21 days after surgery was 0.52 ± 0.05 in the carperitide-treated STZ-diabetic group (n = 8) and significantly higher than that in the vehicle-treated STZ-diabetic group (0.37 ± 0.06, n = 7; $P < 0.05$) (Fig. 2B). The time course of blood flow recovery in each group was shown in Fig. 2A.

In the vehicle-treated STZ-diabetic group, the capillary density was 907 ± 69 counts/mm^2 (n = 6) and was more significantly reduced than in the vehicle-treated nondiabetic group (1406 ± 98 counts/mm^2, n = 6; $P < 0.05$) (Fig. 2, C and D). The capillary density tended to be higher in the carperitide-treated nondiabetic group (1604 ± 108 counts/mm^2, n = 6) than in the vehicle-treated nondiabetic group. Among STZ-diabetic mice, the carperitide administration significantly increased the capillary density to 1180 ± 95 counts/mm^2 (n = 6; $P < 0.05$).

In this study, 4-wk administration of carperitide to mice increased plasma human ANP levels from under the detection limit (10 pg/ml) to 156 ± 79 pg/ml (n = 5 each) and plasma cGMP levels from 8.9 ± 1.1 nM (n = 7) to 20.0 ± 2.9 nM (n = 6, $P < 0.05$). The carperitide administration altered blood pressure from 106 ± 3/73 ± 3 mm Hg to 94 ± 4/62 ± 4 mm Hg (n = 4 each; $P < 0.05$).

Human study

All patients had characteristic symptoms of PAD (Fontaine's class: I, one; II, five; III, two; and IV, five) (Table 2). A patient who was Fontaine's class I had a cold sensation in the lower extremities. The diagnosis was confirmed by ABI measurement, ultrasound velocity spectroscopy, or magnetic resonance angiography.

Hypertension and diabetes were the two most frequent underlying diseases among participants (Table 1). Among diabetic subjects, HbA1c levels were 7.7 ± 0.5%, and disease duration was 16.5 ± 2.1 yr. Seven patients suffered from end-stage renal diseases and were on hemodialysis. Eight patients had a past history of an ischemic heart disease, CHF, or both, and all of them were in stable condition with or without medication Plasma ANP levels were 315 ± 130 pg/ml, and ejection fractions measured by ultrasonic echocardiography were 49.9 ± 6.2%.

Plasma ANP levels were elevated from 224 ± 93 pg/ml at baseline to 400 ± 125 pg/ml during the administration (n = 12; $P < 0.05$; data were lacking in patient 5). Plasma cGMP levels were elevated from 14.4 ± 3.5 to 24.0 ± 4.5 nM (n =

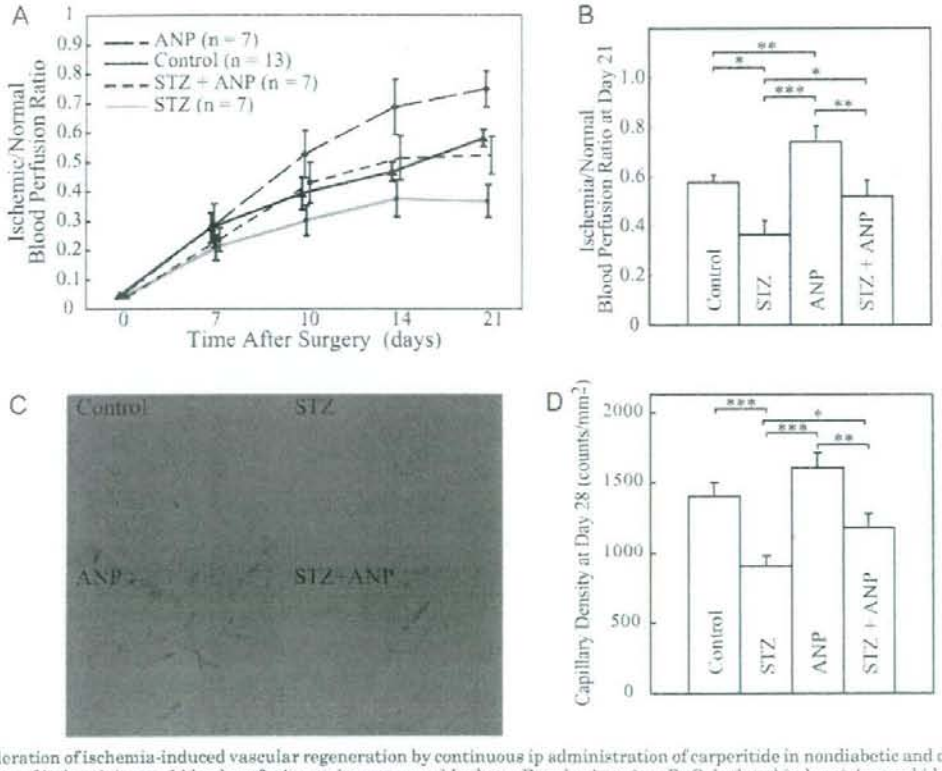

FIG. 2. Acceleration of ischemia-induced vascular regeneration by continuous ip administration of carperitide in nondiabetic and diabetic mice. A, Time course of ischemic/normal blood perfusion ratios measured by laser Doppler imaging; B, Calculated ischemic/normal blood perfusion ratios on d 21; C, immunostaining of the ischemic hind-limb tissue with anti-PECAM-1 antibody (*bright red*) at 28 d after the induction of ischemia; D, quantitative analysis of capillary density assessed by the immunostaining of PECAM-1. Control, Vehicle-treated nondiabetic; STZ, vehicle-treated STZ-diabetic; ANP, carperitide-treated nondiabetic; STZ – ANP, carperitide-treated STZ-diabetic. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 2. Detailed patients' characteristics

Patient no.	Diagnosis	Age (yr)/sex	Fontaine's class	Accompanying disease	Symptoms	RP rating	Exercise tolerance	Plasma ANP levels (pg/ml)	Medication
1	ASO	69/M	III	ESRD, DM, HT, IHD	RP	3	NA	79	Ap, P, C
2	TAO	38/F	II	DM, Ob	IC	NA	290	<5	Ap, P, V
3	ASO	82/F	I	DM, HT, Ob, HL, CHF	CS	NA	NA	16	An, Ap, P, V
4	ASO	77/M	IV	ESRD, HT, CHF	Ul/RP	5	NA	668	An, Ap, P
5	ASO	90/M	IV	DM, HT	Ul/RP	4	NA	152	P, V
6	ASO	85/M	IV	ESRD, DM, HT	Ul/RP	NA	NA	51	An, C, N, V
7	ASO	76/M	II	DM, HT, Ob, HL	IC	NA	240	22	An, Ap, V
8	ASO	75/M	II	DM, HT, HL, IHD	IC	4	200	36	An, Ap, P, V
9	ASO	63/M	III	ESRD, HT	RP	NA	NA	97	An, Ap, P, V
10	ASO	92/M	II	ESRD, DM, HT, CHF	IC	NA	100	922	Ap, N, P
11	ASO	71/M	IV	ESRD, DM, HT, CHF	Ul	NA	NA	645	Ap
12	ASO	57/M	II	DM, HT, HL, IHD	IC	3	5F	14	Ap, C, V
13	ASO	55/M	IV	ESRD, DM, HT, CHF, IHD	Ul/RP		NA	137	An, Ap, P, V

For patient 12, exercise tolerance was assessed by a stair-climb test, the floor number of stair-climbing without pain was 5. Medications were continued during carperitide injection without a change. An, Angiotensin-converting enzyme inhibitor or angiotensin receptor blocker; Ap, antiplatelet; ASO, arteriosclerosis obliterans; C, cilostazaol; CS, cold sensation of the peripheral; DM, type 2 diabetes mellitus; F, female; 5F, five floors; HL, hyperlipidemia; HT, hypertension; IC, intermittent claudication; IHD, ischemic heart disease; M, male; N, nitrate; NA, not applicable; Ob, obesity; P, prostanoid; RP, rest pain; TAO, thromboangitis obliterans; Ul, gangrane or non-healing ulcer(s); V, vasodilator.

9; $P < 0.01$). No significant differences were seen in plasma VEGF levels: 92.2 ± 25.4 pg/ml at the baseline and 65.2 ± 11.1 pg/ml in the course of administration (n = 8). The blood pressure of patients (excepting those on hemodialysis) fell from 143 ± 8/74 ± 2 mm Hg to 123 ± 7/69 ± 3 mm Hg (n = 5; $P < 0.05$). An excessive decrease in systolic blood pressure to less than 90 mm Hg was observed in a few patients on hemodialysis and could be quickly reversed by reducing the carperitide infusion rate. Medications except for injections were continued during carperitide injection without any changes. Details of medications especially for PAD are shown in Table 2. Alprostadil (prostaglandin E) had been iv administrated daily for a week to patients 2 and 3, and for a month to patients 6 and 11, and was stopped at least 3 d before the beginning of carperitide administration. Phosphodiesterase inhibitors other than cilostazaol were not used in patients enrolled in this study. Smoking status was not changed in five never-smokers (patients 1, 3, 5, 12, and 13) and seven former smokers (patients 2, 4, 7, 8, 9, 10, and 11) during this study. One patient (no. 6) was a current smoker (20 cigarettes/d) at the enrollment and stopped smoking 7 d before the administration.

The ABI of the affected limb (or worse side when both limbs affected) was significantly elevated from 0.61 ± 0.08 at the baseline to 0.72 ± 0.09 on the 14th day of administration (n = 12; $P < 0.05$) except for patient 5, for whom the administration was stopped within a week (Table 3 and Fig. 3b). Brachial systolic blood pressure values for ABI calculations before and on the 14th day of administration were 140 ± 10 and 132 ± 8 mm Hg, respectively (n = 12; $P = 0.5$). Ankle systolic blood pressure values at affected limb were 84 ± 13 mm Hg before administration and were increased to 94 ± 11 mm Hg on the 14th day of administration (n = 12; $P = 0.4$).

Pain was assessed with a numerical rating scale in six patients who complained of rest pain (Table 2). Rest pain disappeared in three of the six patients (patient 6, 4/0; patient 9, 4/0; and patient 13, 3/0, as before/after the administration of carperitide) and was reduced in another patient (no. 1, 3/1). In patient 4, although the pain once worsened in the early phase of administration (from 4 to 6), the injections were continued, and the pain was reduced to level 1 within a week. In another patient (no. 5), the carperitide infusion was stopped at d 7 because rest pain had worsened (4 to 6) (Fig. 3A). All patients who felt the rating score of rest pain reduced could stop to use pain relievers or hypnotics.

Exercise performance was carried out on all patients with intermittent claudication except for those who could not walk as a result of rest pain or weakness (patients 2, 7, 8, 10, and 12) (Fig. 3C). The pain-free walking distance was assessed in four patients and prolonged in all of them after the carperitide administration (patient 2, 290 to 380 m; patient 7, 240 to 560 m; patient 8, 200 to 800 m; patient 10, 100 to 200 m). In another patient with a stair-climb test, the floor number of pain-free stair climbing was increased from five to seven.

Five patients had multiple foot ulcers, and dermatologists in our hospital had recommended foot amputation. Al-

TABLE 3. Changes in ABI by 14 d administration of carperitide

Patient no.	Systolic BP (mm Hg)				ABI	
	Brachial		Ankle			
	Before	2 wk	Before	2 wk	Before	2 wk
1	96	182	35	106	0.36	0.58
2	141	101	115	89	0.82	0.88
3	159	140	69	81	0.43	0.58
4	88	115	83	140	0.94	1.22
5	138	NA	86	NA	0.62	NA
6	176	151	188	154	1.07	1.02
7	150	123	112	108	0.75	0.88
8	113	117	97	78	0.86	0.67
9	162	100	0	0	0	0
10	101	99	60	90	0.59	0.91
11	143	161	66	111	0.46	0.69
12	153	132	80	83	0.52	0.63
13	201	164	107	91	0.53	0.55
Mean	140	132	84	94	0.61	0.72
(SEM)	10	8	13	11	0.08	0.09

Values of brachial and ankle brachial pressure and ABI in each patient before and on the d 14 of administration. The administration was interrupted on the d 7 in patient 5. Data of patient 5 are excluded for the calculation of mean and SEM. NA, Not assessed.

