pulmonary Emphysema in Mice

Shinsuke Murakami, Noritoshi Nagaya, Takefumi Itoh, Takashi Iwase, Toshiya Fujisato, Keisuke Nishioka, Kaoru Hamada, Kenji Kangawa, and Hiroshi Kimura

Departments of Regenerative Medicine and Tissue Engineering, Biochemistry, and Internal Medicine, National Cardiovascular Center Research Institute, Osaka; Second Department of Internal Medicine, Nara Medical University, Nara; and Department of Transfusion Medicine, Hyogo College of Medicine, Hyogo, Japan

Rationale: **Adrenomedullin, a potent vasodilator peptide, regulates cell growth and survival. However, whether adrenomedullin contributes to lung regeneration remains unknown.** *Objectives:* **To investigate whether adrenomedullin influences the kinetics of bone marrow cells, and whether adrenomedullin promotes regeneration of alveoli and vasculature and thereby improves lung structure and function in elastase-induced emphysema in mice.** *Methods:* **Adrenomedullin or vehicle was randomly administered to C57BL/6 mice for 5 days. We counted the numbers of mononuclear cells and stem cell antigen-1–positive cells in circulating blood. After intratracheal injection of elastase or saline, mice were randomized to receive continuous infusion of adrenomedullin or vehicle for 14 days. Functional and histologic analyses were performed 28 days after treatment.** *Results:* **Twenty-eight days after elastase injection, destruction of the alveolar walls was observed. However, adrenomedullin infusion significantly inhibited the increase in lung volume, static lung compliance, and mean linear intercept in mice given elastase. Adrenomedullin increased the numbers of mononuclear cells and stem cell antigen-1–positive cells in circulating blood. Adrenomedullin significantly increased the number of bone marrow–derived cells incorporated into the elastase-treated lung. Some of these cells were positive for cytokeratin or von Willebrand factor. Infusion of adrenomedullin after the establishment of emphysema also had beneficial effects on lung structure and function.** *In vitro,* **addition of adrenomedullin attenuates elastase-induced cell death in alveolar epithelial cells and endothelial cells.** *Conclusions:* **Adrenomedullin improved elastase-induced emphysema at least in part through mobilization of bone marrow cells and the direct protective effects on alveolar epithelial cells and endothelial cells.**

Keywords: bone marrow cells; differentiation; mobilization; regeneration

Pulmonary emphysema, a major cause of respiratory dysfunction and death worldwide, is defined as abnormal permanent enlargement of airspaces distal to terminal bronchioles (1–3). Because many mediators with overlapping actions are involved in pulmonary emphysema, there is no effective treatment that prevents the progression of this disease. Destruction of the alveolar walls, one of the pathologic changes in pulmonary emphysema, had been considered irreversible. However, recent studies have demonstrated that bone marrow cells are mobilized into the peripheral blood to be involved in regeneration of alveoli (4, 5). Thus lung regeneration by bone marrow cells may be desirable for the treatment of pulmonary emphysema.

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (6). Immunoreactive AM has subsequently been detected in a variety of tissues, including the lungs (7, 8). The AM receptor has been shown to be expressed strongly in the basal cells of the airway epithelium and type II pneumocytes, both of which are involved in epithelial regeneration of the lung (9). Recent studies have shown that AM activates the phosphatidylinositol 3-kinase (PI3K)/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in cell growth (10–13). These findings raise the possibility that AM may play an important role in the regulation of pulmonary homeostasis. However, whether AM is involved in lung regeneration remains unknown.

Thus the purposes of the present study were to (*1*) investigate whether infusion of AM influences the kinetics of bone marrow cells, (*2*) investigate the direct effects of AM on alveolar epithelial cells and endothelial cells, and (*3*) examine whether AM promotes regeneration of alveoli and vasculature in the lung and thereby improves lung structure and function in elastase-induced emphysema in mice.

METHODS

Animal Model

We used 10-week-old female C57BL/6 mice. To investigate whether AM influences the kinetics of bone marrow cells, AM or saline was randomly administered to 48 mice. Transgenic mice (C57BL/6 background) that ubiquitously express green fluorescent protein (GFP) were provided by Prof. Masaru Okabe (Osaka University, Japan) (14). To assess the kinetics of bone marrow cells, 10 GFP-positive bone marrow chimeric mice were established. Four weeks after bone marrow transplantation, the chimeric mice were given intratracheal injection of porcine pancreatic elastase (200 units/kg; Sigma, St. Louis, MO). An additional 42 wild-type mice were used to examine whether AM improves lung structure and function in elastase-induced emphysema. Finally, 10 GFP-positive bone marrow chimeric mice and 42 wild-type mice were used to examine the effects of AM on established emphysema. All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute.

AM Preparation and Administration

Recombinant human AM was obtained from Shionogi Co., Ltd., Osaka, Japan. The homogeneity of AM was confirmed by reverse-phase, high-performance liquid chromatography and amino acid analysis. Forty-two wild-type mice were randomly given intratracheal injection of either elastase or saline and assigned to receive continuous infusion of AM or vehicle. AM was administered by a subcutaneous osmotic minipump (Alzet minipumps #1002; Durect Corp., Cupertino, CA) with a delivery rate of 0.05 μg/kg/minute for 14 days. This protocol resulted in the creation of three groups: sham mice given vehicle (sham group, n =

(*Received in original form September 26, 2004; accepted in final form June 3, 2005*)

Supported by the Research Grant for Cardiovascular Disease (16C-6) from the Ministry of Health, Labor and Welfare, the Industrial Technology Research Grant Program in 2003 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, Health and Labor Sciences Research Grants-genome 005, and the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

Correspondence and requests for reprints should be addressed to Noritoshi Nagaya, M.D., Department of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail: nnagaya@ri.ncvc.go.jp

Am J Respir Crit Care Med Vol 172. pp 581–589, 2005
Originally Published in Press as DOI: 10.1164/rccm.200409-1280OC on June 9, 2005
Internet address: www.atsjournals.org

14), elastase mice given vehicle (vehicle group, n = 14), and elastase mice treated with AM (AM group, n = 14). Ten GFP-positive bone marrow chimeric mice were divided into two groups: elastase mice given vehicle and elastase mice treated with AM (n = 5 each).

