Sarpogrelate Hydrochloride, a Selective 5-HT_{2A} Antagonist, Augments Autologous Bone-Marrow Mononuclear Cell Implantation-induced Improvement in Endothelium-Dependent Vasodilation in Patients with Critical Limb Ischemia

Brief title: Cell therapy, 5-HT_{2A} antagonist and endothelial function

Yukihito Higashi, MD, PhD, FAHA; Masanori Miyazaki, MD, PhD; Chikara Goto, PhD; Hiroaki Sanada, MD, PhD; Taijiro Sueda, MD; Kazuaki Chayama, MD, PhD

Department of Cardiovascular Physiology and Medicine (C.G., Y.H.),
Department of Medicine and Molecular Science (K.C.),
Department of Surgery (M.M, H.S. and T.S.),
Hiroshima University Graduate School of Biomedical Sciences

Address for correspondence: Yukihito Higashi, MD, PhD, FAHA
Department of Cardiovascular Physiology and Medicine
Graduate School of Biomedical Sciences, Hiroshima University
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
Fax: +81-82-257-5124
Phone: +81-82-257-5122
E-mail: yhigashi@hiroshima-u.ac.jp

Abstract

Background: The purpose of this study was to determine the effect of a combination of bone-marrow mononuclear cell (BM-MNC) implantation and sarpogrelate, a selective 5-HT_{2A} antagonist, on endothelial function in patients with critical limb ischemia (CLI).

Methods: We evaluated the leg blood flow (LBF) responses to acetylcholine and sodium nitroprusside (SNP) before and after BM-MNC implantation in 16 patients with CLI. We divided CLI patients into two groups: those co-treated with sarpogrelate orally for 12 weeks (sarpogrelate group, n=8) and those who remained on conventional therapy (control group, n=8). LBF was measured by strain-gauge plethysmography.

Results: BM-MNC implantation improved ankle-brachial pressure index, transcutanous oxygen pressure, and pain-free walking time. There was no significant difference in these parameters between the two groups. Before BM-MNC implantation, LBF responses to acetylcholine were similar in the sarpogrelate group and control group. Twelve weeks of BM-MNC implantation enhanced LBF responses to acetylcholine in the sarpogrelate and control groups. After 12 weeks of BM-MNC implantation, LBF response to acetylcholine was significantly greater in the sarpogrelate group than in the control group. BM-MNC implantation did not alter the LBF responses to SNP in either group.

Conclusion: These findings suggest that BM-MNC implantation improved not only limb ischemic symptoms but also endothelium-dependent vasodilation in patients with CLI. A combination of BM-MNC implantation and sarpogrelate had a more beneficial effect on vascular function in these patients.

Key words: angiogenesis, cell therapy, endothelial function, limb ischemia

Introduction

Bone-marrow mononuclear cell (BM-MNC) implantation increases collateral vessel formation in both ischemic limb models and patients with limb ischemia.[1-3] Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis and plays an important role in the development and maintenance of atherosclerosis.[4] Limb ischemia is generally associated with endothelial dysfunction.[5-8] We have recently shown that autologous BM-MNC implantation improves not only limb ischemic symptoms and findings of angiography but also endothelial function in patients with critical limb ischemia (CLI).[9]

Serotonin (5-hydroxytryptamine, 5-HT) released from activated platelets has various subtypes of receptors.[10,11] In general, serotonin mediates vasodilation by binding to 5-HT_1 receptors on endothelial cells and vasoconstriction as well as promoting platelet aggregation by binding to 5-HT_{2A} receptors on vascular smooth muscle cells and platelets,[12-14] resulting in regulation of a balance of vasodilation and vasoconstriction. In the case of injury of the endothelium, serotonin would predominately bind to 5-HT_{2A} receptors, leading to vasoconstriction and platelet aggregation on vascular smooth muscle cells and platelets. It has been reported that gene expression of the 5-HT_{2A} receptor was enhanced in an animal model of limb ischemia.[15] It has also been reported that patients with peripheral arterial disease (PAD) have higher plasma serotonin concentrations than those in healthy subjects.[16] Sarpogrelate hydrochloride, a selective 5-HT_{2A} antagonist, has been used as an anti-platelet agent for treatment of PAD.[17,18] Sarpogrelate inhibits thrombus formation, suppresses platelet aggregation and inhibits vascular smooth muscle cell proliferation.[19-23] Sarpogrelate improves ischemic symptoms such as intermittent

claudication, pain and cold sensation of the lower extremities, and objective indices such as ankle-brachial pressure index (ABPI).[24] Recently, we have shown that sarpogrelate improves endothelial function in patients with PAD who have Fontaine II or III. [25]

It is expected that a combination of BM-MNC implantation and sarpogrelate will greatly improve much more endothelial function in PAD patinets with CLI. In the present study, we evaluated endothelium-dependent vasodilation induced by acetylcholine (ACh) and endothelium-independent vasodilation induced by sodium nitroprusside (SNP) before and after BM-MNC implantation in the presence and absence of sarpogrelate.