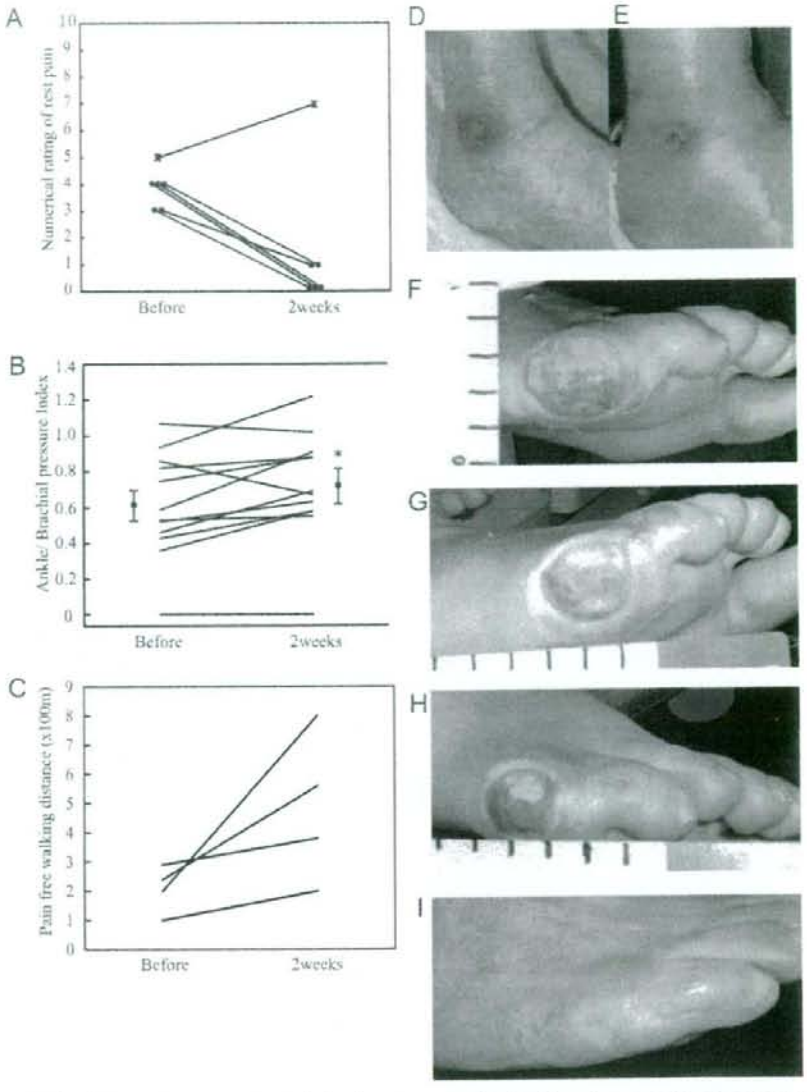

FIG. 3. Changes in symptoms resulting from carperitide infusion. A, Changes of 11-grade numerical rating of rest pain; B, changes in ABI of affected or worse side limb in each patient. Mean values are shown together with *error bars* (SEM) before and 2 wk after the carperitide administration. n = 12; *, $P < 0.05$. The administration was interrupted on the seventh day in patient 5, and ABI was undetectable in the affected limb of patient 9. C, Change in exercise tolerance assessed by pain-free walking distance; D–I, improvement of foot ulcer in patients 4 and 13. Pictures are before (D) and after 8-wk administration of carperitide (E) in patient 4 and before (F) and after 3 (G) and 6 (H) wk administration of carperitide and 4 months after leaving hospital (I) in patient 13. Pitting foot edema was observed in patient 4 (E).

though the ulcers did not change in severity in two cases (patients 5 and 6), they improved in another three cases (patients 4, 11, and 13) for whom foot amputations could be avoided. A representative case is shown in Fig. 3, D–I.

Other changes observed during administration were as follows: hot sensation in lower extremities in eight patients (nos. 1, 2, 4, 5, 6, 7, 8, and 13), transient flush and slight nausea in one patient (no. 2), pitting edema in both feet in five patients on hemodialysis (nos. 1, 4, 6, 11, and 13), and an increase in menstrual bleeding in a patient (no. 2).

Discussion

Diabetic foot is one of the most severe complications of diabetes mellitus and often results in leg amputation. Because it has been shown that an impairment of angiogenesis in patients with diabetes mellitus is a major cause of diabetic gangrene, we tried to generate a mouse model to investigate the mechanism of the impaired angiogenesis in diabetes. We induced diabetes in mice with STZ injections, and the mice were subjected to a femoral artery ligation after exposure to diabetic conditions (a blood glucose level higher than 220 mg/dl) for 4–26 wk. Although a 4-wk exposure to the diabetic condition did not affect blood flow recovery after the femoral artery ligation, exposure to high blood glucose for longer periods (16 or 26 wk) significantly impaired the blood flow recovery. This observation suggests that a quite long period of high blood glucose level is required to impair ischemia-induced collateral vessel formation. We therefore

selected 16 wk after the STZ induction of diabetes as the time point when the femoral artery ligation was performed on mice.

We showed here that carperitide, a recombinant human ANP, significantly accelerated blood flow recovery in a mouse model of ischemia-induced angiogenesis in both nondiabetic and diabetic conditions. The blood flow recovery in carperitide-treated diabetic mice was improved to a level similar to that in vehicle-treated nondiabetic mice. A histological analysis revealed that capillary density in the muscle of the ischemic limb was reduced in diabetic mice. The carperitide infusion significantly recovered capillary density in diabetic mice to the level in vehicle-treated nondiabetic mice. These observations indicate that carperitide can improve ischemia-induced angiogenesis, which accelerates blood flow recovery in diabetic conditions. We have shown that an increase of circulating BNP levels by targeted overexpression of the murine BNP gene in the liver or an overexpression of cGK throughout the body by the transgenic technology can accelerate the restoration of blood flow in limb ischemia experimentally generated by a femoral artery ligation, which results from the promotion of ischemia-induced angiogenesis through the activation of the ERK cascade (14). We have also shown that ANP at a physiological concentration induces proliferation and migration of ECs and enhances endothelial regeneration via activating ERK1/2 and phosphatidylinositol 3-kinase/Akt pathways in an *in vitro* wound healing assay using the cells from either coronary arteries or umbilical veins of humans (11). CNP, another member of the NP family, was shown to enhance migration of ECs and to accelerate reendothelialization in vein grafts after an arterial bypass surgery, although CNP inhibits proliferation and migration of vascular SMCs (16, 17). NPs use particulate GCs as their signaling receptors and share cGMP signaling pathways, especially signaling through cGKI, with NO, which activates soluble GC to generate cGMP (4). It is known that NO is a mediator of VEGF, which is a potent mitogen for vascular ECs and induces angiogenesis (19). A significant portion of VEGF-induced human EC proliferation is reportedly mediated by cGKI (20). In diabetes, hyperglycemia induces formation of reactive oxygen species, which decrease the bioavailability of NO (21). Taken together, deterioration of cGMP signaling appears to be a key process leading to the impaired angiogenesis and PAD in diabetes. In this study, the administration of carperitide could overcome the impairment of cGMP signaling in diabetic conditions, and it would be a new, therapeutic approach to PAD with diabetes. Because the urinary cGMP excretion rate is inversely correlated with the grade of Fontaine's classification in PAD patients (5), an impairment of cGMP signaling appears to be a common feature of PAD. We therefore investigated the therapeutic potential of carperitide administration in PAD patients.

We did not assign participants to a vehicle-treated group for an ethical reason; most cases of participants had been treated with conventional therapies, which had not accomplished appreciable effects. The carperitide administration significantly increased ABI, effectively relieved symptoms including intermittent claudication and rest pain, and promoted the healing of foot ulcers in PAD patients. The dosage of carperitide we used in the human study was optimized for each patient according to the maximum permissible dosage, which is the highest dose possible without causing an excessive fall in systolic blood pressure, because sensitivity to exogenously administered ANP differs among patients depending, presumably, upon basal plasma ANP levels. Although doses of carperitide administration were lower than those usually given in the treatment of CHF, plasma cGMP levels were increased twice as much as basal levels, and relief from the characteristic signs and symptoms of PAD became possible. This observation suggests that a blood pressure fall would not limit the therapeutic use of carperitide for PAD patients.

It is reported that asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial NO synthase, is accumulated in patients with end-stage renal disease (ESRD) and a high plasma ADMA level is a strong indicator of risks for all-cause mortality and cardiovascular events (22). It might be speculated that responses to the carperitide administration are better in ESRD patients than in non-ESRD patients, because carperitide is supposed to restore cGMP signaling, which is impaired by ADMA, via an activation of GC-A. Considering heterogeneity of patients' clinical characteristics, a larger number of participants will be needed to address this issue.

All patients, for whom exercise tolerance was evaluated, had been treated with conventional therapies using per os and per cutaneous medications under hospitalization and been encouraged to walk for at least 3 wk without any increases of pain-free walking distances. A 2-wk carperitide administration was then added to the conventional therapies and resulted in significantly improved exercise tolerance. The improvement, therefore, cannot be explained by a training effect only.

NPs have various biological effects on vascular functions other than the promotion of angiogenesis, and some of them appear favorable to treating PAD. NPs regulate vascular tone, and CNP, especially, is a candidate for endothelial-derived hyperpolarizing factor, which plays a fundamental role in the regulation of local blood flow and systemic blood pressure (23). In the clinical investigation of this paper, changes in symptoms and ABI appeared within a few days or a week of the administration. The effect of carperitide on symptoms in the early phase might be due to a vasodilatory action of ANP to some extent, because the changes appeared too early to be regarded as effects of vascular regeneration. On the other hand, the elongation of pain-free walking distance persisted after the cessation of the administration was ceased, and ABI remained elevated for several months after the end of administration. If the vasodilatory action of ANP is the only mechanism of the improvement, the effects of carperitide should disappear promptly at the cessation of the infusion, because the half life of ANP in circulation is a couple of minutes (24).

In patients with advanced arteriosclerosis, severe calcification of arterial walls in lower extremities can cause an overestimation of ankle blood pressure. Where vasodilators such as carperitide were used in such patients, ABI might be increased solely due to a decrease in brachial blood pressure. In this study, we observed slight decreases in brachial blood

pressure, but we could observe increases in ankle blood pressure although the changes were not statistically significant. Increases in ABI, therefore, should not be false and should be, at least in part, the result of blood flow recovery.

The improvement in exercise tolerance and ABI might, therefore, be achieved by modifying vascular endothelial structure or promoting vascular regeneration. Plasma VEGF levels were not significantly elevated by the carperitide infusion in this study, indicating that VEGF is not an essential mediator of carperitide's effects on PAD symptoms. It is reported that NPs elicit antiinflammatory and antithrombotic effects in animals (17, 25, 26), and further investigation will be needed to see whether such effects of NPs are clinically significant.

Carperitide is often used to treat CHF patients in Japan, and its safety is clinically proven. No critical side effects were observed in this study. An increase in menstrual bleeding observed in a participant could be accidental or a result of ANP's vasodilatory action, because the symptom faded soon after the cessation of the infusion. There are, however, several reports indicating the physiological significance of CNP/GC-B signaling in the control of ovarian cycling (27, 28). A close observation would be needed where carperitide infusion would be applied to women of reproductive age for a long duration (more than 2 wk). Leg edema appeared in three patients, who were in relatively serious states of the foot disease. Many PAD patients develop postoperative edema after surgeries of revascularization (29), which indicates that they have circulatory inadequacy for autoregulating blood hydrostatic pressure. Because ANP reportedly plays an essential role in maintaining vascular permeability via GC-A on vascular ECs (30), edema might result from this direct action on vascular endothelium.

Conclusion

This study revealed that a long-duration diabetic condition impaired ischemia-induced angiogenesis and blood flow recovery in a mouse model of hind-limb ischemia and that ANP as a therapeutic agent for CHF can restore the ischemia-induced angiogenesis in diabetic mice. Based on this observation, we applied carperitide administration to 13 PAD patients and found that carperitide infusion at doses lower than those for CHF could safely improve signs and symptoms. Carperitide administration, therefore, can be a new therapeutic strategy for PAD, and it appears effective in patients for whom conventional therapies do not work well.