To investigate the effects of AM on established emphysema, the mice were randomly given intratracheal injection of either elastase or saline according to a previously described protocol by Massaro and colleagues (15). Twenty-five days after elastase or saline injection, the mice received continuous infusion of AM or vehicle for 12 days (sham, vehicle, and AM groups, n = 14 each). These mice were evaluated on Day 37. In addition, 10 GFP-positive chimeric mice were investigated by a similar protocol to assess the kinetics of bone marrow cells.

Isolation of Mononuclear Cells and Flow Cytometric Analysis

AM (1, 2, or 5 μg in 100 μl saline) was randomly administered to C57BL/6 mice (n = 8 each) by intraperitoneal injection daily for 5 days. Control mice received 100 μl saline according to the same schedule (n = 8). In addition, AM or vehicle was administered to other mice (n = 8 each) by a subcutaneous osmotic minipump with a delivery rate of 0.05 μg/kg/minute for 5 days. Blood samples were obtained on Day 5 and mononuclear cells were separated using Histopaque-1083 (Sigma), as described previously (16). The mononuclear cells were counted manually and analyzed for the expression of stem cell antigen-1 (Sca-1)–fluorescein isothiocyanate (BD Pharmingen, San Diego, CA). Immunofluorescence-labeled cells were analyzed by quantitative flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). An isotype-identical antibody (BD Pharmingen) served as a control.

Bone Marrow Chimeric Mice

Bone marrow chimeric mice were created as described previously (17). In brief, wild-type recipient C57BL/6 mice were lethally irradiated (900 cGy) and transplanted with GFP mouse-derived bone marrow cells (3×10^6 cells/300 μl) via the tail vein. To quantify the reconstitution of mouse bone marrow, peripheral blood mononuclear cells and bone marrow cells were analyzed by flow cytometry 4 weeks after bone marrow transplantation.

Assessment of Bone Marrow Cell Homing and Differentiation

Cryosections were obtained from the lungs of GFP-positive chimeric mice 28 days after elastase injection. The number of GFP-positive cells in the alveolar walls was counted and expressed as the number per 100 alveoli. Alveolar epithelial cells were identified using a murine monoclonal antibody anti–cytokeratin 5 and 8 (Chemicon, Temecula, CA) and a goat Alexa fluor 633–conjugated antimouse antibody (Molecular Probes, Eugene, OR) (4). Vascular endothelial cells were identified using a rabbit polyclonal antibody raised against von Willebrand factor (vWF; Dako, Copenhagen, Denmark) and a goat Alexa fluor 633–conjugated antirabbit antibody (Molecular Probes). The numbers of GFP/cytokeratin double-positive cells and GFP/vWF double-positive cells were counted and expressed as the number per 100 alveoli. Histologic examinations were performed in a blinded fashion by three observers.

Morphologic and Functional Analyses

Twenty-eight days after elastase or saline injection, the mice were paralyzed and static lung compliance was measured using a computer-controlled small animal ventilator (flexiVent; Scireq, Montreal, PQ, Canada; n = 7 each). The lungs were removed and fixed at a constant transpulmonary pressure of 25 cm H_2O for 24 hours (n = 7 each). The lung volume was measured by the method of Scherle (18). The mean linear intercept, a morphometric parameter of emphysema, was calculated by light microscopy on 20 randomly selected fields as described previously (19). Morphologic examinations were performed in a blinded fashion by three observers.

Assessment of Vascular Density

Paraffin sections were obtained from the lungs (n = 7 each). To investigate whether AM induces angiogenesis in the elastase-treated lung, tissue sections were stained for vWF (20). The number of vWF-positive vessels per millimeter squared was counted in a blinded fashion by three observers.

Assessment of Cell Proliferation

Cryosections were obtained from the lungs of GFP-positive chimeric mice. To investigate whether AM promotes cell proliferation, tissue sections were stained for Ki-67, a marker for cell proliferation, using a rat antimouse Ki-67 antibody (Dako) and a goat Alexa fluor 633–conjugated anti-rat antibody (Molecular Probes). The number of Ki-67–positive cells per millimeter squared was counted in a blinded fashion by three observers.

In Vitro Study

A549 human alveolar type II epithelial cells (American Type Culture Collection, Rockville, MD) were cultured in F-12K medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corp.) and 1% penicillin-streptomycin (Invitrogen Corp.). Human umbilical vein endothelial cells (HUVEC) (Cambrex Corp., East Rutherford, NJ) were cultured in Medium 199 (Invitrogen Corp.) supplemented with 20% serum and 1% penicillin-streptomycin. To investigate whether AM attenuates elastase-induced cell death, cells were treated with elastase (0.3 units/ml) along with various concentrations of AM (10^{-10} to 10^{-7} M) (21). Twenty-four hours after elastase administration, cell number and DNA fragmentation were assessed. Cell number was assessed using a CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, Madison, WI) according to the manufacturer's directions (n = 4 each). DNA fragmentation was detected by terminal deoxynucleotidyl transferase–mediated dUTP biotin nick-end labeling (TUNEL) assay with a commercially available kit (ApopTag; Chemicon). The cells were then mounted in medium containing 4′,6-diamidino-2-phenylindole. The ratio of TUNEL-positive cells to total cells was calculated by counting at least 300 cells/well (n = 4 each). Finally, 6 hours after elastase administration, caspase-3 activity was measured with a commercially available kit (Promega) according to the manufacturer's directions (n = 4 each).