Methods

Subjects

Sixteen patients with CLI (15 men and 1 woman; mean age: 64±9 years) who had rest pain and non-healing ulcers and who were not candidates for angioplasty or surgical revasularisation were enrolled in this study. The diagnosis of limb ischemia was confirmed by angiography. CLI was classified according to the guideline of Trans Atlantic Inter-Societal Consensus II.[26] All enrolled patients received antiplatelet agents (100 mg of aspirin once per day or 100 mg of ticlopidine three times per day) before the study. All patients underwent BM-MNC implantation. The patients were randomly divided into two groups: a sarpogrelate treatment group (n=8; 8 men; mean age, 65±8 years), in which patients were treated with sarpogrelate hydrochloride (Mitsubishi-Tanabe Pharma Co., Osaka, Japan) at a dose of 100 mg three times per day for 24 weeks in addition to previous antiplatelet agents, and a control group (n=8; 7

men and 1 woman; mean age, 64±9 years), in which treatment with previous antiplatelet agents was continued for 24 weeks. All patients continued to receive conventional therapy throughout the study. Lifestyle also was regulated throughout the study. Data for one patient in the control group who dropped out of the study were excluded from the primary analysis. The study protocol was approved by the Ethics Committee of Hiroshima University Graduate School of Medicine. Written informed consent for participation in the study was obtained from all subjects.

BM-MNC implantation

BM-MNCs were sorted and implanted in patients with CLI, as previously described.[2,9] Briefly, we aspirated about 500 mL of bone marrow from the ileum of each patients under general anaesthesia and immediately isolated BM-MNC using a CS3000-Plus blood-cell separator (Baxter, Deerfield, IL, USA) to obtain a final volume of about 50 mL. Then we intramuscularly implanted about 0.75 mL of BM-MNCs into each injection site (total of 40 sites, 1.5 cm in depth) in the gastrocnemius of each ischemic leg with a 3 x 3 cm grid using a 22-guage needle.

Effect of BM-MNC implantation on endothelial function in patients with CLI

Leg vascular responses to ACh chloride (Daiichi Pharmaceutical Co., Tokyo, Japan) and SNP (Maluishi Pharmaceutical Co., Tokyo, Japan) were evaluated using a mercury-filled silastic strain-gauge plethysmography (EC-5R, D.E. Hokanson, Inc., Issaquah, WA, USA) before and at 12 weeks after BM-MNC implantation in all subjects. They were kept in the supine position in a quiet, dark, air-conditioned room (temperature, 22°C to 25°C) throughout the study. A 23-gauge polyethylene catheter

was inserted into the BM-MNC-implanted femoral artery for the infusion of ACh and SNP under local anesthesia. After each patient had spent 30 minutes in the supine position, we measured leg blood flow (LBF) and arterial blood pressure. Then, the effects of the ACh and SNP infusion on leg hemodynamics were measured. ACh (7.5, 15, and 30 μg/min) and SNP (0.75, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 minutes at each dose. The infusions of ACh and SNP were carried out in a random order. Each study proceeded after the LBF had returned to baseline.

Baseline fasting serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, malondialdehyde (MDA)-modified LDL, triglycerides, glucose, insulin, electrolytes, interleukin-6, and high-sensitivity C-reactive protein (hs-CRP) were obtained after a 30-minute rest period before the study. The 24-hr urinary excretion of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined.

Measurement of LBF

The blood flow was measured using a mercury-filled Silastic strain-gauge plethysmography (EC-5R, D.E. Hokanson, Inc.), as previously described.[9,27]

Analytical methods

Samples of venous blood were placed in tubes containing sodium EDTA (1 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. Plasma was immediately separated by centrifugation at 3100g for 10 min at 4°C, and serum was separated by centrifugation at 1000g for 10 min at room temperature. Samples were stored at -80°C until the time of assay. Serum concentrations of total

cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, glucose, and electrolytes were determined by routine chemical methods. Serum concentration of hs-CRP was measured by a high sensitive nephelometry assay using a CRP kit (Dade Behring, Deerfield, IL, USA). Serum concentration of interleukin-6 was measured by a high sensitivity ELISA (R&D Systems, Minneapolis, MN, USA). The serum concentration of MDA-LDL was assayed by ELISA (anti-MDA-modified LDL antibody, SRL Co., Atsugi, Japan). The urinary excretion of 8-OHdG also was assayed by ELISA using 8-OHdG kits (Nihon Yushi, Fukuroi, Japan).

Statistical Analysis

Results are presented as the mean±SD. All reported P values were two-tailed. Values of $P<0.05$ were considered significant. The Mann-Whitney U test was used to evaluate the differences in variables at the baseline between the sarpogrelate treatment group and the control group. Comparisons of parameters before and after BM-MNC implantation were performed with adjusted means by analysis of covariance using baseline data as covariates. Comparisons of time course curves of parameters during the infusions of ACh and SNP were analyzed by two-way analysis of variance for repeated measures on one factor followed by the Bonferroni correction for multiple-paired comparisons. Data were processed using the software package Statview (SAS Institute, Cary, NC, USA) or Super ANOVA (Abacus Concepts, Berkeley, CA, USA).