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References

1. **Rivard A, Silver M, Chen D, Kearney M, Magner M, Annex B, Peters K, Isner JM** 1999 Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF. Am J Pathol 154:355–363
2. **Martin A, Komada MR, Sane DC** 2003 Abnormal angiogenesis in diabetes mellitus. Med Res Rev 23:117–145
3. **Tesfamariam B, Cohen RA** 1992 Free radicals mediate endothelial cell dysfunction caused by elevated glucose. Am J Physiol 263:H321–H326
4. **Tamura N, Chrisman TD, Garbers DL** 2001 The regulation and physiological roles of the guanylyl cyclase receptors. Endocr J 48:611–634
5. **Boger RH, Bode-Boger SM, Thiele W, Junker W, Alexander K, Frolich JC** 1997 Biochemical evidence for impaired nitric oxide synthesis in patients with peripheral arterial occlusive disease. Circulation 95:2068–2074
6. **Sugawara A, Nakao K, Morii N, Yamada T, Itoh H, Shiono S, Saito Y, Mukoyama M, Arai H, Nishimura K, Obata K, Yasue H, Ban T, Imura H** 1988 Synthesis of atrial natriuretic polypeptide in human failing hearts. Evidence for altered processing of atrial natriuretic polypeptide precursor and augmented synthesis of β-human ANP. J Clin Invest 81:1962–1970
7. **Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasaki M, Obata K, Yasue H, Kambayashi Y, Inoue K, Imura H** 1991 Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest 87:1402–1412
8. **Suga S, Nakao K, Hosoda K, Mukoyama M, Ogawa Y, Shirakami G, Arai H, Saito Y, Kambayashi Y, Inouye K** 1992 Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. Endocrinology 130:229–239
9. **Colucci WS, Elkayam U, Horton DP, Abraham WT, Bourge RC, Johnson AD, Wagoner LE, Givertz MM, Liang CS, Neibaur M, Haught WH, LeJemtel TH** 2000 Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. N Engl J Med 343:246–253
10. **Suwa M, Seino Y, Nomachi Y, Matsuki S, Funahashi K** 2005 Multicenter prospective investigation on efficacy and safety of carperitide for acute heart failure in the 'real world' of therapy. Circ J 69:283–290
11. **Kook H, Itoh H, Choi BS, Sawada N, Doi K, Hwang TJ, Kim KK, Arai H, Baik YH, Nakao K** 2003 Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro. Am J Physiol Heart Circ Physiol 284:H1388–H1397
12. **Ogawa Y, Itoh H, Tamura N, Suga S, Yoshimasa T, Uehira M, Matsuda S, Shiono S, Nishimoto H, Nakao K** 1994 Molecular cloning of the complementary DNA and gene that encode mouse brain natriuretic peptide and generation of transgenic mice that overexpress the brain natriuretic peptide gene. J Clin Invest 93:1911–1921
13. **Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiss C, Wang GX, Korth M, Aszodi A, Andersson KE, Krombach F, Mayerhofer A, Ruth P, Fassler R, Hofmann F** 1998 Defective smooth muscle regulation in cGMP kinase I-deficient mice. EMBO J 17:3045–3051
14. **Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, Sawada N, Fukunaga Y, Sone M, Yurugi-Kobayashi T, Miyashita K, Tsujimoto H, Kook H, Feil R, Garbers DL, Hofmann F, Nakao K** 2003 Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration. Proc Natl Acad Sci USA 100:3404–3409
15. **Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N, Imura H** 1992 Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. Possible existence of "vascular natriuretic peptide system". J Clin Invest 90:1145–1149
16. **Doi K, Ikeda T, Itoh H, Ueyama K, Hosoda K, Ogawa Y, Yamashita J, Chun TH, Inoue, M, Masatsugu K, Sawada N, Fukunaga Y, Saito T, Sone M, Yamahara K, Kook H, Komeda M, Ueda M, Nakao K** 2001 C-type natriuretic peptide induces redifferentiation of vascular smooth muscle cells with accelerated reendothelialization. Arterioscler Thromb Vasc Biol 21:930–936
17. **Ohno N, Itoh H, Ikeda T, Ueyama K, Yamahara K, Doi K, Yamashita J, Inoue M, Masatsugu K, Sawada N, Fukunaga Y, Sakaguchi S, Sone M, Yurugi T, Kook H, Komeda M, Nakao K** 2002 Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. Circulation 105:1623–1626

18. **Naruko T, Ueda M, van der Wal AC, van der Loos CM, Itoh H, Nakao K, Becker AE** 1996 C-type natriuretic peptide in human coronary atherosclerotic lesions. Circulation 94:3103–3108
19. **Morbidelli L, Chang CH, Douglas JG, Granger HJ, Ledda F, Ziche M** 1996 Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. Am J Physiol 270:H411–H415
20. **Hood J, Granger HJ** 1998 Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. J Biol Chem 273:23504–23508
21. **Ceriello A, Motz E** 2004 Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arterioscler Thromb Vasc Biol 24:816–823
22. **Kielstein JT, Zoccali C** 2005 Asymmetric dimethylarginine: a cardiovascular risk factor and a uremic toxin coming of age? Am J Kidney Dis 46:186–202
23. **Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ** 2003 Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. Proc Natl Acad Sci USA 100:1426–1431
24. **Ingwersen SH, Jorgensen PN, Eiskjaer H, Johansen NL, Madsen K, Faarup P** 1992 Superiority of sandwich ELISA over competitive RIA for the estimation of ANP-270, an analogue of human atrial natriuretic factor. J Immunol Methods 149:237–246
25. **Kiemer AK, Vollmar AM** 2001 The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. Ann Rheum Dis 60(Suppl 3):68–70
26. **Scotland RS, Cohen M, Foster P, Lovell M, Mathur A, Ahluwalia A, Hobbs AJ** 2005 C-type natriuretic peptide inhibits leukocyte recruitment and platelet-leukocyte interactions via suppression of P-selectin expression. Proc Natl Acad Sci USA 102:14452–14457
27. **Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL** 2004 Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci USA 101:17300–17305
28. **Acuff CG, Huang H, Steinhelper ME** 1997 Estradiol induces C-type natriuretic peptide gene expression in mouse uterus. Am J Physiol 273:H2672–2677
29. **Coats P, Wadsworth R** 2005 Marriage of resistance and conduit arteries breeds critical limb ischemia. Am J Physiol Heart Circ Physiol 288:H1044–H1050
30. **Sabrane K, Kruse MN, Fabritz L, Zetsche B, Mitko D, Skryabin BV, Zwiener M, Baba HA, Yanagisawa M, Kuhn M** 2005 Vascular endothelium is critically involved in the hypotensive and hypovolemic actions of atrial natriuretic peptide. J Clin Invest 115:1666–1674

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Augmentation of Neovascularizaiton in Hindlimb Ischemia by Combined Transplantation of Human Embryonic Stem Cells-Derived Endothelial and Mural Cells

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Abstract

Background: We demonstrated that mouse embryonic stem (ES) cells-derived vascular endothelial growth factor receptor-2 (VEGF-R2) positive cells could differentiate into both endothelial cells (EC) and mural cells (MC), and termed them as vascular progenitor cells (VPC). Recently, we have established a method to expand monkey and human ES cells-derived VPC with the proper differentiation stage in a large quantity. Here we investigated the therapeutic potential of human VPC-derived EC and MC for vascular regeneration.

Methods and Results: After the expansion of human VPC-derived vascular cells, we transplanted these cells to nude mice with hindlimb ischemia. The blood flow recovery and capillary density in ischemic hindlimbs were significantly improved in human VPC-derived EC-transplanted mice, compared to human peripheral and umbilical cord blood-derived endothelial progenitor cells (pEPC and uEPC) transplanted mice. The combined transplantation of human VPC-derived EC and MC synergistically improved blood flow of ischemic hindlimbs remarkably, compared to the single cell transplantations. Transplanted VPC-derived vascular cells were effectively incorporated into host circulating vessels as EC and MC to maintain long-term vascular integrity.

Conclusions: Our findings suggest that the combined transplantation of human ES cells-derived EC and MC can be used as a new promising strategy for therapeutic vascular regeneration in patients with tissue ischemia.

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Introduction

Embryonic stem (ES) cells, with their extensive regeneration potential and functional multilineage differentiation capacity, are now highlighted as promising cell sources for regenerative medicine. Previously we reported that mouse ES cells-derived vascular endothelial growth factor receptor-2 (VEGFR2) positive cells could differentiate into both endothelial cells (EC) and mural cells (MC) (pericytes and vascular smooth muscle cells) and reproduce the vascular organization process, which we termed "vascular progenitor cells (VPC)" [1]. Transplanted VPC into tumor-bearing nude mice were incorporated into blood vessels and significantly increased blood flow, which suggests that VPC might be useful for augmenting vessel growth in ischemic tissue [2].

We have demonstrated that human as well as monkey ES cells possessed different differentiation kinetics of VPC derived from mouse ES cells [3,4]. In contrast to mouse ES cells, undifferentiated human ES cells already expressed VEGFR2. After the induction of differentiation on OP9 feeder cells, VEGFR2 positive and tumor rejection antigen-1 (TRA1: a marker indicative of undifferentiated cell phenotype) negative cells appeared at day 8. We confirmed that VEGFR2 positive cells at this stage effectively differentiated into both VE-cadherin positive EC and α-smooth muscle actin (αSMA) positive MC to suffice as human VPC.

Human VPC-derived VEGFR2$^+$ VE-cadherin$^+$ cells, which were considered as EC at an early differentiation stage, formed a network structure on Matrigel-coated dishes.

Based upon these works, in the present study we transplanted human VPC-derived vascular cells; that is, EC and MC in a murine hindlimb ischemia model. By transplantation of these EC and MC differentiated from human VPC, we investigated whether and how they could be incorporated as EC and MC into the sites of neovascularization, compared to human peripheral blood and umbilical cord blood-derived endothelial progenitor cell (EPC) transplantation [5–7]. Furthermore, we specifically asked whether the combined transplantation of human VPC-derived EC and MC could induce stable vascular regeneration to achieve long-term vascular integrity.

Results

Characterization of Transplanted Human VPC-derived Vascular Cells

Flow cytometric analysis disclosed that 20–40% of expanded human VPC-derived EC retained the expression of the endothelial cell-related markers, including VE-cadherin, VEGFR2, CD34, CD31 and CD105, and all of the cells were negative for a panleukocyte marker CD45, monocyte/macrophage marker (CD11b), and stem/progenitor makers (AC133 and c-kit) (Figure 1a). By the double immunostaining of CD31 and αSMA, the cells negative for CD31 were exclusively positive for αSMA (Figure 1b), but weak or negative for staining with other MC markers, including calponin, smooth muscle myosin heavy chain 1 (SM1) and 2 (SM2) (data not shown).

Immunocytochemistry of expanded human VPC-derived MC revealed that all these cells were positive for αSMA, calponin, SM1 and SM2 (Figure 1c). Analysis by reverse transcription-polymerase chain reaction (RT-PCR) also confirmed that mRNA expressions of these MC markers were upregulated in human VPC-derived MC and negative in sorted VE-cadherin$^+$ fraction of expanded human VPC-derived EC (Figure 1d). Although cultured human aortic smooth muscle cells (hAoSMC) expressed a high level of h-caldesmon, its expression in human VPC-derived MC was not detected. Furthermore, mRNA for skeletal (myogenin and MyoD) or cardiac (cardiac troponin T (cTnT) and I (cTnI)) specific marker was not detected in human VPC-derived MC (Figure 1e).

Characterization of Transplanted Human EPC

Flow cytometric analysis of pEPC demonstrated that these cells mainly exhibited two light-scattering properties: one was consistent with a relatively large cell size (gate P1) and the other was found in a smaller gate P2 (Figure 2a). The P1-gated cells were positive for DiI-acLDL uptake and ulex-lectin binding (Figure 2b), and exhibited the reported EPC phenotype [6,8]. However, the smaller P2-gated cells were low positive for DiI-acLDL/ulex-lectin (Figure 2c). Therefore, we performed subsequent fluorescence activated cell sorter (FACS) analysis of pEPC on the P1-gated cells.