Assessment of Nitric Oxide Release and Detection of Soluble Kit-Ligand

In vitro, murine bone marrow stromal cells were prepared, as reported previously (26). Bone marrow stromal cells were cultured in Dulbecco's modified Eagle medium (Invitrogen Corp.) supplemented with 10% serum and 1% penicillin-streptomycin. The cells were treated for 20 minutes with control buffer, AM, or AM plus wortmannin (5×10^{-8} M; Wako Pure Chemical Ind., Ltd., Osaka, Japan), a PI3K inhibitor. To estimate total amounts of nitric oxide (NO) released from bone marrow stromal cells, nitrite content in the culture medium were measured using 2,3-diaminonaphthalene kit (Dojindo Laboratories, Kumamoto, Japan; n = 6 each).

In vivo, 5 days after continuous infusion of AM (0.05 μg/kg/minute) or vehicle, murine bone marrow plasma was collected by flushing femurs and tibias with a total of 0.5 ml saline. After spinning, supernatants were analyzed for soluble kit-ligand, a key molecule for stem cell recruitment, expression by ELISA (R&D Systems, Minneapolis, MN; n = 8 each) (22).

Measurement of Vascular Endothelial Growth Factor

To investigate the effect of AM on endogenous production of vascular endothelial growth factor (VEGF) in the elastase-injected mice, serum VEGF concentration was measured using a mouse VEGF ELISA kit (R&D Systems; n = 7 each).

Statistical Analysis

Numeric values were expressed as mean ± SEM unless otherwise indicated. Comparisons were made by one-way analysis of variance, followed by Newman-Keuls' test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Mobilization of Bone Marrow Cells

Administration of AM dose-dependently increased the number of peripheral blood mononuclear cells on Day 5 (Figure 1A).

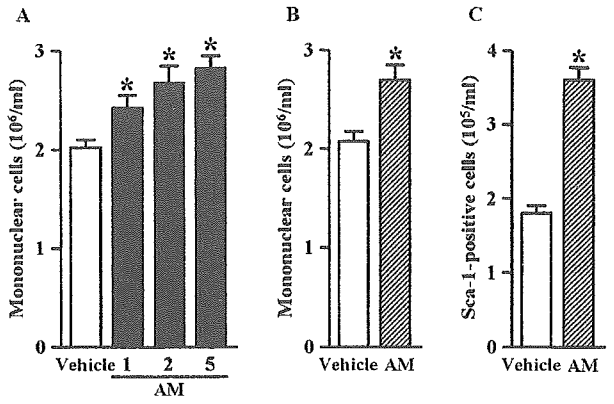

Figure 1. (*A*) Effect of bolus injection of adrenomedullin (AM) on the number of peripheral blood mononuclear cells. (*B*) Effect of continuous administration of AM on the number of peripheral blood mononuclear cells. (*C*) Effect of continuous administration of AM on the number of stem cell antigen-1 (Sca-1)–positive cells. Data are mean ± SEM. *White bars* = vehicle; *black bars* = bolus injection of AM (μg); *striped bars* = continuous administration of AM (0.05 μg/kg/minute). *$p < 0.05$ versus vehicle group.

A significant change was detected from the lowest dose (1 μg/mouse: 120% of vehicle group) to the highest dose (5 μg/mouse: 140% of vehicle group). A significant increase was also detected in mice administered AM by an osmotic minipump (131% of vehicle group; Figure 1B). Furthermore, the frequency of Sca-1–positive cells was significantly increased (200% of vehicle group; Figure 1C).

Regeneration of Pulmonary Epithelium

In the chimeric mice, the proportions of GFP-positive peripheral blood mononuclear cells and bone marrow cells were 87 to 93% and 85 to 90%, respectively, indicating that most of the original stem cell population was replaced by donor cells. Twenty-eight days after elastase injection, GFP-positive cells were detected in the alveolar walls. GFP/cytokeratin double-positive cells were also observed in the alveolar walls (Figure 2A). Semiquantitative analysis demonstrated that the number of GFP-positive cells incorporated into the lung was significantly increased in the AM group compared with that in the vehicle group (Figure 2B). In addition, the number of GFP/cytokeratin double-positive cells was significantly increased in the AM group compared with that in the vehicle group (Figure 2C). Twenty-eight days after elastase injection, the development of airspace enlargement with destruction of the alveolar walls was observed in the vehicle group (Figure 3A). However, administration of AM attenuated the histologic changes in mice given elastase. The mean linear intercept in the vehicle group was significantly increased compared with that in the sham group (Figure 3B), but the increase was significantly attenuated by AM.

Regeneration of Pulmonary Vasculature

GFP/vWF double-positive cells were observed in the alveolar walls (Figure 4A), and the number of these cells in the AM group was larger than that in the vehicle group (Figure 4B). As a whole, the number of vWF-positive pulmonary vessels was decreased 28 days after elastase injection (Figures 4C and 4D). However, AM infusion significantly increased the number of vWF-positive pulmonary vessels in the elastase-treated lung.

Effect of AM on Pulmonary Function

Twenty-eight days after elastase injection, the lung volume was significantly increased in the vehicle group (Figure 5A). The increase in lung volume was significantly attenuated by AM. Static lung compliance was significantly increased in the vehicle group, and the change was significantly attenuated by AM (Figure 5B).