Results

Clinical characteristics

Baseline clinical characteristics before and at 12 weeks after BM-MNC implantation in the sarpogrelate and control groups are summarized in Tables 1 and 2. The numbers of BM-MNCs implanted into ischemic limbs were $1.8x10^9 \pm 0.6x10^9$ in the sarpogrelate group and $1.7x10^9 \pm 0.4x10^9$ in the control group. The numbers of $CD34^+$ cells included in the implanted BM-MNCs were $3.7x10^7 \pm 1.8x10^7$ in the sarpogrelate group and $3.6x10^7 \pm 1.2x10^7$ in the control group. There was no significant difference in the numbers of implanted BM-MNCs and $CD34^+$ cells between the sarpogrelate and control groups. Twelve weeks of sarpogrelate treatment significantly decreased serum concentrations of interleukin-6 and hs-CRP. In the control group, serum concentrations of interleukin-6 and hs-CRP were similar at 0 weeks and 12 weeks of follow-up (Table 1). BM-MNC implantation improved transcutanous oxygen pressure, and pain-free walking time in the sarpogrelate and control groups. There was no significant difference in these parameters between the two groups at 0 weeks and 12 weeks of follow-up (Table 2). BM-NMC implantation did not alter other parameters, including systemic hemodynamics, lipid profiles, glucose metabolism, and oxidative stress markers in the sarpogrelate and control groups.

Effect of BM-MNC implantation on endothelial function in patients with CLI

Intra-arterial infusion of ACh increased LBF in a dose-dependent manner. Before BM-MNC implantation, LBF responses to ACh were similar in the sarpogrelate group and control group (Fig. 1, top). Twelve weeks of BM-MNC implantation enhanced LBF responses to ACh in the sarpogrelate and control groups (Fig. 1, top). After 12 weeks of

BM-MNC implantation, LBF response to ACh was significantly greater in the sarpogrelate group than in the control group (Fig. 1, top). Intra-arterial infusion of SNP also increased LBF in a dose-dependent manner. BM-MNC implantation did not alter the LBF responses to SNP in either group (Fig. 1, bottom). No significant change was observed in arterial blood pressure or heart rate in response to intra-arterial infusion of either ACh or SNP before or after BM-MNC implantation.

There was a significant correlation between interleukin-6 levels and hs-CRP levels ($r=0.47$, $P<0.001$). After 12 weeks of BM-NMC implantation in combination with sarpogrelate, changes in interleukin-6 and hs-CRP were parallel. There was no significant relationship among the vascular responses to ACh and SNP and serum concentration of interleukin-6 or hs-CRP or among the increase in FBF responses to ACh and SNP and change in hs-CRP or interleukin-6.

Discussion

In the present study, BM-MNC implantation improved not only limb ischemic symptoms but also endothelium-dependent vasodilation in patients with CLI. In addition, a combination of BM-MNC implantation and treatment with the 5-HT_{2A} receptor antagonist sarpogrelate had more beneficial effect on vascular function in these patients.

Several possible mechanisms by which sarpogrelate greatly improves endothelial function in patients with CLI who have undergone with BM-MNC implantation have been proposed. Selective blockade of 5-HT_{2A} receptors on vascular smooth muscle cells or platelets by sarpogrelate may inhibit serotonin-induced vasoconstriction or that serotonin may stimulate 5-HT_{1B} and/or 5-HT_{2B} receptors on the endothelial cells, resulting in enhanced NO production in the endothelium.

Interestingly, sarpogrelate significantly decreased circulating levels of inflammatory markers, interleukin-6 and hs-CRP, in patients with CLI. Several lines of evidence have shown that PAD is associated with systemic inflammation.[28,29] In addition, it is well known that there is an association between inflammation and endothelial dysfunction. Under the condition of chronic inflammation, production of proinflammatory cytokines results in activation of endothelial cells, leading to excessive induction of adhesion molecules, cytokines, growth factors, and vasoconstrictors.[30,31] In addition, it has been reported that proinflammatory cytokines downregulate the expression of eNOS.[32] Administration of these cytokines, including interleukin-6, attenuates endothelium-dependent vasodilation in human veins in vivo.[33] CRP also directly decreases eNOS mRNA and protein levels and enzymatic activity in human aortic endothelial cells.[34] It is thought that endothelial dysfunction promotes