As shown in Figure 2d, nearly all pEPC expressed the hematopoietic markers CD45 (99.9%) and CD54 (99.9%) and the monocyte/macrophage markers CD14 (99.0%), CD11b (98.7%), and CD11c (98.9%). The monocyte/macrophage or endothelial markers CD31 (58.3%) and CD105 (70.1%) were also expressed. A much lower percentage of these cells expressed the endothelial cell-related markers VE-cadherin (1.6%), VEGFR2 (5.4%), and von Willebrand Factor (vWF) (0.3%), or the stem/progenitor cell markers AC133 (1.0%), c-kit (0.4%), and CD34 (0.2%).

Flow cytometric analysis of magnetic cell separation system (MACS)-sorted uEPC showed more than 80% of these cells were positive for CD34 (data not shown). Similar to pEPC, almost all CD34$^+$ fraction of uEPC expressed the hematopoietic markers CD45 (99.0%) and CD54 (84.9%) (Figure 2e). However, the expression of monocyte/macrophage markers was limited in uEPC (CD14 5.7%, CD11b 99.7%, CD11c 21.3%), and significant number of these cells was positive for the endothelial cell-related markers, including VE-cadherin (11.2%), VEGFR2 (8.1%), and vWF (7.9%). In addition, these CD34$^+$ uEPC expressed the stem/progenitor markers AC133 (80.6%) and c-kit (95.3%).

Long-term Improvement of Blood Flow of Ischemic Hindlimb by Human VPC-derived Vascular Cell Transplantation

To examine the comparative effectiveness of transplanted human VPC-derived vascular cells for vascular regeneration, we set up six groups as follows (Figure 3);

1) EC+MC group (n = 9): the mixture of 0.5×10^6 human VPC-derived EC and 0.5×10^6 MC, with the total cell number of 1×10^6,
2) EC group (n = 20): 0.5×10^6 human VPC-derived EC,
3) MC group (n = 18): 0.5×10^6 human VPC-derived MC,
4) uEPC group (n = 10): 1×10^6 umbilical cord-derived CD34$^+$ cells,
5) pEPC group (n = 16): 1×10^6 peripheral mononuclear cells (MNC)-derived EPC,
6) Control group (n = 17): only 100 μl PBS.

To analyze subcutaneous hindlimb perfusion, laser Doppler perfusion image (LDPI) analysis was performed (Figure 4a). Throughout the 42 day follow-up period, significantly accelerated limb perfusion improvement was observed in the VPC-derived EC+MC-transplanted group, compared to the EPC and control groups (Figure 4b, $P<0.001$ vs. control, pEPC, uEPC, and MC groups, $P = 0.002$ vs. EC group, repeated measures ANOVA followed by Bonferoni's multiple comparison test).

At day 14, blood flow of the mice transplanted with EPC (the ratio of ischemic/non-ischemic blood flow: 0.907 ± 0.058 in pEPC and 0.942 ± 0.075 in uEPC) ($P = 0.035$ and 0.028, compared to the control group), as well as MC (0.957 ± 0.056) ($P = 0.006$) and EC (0.901 ± 0.063) ($P = 0.032$) showed significant increase, compared to the control group (0.730 ± 0.042) (Figure 4b). In the EC+MC group, the ratio of ischemic/non-ischemic blood flow markedly elevated to 1.187 ± 0.083 ($P<0.0001$), compared to other groups.

Blood flow in the pEPC group, however, did not increase thereafter and no significant difference in the blood flow between the pEPC and control group was seen at days 28 and 42 (Figure 4b). In the uEPC group, significant blood flow recovery was seen at day 42 (0.990 ± 0.054) ($P = 0.009$), compared to the control group (0.749 ± 0.039). The blood flow in the VPC-derived vascular cells-transplanted groups progressively increased. At day 42, the calculated perfusion ratio of ischemic to non-ischemic hindlimb significantly elevated to 0.943 ± 0.057 for the MC ($P = 0.013$), 1.038 ± 0.059 for the EC ($P = 0.0002$), and 1.231 ± 0.067 for the EC+MC group ($P<0.0001$) compared to the control group (0.749 ± 0.039). Between the cell mixture transplantation (EC+MC) group and the single cell transplantation (EC or MC) groups, the blood flow of ischemic hindlimbs was significantly different at day 42 ($P<0.05$).

Effective Contribution of Human VPC-derived Vascular Cells for Vascular Regeneration

Fixed tissues harvested from ischemic hindlimbs at day 7 were inspected by the fluorescence stereomicroscope (Leica, Wetzlar,

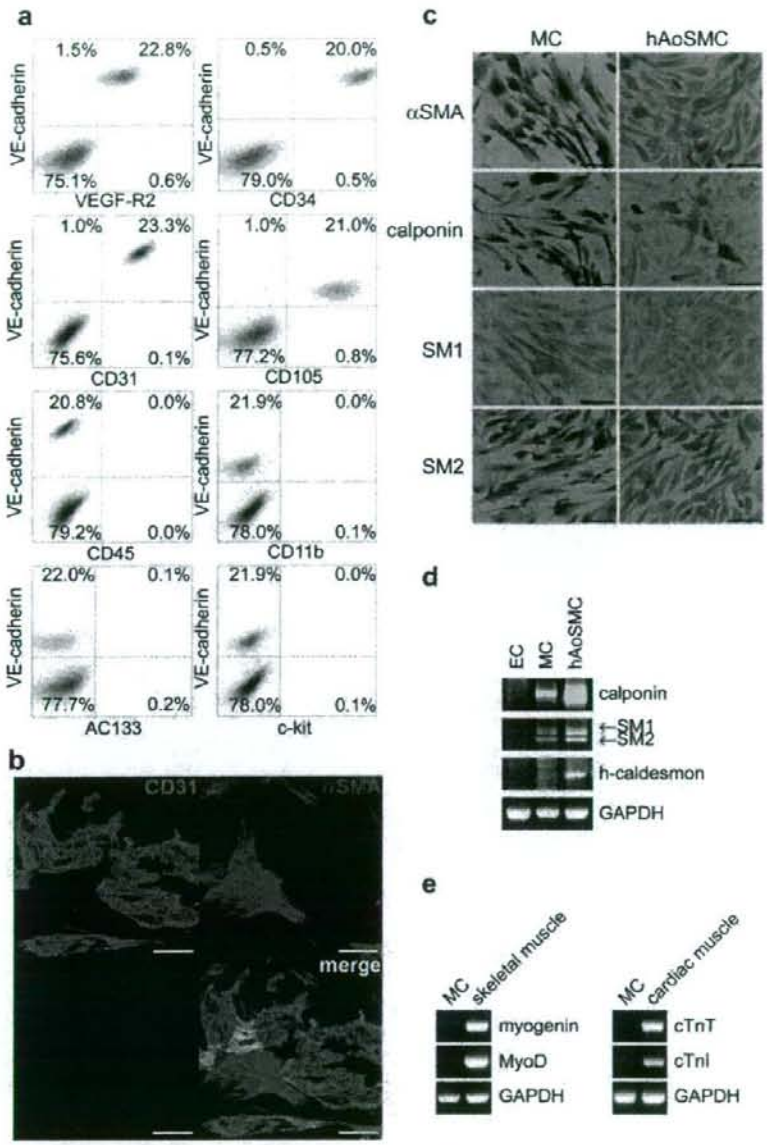

Figure 1. Characterization of transplanted human VPC-derived vascular cells. a) Flow cytometric analysis of cell surface markers on expanded human VPC-derived VEGF-R2$^+$VE-cadherin$^+$ cells (=EC). b) Immunofluorescence image of CD31 (green) and αSMA (red) with nuclear staining (blue) in expanded EC. Scale bar: 100 μm. c) Immunostaining of mural cell markers (brown) with hematoxyline counter-staining of expanded VPC-derived VEGF-R2$^+$VE$^-$cadherin- cells (=MC). Scale bar: 100 μm. d, e) RT-PCR analysis of mural cell (d) and skeletal/cardiac specific (e) markers in human VPC-derived vascular cells.
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Germany). Extended distribution of DiI-positive transplanted cells was clearly seen in both VPC-derived EC+MC and pEPC-transplanted hindlimbs (Figure 5a). We also detected some DiI-positive vessel-like formation in the lung and spleen, but no obvious tumor-like structures were seen (data not shown). Ischemic hindlimbs at day 14 were sectioned and treated with streptavidin conjugated dye to stain intravenously injected biotinylated isolectin B_4, followed by anti-human CD31 antibody, and scanned for the incorporation of transplanted cells into circulating vessels. In the EC+MC group, we found that human

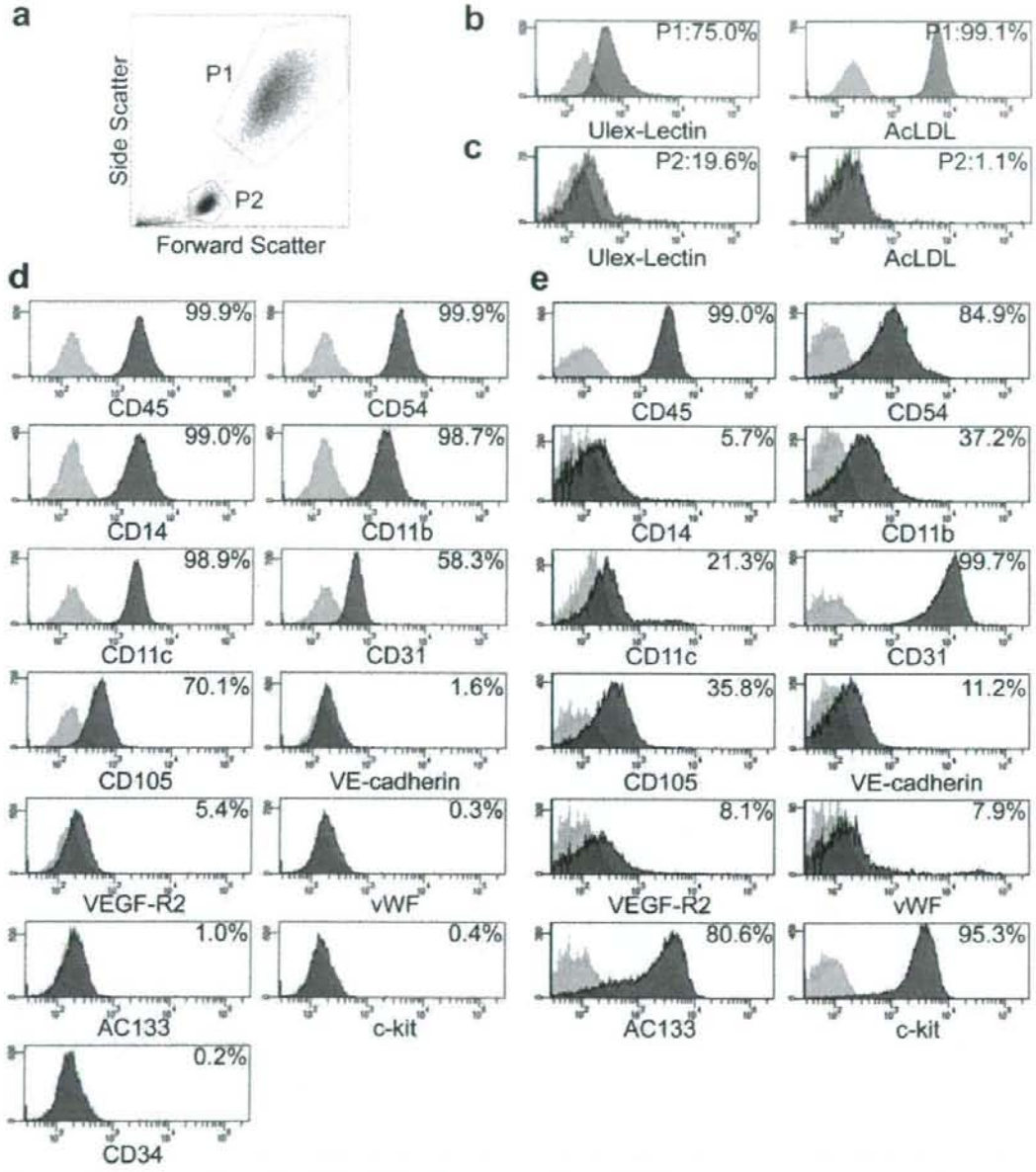

Figure 2. Characterization of peripheral blood and umbilical cord-derived EPC (pEPC and uEPC, respectively) by flow cytometer. a) Representative forward and side scatter profile of cultured pEPC. b-d) Flow cytometric analysis of ulex-lectin binding/acLDL uptake (b, c) and various cell surface markers (d) in pEPC. e) Flow cytometric analysis of cell surface markers in uEPC.
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CD31 positive cells formed capillaries with host EC, which were stained with isolectin B_4 (Figure 5b: arrowhead). Furthermore, some human CD31 positive cells solely formed capillary vessel (Figure 5b: arrow), which might indicate de novo vessel formation from human VPC-derived EC. We also detected human CD31 positive cells in the pEPC and uEPC group; however, many of these cells were located within the lumen of host capillaries (Figure 5c, arrow).