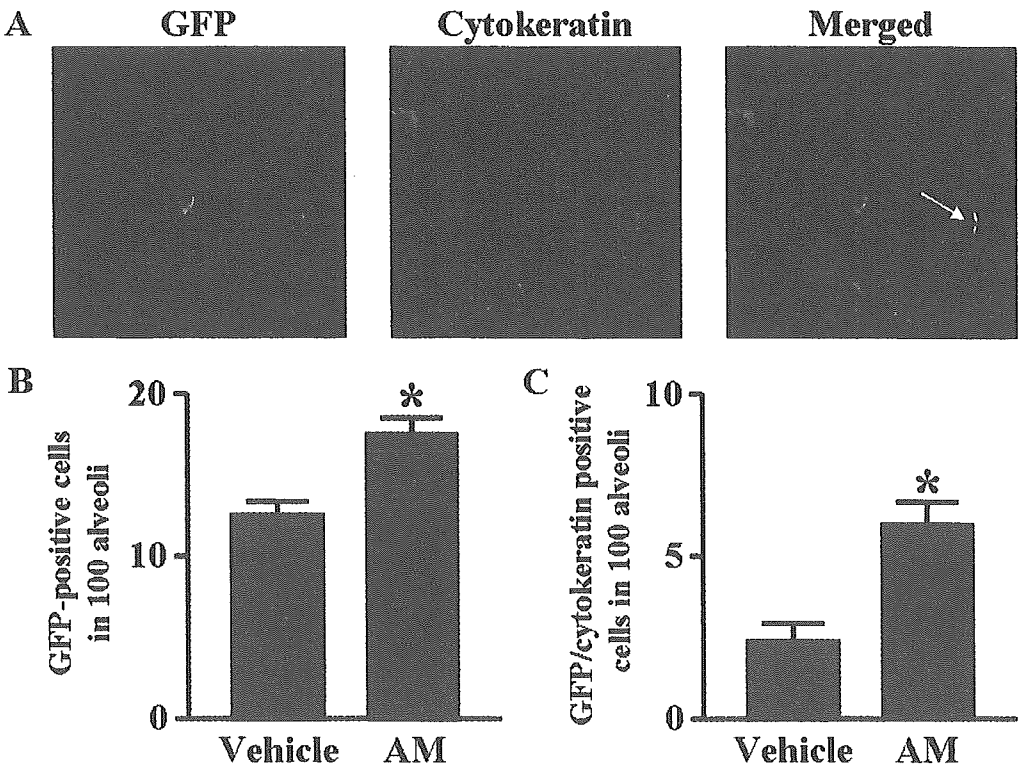

Figure 2. (*A*) Representative examples of bone marrow cell differentiation into epithelial lineage. Green fluorescence indicates green fluorescent protein (GFP); red fluorescence indicates cytokeratin, a marker for epithelial cells. Original magnification ×400. (*B*) Semiquantitative analysis of cell migration. The number of GFP-positive cells in the alveolar walls was significantly higher in the AM group than that in the vehicle group. (*C*) Semiquantitative analysis of epithelial differentiation. The number of GFP/cytokeratin double-positive cells was significantly higher in the AM group than that in the vehicle group. Data are mean ± SEM. *$p < 0.05$ versus vehicle group.

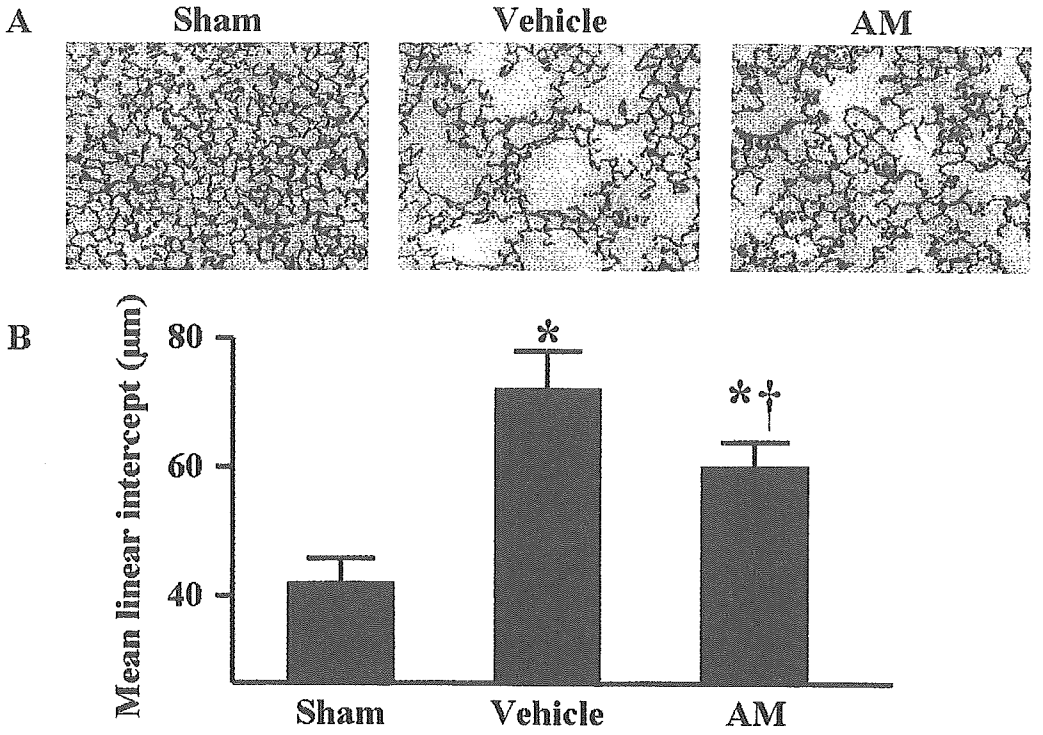

Figure 3. (*A*) Representative photographs of lung tissue stained with hematoxylin and eosin. Intratracheal injection of elastase induced the development of airspace enlargement with destruction of the alveolar walls in murine lungs (vehicle group). AM infusion attenuated the elastase-induced emphysematous changes (AM group). Original magnification ×100. (*B*) Semiquantitative analysis of lung tissue using the mean linear intercept, a morphometric parameter of pulmonary emphysema. Data are mean ± SEM. *$p < 0.05$ versus sham group; †$p < 0.05$ versus vehicle group.