inflammation of the vascular wall, leading to a vicious circle between endothelial dysfunction and inflammation. We have recently reported that chronic infection with *Helicobacter pylori* impairs endothelium-dependent vasodilation in healthy male subjects.[35] Periodontitis, which is an ideal model for determining how endothelium-dependent vasodilation is affected by inflammation, has been shown to be associated with endothelial dysfunction in subjects without cardiovascular risk factors as well as hypertensive patients through a decrease in NO bioavailability.[36] Periodontal therapy has been shown to improve endothelium-dependent vasodilation in these patients.[41] These findings suggest that systemic inflammation might be, at least in part, a cause of endothelial dysfunction. In the present study, there was no correlation between changes in vascular response to ACh and changes in levels of inflammatory markers, interleukin-6 and hs-CRP, suggesting that the effects of sarpogrelate on endothelial function are at least partly independent of these inflammatory markers. In addition, there was no significant relationship between basal levels of interlukin-6 and hs-CRP and vascular response to ACh. However, we cannot deny the possibility that reduction in inflammation contributes to the improvement in endothelium-dependent vasodilation in patients with PAD. Discovery and rigid validation of potential biomarkers of inflammation-related endothelial dysfunction would enable a more specific conclusion concerning the role of inflammation in endothelial function to be drawn.

It has been reported that 5-HT stimulates interleukin-6 synthesis in aortic endothelial cells, leading to vascular inflammation in the process of development of atherosclerosis.[37] It has also been shown that interleukin-6 production in human vascular smooth muscle cells is due to the action of 5-HT on 5-HT_{2A} receptors through,

at least in part, a PKC-dependent pathway and that the interleukin-6 production is significantly inhibited by sarpogrelate.[38] It is likely that 5-HT also plays an important role in vascular inflammation associated with atherosclerosis. Therefore, we believe that sarpogrelate lowers interleukin-6 and CRP in the absence of BM-MNC implantation. Yamanaka et al.[39] have shown that circulating interkeulin-6 levels tend to be decreased, but not significantly, during 2-month treatment with sarpogrelate at a dose of 300 mg/d in diabetic patients with atherosclerotic PAD. Further studies are needed to confirm the effects of sarpogrelate on systemic inflammation in patients with PAD of various degrees in the presence and absence of interventions, including BM-MNC implantation.

Several lines of evidence have shown that sarpogrelate decreases the production of reactive oxygen species from macrophages and neutrophils through inhibition of the adhesion and proliferation of macrophages and suppresses neutrophil function.[40,41] Therefore, sarpogrelate may inhibit the degradation of NO by inhibition of the production of reactive oxygen species. However, in the present study, BM-MNC implantation per se and a combination of BM-MNC implantation and sarpogrelate did not alter oxidative stress markers such as urinary 8-OHdG excretion and serum MDA-LDL concentration in patients with CLI.

Study limitations

Although significant improvement in endothelial function was observed following a combination of BM-MNC implantation and treatment with sarpogrelate, the number of subjects enrolled in this study was small. This phase 1 clinical trial was not placebo-controlled. Controlled studies using large populations of patients and with long

observation periods are needed to determine the role of a combination of BM-MNC implantation and treatment with sarpogrelate in endothelial function in patients with CLI. In addition, evaluation of a large number of subjects may enable more specific conclusions concerning the roles of inflammation, especially inflammatory markers, in endothelial function after combination therapy of BM-MNC implantation and sarpogrelate to be drawn.

In conclusion, a combination of angiogensis with cell therapy or gene therapy and interventions that are known to improve or augment endothelial function should greatly improve endothelial function in patients with PAD, leading to prevention of cardiovascular or cerebrovascular complications. It is clinically important to select appropriate interventions for improving endothelial function in patients with severe atherosclerosis.

Acknowledgments

We thank Megumi Wakisaka and Satoko Michiyama for their excellent secretarial assistance.

Sources of Founding

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry Education, Culture, Sports, Science, and Technology, Japan (1859081500 and 21590898).

Conflict of Interest

None.

References

1. Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T. Augmentation of postnatal neovascularization with autologus bone marrow transplantation. *Circulation*. 2001; 103: 897-895.
2. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T; Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Theraputic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. *Lancet*. 2002; 360: 427-435.
3. Rajagopalan S, Shah M, Luciano A, Crystal R, Nabel EG. Adenovirus-mediated gene transfer of $VEGF_{121}$ improves lower-extremity endothelial function and flow reserve. *Circulation*. 2001; 104: 753-755.
4. Ross R. The pathogenesis of atherosclerosis. *N Engl J Med.* 1999; 340: 115-126.
5. Brevetti G, Silvestro A, Schiano V, Chiariello M. Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation*. 2003; 108: 2093-2098.
6. Böger RH, Bode-Böger SM, Brandes RP, Phivthong-ngam L, Böhme M, Nafe R, Mügge A, Frölich JC. Biochemical evidence for impaired nitric oxide synthase in patients with peripheral arterial occlusive disease. *Circulation*. 1997; 95: 2068-2074.
7. Harris LM, Faggioli GL, Shah R, Koerner N, Lillis L, Dandona P, Izzo JL, Snyder B, Ricotta JJ. Vascular reactivity in patients with peripheral vascular disease. *Am J Cardiol*. 1995; 76: 207-212.
8. Gokce N, Keaney JF Jr, Hunter LM, Watkins MT, Nedeljkovic ZS, Menzoian JO, Vita JA. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol*. 2003; 41: 1769-1775.
9. Higashi Y, Kimura M, Hara K, Goto C, Noma K, Jitsuiki D, Nakagawa K, Oshima T, Chayama K, Sueda T, Matsubara H, Murohara T, Yoshizumi M. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. *Circulation.* 2004; 109: 1215-1218.

10. Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP. International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol Rev*. 1994; 46: 157-201.
11. Nagatomo T, Rashid M, Abul Muntasir H, Komiyama T. Functions of 5-HT_{2A} receptor and its antagonists in the cardiovascular system. *Pharmacol Ther*. 2004; 104: 59-81.
12. Roth BL, Willins DL, Kristiansen K, Kroeze WK. 5-hydroxytryptamine2-family receptors (5-hydroxytryptamine2A, 5-hydroxytryptamine2B, 5-hydroxytryptamine2C): where structure meets function. *Pharmacol Ther*. 1998; 79: 231-257.
13. Banes A, Florian JA, Watts SW. Mechanisms of 5-hydroxytryptamine(2A) receptor activation of the mitogen-activated protein kinase pathway in vascular smooth muscle. *J Pharmacol Exp Ther*. 1999; 29: 1179-1187.
14. Keegan A, Morecroft I, Smillie D, Hicks MN, MacLean MR. Contribution of the 5-HT_{1B} receptor to hypoxia-induced pulmonary hypertension. Converting evidence using 5-HT-receptor knockout mice and the 5-$HT_{1B/1D}$-receptor antagonist GR. *Circ Res*. 2001; 89: 1231-1239.
15. Li TS, Furutani A, Takahashi M, Ohshima M, Qin SL, Kobayashi T, Ito H, Hamano K. Impaired potency of bone marrow mononuclear cells for inducing therapeutic angiogenesis in obese diabetic rats. *Am J Physiol Heart Circ Physiol*. 2006; 290: H1362-1369.
16. Barradas MA, Gill DS, Fonseca VA, Mikhailidis DP, Dandona P. Intraplatelet serotonin in patients with diabetes mellitus and peripheral vascular disease. *Eur J Clin Invest*. 1988; 18: 399-404.
17. Ito K, Notsu T. Effect of sarpogrelate hydrochloride (MCI-9042) on peripheral circulation of chronic arterial occlusive disease. *J Clin Ther Med*. 1991; 7: 1243-1251.
18. Nakamura K, Kariyazono H, Masuda H, Sakata R, Yamada K. Effects of sarpogrelate hydrochloride on adenosine diphosphate- or collagen-induced platelet responses in arteriosclerosis obliterans. *Blood Coagul Fibrinolysis*. 2001; 12: 391-397.
19. Hara H, Kitajima A, Shimada H, Tamao Y. Antithrombic effect of MCI-9042, a new

antiplatelet agent on experimental thrombosis models. *Thromb Haemost*. 1991; 66: 484-488.

20. Yamashita T, Kitamori K, Hashimoto M, Watanabe S, Giddings JC, Yamamoto J. Conjunctive effects of 5HT2 receptor antagonist, sarpogrelate, on thrombolysis with modified tissue plasminogen activator in different laser-induced thrombosis models. *Haemostasis*. 2000; 30: 321-332.
21. Hara H, Osakabe M, Kitajima A, Tamao Y, Kikumoto R. MCI-9042, a new antiplatelet agent is a selective S2-serotonergic receptor antagonist. *Thromb Haemost*. 1991; 65: 415-420.
22. Nakamura K, Kariyazono H, Moriyama Y, Toyohira H, Kubo H, Yotsumoto G, Taira A, Yamada K. Effects of sarpogrelate hydrochloride on platelet aggregation, and its relation to the release of serotonin and P-selectin. *Blood Coagul Fibrinolysis*. 1999; 10: 513-519.
23. Sharma SK, Zahradka P, Chapman D, Kumamoto H, Takeda N, Dhalla NS. Inhibition of serotonin-induced vascular smooth muscle cell proliferation by sarpogrelate. *J Pharmacol Exp Ther*. 1999; 290: 1475-1481.
24. Takei I, Nakamoto S, Yamauchi Y. Effects of the 5-HT2 receptor antagonist sarpogrelate on diabetic vascular disease. *Diabetes Res*. 1999; 34: 239-246.
25. Miyazaki M, Higashi Y, Goto C, Chayama K, Yoshizumi M, Sanada H, Sueda T. Sarpogrelate hydrochloride, a selective 5-HT_{2A} antagonist, improves vascular function in patients with peripheral arterial disease. *J Cardiovasc Pharm*. 2007; 49: 221-227.
26. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG; TASC II Working Group, Bell K, Caporusso J, Durand-Zaleski I, Komori K, Lammer J, Liapis C, Novo S, Razavi M, Robbs J, Schaper N, Shigematsu H, Sapoval M, White C, White J, Clement D, Creager M, Jaff M, Mohler E 3rd, Rutherford RB, Sheehan P, Sillesen H, Rosenfield K. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg. 2007; 33 Suppl 1: S1-S75.
27. Higashi Y, Sasaki S, Nakagawa K, Matsuura H, Oshima T, Chayama K. Endothelial function and oxidative stress in renovascular hypertension. *N Engl J Med*. 2002; 346: 1954-1962.
28. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Plasma