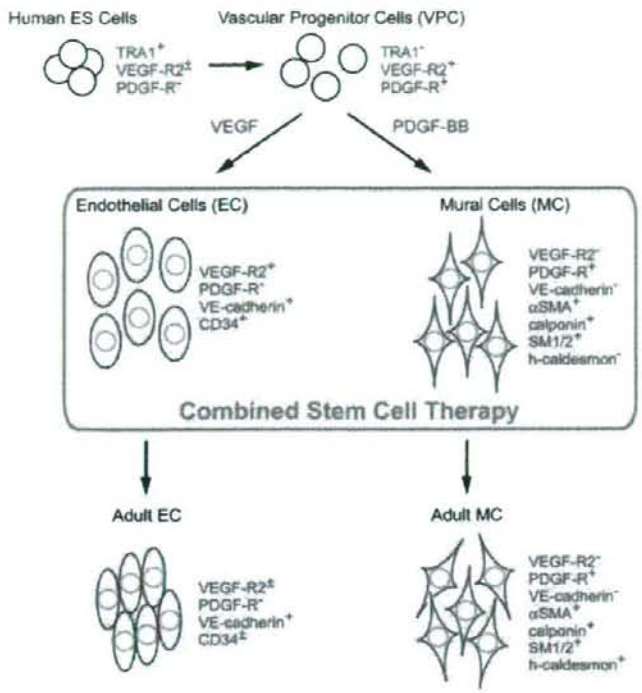

Figure 3. Possible differentiation pathway of vascular cells from human ES cells via VPC.
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We further investigated the contribution of transplanted VPC-derived MC to the recruitment of mural cells. We stained the sections of ischemic hindlimbs at day 14 with anti-human SM1 and αSMA antibodies. In EC+MC-transplanted mice, we found some human SM1 and αSMA double positive cells, which were localized within the αSMA positive host vessel wall (Figure 5d: arrow).

Quantification of Transplanted VPC-derived Vascular Cell-induced Vascular Regeneration in Ischemic Hindlimb

The sections of ischemic hindlimbs of the EC+MC group at day 42 were stained with anti-human and mouse CD31 antibodies. Mouse CD31 positive capillary density was significantly high in the EC+MC group ($1775.3\pm54.2/mm^2$), compared to other groups ($P<0.0001$ vs. control group: $1318.6\pm73.0/mm^2$) (Figure 6b). Human CD31 positive capillary density in mice transplanted with human VPC-derived EC (EC ($149.9\pm12.3/mm^2$) and EC+MC ($135.7\pm13.7/mm^2$) was significantly higher than that in mice transplanted with EPC ($95.7\pm8.5/mm^2$ in the pEPC and $115.2\pm12.0/mm^2$ in the uEPC group) ($P<0.05$). Compatible with the result of blood flow measurement, mouse and/or human CD31 positive capillary density markedly increased in mice that received human VPC-derived EC+MC ($1856.8\pm57.0/mm^2$) ($P<0.0001$, compared to the control group ($1318.6\pm73.0//mm^2$)), and also to other groups. Among the single cell transplantation groups, mouse and/or human CD31 positive capillary density increased in the EC group ($1601.4\pm51.4/mm^2$) ($P=0.0016$) compared to the control group, but did not increase in the MC ($1471.8\pm42.4/mm^2$) or EPC groups ($1403.5\pm84.4/mm^2$ in the pEPC and $1524.8\pm108.2/mm^2$ in the uEPC group).

To confirm the maturity of newly formed vessels, we performed the immunostaining of the ischemic tissues with anti-αSMA antibody, which could stain both human and mouse MC (Figure 6c). We confirmed that αSMA positive capillary density was significantly increased in the human VPC-derived vascular cells-transplanted groups (MC ($1317.6\pm45.4/mm^2$), EC ($1357.7\pm27.3/mm^2$) and EC+MC ($1554.9\pm48.8/mm^2$)) ($P<0.0001$), compared to the control group ($1021.3\pm46.3/mm^2$) (Figure 6d). Among the EPC groups, αSMA positive capillary density was significantly increased in the uEPC group ($1185.7\pm42.2/mm^2$) ($P<0.0076$) compared to the pEPC ($1118.9\pm36.8/mm^2$) and control group. We further investigated the extent of arteriogenesis in these groups using αSMA immunostaining sections. Many αSMA positive arterioles with more than 20 μm in diameter were detected in the EC+MC group, but not in the control group (Figure 6c: arrowhead). The number of αSMA positive arterioles significantly increased in the human VPC-derived vascular cells-transplanted groups, especially in the EC+MC group (the MC group:$4.0\pm0.3/mm^2$ and the EC group:$3.7\pm0.2/mm^2$; $P<0.001$, compared to the control group: $2.0\pm0.2/mm^2$, the EC+MC group: $5.5\pm0.7/mm^2$; $P<0.0001$, compared to all other groups) (Figure 6e). However, no significant difference in the number of αSMA positive arterioles was seen between the EPC (the pEPC group:$1.9\pm0.2/mm^2$ and the uEPC group:$2.0\pm0.2/mm^2$) and control groups.

Discussion

The present study demonstrated that the transplantation of human VPC-derived vascular cells at the proper differentiation

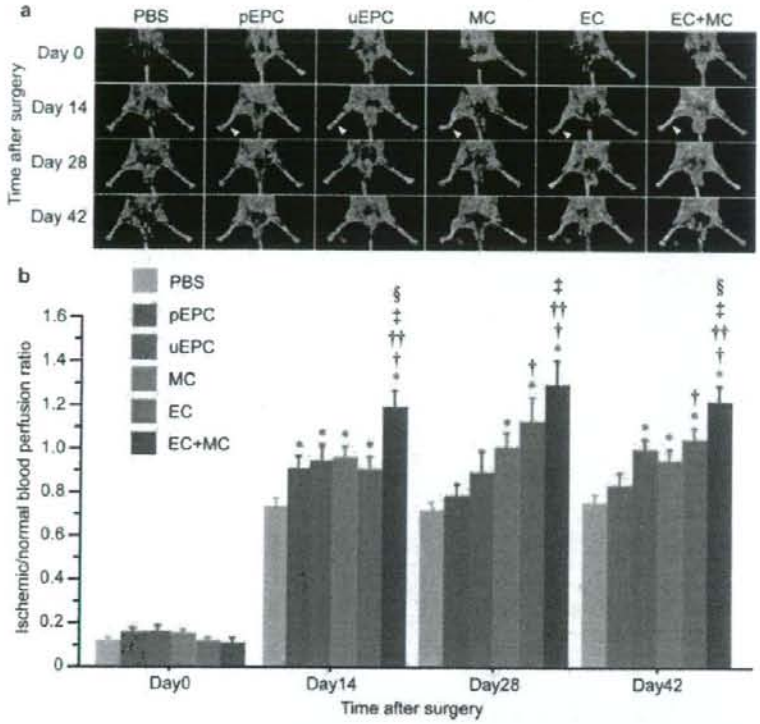

Figure 4. Augmented vascular regeneration by intra-arterial transplantation of human VPC-derived vascular cells in a murine hindlimb ischemia model. a) Serial LDPI analysis in hindlimb ischemia mice. At day 14, the blood flow of ischemic limbs in all cell transplanted groups increased significantly compared to the control group (white arrowhead). After 42 days, significant blood flow recovery was observed in the uEPC and human VPC-derived EC and/or MC-transplanted groups (red arrowhead), but not in pEPC. b) Quantitative analysis of hindlimb blood flow by calculating the ischemic/normal limb perfusion ratios after the induction of hindlimb ischemia. *$P<0.05$ vs. control, †$P<0.05$ vs. pEPC, ††$P<0.05$ vs. uEPC, ‡$P<0.05$ vs. MC, §$P<0.05$ vs. EC.
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stage successfully promoted vascular regeneration in the setting of tissue ischemia. After the expansion of human VPC-derived EC and MC, when intra-arterially administered, these cells significantly augmented neovascularization in an animal model of experimentally-induced hindlimb ischemia, compared to human peripheral blood and umbilical cord-derived EPC (pEPC and uEPC). Furthermore, the combined transplantation of human VPC-derived EC and MC could markedly induce vascular regeneration, compared to the single fraction transplantation of VPC-derived vascular cells (EC or MC). We also succeeded in demonstrating that transplanted human VPC-derived vascular cells were incorporated into the host circulation as both EC and MC. These results indicate that the combined transplantation of human VPC-derived EC and MC may have utility as a novel strategy for vascular regenerative medicine.

In the present study we used human VPC-derived VEGFR2$^+$ VE-cadherin$^+$ cells for the expansion and transplantation of EC. VEGFR2$^+$VE-cadherin$^+$ cells, obtained at day 10 of differentiation, were also positive for CD34 and therefore considered to be EC at the early differentiation stage (Figure 3) [9]. Even after 6 passages, 20~40% of these cells exhibited the expression of VEGFR2, VE-cadherin, and CD34, which indicated that they still retained the phenotype of EC at the early differentiated stage. Compared to EPC, transplantation of these EC significantly augmented ischemia-induced neovascularization. In contrast, we found that ischemia-induced neovascularization was not improved in mice receiving human aortic endothelial cells [4]. Therefore, human VPC-derived EC at the early differentiation stage might possess vascular regenerative capacity and these EC can be a valuable source for promoting vascular regeneration.

After expansion of human VPC-derived VEGFR2$^+$VE-cadherin$^+$ cells, about 70% of the expanded cells were αSMA positive. However, these cells were negative for the mature mural cell markers, including calponin, SM1, SM2, and h-caldesmon (data not shown). In contrast, expanded VEGFR2$^+$ VE-cadherin$^-$ cells obtained from human VPC under platelet derived growth factor (PDGF)-BB stimulation were positive for αSMA, calponin, SM1, and SM2, but negative for h-caldesmon. HAoSMC was positive for all of the mature MC markers, including h-caldesmon. In another series of our experiments, the mice receiving hAoSMC transplantation exhibited no significant improvement of neovascularization after the induction of ischemic hindlimbs (data not shown). Because h-caldesmon and calponin were reported to be expressed relatively late in SMC differentiation [10], human VPC-derived MC might be at a rather early "immature" differentiation

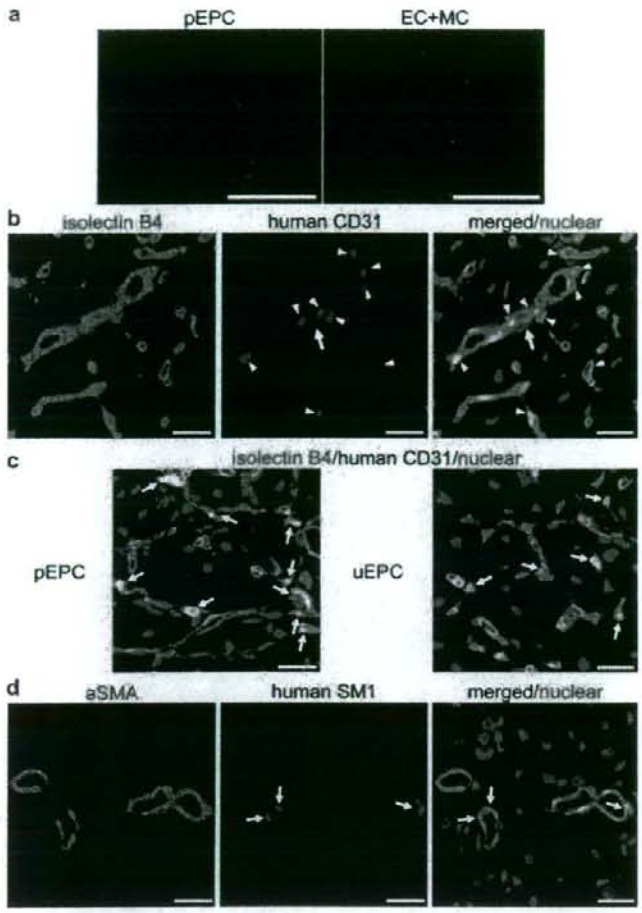

Figure 5. Incorporated human VPC-derived vascular cells at the sites of vascular regeneration. a) Transplanted CM-DiI (red) labeled pEPC or VPC-derived vascular cells in ischemic hindlimbs at day 7 were detected by the fluorescence stereomicroscope. Scale bar: 500 μm. b, c) Immunostaining of frozen sections harvested from ischemic limb tissues at day 14. Fluorescence staining of GSL I-isolectin B4 (green) and human CD31 (blue) with nuclear staining (red) in human VPC-derived EC+MC (b), pEPC, and uEPC (c) transplanted mice. Scale bar: 20 μm. d) Immunostaining of αSMA (green)/human SM1 (blue) with nuclear staining (red) in human VPC-derived EC+MC-transplanted mice at day 14. Scale bar: 20 μm.
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stage compared to hAoSMC, and thus, MC could be incorporated into the site of neovascularization.