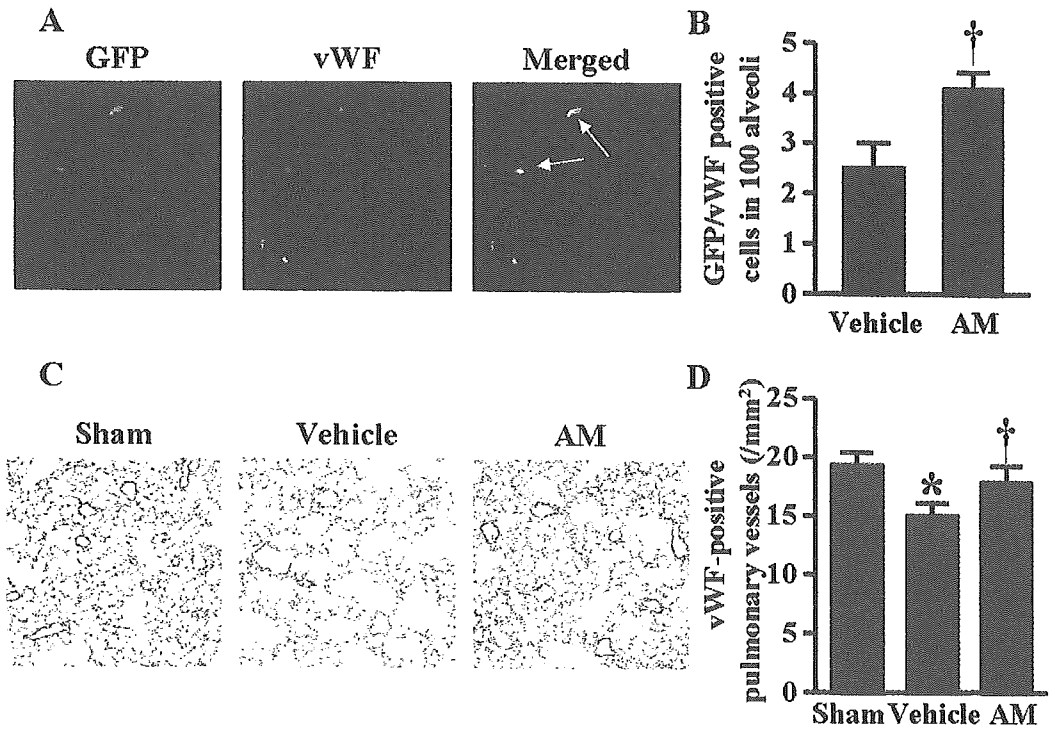

Figure 4. (*A*) Representative examples of bone marrow cell differentiation into endothelial lineage. Green fluorescence indicates GFP; red fluorescence indicates von Willebrand factor (vWF), a marker for endothelial cells. Original magnification ×400. (*B*) Semiquantitative analysis of endothelial differentiation. The number of GFP/vWF double-positive cells was significantly higher in the AM group than in the vehicle group. (*C*) Immunohistochemical analysis of vWF. Although vWF-positive vessels were less frequently observed in elastase-injected mice given vehicle, they were increased by AM infusion. Original magnification ×100. (*D*) Semiquantitative analysis demonstrated that the number of vWF-positive vessels was decreased 28 days after elastase injection (vehicle group). However, AM infusion significantly increased the number of vWF-positive vessels in elastase-treated lung (AM group). Data are mean ± SEM. *$p < 0.05$ versus sham group; †$p < 0.05$ versus vehicle group.

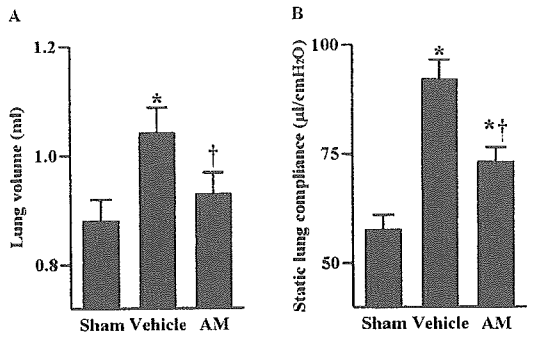

Figure 5. Effects of AM on lung volume (*A*) and static lung compliance (*B*) in mice 28 days after elastase injection. Elastase injection significantly increased lung volume and compliance in mice (vehicle group). AM infusion significantly suppressed the changes in elastase-injected mice (AM group). Data are mean ± SEM. *p < 0.05 versus sham group; †p < 0.05 versus vehicle group.

Protective Effects of AM on Epithelial and Endothelial Cells

In vitro, exposure of elastase significantly decreased the numbers of A549 cells (Figure 6A) and HUVEC (Figure 6B). However, AM dose-dependently attenuated elastase-induced cell death. In addition, elastase increased the frequency of TUNEL-positive cells in both A549 cells (Figure 6C) and HUVEC (Figure 6D). However, AM (10^{-8} M) significantly decreased the frequency of these TUNEL-positive cells (A549 cells: elastase alone, 26.5 ± 2.2, elastase + AM, 20.0 ± 1.5%; $p < 0.05$; HUVEC: elastase alone, 27.3 ± 2.3, elastase + AM, 19.8 ± 1.6%; $p < 0.05$). Elastase induced caspase-3 activation in both A549 cells (Figure 6E) and HUVEC (Figure 6F). However, AM (10^{-8} M) significantly attenuated elastase-induced caspase-3 activation in both type of cells.

In vivo, Ki-67–positive cells were observed in both GFP-positive and GFP-negative populations after elastase injection (Figure 7A). Semiquantitative analysis demonstrated that the number of Ki-67–positive cells in GFP-negative populations was significantly increased in the AM group compared with that in the vehicle group (Figure 7B).

Mechanism of Effect of AM on Bone Marrow Cell Mobilization

In vitro, administration of AM (10^{-8} M) stimulated NO release from bone marrow stromal cells (Figure 8A). The effect of AM on NO release was attenuated by pretreatment with wortmannin, a PI3K inhibitor (5×10^{-8} M). *In vivo*, continuous infusion of AM for 5 days significantly increased the concentration of soluble kit-ligand in bone marrow (Figure 8B).

VEGF Concentration

There was no significant difference in circulating VEGF concentration between the sham group (11.7 ± 1.0 pg/ml) and vehicle group (13.8 ± 1.5 pg/ml). However, AM infusion significantly increased the VEGF concentration (19.7 ± 2.0 pg/ml; $p < 0.05$ vs. the vehicle group).