concentration of C-reactive protein and risk of developing peripheral vascular disease. *Circulation*. 1998; 97: 425-428.

29. Erren M, Reinecke H, Junker R, Fobker M, Schulte H, Schurek JO, Kropf J, Kerber S, Breithardt G, Assmann G, Cullen P. Systemic inflammatory parameters in patients with atherosclerosis of the coronary and peripheral arteries. *Arterioscler Thromb Vasc Biol*. 1999; 19: 2355-2363.
30. Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation*. 2002; 106: 913-919.
31. Huang AL, Vita JA. Effects of systemic inflammation on endothelium-dependent vasodilation. *Trends Cardiovasc Med*. 2006; 16:15-20.
32. Kofler S, Nickel T, Weis M. Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clin Sci (Lond)*. 2005; 108: 205-213.
33. Bhagat K, Vallance P. Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo. *Circulation*. 1997; 96: 3042-3047.
34. Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I. Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. *Circulation*. 2002; 106:1439-1441.
35. Oshima T, Ozono R, Yano Y, Oishi Y, Teragawa H, Higashi Y, Yoshizumi M, Kambe M. Association of helicobacter pylori infection with systemic inflammation and endothelial dysfunction in healthy male subjects. *J Am Coll Cardiol*. 2005; 45: 1219-1222.
36. Higashi Y, Jitsuiki D, Goto C, Umemura T, Nishioka K, Hidaka T, Takemoto T, Nakamura S, Nakagawa K, Oshima T, Chayama K, Yoshizumi M, Taguchi A. Periodontal infection is associated with endothelial dysfunction in humans. *Hypertension.* 2008; 51: 446-453.
37. Katz MF, Farber HW, Dodds-Stitt Z, Cruikshank WW, Beer DJ.Serotonin-stimulated aortic endothelial cells secrete a novel T lymphocyte chemotactic and growth factor. *J Leukoc Biol*. 1994; 55: 567-573.
38. Ito T, Ikeda U, Shimpo M, Yamamoto K, Shimada K. Serotonin increases interleukin-6 synthesis in human vascular smooth muscle cells. *Circulation*. 2000;

102: 2522-2527.

39. Yamakawa J, Takahashi T, Saegusa S, Moriya J, Itoh T, Kusaka K, Kawaura K, Wang XQ, Kanda T. Effect of the serotonin blocker sarpogrelate on circulating interleukin-18 levels in patients with diabetes and arteriosclerosis obliterans. *J Int Med Res*. 2004; 32: 166-169.
40. Norgren L, Jawien A, Matyas L, Riegerd H, Arita K; European MCI-9042 Study Group. Sarpogrelate, a 5-HT2A receptor antagonist in intermittent claudication. A phase II European study. *Vasc Med*. 2006; 11: 75-83.
41. Mikawa K, Akamatsu H, Nishina K, Shiga M, Maekawa N, Obara H, Niwa Y. The effects of sarpogrelate on superoxide production by human neutrophils. *Reg Anesth Pain Med*. 2000; 25: 181-186.

Figure Legend

Fig. 1. Comparison of the leg blood flow (as % change from basal flow) response to acetylcholine administration (top) and sodium nitroprusside administration (bottom) before and after BM-NMC implantation of 12 weeks of follow-up in the sarpogrelate and control groups.

Table 1. Clinical Characteristics in the Supportive and Control Groups before (0 weeks) and after 12 Weeks of Bone Marrow Mononuclear Cell Implantation