Recently, Ferreira et al. reported that transplantation of human ES cells-derived EC into nude mice using Matrigel as scaffold contributed for the formation of blood vessels [11]. However, they did not show the direct integration of transplanted human ES cells-derived EC into host blood vessels. Judging from the double staining using intravenously injected isolectin B_4 and anti-human specific CD31 antibody, we found that the transplanted human VPC-derived EC incorporated into host circulating vessels. These transplanted EC could solely form de novo capillaries. In addition, by the double immunostaining of human SM1 and αSMA, we confirmed that transplanted human VPC-derived MC was also incorporated into host vessel walls. Therefore, transplanted human VPC-derived EC and MC structurally contributed to form new vessels in the process of vascular regeneration.

Interaction between EC and MC is essential for vascular development and maintenance of vascular stability [12,13]. Compared to only EC or MC-transplanted mice, the mice transplanted with the combined transplantation of EC and MC showed significant improvement after the induction of ischemic hindlimb. At day 42, the blood flow in the EC+MC group was significantly higher compared to only the EC or MC-transplanted groups. Not only mouse and/or human CD31 but also αSMA positive capillary density at day 42 significantly increased in the EC+MC group. We also found that the density of αSMA positive arterioles also significantly increased in the EC+MC group. These results indicated that combined transplantation of human VPC-derived EC and MC could synergistically contribute to vascular

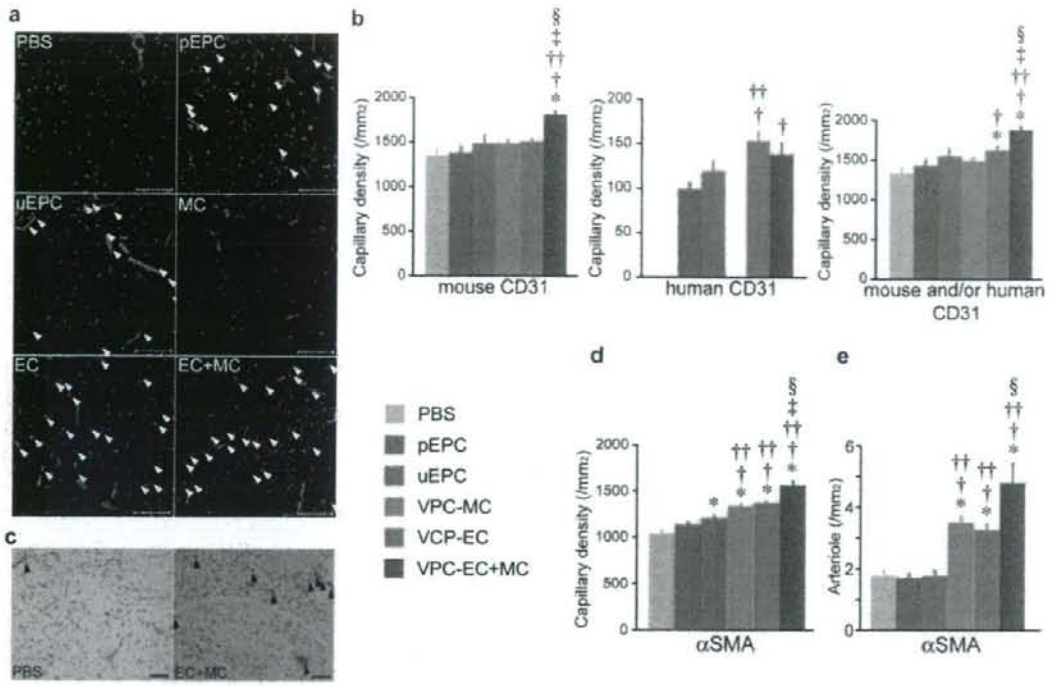

Figure 6. Immunohistochemical analysis of human VPC-derived vascular cells-transplanted murine hindlimb tissues. a) Representative fluorescent photographs of ischemic hindlimb stained for human (red) and mouse (green) CD31 at day 42. Overlapped-stained capillaries are shown in arrowhead. Scale bar: 100 μm. b) Quantitative analysis of the endothelial cell marker positive capillary density in ischemic hindlimbs at day 42. c) Representative αSMA immunostaining (brown) of ischemic hindlimbs at day 42. Scale bar. 100 μm. d) Quantitative analysis of αSMA positive capillary density in ischemic hindlimbs at day 42. e) Quantitative analysis of αSMA positive arterioles (black arrowhead) at day 42. *$P<0.05$ vs. control, †$P<0.05$ vs. pEPC, ††$P<0.05$ vs. uEPC, ‡$P<0.05$ vs. MC, §$P<0.05$ vs. EC.
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regeneration, and these MC could make mature blood vessels with adequate MC coating.

VEGFR2 is one of the most specific markers involved in the earliest stage of vascular endothelial and hematopoietic differentiation [14]. Recent reports suggest that VEGFR2$^+$ mesodermal progenitor cells also contribute muscle lineages including vascular smooth, skeletal, and cardiac muscles [1,15]. This evidence indicates the possibility that human VPC-derived MC, which were expanded from VEGFR2$^+$TRA1$^-$VE-cadherin$^-$ cells, might contain skeletal or cardiac muscle cells. However, 40-cycle RT-PCR was confirmed negative for skeletal and cardiac specific markers in expanded human VPC-derived MC. We cultured VPC-derived MC on dishes coated with collagen type IV, which is the major component of basement membrane. Previous reports described that basement membrane played an essential role in endothelial and smooth muscle cell differentiation [16]. Recently, Xiao et al. demonstrated that pretreatment of mouse ES cells with antibodies against collagen IV significantly inhibited smooth muscle cell differentiation [17]. They also demonstrated PDGF receptor-β signaling pathway plays a crucial role in ES cell-derived smooth muscle cell differentiation using PDGF receptor-β siRNA knockdown studies. Therefore, we suspected that, under the presence of collagen type IV and PDGF-BB, our human VPC-derived VE-cadherin negative cells could only differentiate to MC.

Human VPC-derived EC+MC-transplanted KSN nude mice showed considerable blood flow recovery, which led to more than 1.2 in the perfusion ratio of ischemic/non-ischemic limb. When we transplanted human VPC-derived vascular cells to immunosuppressed C57BL/6 mice, the perfusion ratio elevated to nearly 1 (data not shown). Therefore, the tendency of the blood flow recovery in C57BL/6 mice was consistent with the data of KSN nude mice, the absolute value of blood flow ratio after hindlimb ligation was different. Because both KSN nude and C57BL/6 mice received the same procedure for hindlimb ischemia, the degree of perfusion recovery after induction of hindlimb ischemia between these mice might reflect their difference in genetic background for angiogenesis, as reported by Fukino et al [18]. They demonstrated that the VEGF and VEGFR1/2 expression in response to ischemia was impaired in BALB/c mice, compared to other mouse strains (i.e, C57BL/6J or C3H/He mice). These results indicate that, because of the difference in genetic background, spontaneous collateral formation might be accelerated in our KSN nude mice compared to other strain mice.

In transplantation experiments, the number of mouse and/or human CD31 and mouse CD31-positive capillary density in the EC group was 1601.4±51.4/mm^2 and 1470.1±41.6/mm2, respectively. This difference in capillary density (1601.4–1470.1 = 131.3) was consistent with the number of human CD31-positive capillary density (149.9±12.3/mm^2). However,

compared to the EC group, the EC+MC group showed significant augmentation in mouse and/or human CD31 positive capillary density without the increase of human CD31 positive capillary density. One possible reason for this discrepancy is paracrine effects of transplanted human VPC-derived vascular cells might accelerate angiogenesis in ischemic tissues. We demonstrated that cultured human VPC-derived vascular cells expressed several angiogenic factors including VEGF, bFGF, HGF and PDGF-BB, and the release of VEGF from human VPC-derived vascular cells was significantly upregulated after transplantation (data not shown) [4]. Therefore, in addition to the structural contribution of transplanted human VPC-derived vascular cells into the host vascular network, the paracrine effects of these cells might enhance vascular regeneration in tissue ischemia.

Several reports described the contribution of pEPC or uEPC to neovascularization in tissue ischemia [6,7]. However, it has not been clearly demonstrated whether transplanted EPC augment neovascularization through differentiation and proliferation into mature EC or indirectly through paracrine stimulation of resident EC proliferation. Rehamn et al. demonstrated that the majority of pEPC, which were positive for acLDL and ulex-lectin, expressed monocyte/macrophage markers, and only a minority cell fraction expressed the specific endothelial or stem/progenitor markers [8]. They also demonstrated that pEPC did not proliferate, but released several potent angiogenic growth factors. In this study, we confirmed that a low percentage of cultured pEPC and uEPC expressed endothelial makers. A considerable number of pEPC or uEPC were localized inside the capillary lumen, not in the vessel wall. In addition, we found that VEGF mRNA expression in transplanted EPC was significantly higher compared with before transplantation (data not shown). These results suggest that the majority of EPC might have little ability to proliferate or differentiate to endothelial linage, and their angiogenic effects could be attributed to angiogenic factors secreted from transplanted EPC.

In conclusion, we have shown that human VPC-derived cells could effectively differentiate and be expanded to EC and MC. Combined transplantation of these "immature" VPC-derived vascular cells, unlike "mature" somatic EC and MC, augmented reparative neovascularization and contributed to make newly formed vessels in the murine hindlimb ischemia model far more effectively compared to EPC transplantation. Thus, human ES cells-derived EC and MC can be used as the new promising cell source for therapeutic vascular regeneration in patients with tissue ischemia in order to realize a novel combined stem cell therapy.

Materials and Methods

Differentiation of Human VPC-derived EC and MC

Maintenance of human ES cell line (HES3) was as previously described [19]. To induce VPC, undifferentiated ES cells were cultured on an OP9 feeder cell line as reported [3,4]. To obtain human VPC-derived EC, VEGFR2$^+$TRA1$^-$VE-cadherin$^+$ cells were sorted by fluorescence activated cell sorter (FACSAria; Becton Dickinson, Bedford, MA) at day 10 of differentiation, and cultured on type IV collagen-coated dishes (Becton Dickinson) in the presence of 10% FCS and 50ng/ml VEGF (human VEGF165, Peprotech Inc, Rocky Hill, NJ). After 6 passages of these cells, we re-sorted VE-cadherin$^+$ cells for transplantation of human VPC-derived EC. To expand human VPC-derived MC, sorted VEGFR2$^+$TRA1$^-$VE-cadherin$^-$ cells derived from VPC at day 8 were re-cultured on type IV collagen-coated dishes with 1% FCS and 20ng/ml human PDGF-BB (Peprotech Inc). We transplanted these human VPC-derived MC after 6 passages.