Delayed Therapy

Morphometric analysis demonstrated that delayed AM therapy slightly, but significantly attenuated the increase in mean linear intercept in elastase-injected mice (Figures 9A and 9B). Delayed AM therapy significantly attenuated the increases in lung volume (Figure 9C) and static lung compliance (Figure 9D). In addition, the number of bone marrow–derived cells (GFP-positive cells) incorporated into the lung was significantly increased in the AM group in established emphysema (16.2 ± 0.9 vs. 11.5 ± 0.7 in 100 alveoli; $p < 0.05$). The numbers of bone marrow–derived epithelial cells (GFP/cytokeratin double-positive cells) and bone marrow–derived endothelial cells (GFP/vWF double-positive cells) were also significantly increased in the AM group in established emphysema (5.1 ± 0.3 vs. 2.2 ± 0.2, 3.8 ± 0.2 vs. 1.7 ± 0.1 in 100 alveoli, respectively; $p < 0.05$).

DISCUSSION

In the present study, we demonstrated *in vivo* that (*1*) infusion of AM increased the numbers of mononuclear cells and Sca-1–positive cells in circulating blood, (*2*) AM increased the number of bone marrow–derived cells incorporated into the elastase-treated lung, and (*3*) AM promoted regeneration of alveoli and vasculature in the lung. We also demonstrated *in vitro* that (*4*) AM improved cell survival after elastase exposure. Finally, we demonstrated that (*5*) AM improved lung structure and function in mice given elastase.

AM has a variety of effects on the vasculature that include vasodilation, inhibition of endothelial cell apoptosis, and regulation of smooth muscle cell proliferation (6, 10, 23–28). Recently, AM has been shown to induce angiogenesis through the PI3K/Akt-dependent pathway (12, 13). However, the effect of AM on bone marrow cell mobilization has remained unknown. In the present study, infusion of AM significantly increased the numbers of mononuclear cells and Sca-1–positive cells in circulating blood. Circulating endothelial progenitor cells have been shown to exist in a fraction of Sca-1–positive mononuclear cells (29). Thus, AM might induce release of progenitor cells from the bone marrow into the circulation. Earlier studies have shown that VEGF and stromal-derived factor-1 mobilize endothelial progenitor cells from the bone marrow (16, 30). Bone marrow cell mobilization has been shown to be dependent on local secretion of matrix metalloproteinase-9 and subsequent release of soluble kit-ligand, a key molecule for stem cell recruitment (31). A recent study has shown that NO activates matrix metalloproteinase-9 by S-nitrosylation (32). Moreover, endothelial NO synthase has been shown to be essential for mobilization of stem and progenitor cells (22). In the present study, AM stimulated NO release from bone marrow stromal cells, and the effect of AM on NO release was attenuated by pretreatment with wortmannin, a PI3K inhibitor. These findings suggest that infusion of AM stimulates NO release from bone marrow stromal cells at least in part through the PI3K/Akt-dependent pathway. *In vivo*, continuous infusion of AM significantly increased expression of soluble kit-ligand in murine bone marrow plasma. Taken together, AM may mobilize bone marrow cells at least in part through NO production and release of soluble kit-ligand.

Recent studies have shown that bone marrow progenitor cells contribute to regeneration of alveoli and vasculature (4, 16, 33, 34). These progenitor cells mobilized from the bone marrow have been shown to migrate to the site of damage and differentiate into alveolar epithelium or vascular endothelial cells. In the present study, to observe the behavior of bone marrow cells during tissue regeneration, we generated a chimeric mouse in which the bone marrow is reconstituted with GFP-positive cells. GFP-positive cells were observed in the alveolar walls of elastase-injected mice. Some of these GFP-positive cells were flat and stained for cytokeratin, a marker of epithelial cells. Interestingly, in the present study, AM infusion increased the number of GFP-positive cells in the alveolar walls. Moreover, the number of GFP/cytokeratin double-positive cells was significantly increased after AM infusion. These results suggest that AM promotes lung regeneration by increasing the number of bone marrow–derived alveolar epithelial cells in elastase-treated lung. It has been well