	Supportive group (n=8)		Control group (n=7)	
Variable	Before	12 weeks	Before	12 weeks
Body mass index (kg m2)	21.3 ± 2.2	21.4 ± 2.3	22.0 ± 2.2	22.0 ± 2.1
Systolic blood pressure (mm Hg)	133.8 ± 11.2	130.3 ± 14.8	128.8 ± 12.5	127.7 ± 19.4
Diastolic blood pressure (mm Hg)	75.0 ± 9.6	70.5 ± 12.9	72.2 ± 12.1	70.7 ± 14.5
Heart Rate (bpm)	72.1 ± 8.7	71.4 ± 9.1	69.8 ± 8.8	71.0 ± 9.5
Total cholesterol (mg dL)	198.7 ± 25.7	192.4 ± 20.3	207.2 ± 12.6	211.0 ± 21.2
HDL cholesterol (mg dL)	45.2 ± 6.1	46.3 ± 5.8	44.8 ± 6.5	45.1 ± 7.6
LDL cholesterol (mg dL)	101.2 ± 19.8	98.7 ± 20.1	104.7 ± 18.6	102.5 ± 17.2
Triglycerides (mg mL)	121.5 ± 17.3	122.3 ± 13.3	116.4 ± 15.2	114.2 ± 22.1
Glucose (mg mL)	122.4 ± 33.7	114.6 ± 25.4	119.8 ± 41.4	118.3 ± 45.3
Plasma renin activity (ng mL hr)	2.1 ± 1.9	1.9 ± 2.2	2.2 ± 1.8	2.3 ± 2.1
Plasma angiotensin II (pg mL)	17.2 ± 7.3	17.9 ± 9.8	18.6 ± 13.1	20.1 ± 12.9
Interleukin 6 (mg L)	2.2 ± 1.9	1.4 ± 2.0**	2.1 ± 2.3	1.9 ± 2.3
High sensitivity CRP (mg L)	2.1 ± 2.2	1.2 ± 1.3**	2.2 ± 2.1	2.0 ± 2.1
MDA LDL (U L)	73.2 ± 27.8	68.7 ± 29.1	67.2 ± 25.1	65.8 ± 30.1
Urinary 8 OHdG (ng mg Cr)	12.8 ± 5.4	12.3 ± 4.6	11.7 ± 4.7	11.1 ± 4.1
Feminine classification (no.)				
IV	8		7	
Previous treatment (no.)				
Bypass graft	2		3	
PTA	3		3	
Complications (no.)				
Hypertension	4		5	
Diabetes mellitus	4		5	
Dyslipidemia	3		3	
Medication (no.)				
Anti platelet	8		7	
ACE inhibitors	2		1	
ARBs	4		4	
Calcium channel blockers	3		2	
Statins	2		3	
Sulfonylurea and or metformin	4		5	
Smoker (no.)	6		5	

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; MDA, malondialdehyde; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Cr, creatinine; PTA, percutaneous transluminal angioplasty; ACE, angiotensin-converting enzyme; ARB, angiotensin type 1 receptor blocker. All results are presented as mean±SD.

*P<0.05 vs. before 18 weeks in the same group.

†P<0.05 vs. control at the same follow-up period.

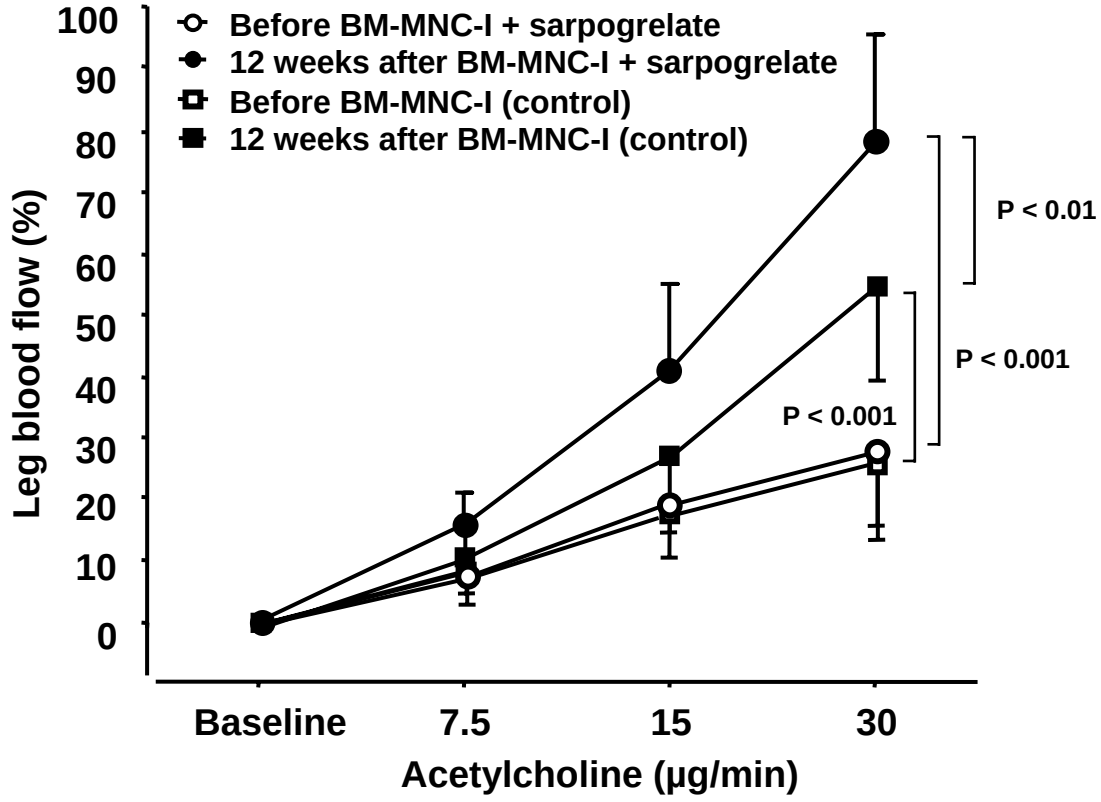
Table 2. Parameters of Limb Ischemia in the Saphenectomy and Control Groups before (0 weeks) and after 12 Weeks of Bone Marrow Mononuclear Cell Implantation