Preparation of Human EPC

Peripheral MNC-derived EPC (pEPC) were obtained from healthy volunteer, as previously described [6]. To confirm EPC phenotype, cells were detached with cell dissociation buffer (Invitrogen, Carlsbad, CA) and incubated with DiI-labeled acLDL (Invitrogen) and FITC-labeled Ulex europaeus agglutinin I (ulex-lectin) (Sigma-Aldrich, St. Louis, MO) for 1 hour. These cells were analyzed by FACSAria to be confirmed as EPC [6,8].

Umbilical cord blood-derived CD34$^+$ EPC (uEPC) were isolated from human umbilical cord blood, which were obtained from Cell Bank, RIKEN BioResource Center (Tukuba, Japan). CD34$^+$ cells were separated by a magnetic bead separation method using autoMACS system with direct CD34$^+$ progenitor cell isolation kit (Miltenyi Biotec GmbH, Gladbach, Germany) [7]. Protocols for using human umbilical cord blood were approved by the Ethics Committee of Kyoto University Graduate School of Medicine.

Characterization of VPC-derived Vascular Cells and EPC

To evaluate the surface marker phenotype of VPC-derived vascular cells and EPC, these cells were detached by cell dissociation buffer with or without collagenase (Wako Pure Chemical Industries, Osaka, Japan) and labeled for 15 minutes at 4°C with various fluorescence-conjugated monoclonal antibodies (Table 1) [20]. Cells were washed and analyzed on FACSAria flow cytometer with ≥30,000 events stored.

For the staining of cultured VPC-derived vascular cells on dishes, cells were stained with anti-human CD31 (WM59) (Becton Dickinson) antibody and several smooth muscle specific markers, as shown in Table 2. Cultured hAoSMC (Cambrex, East Rutherford, NJ) were used to obtain positive control staining.

For RT-PCR analysis, total RNA was prepared with RNeasy Mini Kit (QIAGEN Inc., Valencia, CA), and RT-PCR was performed by TaKaRa One Step RNA PCR Kit (TaKaRa Bio Inc., Otsu, Japan). Total RNA from human heart and skeletal muscle were purchased from Clontech (Mountain View, CA). Primers are listed in Table 3 [21–23].

Hindlimb Ischemia Model and Cell Transplantation

After 8-week-old male KSN/Slc nude mice (Japan SLC, Shizuoka, Japan) were anesthetized with pentobarbital (80mg/kg, i.p.), the right femoral vein was ligated. To transplant vascular cells intra-arterially, we injected these cells in 100 μl PBS into the right femoral artery. Immediately after the cell injection, the right femoral artery and vein were ligated and excised [24]. Animal procedures were performed according to Kyoto University standards for animal care.

Assessment of Transplanted Animals

The measurement of hindlimb blood flow was performed with a LDPI analyzer (Moor Instruments, Devon, United Kingdom), as previously described [24].

At arbitrary time points, biotin conjugated Griffonia simplicifolia lectin (GSL) I-isolectin B_4 (Vector Laboratories, Burlingame, CA) in 100 μl PBS was injected into the portal vein 15 minutes before sacrifice. Cryostat sections (10 μm thick) of the ischemic lower legs were stained with anti-mouse/human CD31 (clone WM59/Mec13.3) (Becton Dickinson) or anti-αSMA/human SM1 (clone 1A4/3F8) (DakoCytomation, Glostrup, Denmark/Yamasa Co., Tokyo, Japan) antibodies. For biotinylated isolectin B_4 staining to detect circulating vessels, sections were incubated with streptoavidin conjugated Alexa Fluor dye (Invitrogen).

Capillary densities were examined by counting the number of capillaries stained with anti-human and/or mouse CD31 or anti-

Table 1. Fluorescence-conjugated monoclonal antibodies used for FACS analysis

Antibody	Specificity	Clone	Conjugated fluorescence	Supplier
VEGF-R2	Endothelial cells	KM1998	Alexa Fluor 647	A generous gift of Prof. M. Shibuya, Tokyo University (Ref.20)
VE-cadherin	Endothelial cells	55-7H1	FITC or PE	Becton Dickinson, Bedford, MA
von Willebrand Factor (vWF)	Endothelial cells	2F2-A9	Alexa Fluor 488	Becton Dickinson, Bedford, MA
CD31 (PECAM1)	Endothelial cells or Monocytes	WM59	Alexa Fluor 488	eBioscience, San Diego, CA
CD105 (Endoglin)	Endothelial cells or Monocytes	266	Alexa Fluor 647	Becton Dickinson, Bedford, MA
CD11b (Mac1)	Monocytes	ICRF44	PE	eBioscience, San Diego, CA
CD11c	Monocytes	B-ly6	FITC	Becton Dickinson, Bedford, MA
CD14	Monocytes	M5E2	APC	Becton Dickinson, Bedford, MA
CD45	Panleukocytes	HI30	PE	Becton Dickinson, Bedford, MA
CD54 (ICAM-1)	Panleukocytes	581	PE	Becton Dickinson, Bedford, MA
AC133	Stem/Progenitor cells	AC133	PE	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
c-kit	Stem/Progenitor cells	YB5.B8	APC	Becton Dickinson, Bedford, MA
CD34	Stem/Progenitor cells	581	FITC	Becton Dickinson, Bedford, MA

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Table 2. Smooth muscle specific antibodies used for analysis

Antibody	Specificity	Clone	Supplier
Alpha smooth muscle actin (αSMA)	Human & mouse	1A4	DakoCytomation Denmark A/S, Glostrup, Denmark Sigma-Aldrich, St. Louis, MO
Calponin	Human	CALP	DakoCytomation Denmark A/S, Glostrup, Denmark
Smooth muscle myosin heavy chain 1 (SM1)	Human	3FB	Yamasa Co., Tokyo, Japan
Smooth muscle myosin heavy chain 2 (SM2)	Human & mouse	1G12	Yamasa Co., Tokyo, Japan

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Table 3. Primers for reverse transcription-polymerase chain reaction

Gene		Sequence	Length (bp)
Calponin[1]	Sense	5'-CTTCATGGACGGCCTCAAAGA-3'	713
	Antisense	5'-GTAGTTGTGTGCGTGGTGGTT-3'	
Smooth muscle myosin heavy chain 1 (SM1) and 2 (SM2)[1, 2]	Sense	5'-ATGAGGCCACGGAGAGCAACGA-3'	178 (SM1)
	Antisense	5'-CCATTGAAGTCTGCGTCTCGA-3'	217 (SM2)
h-caldesmon[1]	Sense	5'-AGACAAGGAAAGAGCTGAGGCA-3'	395
	Antisense	5'-GCTGCTTGTTACGTTTCTGCTC-3'	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1]	Sense	5'-ACCACAGTCCATGCCATCAC-3'	452
	Antisense	5'-TCCACCACCCTGTTGCTGTA-3'	
Myogenin[3]	Sense	5'-GTGGGCGTGTAAGGTGTGTA-3'	141
	Antisense	5'-TGGTTGGGGTTGAGCAGGGT-3'	
MyoD[3]	Sense	5'-CCAAATGTAGCAGGTGTAAC-3'	142
	Antisense	5'-AGAGATAAATACAGCCCCAG-3'	
Cardiac troponin T (cTnT)[4]	Sense	5'-GGCAGCGGAAGAGGATGCTGAA-3'	150
	Antisense	5'-GAGGCACCAAGTTGGGCATGAACGA-3'	
Cardiac troponin I (cTnI)[4]	Sense	5'-CCCTGCACCAGCCCCAATCAGA-3'	250
	Antisense	5'-CGAAGCCCAGCCCGGTCAACT-3'	

[1]Ref. 21.
[2]We used a single pair of PCR primers that cover the sequence specific to SM2, because these two isoforms are produced from a single gene by alternative splicing.
[3]Ref. 22.
[4]Ref. 23.
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αSMA antibodies. Twenty (for CD31) or ten (for αSMA) random fields on two different sections (approximately 3mm apart) from each mouse were photographed and analyzed by NIH image as previously described [24].

Statistical Analysis

Results are presented as means±S.E.M. The serial changes of the hindlimb blood flow were assessed by repeated measures ANOVA, followed by Bonferoni's multiple comparison test. Comparisons among groups were tested by one-way ANOVA followed by Bonferoni's multiple comparison test. A P value <0.05 was considered significant.

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Author Contributions

Conceived and designed the experiments: KY HI KN MS JY TY TC KH DT KM KP NO NS NT YF. Performed the experiments: KY MS KH. Analyzed the data: KY. Wrote the paper: KY HI.

References

1. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, et al. (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 408: 92–96.
2. Yurugi-Kobayashi T, Itoh H, Yamashita J, Yamahara K, Hirai H, et al. (2003) Effective contribution of transplanted vascular progenitor cells derived from embryonic stem cells to adult neovascularization in proper differentiation stage. Blood 101: 2675–2678.
3. Sone M, Itoh H, Yamashita J, Yurugi-Kobayashi T, Suzuki Y, et al. (2003) Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells. Circulation 107: 2085–2088.
4. Sone M, Itoh H, Yamahara K, Yamashita JK, Yurugi-Kobayashi T, et al. (2007) Pathway for differentiation of human embryonic stem cells to vascular cell components and their potential for vascular regeneration. Arterioscler Thromb Vasc Biol 27: 2127–2134.
5. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, et al. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275: 964–967.
6. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, et al. (2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 97: 3422–3427.
7. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, et al. (2000) Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 105: 1527–1536.
8. Rehman J, Li J, Orschell CM, March KL (2003) Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 107: 1164–1169.
9. Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, et al. (1990) Expression of the CD34 gene in vascular endothelial cells. Blood 75: 2417–2426.
10. Frid MG, Shekhonin BV, Koteliansky VE, Glukhova MA (1992) Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. Dev Biol 153: 185–193.
11. Ferreira LS, Gerecht S, Shieh HF, Watson N, Rupnick MA, et al. (2007) Vascular progenitor cells isolated from human embryonic stem cells give rise to endothelial and smooth muscle like cells and form vascular networks in vivo. Circ Res 101: 286–294.
12. Chan-Ling T, Page MP, Gardiner T, Baxter L, Rosinova E, et al. (2004) Desmin ensheathment ratio as an indicator of vessel stability: evidence in normal development and in retinopathy of prematurity. Am J Pathol 165: 1301–1313.
13. Hughes S, Chan-Ling T (2004) Characterization of smooth muscle cell and pericyte differentiation in the rat retina in vivo. Invest Ophthalmol Vis Sci 45: 2795–2806.
14. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H (1998) Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development 125: 1747–1757.
15. Motoike T, Markham DW, Rossant J, Sato TN (2003) Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. Genesis 35: 153–159.
16. Sakata N, Kawamura K, Takebayashi S (1990) Effects of collagen matrix on proliferation and differentiation of vascular smooth muscle cells in vitro. Exp Mol Pathol 52: 179–191.
17. Xiao Q, Zeng L, Zhang Z, Hu Y, Xu Q (2007) Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways. Am J Physiol Cell Physiol 292: C342–352.
18. Fukino K, Sata M, Seko Y, Hirata Y, Nagai R (2003) Genetic background influences therapeutic effectiveness of VEGF. Biochem Biophys Res Commun 310: 143–147.
19. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18: 399–404.
20. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, et al. (2001) Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood 97: 785–791.
21. Miwa Y, Sasaguri T, Inoue H, Taba Y, Ishida A, et al. (2000) 15-Deoxy-Delta(12,14)-prostaglandin J(2) induces G(1) arrest and differentiation marker expression in vascular smooth muscle cells. Mol Pharmacol 58: 837–844.
22. Kato H, Ohta S, Koshida S, Narita T, Taga T, et al. (2003) Expression of pericyte, mesangium and muscle markers in malignant rhabdoid tumor cell lines: differentiation-induction using 5-azacytidine. Cancer Sci 94: 1059–1065.
23. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, et al. (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108: 407–414.
24. Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, et al. (2003) Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration. Proc Natl Acad Sci U S A 100: 3404–3409.