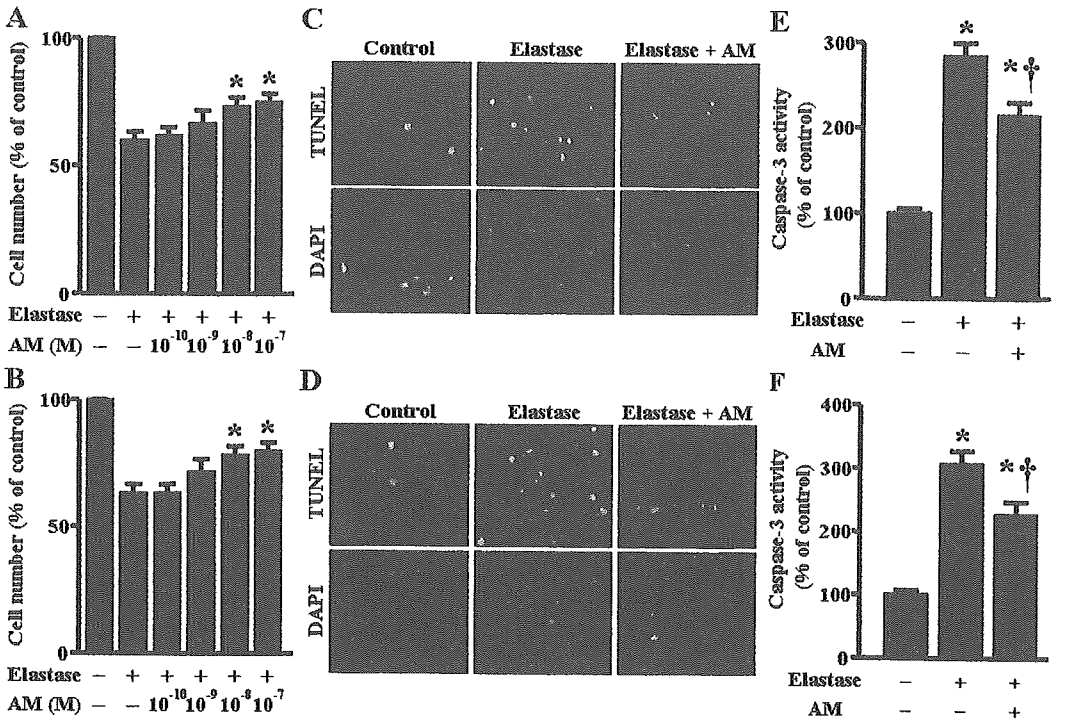

Figure 6. Effects of AM on the numbers of A549 cells (*A*) and human umbilical vein endothelial cells (HUVEC; *B*) 24 hours after treatment with elastase. Cell number was expressed as % of control (medium alone). Data are mean ± SEM. *$p < 0.05$ versus elastase alone. Terminal deoxynucleotidyl transferase–mediated dUTP biotin nick-end labeling (TUNEL) staining (*green*) of A549 cells (*C*) and HUVEC (*D*) 24 hours after treatment with elastase. Nuclei of cells were stained with 4′,6-diamidino-2-phenylindole (*blue*). AM (10^{-8} M) significantly decreased the frequency of these TUNEL-positive cells. Caspase-3 activity in A549 cells (*E*) and HUVEC (*F*) 6 hours after treatment with elastase. AM (10^{-8} M) significantly attenuated elastase-induced caspase-3 activation in these cells. Data are mean ± SEM. *$p < 0.05$ versus control (medium alone); †$p < 0.05$ versus elastase alone.

established that intratracheal injection of proteolytic enzymes induces emphysema in laboratory animals (35). In fact, in the present study, intratracheal injection of elastase induced emphysematous changes in the lung, as indicated by lung function (increased lung volume and static lung compliance) and morphologic findings (increased mean linear intercept). These findings were consistent with the results from earlier studies (36, 37). Interestingly, 14-day infusion of AM significantly attenuated the increase in lung volume, static lung compliance, and mean linear intercept in mice given elastase. Taken together, AM infusion

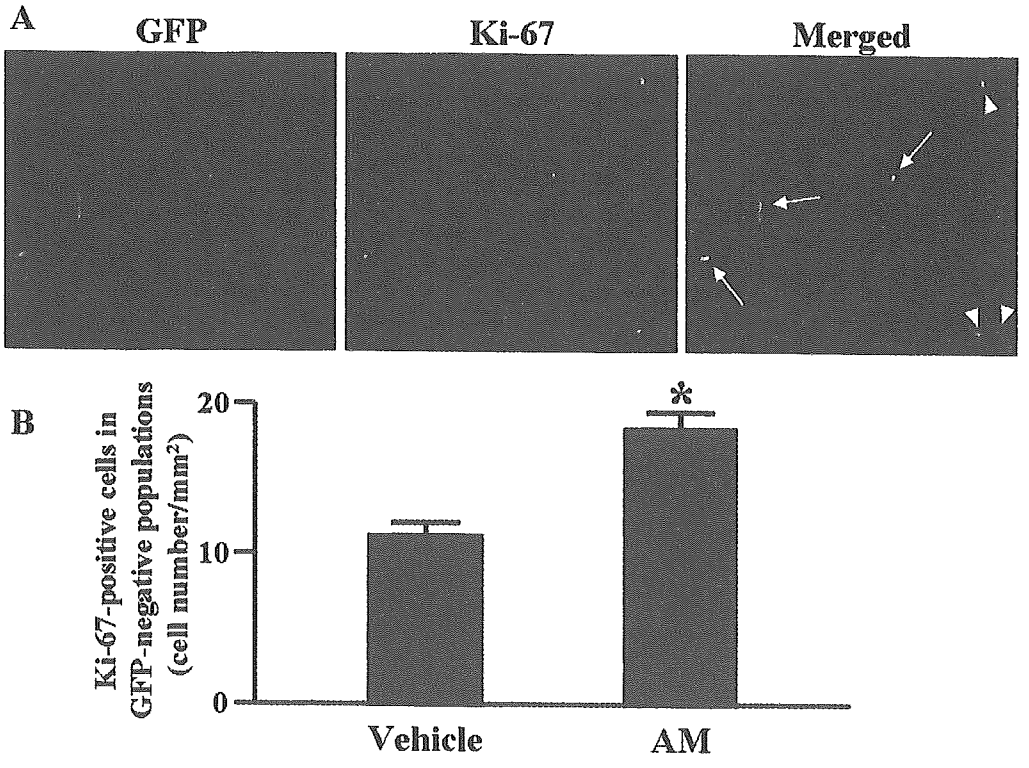

Figure 7. (*A*) Immunohistochemical analysis of Ki-67 antigen, a marker for cell proliferation. *Green fluorescence* indicates GFP; *red fluorescence* indicates Ki-67. Ki-67–positive cells were observed in both GFP-positive (*arrows*) and GFP-negative populations (*arrowheads*) in the alveolar walls after AM infusion. Original magnification ×400. (*B*) Semiquantitative analysis demonstrated that the number of Ki-67–positive cells in the GFP-negative populations was significantly increased in the AM group. Data are mean ± SEM. *$p <$ 0.05 versus vehicle group.