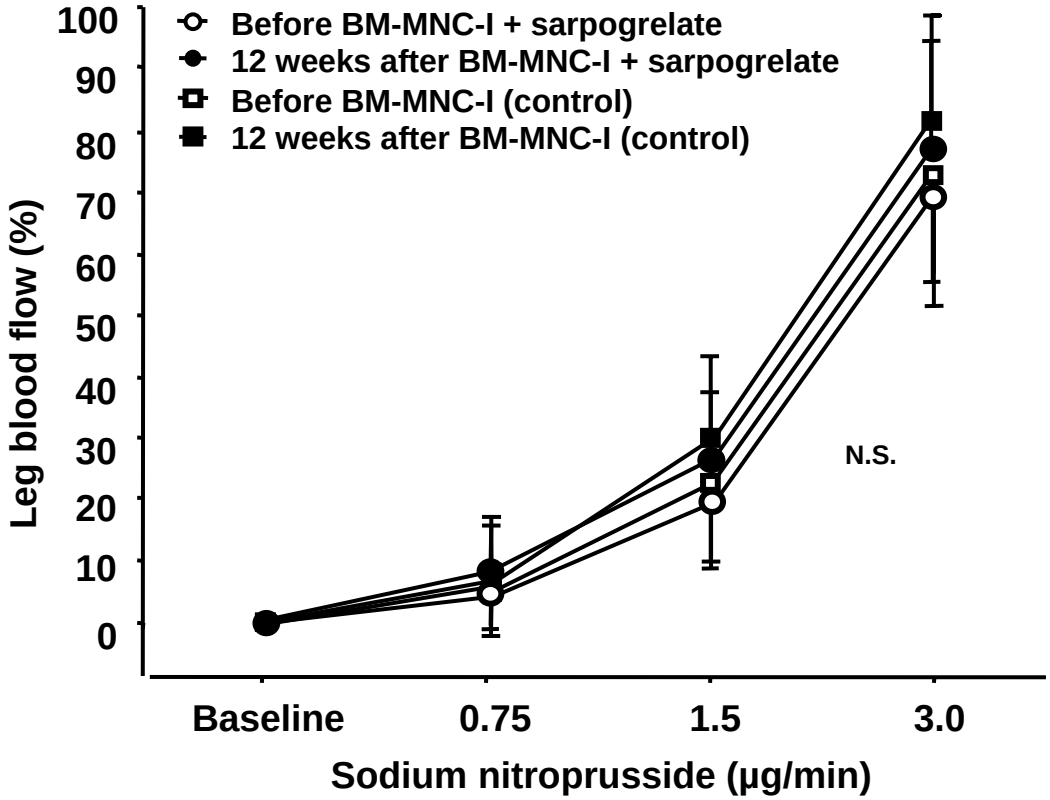
	Saphenectomy group (n=5)		Control group (n=7)	
Variable	Before	12 weeks	Before	12 weeks
Ankle brachial pressure index	0.51 ± 0.09	0.66 ± 0.12	0.52 ± 0.13	0.64 ± 0.15
Transcutaneous oxygen pressure (mm Hg)	27.9 ± 10.7	36.9 ± 8.7*	30.1 ± 9.6	41.1 ± 9.8*
Pain free walking time (min)	0.7 ± 0.6	2.8 ± 2.1*	0.8 ± 0.7	3.0 ± 1.7*
Basal LBF (mL min 100 mL tissue)	1.8 ± 1.0	2.2 ± 1.2	1.7 ± 1.3	2.0 ± 1.4

LBF indicates leg blood flow.

All results are presented as mean±SD. *P<0.05 vs. before (0 weeks) in the same group.

Figure 1


Before BM-MNC-I + sarpogrelate
12 weeks after BM-MNC-I + sarpogrelate
Before BM-MNC-I (control)
12 weeks after BM-MNC-I (control)
Leg blood flow (%)
100
90
80
70
60
50
40
30
20
10
0
P < 0.01
P < 0.001
P < 0.001
Baseline
7.5
15
30
Acetylcholine (μg/min)


Before BM-MNC-I + sarpogrelate
12 weeks after BM-MNC-I + sarpogrelate
Before BM-MNC-I (control)
12 weeks after BM-MNC-I (control)
Leg blood flow (%)
100
90
80
70
60
50
40
30
20
10
0
N.S.
Baseline
0.75
1.5
3.0
Sodium nitroprusside (μg/min)