Circulating CD34-Positive Cell Number Is Associated With Brain Natriuretic Peptide Level in Type 2 Diabetic Patients

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Patients with type 2 diabetes often suffer from asymptomatic left ventricular (LV) injury, including increased LV mass, without apparent myocardial ischemia. The mechanisms underlying diabetic LV injury remain unclear; however, it has been suggested that endothelial dysfunction plays a role. Accumulating evidence indicates that bone marrow–derived endothelial progenitor cells (EPCs) contribute to neovascularization of ischemic tissue and endothelialization of denuded endothelium. Recent studies have shown that circulating bone marrow–derived immature cells, including $CD34^+$ cells, contribute to the maintenance of the vasculature, both as a pool of EPCs and as the source of growth/angiogenesis factors (1). We hypothesized that circulating $CD34^+$ cells might be associated with LV dysfunction in patients with type 2 diabetes. Therefore, we studied the correlation between circulating $CD34^+$ cell levels and plasma brain natriuretic peptide (BNP) levels, an LV dysfunction marker, in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

The institutional review board of the National Cardiovascular Center approved this study, and all subjects provided informed consent. We examined 26 patients with type 2 diabetes (12 men and 14 women, duration of diabetes 16.1 ± 10.7 years) who were over 60 years of age (70.5 ± 6.4 years). Statin was given to nine subjects. ACE inhibitor or angiotensin receptor blocker was given to nine subjects, and thiazolidinedione was given to two subjects. Subjects were excluded from the study if they had known cardiovascular disease or chronic renal failure (defined as serum creatinine ≥180 μmol/l). No study subject showed hypokinesis by echocardiography or electrocardiogram change, indicating myocardial ischemia. Systolic (SBP) and diastolic (DBP) blood pressure and anthropometric parameters were determined. Blood samples were taken after 12-h fasting to measure circulating $CD34^+$ cells, plasma BNP, fasting plasma glucose (FPG), and A1C. Circulating $CD34^+$ cells were quantified by flow cytometry according to the manufacturer's protocol (ProCOUNT; Becton Dickinson Biosciences) as previously reported (2). BNP was quantified by enzyme immunoassay (Tohso, Tokyo, Japan). We further examined LV fractional shortening (LVFS), LV mass index (LVMI) (3), and peak flow velocity of the early filling wave (E), the late filling wave (A), and the E/A-wave ratio (E/A) by echocardiography. All echocardiograms were performed by several expert physicians who were blinded to $CD34^+$ cell level.

All statistical analyses were performed using JMP version 5.1.1 software (SAS Institute). Data are expressed as means ± SD. Comparisons of number of $CD34^+$ cells by sex were made using the two-tailed unpaired *t* test. Correlations between number of $CD34^+$ cells and clinical parameters were assessed by univariate liner regression analysis and multiple regression analysis. LVMI and plasma BNP concentrations were analyzed after logarithmic transformation.

RESULTS

FPG levels, A1C levels, and BMIs in the study subjects were measured to be 9.5 ± 2.6 mmol/l, 9.2 ± 1.8%, and 26.4 ± 4.3 kg/m^2, respectively. A total of 88% of the patients had hypertension (SBP 142 ± 18 mmHg, DBP 75.7 ± 13.5 mmHg). Plasma BNP levels were measured to be 95 ± 319 pg/ml. Although it has been reported that the level of BNP ≥100 pg/ml has a sensitivity of 90% of diagnosing congestive heart failure (CHF) in patients with CHF symptoms (4), none of the subjects in this study, including subjects with ≥100 pg/ml of BNP, showed symptoms of CHF. The level of circulating $CD34^+$ cells was measured to be 0.76 ± 0.39 cells/μl, and there was no significant difference between sexes. The range of LVMI was 73.3–340.2, and 11 subjects applied to the definition of LV hypertrophy (LVMI ≤131 in men and ≤100 in women) (3).

Plasma BNP levels had a significant inverse correlation with the number of circulating $CD34^+$ cells (Fig. 1*A*), whereas FPG, A1C, BMI, SBP, DBP, and age showed no significant correlations. There was a significant correlation between the number of circulating $CD34^+$ cells and LVMI by echocardiography (Fig. 1*B*). LVFS and E/A were not associated with circulating $CD34^+$ cell numbers (LVFS $r = -0.07$, $P = 0.72$; E/A $r = -0.11$, $P = 0.59$). There was also a significant correlation between BNP levels and LVMI ($r = 0.59$, $P = 0.001$).

In multiple regression analysis, the

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Abbreviations: BNP, brain natriuretic peptide; CHF, congestive heart failure; DBP, diastolic blood pressure; EPC, endothelial progenitor cell; FPG, fasting plasma glucose; LV, left ventricular; LVFS, LV fractional shortening; LVMI, LV mass index; SBP, systolic blood pressure.

A table elsewhere in this issue shows conventional and Systeme International (SI) units and conversion factors for many substances.

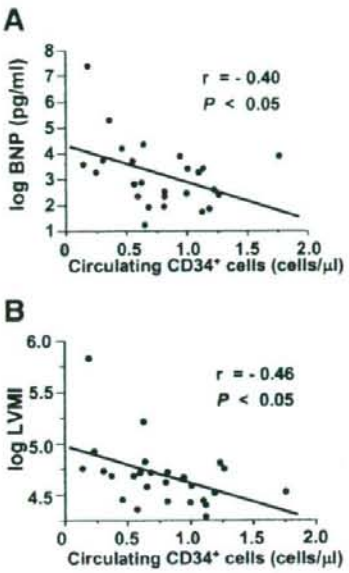

Figure 1—*Correlation between CD34⁺ cell numbers and plasma BNP levels* (A) *and correlation between CD34⁺ cell numbers and LVMI* (B) *in type 2 diabetic patients* (n = 26).

level of CD34⁺ cells was an independent correlate of both BNP ($\beta = -1.64$, $P = 0.017$) and LVMI ($\beta = -0.337$, $P = 0.031$) in the model including age, A1C, SBP, BMI, and medication (ACE inhibitor/angiotensin receptor blocker, statin, and thiazolidinedione).

CONCLUSIONS— In this study, circulating CD34⁺ cell number was found to significantly correlate with plasma BNP level, a marker of LV dysfunction. To the best of our knowledge, this is the first report that circulating bone marrow–derived cells are associated with diabetic LV abnormality. Circulating CD34⁺ cell numbers also significantly correlated with LVMI, whereas they did not correlate with LVFS (an LV systolic function marker) or E/A (an LV diastolic function marker). LV hypertrophy is a well-known predictor of cardiovascular events independent of coronary artery disease. The Framingham Heart Study identified an association between diabetes and increased LV wall thickness and mass (5). Although the precise mechanisms underlying the association between diabetes and LV hypertrophy remain unknown, our results suggest that reduced circulating CD34⁺ cell numbers may be involved in the progression of LV hypertrophy in diabetic patients. However, further investigations are necessary to demonstrate this hypothesis.

We measured the level of CD34⁺ cells in this study but not the levels of circulating CD34⁺/kinase insert domain receptor (KDR)⁺ cells that are regarded as EPCs. Circulating CD34⁺ cell levels are associated with ischemic stroke (6), and administration of CD34⁺ cells ameliorates cerebral ischemia in mice (7). This indicates that CD34⁺ cells may be involved in cardiovascular disease. Indeed, another recent report indicated that levels of circulating CD34⁺ cells are more strongly correlated with cardiovascular risk than levels of EPCs (8). Therefore, our results suggest that measurement of CD34⁺ cells may provide an indicator for diabetic LV hypertrophy.

Our study had several limitations. First, the study was performed only by cross-sectional analysis; therefore, a prospective study is needed to clarify whether circulating CD34⁺ cell numbers predict LV injury in diabetic patients. Second, although systemic blood pressure did not significantly associate with CD34⁺ cell numbers, further investigation of normotensive diabetic patients is needed to exclude the possible effects of hypertension on circulating CD34⁺ cell numbers, as most of the subjects in this study were hypertensive. Despite this caveat, these results may be of practical use in elderly patients with type 2 diabetes, as hypertension is a very common comorbid condition in this population.

In conclusion, reduced circulating CD34⁺ cell numbers are significantly associated with plasma BNP concentration and LVMI in elderly patients with type 2 diabetes. These results suggest that decreased circulating CD34⁺ cells may be involved in LV hypertrophy and that measurement of circulating CD34⁺ cell numbers may be useful for the identification of diabetic patients at high risk of LV injury.

References

1. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, Ratajczak MZ: Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. *Blood* 97:3075–3085, 2001
2. Kikuchi-Taura A, Soma T, Matsuyama T, Stern DM, Taguchi A: A new protocol for quantifying CD34(+) cells in peripheral blood of patients with cardiovascular disease. *Tex Heart Inst J* 33:427–429, 2006
3. Devereux RB, Reichek N: Echocardiographic determination of left ventricular mass in man: anatomic validation of the method. *Circulation* 55:613–618, 1977
4. McCullough PA, Nowak RM, McCord J, Hollander JE, Herrmann HC, Steg PG, Duc P, Westheim A, Omland T, Knudsen CW, Storrow AB, Abraham WT, Lamba S, Wu AHB, Perez A, Clopton P, Krishnaswamy P, Kazanegra R, Maisel AS, BNP multinational study investigators: B-type natriuretic peptide and clinical judgment in emergency diagnosis of heart failure: analysis from Breathing Not Properly (BNP) Multinational Study. *Circulation* 106:416–422, 2002
5. Galderisi M, Anderson KM, Wilson PW, Levy D: Echocardiographic evidence for existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *Am J Cardiol* 68:85–89, 1991
6. Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, Todo K, Mori K, Stern DM, Soma T, Naritomi H: Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation* 109:2972–2975, 2004
7. Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, Tsukamoto Y, Iso H, Fujimori Y, Stern DM, Naritomi H, Matsuyama T: Administration of CD34 + cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest* 114:330–338, 2004
8. Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A, Avogaro A: Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. *Eur Heart J* 27:2247–2255, 2006

Original Article

Impact of Metabolic Syndrome Components on the Incidence of Cardiovascular Disease in a General Urban Japanese Population: The Suita Study

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Abdominal obesity is a prerequisite for some definitions of metabolic syndrome (MetS). We investigated the impact of MetS defined by two different criteria, which either did or did not require abdominal obesity as a prerequisite, on cardiovascular disease (CVD) incidence in an urban Japanese cohort study. We studied 5,332 Japanese (aged 30–79 years, without CVD at baseline), who completed a baseline survey (September 1989 to March 1994) and were followed up through December 2005. MetS was defined by the NCEP-ATPIII (modified by Asian obesity criteria) and the Japanese criteria. After 61,846 person-years of follow-up, we documented 317 CVD incidences. The MetS frequencies of the Japanese and of the modified NCEP-ATPIII criteria were 17.7% and 25.1% for men and 5.0% and 14.3% for women, respectively. The multivariate hazard ratios (HRs; 95% confidence intervals [CI]) of CVD incidence for MetS by the modified NCEP-ATPIII criteria were 1.75 (1.27–2.41) in men and 1.90 (1.31–2.77) in women, and those for MetS by the Japanese criteria were 1.34 (0.96–1.87) in men and 2.20 (1.31–3.68) in women. The multivariate HRs of CVD incidence for MetS for the Japanese and for the modified NCEP-ATPIII criteria were 2.92 (1.54–5.55) and 1.94 (0.98–3.82) in men under 60 years old, respectively. The CVD incidence risks increased according to the number of MetS components. The risks were similar among participants with the same number of MetS components, regardless of abdominal obesity. In conclusion, the number of MetS components (modified NCEP-ATPIII criteria) may be more strongly associated with CVD incidence than the abdominal obesity essential criteria (the Japanese criteria) in a general urban Japanese population. (*Hypertens Res* 2008; 31: 2027–2035)

***Key Words*: metabolic syndrome, cardiovascular risk factor, cohort study, general population**

Introduction

Metabolic syndrome (MetS) is a clustering of impaired glucose metabolism, abdominal fat accumulation, dyslipidemia, and elevated blood pressure (*1*). Previous papers have shown an association between MetS and cardiovascular disease (CVD) (*2*), but most studies conducted thus far have been based on Western populations. There have been several well-designed prospective studies of Asian populations, and those

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