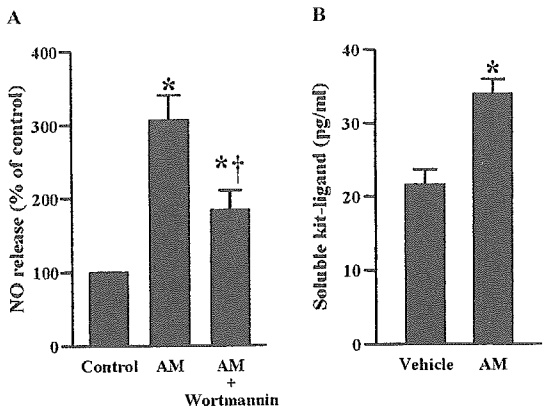

Figure 8. (*A*) Effects of AM (10^{-8} M) on nitric oxide (NO) release from murine bone marrow stroma cells. Pretreatment with 5×10^{-8} M wortmannin, a PI3K inhibitor, attenuated the effect of AM. Data are mean ± SEM. *$p < 0.05$ versus control (medium alone); †$p < 0.05$ versus AM alone. (*B*) Soluble kit-ligand level in bone marrow plasma. Continuous infusion of AM significantly increased soluble kit-ligand expression. Data are mean ± SEM. *$p < 0.05$ versus vehicle group.

may improve elastase-induced emphysema at least in part through regeneration of alveoli.

In the progression of emphysema, the capillary net cannot escape being destroyed because capillaries make up a substantial fraction of the volume of the alveolar walls. Accordingly, to repair emphysematous changes, it is important to regenerate not only alveoli, but also vasculature. In the present study, infusion of AM increased the number of GFP/vWF double-positive cells, suggesting that AM increases bone marrow–derived vascular endothelial cells. Furthermore, recent studies have shown that AM exerts angiogenic activities through activation of Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2, and focal adhesion kinase in endothelial cells (12, 13). Expectedly, in the present study, AM significantly increased the number of pulmonary vessels in the mice given elastase. Thus AM-induced angiogenesis in the lung may contribute to pulmonary regeneration in mice given elastase. AM infusion significantly increased the circulating VEGF level. Considering that VEGF signaling is required for cell growth and survival, it is interesting to speculate that VEGF upregulated by AM also induces angiogenesis in the lung.

In the present study, exposure of elastase reduced the numbers of cultured alveolar epithelial cells and vascular endothelial cells. Furthermore, elastase induced DNA fragmentation and caspase-3 activation in both type of cells. Interestingly, AM attenuated elastase-induced cell death in alveolar epithelial cells and vascular endothelial cells. Recently, AM has been shown to promote vascular endothelial cell proliferation (38). In the present study, AM significantly increased the number of Ki-67–positive cells even in GFP-negative populations, implying that AM may also induce proliferation of host lung cells. Thus, not only mobilization of bone marrow cells but also protective effects of AM on alveolar epithelium and endothelium may contribute to improvement in lung structure and function in elastase-injected mice. An inadequate repair process contributes to the development of emphysema (39, 40). Moreover, apoptosis of alveolar cells may be involved in pathogenesis of emphysema (41, 42). These findings raise the possibility that AM may prevent loss of alveoli via modulation of alveolar repair and inhibition of alveolar cell apoptosis.

Finally, to confirm the effect of AM on elastase-induced emphysema via its bone marrow action, we administered AM to mice with established emphysema. AM slightly but significantly improved lung structure and function in mice given elastase. In addition, AM increased the number of bone marrow–derived alveolar epithelial cells and vascular endothelial cells in GFP-positive bone marrow chimeric mice. Thus, continuous infusion

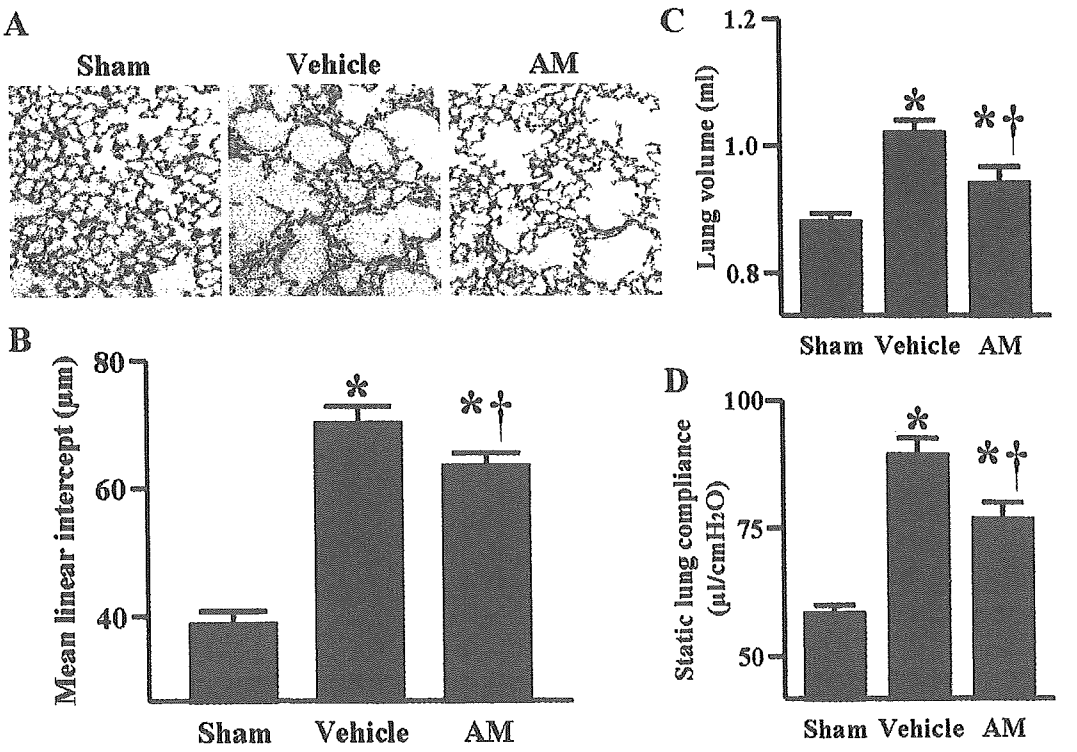

Figure 9. Effects of AM on established emphysema. AM administration was started 25 days after elastase injection. (*A*) Representative photographs of lung tissue. (B) Semiquantitative analysis of lung tissue using the mean linear intercept. (C) Lung volume. (*D*) Static lung compliance. Data are mean ± SEM. *$p < 0.05$ versus sham group; †$p < 0.05$ versus vehicle group.